A Novel Function of Giant Ankyrin-G in Promoting the Formation of Somatodendritic 
GABA_A Receptor Synaptogenesis

by

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Christopher Nicchitta

Dissertation submitted in partial fulfillment of 
the requirements for the degree of Doctor 
of Philosophy in the Department of 
Pharmacology and Cancer Biology in the Graduate School 
of Duke University

2014
ABSTRACT

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Abstract

The formation and retention of distinct membrane domains in the fluidic membrane bilayer are the key processes in establishing spatial organization for mediating physiological functions in metazoans. The spectrin-ankyrin network organizes diverse membrane domains including T-tubule and intercalated disc of cardiomyocytes, basolateral membrane of epithelial cells, costameres of striatal muscle, and axon initial segments and nodes of Ranvier in nervous system. This thesis identifies a novel function of 480 kDa ankyrin-G, an alternatively spliced isoform of the ankyrin family, in promoting somatodendritic GABA_A receptor synaptogenesis both in vitro and in vivo. In the nervous system, an insertion of a neuronal specific exon (exon 37) occurs in ankyrin-G polypeptide which results in a 480 kDa isoform. 480 kDa ankyrin-G (giant ankyrin-G) has been shown to coordinate formation and maintenance of the axon initial segment (AIS) and nodes of Ranvier. This thesis research began with the discovery that giant ankyrin-G, previously thought to be confined to the axon initial segment, forms developmentally-regulated and cell-type specific somatodendritic “outposts” on the plasma membrane of pyramidal neurons. This somatodendritic 480 kDa ankyrin-G outpost forms micron-scale membrane domains where it associates with canonical AIS binding partners including voltage-gated sodium channel and neurofascin. This thesis further discovered that the giant insert of 480 kDa ankyrin-G interacts with GABARAP,
a GABA<sub>α</sub> receptor-associated protein. Both the interaction with GABARAP and the membrane association through palmitoylation of giant ankyrin-G are required for the formation of somatodendritic GABAergic synapses. This work further found that ankyrin-G associates with extrasynaptic GABA<sub>α</sub> receptors and stabilizes receptors on the extrasynaptic membrane through opposing endocytosis. This story demonstrates for the first time the existence of giant ankyrin-G somatodendritic outpost as well as its function in directing the formation of GABAergic synapses that provides a rationale for studies linking ankyrin-G genetic variation with psychiatric disease and neurodevelopmental disorders.

Additional work presented in the Appendix characterized novel ankyrin-G full length transcripts in the heart and kidney with unique domain compositions though alternative splicing. The preliminary work further identified biochemical properties and potential role of an insert C in the C-terminus of ankyrin-G in mediating cytokinesis and cellular migration in mouse fibroblasts. Together, this thesis work expands the knowledge of giant ankyrin-G functions in the nervous system and offers insights into the diversified roles of distinct ankyrin-G peptides acquired from alternative splicing in organizing specific membrane domains and interacting with defined intracellular pathways in different tissues.
Dedication

This is dedicated to my family and my husband Juanchi for their endless support and love.
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<th>Description</th>
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<tbody>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>vGAT</td>
<td>vesicular GABA transporter</td>
</tr>
<tr>
<td>GABARAP</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt; receptor associated protein</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>PND</td>
<td>Postnatal day</td>
</tr>
<tr>
<td>DIV</td>
<td>Day <em>in vitro</em></td>
</tr>
<tr>
<td>AIS</td>
<td>Axon initial segment</td>
</tr>
<tr>
<td>VGSC</td>
<td>voltage-gated sodium channel</td>
</tr>
<tr>
<td>WT</td>
<td>wild type</td>
</tr>
<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
</tr>
<tr>
<td>BFP</td>
<td>blue fluorescent protein</td>
</tr>
<tr>
<td>CV</td>
<td>column volume</td>
</tr>
<tr>
<td>MBD</td>
<td>Membrane-binding domain</td>
</tr>
<tr>
<td>SBD</td>
<td>Spectrin-binding domain</td>
</tr>
<tr>
<td>MDCK</td>
<td>Madin-Darby canine kidney</td>
</tr>
<tr>
<td>HBE</td>
<td>Human Bronchial Epithelial</td>
</tr>
<tr>
<td>ZU5</td>
<td>Domain present in ZO-1 and Unc5-like netrin receptors</td>
</tr>
<tr>
<td></td>
<td>Description</td>
</tr>
<tr>
<td>---</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>ZO-1</td>
<td>Zona occludens protein 1</td>
</tr>
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Chapter 1. Background and Overview

1.1 Ankyrins: what are they?

In multicellular organisms, plasma membranes are organized into well-defined micron-scale domains which possess functional diversity spanning processes such as vectorial transport, localized scaffolding, and electrical signaling (Bennett and Healy, 2009; Bennett and Lorenzo, 2013). Spectrins and ankyrins are proteins that interact with membrane-spanning proteins and coordinate the segregation of diverse proteins into specialized domains according to their functions. Given the evidence from evolutionary analysis, the spectrin-ankyrin network gained its ability to organize membrane-spanning proteins during the evolution of bilaterians when the multicellular organisms require more complicated tissue and mechanisms for precise spatial patterning in the plasma membrane. The ankyrin gene family in mammals has three members: ankyrin-R (R for restricted; ankyrin1), ankyrin-B (B for broadly expressed; ankyrin2), and ankyrin-G (G for general; ankyrin3) (Bennett and Baines, 2001; Kordeli et al., 1995; Lambert and Bennett, 1993; Lambert et al., 1990; Otto et al., 1991) (Figure 1). A canonical ankyrin molecule starts with a membrane binding domain composed of ANK repeats followed by spectrin binding domain, UPA domain, death domain, and a C-terminus tail. Ankyrins binds to their membrane partners though ANK repeats that are folded into extended solenoids suitable for protein-protein interaction (Michaely et al., 2002). The ankyrin-binding motifs identified so far lack defined primary sequence but share
common features as short, extended peptide without extensive secondary structure. The intrinsically unstructured nature of ankyrin-binding code thus offers evolutionary advantages such as the rapid acquisition of the new partners for vertebrate adaptation as well as the capacity for integrating novel pathways through multiple binding partners (Bennett and Lorenzo, 2013).

As its name indicates, ankyrin “anchors” various membrane integral proteins, channels, adhesion molecules, and transporters to an extended spectrin network associated with the plasma membrane for advanced mechanical support (Bennett and Lorenzo, 2013) (Figure 2). The first ankyrin-spectrin-based assembly of membrane elements was elucidated in human erythrocyte, where the membrane-cytoskeleton connection is organized through the interaction between ankyrin-R, spectrin lattice, and the anion exchanger on the membrane (Bennett and Baines, 2001). Another two members of ankyrin family, ankyrin-B and ankyrin-G, express in most other tissues with non-redundant functions and collaborative roles. The ankyrin-B/G-spectrin network has been implicated in the organization and stabilization of numerous membrane domains (Bennett and Healy, 2009; Bennett and Lorenzo, 2013) (Figure 2). In costameres of striatal muscle, ankyrin-B is required for sarcolemma localization of dystrophin and dynactin-4 (Ayalon et al., 2008). In rod photoreceptors, ankyrin-B is expressed in the inner segment while ankyrin-G exclusively localizes at the outer segment and plays essential roles in segregating cyclic nucleotide-gated channels (Kizhatil et al., 2009). In cardiomyocytes,
ankyrin-B directs beta-2 spectrin to an intracellular compartment, while ankyrin-G is required for the localization of voltage-gated sodium channel in intercalated discs and T-tubules (Lowe et al., 2008; Mohler et al., 2004a; Mohler et al., 2004b). In neurons, ankyrin-G and beta IV spectrin colocalize in axon initial segments and nodes of Ranvier and are required to cluster voltage-gated channels and adhesion molecules (Hedstrom et al., 2008; Jenkins et al., 2014; Jenkins and Bennett, 2001; Yang et al., 2007), whereas ankyrin-B and beta-2 spectrin assemble a distal axonal cytoskeleton that limits ankyrin-G localization (Galiano et al., 2012). In this thesis, we focus on the role of an alternatively-spliced isoform of ankyrin-G in the nervous system, demonstrating a novel localization and function of giant ankyrin-G in directing somatodendritic GABAergic synaptogenesis.
Figure 1: Domain organization of ankyrin molecules

(From Bennett and Lorenzo, 2013)
Figure 2: Spectrin-ankyrin-based plasma membrane domains

(From Bennett and Lorenzo, 2013)
1.2 Ankyrin-G

Ankyrin was first identified as the protein co-purified with voltage-gated sodium channel from rat brain membrane lysates (Srinivasan et al., 1988). The localization of an ankyrin variant was then defined as being co-distributed with voltage-gated sodium channel at the nodes of Ranvier and myelinated central and peripheral nerves that is distinct from ankyrin-B and erythrocyte ankyrin (Kordeli and Bennett, 1991; Kordeli et al., 1990). Ankyrin-G protein was subsequently cloned and characterized as a component of epithelia and axon initial segments essential for clustering voltage-gated sodium channel and firing of action potential (Jenkins and Bennett, 2001; Kordeli et al., 1995; Peters et al., 1995). Since then, Ankyrin-G has been recognized as the coordinator for diverse functional domains in vertebrate systems including the intercalated disc in cardiomyocytes, the basolateral membrane in epithelium, the node of Ranvier and the axon initial segment (AIS) in neurons (Bennett and Lorenzo, 2013; Dzhashiashvili et al., 2007; Kizhatil and Bennett, 2004; Lowe et al., 2008; Mohler et al., 2004a; Zhou et al., 1998). In different tissues, ankyrin-G is known to be subjective to extensive alternative splicing, generating isoforms ranging from 480 kDa to 120 kDa capable for diverse functions and protein interactions in specialized microdomains. The canonical epithelial 190 kDa ankyrin-G isoform is composed of ANK repeats, a spectrin binding domain, an UPA domain, a death domain, and a C-terminal tail (Bennett and Baines, 2001; Wang et al., 2012b) (Figure 1). The neuronal specific ankyrin-G is discussed in detail below. For
smaller ankyrin-G isoforms lacking parts of defined domains from alternative splicing, see Appendix.

Neuronal-specific ankyrin-G contains an alternatively spliced giant insert encoded by a single exon #37, resulting in a 480 kDa isoform (Bennett and Lorenzo, 2013; Kordeli et al., 1995) (Figure 1). Despite the discovery of 480 kDa ankyrin-G 20 years ago, little is known about the necessity and significance of 480 kDa ankyrin-G in forming functional AIS. A recent study has been dedicated to determining the nature of this giant exon-37 and the function of 480 kDa ankyrin-G by generating exon-37 KO mice (Jenkins et al., 2014). Exon-37 encodes 2600 residues configured as a fibrous polypeptide with sequence similarity to I-connectin, and the origin of this exon is likely due to exon swapping (Jenkins et al., 2014). Exon-37 KO caused a complete loss of AIS components including VGSC, neurofascin, and beta-4 spectrin. However, this loss of AIS is compatible with animal survival to weaning and the preservation of the action potential, probably due to the compensation from upregulated levels of 190 kDa ankyrin-G from in-frame splicing of exon 37. The 190 kDa isoform appears to be upregulated approximately five times compared to control mice (Figure 26). Collectively these results further confirm the requirement for 480 kDa ankyrin-G in forming a functional AIS, though action potentials may trigger from somewhere outside of the initial segment.

The importance of ankyrin-G in establishing organized functional domains has been extensively reported in cardiac tissue. Ankyrin-G is required for the expression and
localization of the major cardiac voltage-gated sodium channel Nav1.5 at specialized membrane domains (Lowe et al., 2008; Makara et al., 2014) (Figure 2). Blocking this interaction through a human variant in gene encoding Nav1.5 leads to the loss of sodium channel on the membrane and Brugada syndrome (Mohler et al., 2004a). Ankyrin-G has also been shown as a key component of the intercalated disc where it interacts with Plakophilin-2 and Connexin43, linking voltage-gated sodium channel, gap junction, and desmosome complex together into a functional “hub” for controlling excitability, molecular adhesion, and intercellular communication (Agullo-Pascual et al., 2013; Makara et al., 2014; Sato et al., 2011). Regardless of functional studies of ankyrin-G in cardiac tissue, ankyrin-G isoforms expression profile and subcellular localization have not been characterized (Malhotra et al., 2002; Mohler et al., 2004a). Therefore in the Appendix, we used crude RNA from mice hearts for RT-PCR in the attempt of identifying alternatively spliced full length ankyrin-G constructs which might have yet to be determined specific functions and localizations in cardiac tissue.

Another tissue of interest in this study is kidney. As the primary sites for solute, salt, mineral, and liquid reabsorption, proximal and distal convoluted tubules of kidney are lined with polarized epithelial cells. The spatial compartmentalization of these epithelial cells consists of apical and basolateral membrane domains, which are specialized in unidirectional transport of ions and organic nutrients. One of the essential basolateral membrane transporter for maintaining the gradient of positive ions is Na⁺K⁺
ATPase, whose assembly and stabilization on the membrane is achieved through direct interaction with ankyrin-G and spectrin-based cytoskeleton (Devarajan et al., 1994; Nelson and Veshnock, 1987) (Figure 2). Similarly, in human bronchial epithelial cells, ankyrin-G and spectrin network is required for retaining Na\(^+\)K\(^+\) ATPase to the lateral membrane as well as for the biogenesis of basolateral membrane (Kizhatil and Bennett, 2004; Kizhatil et al., 2007). Another important membrane protein of connecting tubule and collecting duct cells, the ammonium transporter RhBG responsible for ammonium secretion, also displays polarized targeting to the basolateral membrane which requires the association with ankyrin-G on the cytoplasmic domain (Lopez et al., 2005). Moreover, a mutation disrupting the binding of RhBG with ankyrin-G resulted in nonfunctional gas channel in epithelial kidney cells, suggesting that ankyrin-G-spectrin mediated membrane targeting is essential for the function of ammonium transporter (Sohet et al., 2008). Recently, distal convoluted tubule-localized voltage-gated potassium channel Kv1.1 has been described to interact with ankyrin-G for the potential role in channel biophysical properties and magnesium regulation (San-Cristobal et al., 2014). Given the presence of multiple alternatively spliced ankyrin-G isoforms in kidney lysate immunoblotting and the diverse localization of distinct ankyrin-G peptides (Doctor et al., 1998; Peters et al., 1995), our study thus aims to fully recover the actual exon usage of various ankyrin-G transcripts for further elucidation of potential isoform-specific functions in kidney tissue.
Chapter 2. New role of giant ankyrin-G in the formation of somatodendritic GABAergic synapses

2.1 Introduction

In a structurally and functionally polarized neuron, the AIS is a specialized excitable membrane in the proximal axon responsible for both generation and modulation of action potentials (Kole et al., 2008; Yoshimura and Rasband, 2014). The formation, assembly, and maintenance of the AIS critically depend on ankyrin-G, as ankyrin-G is required for the clustering of voltage-gated sodium channel (VGSC), KCNQ2/3 channel, cell adhesion molecule neurofascin, and cytoskeletal beta-4 spectrin (Ango et al., 2004; Jenkins and Bennett, 2001; Pan et al., 2006; Yang et al., 2007; Zhou et al., 1998). Consequently, in both cultured neurons and in mouse brain neurons devoid of ankyrin-G have been reported to develop proximal axons that contain dendritic properties (Hedstrom et al., 2008; Sobotzik et al., 2009).

In addition to the canonical role in initiating action potential, AIS has been shown to harbor the exclusive axon-axonic neurotransmission specialized interneurons (Kole and Stuart, 2012; Muir and Kittler, 2014; Somogyi et al., 1998). For instance, basket and stellar interneurons project specific axon terminals to ensheath the initial segment of Purkinje cells, forming the so called GABAergic “pinceau” synapses (Blot and Barbour, 2014; Sotelo and Llinas, 1972). The proper formation of pinceau synapses depends on a sharp gradient of cell adhesion molecule neurofascin, which is expressed at low level in the somatic membrane and high in the AIS of Purkinje neurons (Ango et al., 2004).
Remarkably, both neurofascin gradient and GABAergic synapses on the AISs of Purkinje neurons are lost in mice with conditional cerebellar knockdown of ankyrin-G (Ango et al., 2004). It would thus be of interest to further explore the role of ankyrin-G in directing inhibitory synapses in the AIS, as well as in other compartments of neurons where GABAergic synapses are enriched. As of today, no report has been made about the presence of ankyrin-G outside of the AIS. Interestingly, voltage-gated sodium channels have been detected on the soma and dendrite of hippocampal and neocortical neurons, where they carry action potentials back propagating for nonlinear synaptic integration and dendritic sodium spike generation (Larkum et al., 2009; Lorincz and Nusser, 2010; Losonczy et al., 2008; Migliore and Shepherd, 2002). The molecular mechanisms for coordinating these membrane spanning proteins as well as their precise spatial patterning on the plasma membrane remain unknown. Here we report the discovery of a 480 kDa ankyrin-G “outpost” on the somatodendritic membrane, where it serves as a master organizer for the formation of membrane microdomains with known ankyrin binding partners as well as novel interactions with components of GABAergic synapses.

Neuronal activity in adult brain is orchestrated by the crosstalk and balance between excitatory and inhibitory synapses (Uchizono, 1965). While excitatory synapse transmission increases the probability of an action potential occurring in postsynaptic cells, inhibitory synapses hyperpolarizes the postsynaptic membrane thus counteracting
excitatory signals (Purves and Williams, 2001). Distinct from excitatory synapses in which glutamate is the major neurotransmitter, inhibitory synapses utilize γ-aminobutyric acid (GABA) and glycine in modulating intricate neuronal transmission at multiple levels of brain function (Macdonald and Olsen, 1994; Sheng and Hoogenraad, 2007). The action of GABA is mediated by ionotropic (GABA\(_A\)) and metabotropic (GABA\(_B\)) receptors (Olsen and Sieghart, 2008). Ionotropic ligand-gated GABA\(_A\) receptors modulate fast inhibitory neurotransmission in the vertebrate CNS via the influx of Cl\(^{-}\) ions through an integral membrane channel (Luscher et al., 2011; Sieghart and Sperk, 2002). Clinically, GABA\(_A\) receptors are defined through activation by selective agonist muscimol, inhibition by bicuculline and picrotoxin, and modulation by benzodiazepines, barbiturates, and certain other CNS anti-convulsant and sedative-hypnotic agents (Macdonald and Olsen, 1994; Rudolph and Knoflach, 2011; Sieghart, 1995; Sieghart, 2000). Alterations in receptor subunit composition and dynamics have been implicated in the etiology of epilepsy and other psychological abnormalities, including schizophrenia and bipolar disorder (Benarroch, 2007; Charych et al., 2009; Lewis et al., 2005; Rudolph and Mohler, 2004).

GABA\(_A\) receptors belong to the superfamily which includes nicotinic acetylcholine receptors and serotonin receptors (Grenningloh et al., 1987; Maricq et al., 1991; Schofield et al., 1987). In vertebrates, 18 GABA\(_A\) receptor subunits have been characterized and divided into seven classes: \(\alpha\) (1-6), \(\beta\) (1-3), \(\gamma\) (1-3), \(\delta\), \(\varepsilon\) (1-3), \(\theta\), and \(\pi\).
Despite the complexity in subunit combinations, most GABA\textsubscript{A} receptors are composed of two α subunits, two β subunits, and one γ (or δ) subunit (Rudolph and Mohler, 2004). This heteropentameric structure encompasses a large extracellular amino terminus, four membrane-spanning domains, and a large intracellular domain (Unwin, 1989). The intracellular loops of the receptor harbor the binding sites for protein-protein interactions involved in synaptic localization and intracellular trafficking (Jacob et al., 2008; Maricq et al., 1991; Moss and Smart, 2001).

Numerous proteins have been identified as GABA\textsubscript{A} receptor associating partners from yeast two-hybrid assays. Among these, GABA\textsubscript{A} receptor associated protein, GABARAP, was one of the first to be cloned and the best studied as a member of an ubiquitin-like family and a more distantly-related MAP1 LC3 family (Chen and Olsen, 2007; Wang et al., 1999). Other paralogs of GABARAP in the family include GEC-1 (guinea-pig endometrial cells-1, or GABARAPL1), GATE-16 (Golgi-associated ATPase enhancer of 16 kDa, or GABARAPL2), GABARAPL3, and GABARAPL4 (Chen and Olsen, 2007; Luscher et al., 2011). In cultured neurons, GABARAP mainly localizes to intracellular compartments including endoplasmic reticulum and Golgi, with only a fraction colocalized with GABA\textsubscript{A} receptor on the membrane (Chen and Olsen, 2007; Kneussel et al., 2000; Leil et al., 2004). Overexpression of GABARAP in hippocampal neurons and Cos7 cells facilitates the translocation of GABA\textsubscript{A} receptors to the surface.
(Leil et al., 2004). GABARAP interacts with several other proteins known to be associated with GABAergic and glutamatergic synapses for receptor trafficking and modification such as PDZ-domain containing GRIP, phospholipase C-related catalytically inactive proteins 1 and 2 (PRIP1/2, PRIP1, or p130), and N-ethylmaleimide-sensitive factor (NSF, ATPase and chaperone of SNARE complexes) (Kanematsu et al., 2002; Kittler et al., 2004; Li et al., 2005; Marsden et al., 2007; Zhao et al., 2007). Interestingly, GABARAP also binds to microtubule-dependent molecular motor KIF5A in the regulation of GABA<sub>A</sub> receptor trafficking from post-Golgi to the neuronal surface in dendrites (Nakajima et al., 2012). In addition to neuronal factors, GABARAP has also been recognized as the binding partner of a broad range of proteins, such as tubulin, transferrin receptor, clathrin heavy chain, Rho/Cdc42/Rac GTPase-activating protein, and more (Green et al., 2002; Mohrluder et al., 2007; Mohrluder et al., 2009; Nakamura et al., 2008; Wang and Olsen, 2000). The steadily expanding number of GABARAP interacting partners has strengthened the idea that GABARAP not only plays a role in the trafficking of GABA<sub>A</sub> receptors, but also participates in other biological processes of both neuronal and non-neuronal cells, such as general vesicular transport and fusion events, autophagy and apoptosis (Mohrluder et al., 2009).
Figure 3: GABA_\textsubscript{A} receptors are members of ligand-gated ion channel family. 

(From Jacob et al., 2008)
The importance of GABA<sub>A</sub> receptors in GABAergic innervations has drawn much interest as to the precise cellular mechanisms of receptor accumulation on the neuronal membrane. One primary determinant is receptor endocytosis, as the number of the receptors on the membrane directly affects the efficacy of synaptic inhibition and hence neuronal excitation. GABA<sub>A</sub> receptor endocytosis upon GABA transmission and the dynamic exchange between synaptic and postsynaptic sites have been linked to the maintenance of receptor surface levels through a clathrin-mediated dynamin-dependent pathway (Jacob et al., 2008; Kittler et al., 2005; Kittler et al., 2000; Luscher et al., 2011; Thomas et al., 2005). In addition to its natural agonist, GABA<sub>A</sub> receptors undergo regulated endocytosis upon activation by other molecules such as Wnt ligand, TNF-α, and BDNF (Bernstein and Quick, 1999; Cuitino et al., 2010; Hewitt and Bains, 2006; Pribiag and Stellwagen, 2013). Impaired receptor endocytosis has a profound influence on the physiological and pathological status of epilepsy and cognitive deficits (Chaumont et al., 2013; Jurd and Moss, 2010; Zhou et al., 2013). Histochemically and functionally, GABA<sub>A</sub> receptors are compartmentalized into the somatodendritic membrane or axon initial segment (AIS) (Kullmann et al., 2005; Rojas et al., 2011). Though GABAergic clustering, receptor lateral diffusion, and synapse dynamics have been extensively characterized in dendrites and the AIS (Bannai et al., 2009; Muir et al., 2010; Muir and Kittler, 2014; Thomas et al., 2005), the details of how GABAergic synapses accumulate on the somatic membrane as well as whether there is any overlap...
with the previously described GABARAP-mediated processes or receptor endocytosis remain unclear. Here, we show that ankyrin-G outside of the AIS is required for the formation of GABAergic synapses on the somatodendritic membrane by impeding receptor endocytosis through a GABARAP-association dependent process.
2.2 Methods and Materials

Tissue preparation and immunohistochemistry

Mice at postnatal day 20 were anesthetized and sacrificed by cardiac perfusion prior to removal of brain and fixation in 4% paraformaldehyde overnight. Paraffin embedding protocol was then applied for dehydration through increasing concentration of ethanol and clearing in xylene followed by infiltration with molten paraffin in a vacuum oven. For long term storage and preservation, paraffin sections were cut at 7 µm using a Leica RM2155 rotary microtome and then mounted onto microscope slides.

Deparaffinization protocol was adapted from Rosen Lab, Baylor College of Medicine (https://www.bcm.edu/rosenlab/index.cfm?pmid=12982). Generally, sections were removed of embedding material by xylene and rehydrated using decreasing concentration of ethanol followed by PBS. For antigen retrieval, 10mM sodium citrate was used to unmask the epitopes under boiled condition from microwaving for 20 minutes. Slides were then cooled, washed in PBS, blocked using blocking buffer, and incubated with primary antibodies in blocking buffer. One the following day, sections were washed by PBS-0.2% Tween 20 (Calbiochem) (PBST) and incubated with secondary fluorescent antibodies in blocking buffer at room temperature for 2 hours. Finally, sections were washed in PBST, mounted with Prolong Gold Antifade reagent (Life Technologies), and stored at 4°C.

DNA constructs
190 kDa (Kizhatil and Bennett, 2004) and 480 kDa (Jenkins et al., 2014) ankyrin-G-GFP were previously described. W1989R 480 kDa ankyrin-G-GFP was generated using the Quikchange II XL mutagenesis kit (Agilent). CAG-Cre-2A-BFP plasmid was generated by cloning a Cre recombinase, a viral 2A peptide (He et al., 2012), and a TagBFP (a gift from Dr. James Bear, University of North Carolina-Chapel Hill) into pLenti6-V5-DEST viral vector (Invitrogen) with its promoter replaced by CAG (a gift from Dr. Scott Soderling, Duke University). CAG-pEGFP-N1 plasmid was acquired by replacing CMV promoter from pEGFP-N1 (Addgene) with CAG promoter. CAG-pEBFP-N1 plasmid was generated by replacing GFP with TagBFP in CAG-pEGFP-N1 vector. CAG-Cre-2A-GFP plasmid was obtained by cloning a Cre and a 2A peptide into CAG-pEGFP-N1 vector. Full length GABARAP was pulled out from mouse brain library using yeast two-hybrid and cloned into pGEX/MAL expression vector. WT or W1989R giant insert region from residue 1819-2535 (see Figure 17) was cloned into pGEX/MAL expression vector.

**Antibodies**

Rabbit anti-480 kDa ankyrin-G, rabbit anti-beta-4 spectrin (Jenkins et al., 2014), goat anti-C-terminal (total) ankyrin-G (He et al., 2014), rabbit anti-neurofascin FNIII (Davis et al., 1996), and rabbit anti-GFP (Kizhatil et al., 2009) antibodies were previously described. Rabbit anti-beta-2 spectrin antibody was made from epitope consists of human beta-2 spectrin repeat 4-9. Chicken anti-MAP2 (ab5392) and anti-GFP (ab13970)
antibodies were from Abcam. Mouse anti-pan NaCh (S8809), mouse anti-MAP2 (M4403), and mouse anti-calbindin (C9848) antibodies were from Sigma-Aldrich. Mouse anti-GABAA receptor β2/3 subunit (MAB341) antibody was from EMD Millipore. Guinea pig anti-VGAT (131 004) and mouse anti-gephyrin (147 021) antibodies were from Synaptic Systems. Rabbit anti-GABARAP (FL-117) antibody was from Santa Cruz. All AlexaFluor-conjugated secondary antibodies were from Life Technologies.

Neuronal culture

The methods for obtaining and maintaining the hippocampal cultures used in our laboratory have been described previously (He et al., 2013). Hippocampi of P0 mouse pups were dissected in cold 1X Hank’s Balanced Salt Solution (HBSS) / 10mM Hepes (both from Life Technologies) and trypsinized for 15-20 minutes at 37°C with 0.25% trypsin and 100 µg/mL DNase (Sigma-Aldrich). Trypsinized hippocampi were then washed 2 x with Neurobasal-A plating media (Life Technologies) which consist of 10% fetal bovine serum (FBS), 1X B27 supplement, 2mM glutamine, and 1X Penicillin/Streptomycin (Pen/Strep) (all from Life Technologies). After washed, hippocampi were triturated with fire-polished glass pipettes, and filtered through 100 µm cell strainers to obtain dissociated cells in suspension. Cells were plated onto poly-D-lysine- and laminin- (both from Sigma-Aldrich) coated Mat-Tek dishes. On the following day, neurons were first washed twice with plain Neurobasal-A media to remove large unattached debris and then incubated with growth media containing B27,
glutamine, Pen/Strep, and 1% FBS in Neurobasal-A in humidified 5% CO₂ chamber at 37°C.

For tetrodotoxin experiments, 1µM tetrodotoxin (Tocris Bioscience) was added to growth media at DIV3 after transfection and replenished every 5 days.

**Transfection and rescue**

Calcium phosphate transfection technique was applied to introduce DNA/Ca²⁺ phosphate complex directly onto the cell layer at days 3 *in vitro* (DIV3) (Jiang and Chen, 2006). Generally, 1 µg cDNA in 25 µL CaCl₂/water solution was mixed with 2X Hank’s Balanced Salt Solution followed by gentle vortexing (Clontech). The DNA-Ca²⁺-phosphate complex was formed after 15 minutes at room temperature and then added dropwise into Mat-Tek plates containing days *in vitro* (DIV) 3 neurons pre-washed by Neurobasal. Cells were incubated in humidified 5% CO₂ chamber at 37°C for 1 hour followed by incubation with acidified Neurobasal (pre-equilibrated in 10%CO₂) in 5% CO₂ chamber at 37°C for 20 minutes to dissolve DNA/Ca²⁺ phosphate complex. At the end cells were incubated back with original growth media plus 2.5 µg/mL Ara-C (Cytosine β-D-arabinofuranoside, Sigma-Aldrich) and maintained until DIV 21 for immunofluorescence staining as described below.

Neurons dissociated from homozygous exon 22-23 flox/flox pups were cultured as described to introduce ankyrin-G KO background by transfecting with 1µg CAG-Cre-2A-BFP or -GFP plasmid to excise Ank3 *in vitro*. For rescue experiments, 0.5µg CAG-
Cre-2A-BFP plus 0.5µg CAG-GFP rescue plasmid were used. For control experiments, 1µg CAG-pEBFP-N1 or CAG-pEGFP-N1 plasmid was used for cell-filling effect in exon 22-23 flox/flox neurons.

Immunofluorescence staining

The method for immunofluorescence staining was reported previously (He et al., 2013). Dissociated neurons at DIV21 were fixed for 15 minutes at room temperature with 4% paraformaldehyde + 4% sucrose in PBS. After washed by PBS, neurons were then permeabilized for 15 minutes with 0.05% Triton X-100 (MP Biomedicals) in PBS and blocked with blocking buffer 5% BSA in PBST. After blocking, neurons were incubated with primary antibodies diluted in blocking buffer at 4°C overnight. On the following day, neurons were washed with PBST 3 times 10 minutes each, and incubated with appropriate Alexa Fluor secondary antibodies in blocking buffer for 2 hours at room temperature. Finally cells were washed with PBST 3 times 10 minutes each and then mounted with Prolong Gold Antifade reagent.

For VGSC staining, neurons were fixed for 15 minutes at room temperature with 4% paraformaldehyde + 4% sucrose in PBS and then permeabilized / blocked with 5% fish gelatin (Sigma-Aldrich) / 0.1% Triton X-100 in PBS for 30 minutes at room temperature. Primary antibodies were diluted in gelatin buffer and incubated with neurons for 2 hours at room temperature prior to the secondary antibody staining as described.
The following antibody dilutions were used: rabbit anti-480 kDa ankyrin-G (1:500), goat anti-ankyrin-G (1:1000), chicken or mouse anti-MAP2 (1:2000), mouse anti-pan NaCh (1:100), rabbit anti-beta-4 spectrin (1:1000), rabbit anti-beta-2 spectrin (1:500), rabbit or chicken anti-GFP (1:1000), rabbit anti-GABARAP (1:100), rabbit anti-neurofascin (1:250), mouse anti-gephyrin (1:500), mouse anti-GABA\(A\) receptor (1:250), guinea pig anti-VGAT (1:1000), mouse anti-calbindin (1:1000), and all secondary Alexa Fluor antibodies (1:250).

**Electrophysiology**

Whole-cell voltage-clamp recordings were made from genetically labeled hippocampal neurons at ~30°C in oxygenated ACSF containing (mM): 143 NaCl, 5 KCl, 5 HEPES, 10 dextrose, 1 MgCl\(_2\), and 2 CaCl\(_2\) (pH 7.3, adjusted with NaOH; 296 mOsm). The pipette solution contained (mM): 130 CsCl, 10 NaCl, 10 HEPES, 10 EGTA, 1 MgCl\(_2\), 0.5 CaCl\(_2\), 4 ATP-Mg, 0.4 GTP-Na\(_3\), and 0.05 Alexa 594 hydrazide (pH 7.3, adjusted with CsOH; 295 mOsm). The pipettes were fabricated from borosilicate glass tubes and pipette resistances were 3–6 M\(\Omega\).

Data were acquired with Multiclamp 700B, low-pass filtered at 2.8 kHz, and digitized at 10 kHz. Recordings with unstable resting currents or high series resistance of > 30 M\(\Omega\) were excluded from the analysis. The series resistance was compensated by 30%. After confirming that massive spontaneous excitatory postsynaptic currents could be observed in recorded hippocampal neurons, tetrodotoxin citrate (0.3 \(\mu\)M; Tocris),
DNQX disodium salt (10 µM; Tocris), D-AP5 (25 µM; Tocris), and strychnine (1 µM; Sigma-Aldrich) were applied for ~5 minutes before recording GABAergic spontaneous inhibitory postsynaptic currents (IPSCs) for > 4 minutes at −70 mV. Recorded currents were smoothed and events that crossed the threshold of −5 pA/ms were designated as the onset of IPSCs. From these IPSCs, miniature IPSCs (mIPSCs) that had fast rise time and exponential decay were manually extracted. The frequency of mIPSCs was calculated as the number of mIPSCs divided by the recording period. The amplitude of mIPSCs was measured as the peak amplitude from the baseline, which was defined as the average of 20 ms currents just before the onset of mIPSCs. The rise time of mIPSCs was the period between 10–90% of the peak amplitude during the rise phase of mIPSCs. The decay tau was the time constant of a single exponential function that was fit to the averaged waveform of peak-normalized mIPSCs in the period between its peak and the time when it returns to the baseline-level. Neurons that generated only < 3 mIPSCs during the recording were excluded from the analysis of amplitude, rise time, and decay tau of mIPSCs. After recording mIPSCs, 1(S)-9(R)-(−)-bicuculline methiodide (10 µM; Sigma-Aldrich) was incubated in a subset of recordings, which almost completely abolished mIPSCs, indicating that the recorded mIPSCs were mediated exclusively by GABA\textsubscript{A} receptors.

Protein purification and Isothermal Titration Calorimetry
His- and maltose binding protein double-tagged GABARAP and WT / W1989R unstructured region of 480 kDa ankyrin-G (residue 1819-2535) were expressed in BL-21 cells under the induction of 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) and frozen at -80°C overnight. On day 2, the cell pellets were solubilized and sonicated in NiNTA buffer (50 mM phosphate buffer pH=7.4, 0.3M NaBr, 20mM imidazole, 1mM NaN₃, 0.5mM EDTA, 0.5mM DTT, 100 µg/ml AEBSF, 100 µg/ml benzamidine, 20 µg/ml leupeptin, and 10 µg/mL pepstatin) with 1% Triton X-100 and centrifuged at 35000 rpm 4°C for 1 hour. The lysates were collected, incubated with NiNTA sepharose (GE Healthcare), and rotated at 4°C overnight. On day 3, the NiNTA resins were loaded onto columns, washed with 30 column-volume (CV) NiNTA buffer, and eluted with NiNTA buffer with 0.3M imidazole 2mL / fraction. The eluates were pooled and incubated with amylose sepharose beads (NEB Lab) rotating at 4°C overnight. On day 4, amylose beads were washed first with 15 column-volume (CV) NiNTA buffer with 0.3M imidazole and then 15CV precision protease buffer (50mM Tris-Cl pH=7.0, 150mM NaCl, 1mM EDTA, and 1mM DTT). Beads were resuspended in equal volume of precision protease buffer with 100 units Precision Protease (GE Healthcare) at 4°C overnight. On day 5, eluate was collected and incubated with Glutathione S-transferase beads rotating at 4°C for 2 hours to remove Precision Protease, and the suspension was concentrated and applied directly to Isothermal Titration Calorimetry.
Isothermal Titration Calorimetry (ITC) was performed with an ITC-200 (MicroCal) at 20°C using a protocol described previously (Wang et al., 2012a). The concentrations of purified GABARAP and ankyrin-G insert region were calculated by Bradford Reagent (Bio-Rad) using a UV-VIS spectrophotometer (Shimadzu Scientific Inc.) at wavelength 595 nm. Cell solution containing 2 mL of 20 µM WT or W1989R insert region was titrated with 30 injections of 10 µL/ea syringe solution containing 200 µM GABARAP. Experiments were repeated three times to confirm the final thermodynamic parameters and stoichiometry values. The binding curves were fitted from a single site binding model in MicroCal Origina software (Originlab Corporation) to obtain the binding enthalpy (ΔH), entropy (ΔS), stoichiometry (n), and the dissociation constant (K_D).

**Proximity Ligation Assay**

The protocol for Proximity Ligation Assay can be found on Sigma-Aldrich’s website for Duolink® using PLA® Technology with slight modifications. Neurons at DIV21 were fixed and incubated with primary antibodies as described in the section 5-5: immunofluorescence staining. Rabbit anti-GFP or rabbit anti-GABARAP were used in combination with goat anti-ankyrin-G. On the next day neurons were washed 3 x by PBST, and after this step temperature-controlled (37°C) humidified chamber was used for all incubation processes. Neurons were first incubated with a pair of PLA probes diluted 1:10/each in PBST/5% BSA for 2 hours, before washed 3 x 10 minutes with
5mL/ea 1X Washing Buffer-A. Ligase was diluted 1:40 into 1X Ligation buffer and added to neurons for 1 hour ligation, followed by 3 x 5 minutes washes with 5mL/ea 1X Washing Buffer-A. Then polymerase was applied 1:80 to neurons in 1X amplification stock for 2 hours. Neurons were washed 2 x 10 minutes with 5mL/ea 1X Washing Buffer-B, 1 x 2 minutes with 5mL 0.01X Washing Buffer-B, and 1 x 10 minutes with 5mL PBST. Neurons were then incubated with primary antibody chicken anti-GFP 1:1000 at room temperature for 2 hours followed by secondary fluorescence antibody Alexa Fluor 488 donkey-anti-chicken for 1 hour at room temperature before mounted with ProLong Gold antifade reagent.

**Dynasore treatment and receptor Internalization Assay**

Live neurons at DIV21 were incubated with either 80nM dynasore or DMSO (both from Sigma-Aldrich) added in original growth media at 37°C for 2 hours prior to the paraformaldehyde fixation as described. Without permeabilization, neurons were then incubated with antibodies raised against the extracellular epitope of GABA<sub>A</sub> receptor β2/3 subunits (α-GABAR β2/3) in 5% BSA/PBS at 4°C overnight. On the following day, neurons were washed 3 times with PBS 10 minutes each prior to incubation with secondary antibody Alexa Fluor 488 donkey-anti-mouse (Life Technologies) for 1 hour at room temperature. Finally neurons were washed with PBS 3 times 10 minutes each and mounted with ProLong Gold antifade reagent (Life Technologies).
The methods for receptor internalization assay used in this study have been described previously (Goodkin et al., 2005). Live neurons were washed twice by warm Neurobasal media before incubated on ice for 1 hour in the presence of high concentration of α-GABAR β2/3 (20µg/mL). Since receptor endocytosis was inhibited at low temperature, antibody-receptor complexes stayed on the surface during the incubation. Then neurons were washed 3 times with ice-cold Neurobasal media to remove any unbound antibody, and incubated back in normal growth media without antibody at 37°C for various amount of time to allow receptor internalization. Neurons were fixed at 0, 15, 30 minutes with 4% paraformaldehyde + 4% sucrose / PBS at room temperature for 15 minutes, followed by the incubation with excess amount of first secondary fluorescence antibody Alexa Fluor 488 donkey-anti-mouse (20 µg/ml) for 2 hrs at room temperature to saturate all surface-bound primary antibodies. Then neurons were permeabilized with 0.25% Triton X-100 / PBS for 15 minutes and labeled with second fluorescence antibody Alexa Fluor 568 donkey-anti-mouse (8 µg/ml) for 1 hour at room temperature before mounted with ProLong Gold antifade reagent.

**Western Blotting**

Adult mice brains were dissected on ice and homogenized in 5 volume/weight buffer (0.32M sucrose, 10mM phosphate buffer pH=7.4, 1mM EDTA, 1mM NaN₃, 100 µg/ml AEBSF, 100 µg/ml benzamidine, 20 µg/ml leupeptin, and 10 µg/mL pepstatin) using dounce homogenizer. Lysates were mixed 1:1 with 5X PAGE buffer (25% sucrose,
5% SDS, 50mM Tris pH=8, 5mM EDTA, and bromophenol blue), sonicated 10 pulses, and heated to 65-70 °C for 10 minutes. Samples (10 µL/ea) were loaded on a 3.5-17.5% gradient gel in 1X Tris buffer (40mM Tris pH=7.4, 0.2% SDS, 20mM NaOAc, and 2mM EDTA) (Bennett and Davis, 1981) until the dye front diffused out of the bottom. The gel was transferred to nitrocellulose at 300 mA overnight at 4 °C in 0.5X Tris buffer (20mM Tris pH=7.4 and 0.01% SDS). Membranes were blocked with Blot buffer (150 mM NaCl, 10 mM phosphate buffer pH=7.4, 0.2% Triton X-100, 1mM NaN₃, and 1mM EDTA) with 2% bovine serum albumin (Gemini Bioproducts) at room temperature for 1 hour before incubated overnight at 4 °C with primary antibodies diluted in blocking buffer. On the next day, membranes were washed and incubated with I¹²⁵-labeled protein A/G 1:1000 at room temperature for 2 hours before washed and exposed on a phosphor screen. Given that protein A/G has lower affinity toward mouse IgG subclasses, membranes blotted with mouse primary antibody were incubated with a secondary rabbit anti-mouse IgG diluted 1:2500 (Pierce) in blocking buffer before incubated with protein A/G. Radioactive signals were detected using a Typhoon imager (GE Healthcare).

Image acquisition and data analysis

Samples were imaged on a Zeiss LSM 780 with a 40 x 1.3 Plan-Apochromat objective and excitation was accomplished using 405, 488, 561, and 633nm lasers. Each experiment was repeated for at least three independent times.
Measurements were taken using Volocity (PerkinElmer) and ImageJ software. For the quantification of GABAergic synapse density, each background subtracted region of interest was drawn around the soma and proximal dendrites and converted to a 8-bit binary file. The number and the size of synaptic clusters were determined by ImageJ and normalized to the volume corresponding to each region. Same conversion, threshold, and calculation were applied to every image of each antibody staining. Statistical analysis was performed and presented using Graphpad Prism software. Data shown were mean ± SEM. Student’s t test was used for comparisons between two groups, while a one-way ANOVA with Tukey post-hoc test was used to compare three or more groups.
2.3 Results

2.3.1 480 kDa ankyrin-G exists on the somatodendritic membrane

We first used antibody against 480 kDa Ankyrin-G (Jenkins et al., 2014) (Figure 4A) to stain mouse brain slices of different ages. In postnatal day 7 mouse brain, 480 kDa antibody staining was highly enriched in the AIS of pyramidal neurons of all ages (Figure 4A), consistent with the traditional view of ankyrin-G as an AIS protein. Surprisingly in postnatal day 24, in addition to the AIS, 480 kDa ankyrin-G also accumulated on the membrane of soma and proximal dendrites (Figure 4A). These AIS and somatodendritic immunochemical signals were lost in Exon-37 KO mice that lack 480 kDa ankyrin-G isoform, indicating that the specific staining on both places was from 480 kDa ankyrin-G. We then used pyramidal neurons dissociated from total ankyrin-G KO mice (Exon22/23 flox) (Jenkins et al., 2013) to verify this somatodendritic staining in cultured neurons. In neurons expressing soluble blue fluorescent protein (BFP), 480 kDa ankyrin-G was only observed in the AIS at day 7 in vitro, while it was highly enriched in the AIS and readily detected on the somatodendritic compartment at 21 days in vitro (Figure 4B). The ratio of somatodendritic ankyrin-G to AIS ankyrin-G increased about three times in fully developed neurons (from 0.1 to 0.3). By expressing Cre-2A-BFP plasmid in exon 22/23 floxed neurons, we excised all ankyrin-G isoforms containing membrane binding domain and lost the staining of 480 kDa ankyrin-G on both the AIS and somatodendritic membrane (Figure 4B). Collectively, these results demonstrated the
new discovery of developmentally-regulated expression of 480 kDa ankyrin-G “outpost” on the somatodendritic membrane in addition to the AIS in both brain slices and cultured neurons.
Figure 4: Somatodendritic 480 kDa ankyrin-G outpost is developmental regulated.

(A), scheme of 480 kDa ankyrin-G showing neuronal specific insert encoded by exon 37. (B), somatodendritic accumulation of 480 kDa ankyrin-G only appears after PND 24 in WT cortical pyramidal neurons while no 480 kDa ankyrin-G staining is detected in exon 37 KO brain. Arrowhead denotes ankyrin-G outpost on the soma. Scale bar represents 5 µm. (C), in neurons dissociated from exon 22-23 flox/flox mice, 480 ankyrin-G clusters on both the AIS and somatodendritic membrane after DIV 21 in BFP-transfected neurons but not in neurons expressing Cre. Scale bars represent 5 µm in all axes. x-z view shows the height of the somatodendritic membrane delineated by 480 kDa ankyrin-G staining (left). The ratio of somatodendritic membrane to the AIS at different developmental stages is quantified (right). Scale bar represents 5 µm.
We further investigated the micron-scale organization of ankyrin-G on the somatodendritic membrane both *in vivo* and *in vitro*. Z-stack image sequences of neurons were collected, averaged, and summarized for the generation of 3D-rendered images. We then cropped one side of the somatodendritic membrane and deconvolved the immunochemical signal from antibodies raised against total or 480 kDa ankyrin-G to reveal endogenous elongated “microdomains” on the membrane (Figure 5A, B). The high colocalization ratio of signals from both antibodies (Pearson correlation coefficient = 0.8) confirmed that under physiological condition 480 kDa ankyrin-G is the major isoform of somatodendritic ankyrin-G outpost in both *in vivo* slice and cultured neurons (Figure 5B). The small region that was occupied by immunochemical signal from antibody against total ankyrin-G but devoid of signal from antibody against 480 kDa ankyrin-G, on the other hand, marked the sporadic presence of 190 kDa ankyrin-G in the somatodendritic membrane microdomain. Though the function and dynamic of this ankyrin-G microdomain on somatodendritic membrane needs further characterization, this is the first report of the existence of ankyrin-G-positive membrane microdomain in the nervous system.
Figure 5: Ankyrin-G forms micron-scale membrane domains on the somatodendritic membrane both in vivo and in vitro.

(A), schematic presentation of the cropped region on the somatodendritic membrane for imaging deconvolution in a 3D image of a neuron. (B), antibody against 480 kDa ankyrin-G labels microdomains on the somatodendritic membrane of PND 24 cortical pyramidal neurons (upper) and DIV 21 hippocampal neurons (lower) highly colocalized with signals from antibody against total ankyrin-G. Scale bars represent 1 μm in all axes.
2.3.2 Somatodendritic localization of neurofascin and voltage-gated sodium channel depends on ankyrin-G

In order to address whether the newly-discovered somatodendritic outpost was simply an extension of the initial segment, we asked if other known ankyrin-G AIS binding partners were present in the outpost. Both voltage-gated sodium channels and the cell adhesion molecule neurofascin appeared to cluster on the somatodendritic membrane colocalized with ankyrin-G staining (Figure 6A). In the AIS, direct interaction with ankyrin-G is critical for the accumulation of VGSC and neurofascin (Davis and Bennett, 1994; Garrido et al., 2003; Garver et al., 1997; Hedstrom et al., 2007; Jenkins and Bennett, 2001). Using Cre-2A-BFP expressing exon 22/23 floxed neurons, we demonstrated that not only the AIS but also the somatodendritic localization of both proteins were disrupted in the absence of ankyrin-G (Figure 6A). Beta2-spectrin also formed clusters on the somatodendritic membrane, but its membrane localization was not dependent on ankyrin-G likely due to an intact pleckstrin homology domain tethering it to membrane lipids (Figure 6A). Beta4-spectrin, on the other hand, was only restricted to the AIS in wild type (WT) neurons, suggesting that additional mechanisms limit beta4-spectrin to the AIS (Figure 6A). These results demonstrate that ankyrin-G organizes the accumulation of VGSC and neurofascin in the newly-discovered somatodendritic compartment which possesses a unique molecular composition different from the AIS.
Given the existence of ankyrin-G positive microdomains on the somatodendritic membrane, we thus deconvolved the somatic membrane double-stained with total ankyrin-G and neurofascin or VGSC to investigate the microscopic organization of different proteins (Figure 6B). Strikingly, immunochemical signal of these two binding partners displayed high degree of colocalization (Pearson correlation coefficient = 0.6) with endogenous ankyrin-G into patches of elongated microdomains (Figure 6C). This subsequently verified that ankyrin-G microdomains are functional units which consist of interacting proteins responsible for mediating distinct physiological activities on the neuronal somatodendritic membrane.
Figure 6: Somatodendritic accumulation of neurofascin and VGSC is dependent on ankryin-G and forms microdomain on the membrane with ankryin-G.

(A), both VGSC and neurofascin but not beta-4 spectrin accumulate on the somatodendritic membrane and the AIS. Arrowheads indicate neurofascin or VGSC outposts on the soma of WT neurons. Beta-2 spectrin also exists on the somatodendritic membrane, while its accumulation persists in ankryin-G KO neurons. Arrow head denotes beta-2 spectrin clusters on the soma of the WT neuron, and the arrow marks the presence of beta-2 spectrin on the soma of an ankryin-G KO neuron. Scale bars represent 5 µm in all axes. (B), schematic presentation of the cropped region on the somatodendritic membrane for imaging deconvolution. (C), ankryin-G interacting proteins VGSC, neurofascin, and beta-2 spectrin also form microdomains on the membrane organized by ankryin-G. Scale bars represent 1 µm in all axes.
We also examined which ankyrin-G isoform was responsible for the localization of neurofascin and VGSC on the somatodendritic membrane. While GFP-tagged 480 kDa ankyrin-G fully restored the accumulation of both proteins on the somatodendritic membrane and the AIS in ankyrin-G KO neurons, GFP-tagged 190 kDa ankyrin-G, though not clustered on the AIS, rescued the somatodendritic membrane localization but not the AIS enrichment of neurofascin and VGSC (Figure 7). 190 kDa ankyrin-G targets to the plasma membrane through the palmitoylation on a Cysteine-70 residue in the membrane binding domain (He et al., 2012). Combining with the fact that the binding sites of neurofascin and VGSC lie within ankyrin-G membrane binding domain (Michaely and Bennett, 1995; Srinivasan et al., 1992), we reason that 190 kDa ankyrin-G is capable of tethering neurofascin and VGSC to the somatodendritic membrane through the involvement of membrane binding domain. However in the ankyrin-G KO background, the insert region of 480 kDa ankyrin-G is required for the ankyrin-G targeting to the AIS (He et al., 2012; Jenkins et al., 2014). Consistently we could not restore the AIS association of either neurofascin or VGSC in ankyrin-G KO neurons by coexpressing GFP-tagged 190-kDa ankyrin-G (Figure 7). In conclusion, the somatodendritic membrane accumulation of neurofascin and VGSC depends on the association with ankyrin-G membrane binding domain.
Figure 7: 190 kDa ankyrin-G is able to rescue the somatodendritic clustering of neurofascin and VGSC.

190 kDa ankyrin-G is capable of associating with somatodendritic membrane and rescuing somatodendritic accumulation but not axon initial segment enrichment of VGSC and neurofascin.
2.3.3 Somatodendritic 480 kDa ankyrin-G is neuron-type specific.

Interestingly, this somatodendritic 480 kDa ankyrin-G only existed on the membrane of pyramidal cortical neurons in cerebrum while in cerebellar Purkinje neurons it was restricted to the AIS (Figure 10). Instead, in Purkinje neurons, we detected the accumulation of neurofascin on the somatodendritic membrane throughout development (Figure 8), as shown in a previous study. (Ango et al., 2004). We therefore conclude that this somatodendritic ankyrin-G staining is neuron-type specific. In cortical neurons, somatodendritic and AIS ankyrin-G mediates the accumulation of neurofascin in both places. In Purkinje neurons, while ankyrin-G on the AIS is responsible for setting up the subcellular gradient of neurofascin on the somatodendritic compartment, the role of this somatodendritic neurofascin needs further elucidation.

![Image of α-neurofascin and α-total AnkG](image.png)

Figure 8: Neurofascin, instead of ankyrin-G, accumulates on the somatodendritic membrane of Purkinje cells in cerebellum.
2.3.4 GABAergic synapse formation requires somatodendritic 480 kDa ankyrin-G.

After characterizing the composition of somatodendritic ankyrin-G outpost, we investigated what is the function of this ankyrin-G outpost. Using mice lacking 480 kDa ankyrin-G (Exon-37 KO), we first confirmed the loss of GABAergic innervation in the AIS region of Purkinje neurons by examining presynaptic vesicular GABA transporter (vGAT) (Figure 9A and Jenkins et al., 2014), as first described in cerebellar total ankyrin-G KO (Ango et al., 2004). Moreover, we discovered that the loss of vGAT staining was not limited to cerebellum. In hippocampus, vGAT immunostaining was highly reduced in the somatic region of CA1 neurons, while the overall vGAT signal in the dentate gyrus was not altered (Figure 9B). In order to inspect the GABAergic synapse on the scale of single neuron, we chose pyramidal cell in the cortex whose soma is individually-spaced from each other. In WT cortical neurons, vGAT displayed a circular pattern on 480 kDa ankyrin-G positive somatodendritic membrane as well as AIS region. However in Exon 37 mice, vGAT innervations no longer decorated the membrane but instead distributed randomly in the neuropile region (Figure 10). Thus using in vivo approaches, we demonstrated that in addition to the pinceau synapses in Purkinje cells of cerebellum, GABAergic synaptogenesis on the somatodendritic membrane of CA1 and cortical neurons of cerebrum was also dependent on 480 kDa ankyrin-G.
Figure 9: Excision of giant ankyrin-G leads to loss of vGAT clustering at the AIS of Purkinje cells and somatodendritic compartment in hippocampal CA1 region.

(A), loss of vGAT staining on the AIS of exon 37 KO Purkinje neurons. Scale bar represents 5 µm. (B), reduced vGAT accumulation on the CA1 region of exon 37 KO hippocampus, as illustrated by staining (up) and quantification (bottom) of the averaged fluorescence level over 250 µm. Scale bar represents 50 µm.
Figure 10: Excision of giant ankyrin-G in exon 37 KO mice leads to loss of vGAT clustering at both the AIS and the somatodendritic membrane of cortical neurons.

Loss of somatodendritic 480 kDa ankyrin-G and GABA\_ in exon 37 KO pyramidal neurons. Arrowhead denotes ankyrin-G outpost on the soma. Scale bar represents 5 µm.
We then analyzed GABAergic synapse formation in vitro in total ankyrin-G KO cultured neurons dissociated from Exon22/23 flox mice. Compared to BFP-only transfected neurons where numerous GABA\textsubscript{A} receptors accumulated on the membrane, Cre-2A-BFP transfected ankyrin-G KO neurons lost receptor clustering on both somatodendritic membrane and AIS (Figure 11). We also examined two other GABAergic synaptic components including presynaptic vGAT and postsynaptic density protein gephyrin. The clusters of both synaptic proteins disappeared on the membrane of ankyrin-G KO neurons (Figure 11), suggesting that both pre- and post-synaptic molecular machinery were disrupted in the somatodendritic and AIS compartment in the absence of ankyrin-G.
Figure 11: GABAergic innervations are lost from total ankyrin-G KO cultured hippocampal neurons.

Loss of $\text{GABA}_\alpha$ receptor, postsynaptic gephyrin, and presynaptic vGAT in cultured hippocampal ankyrin-G KO neurons. Scale bars represent 5 µm in all axes.
Cultured neurons have the advantage of ease in genetic manipulation by plasmid transfection. By co-transfecting plasmids encoding 480 kDa GFP-tagged ankyrin-G and BFP-Cre we fully rescued the formation of GABAergic synapses on both somatodendritic membrane and AIS as demonstrated by the clustering of GABA\_ receptor, gephyrin, and vGAT (Figure 12, 13). However the plasmid encoding 190 kDa GFP-ankyrin-G, which lacks the insert in 480 kDa ankyrin-G (Figure 3), failed to restore GABAergic synapse localization (Figure 12, 13). Pyramidal neurons dissociated from Exon 37 KO mice, on the other hand, contained upregulated 190 kDa ankyrin-G remained from in-frame splicing of exon 37 (Figure 25). Consistent with the rescue result of 190 kDa ankyrin-G in total ankyrin-G KO neurons, this upregulated levels of 190 kDa ankyrin-G on the somatodendritic membrane of Exon 37 KO mice were not able to congregate GABAergic innervations (Figure 9, 10). Moreover, C70A 480 kDa ankyrin-G, which cannot be palmitoylated by DHHC 5/8 (He et al., 2012), failed to localize to the plasma membrane and also lacked ability to restore GABAergic clusters, as illustrated by the loss of immunostaining of GABA\_ receptors, gephyrin, and vGAT (Figure 14). We then addressed whether the formation of specific ankyrin-G microdomain is required for GABAergic synaptogenesis. Adding a MyrPalm consensus sequence for acylation to the N-terminus of C70A 480 kDa ankyrin-G construct tethered the protein to plasma membrane through lipid modification, resulting in the membrane association of palmitoylation-deficient C70A 480 kDa ankyrin-G (Zacharias et al., 2002). Strikingly,
C70A 480 kDa ankyrin-G MP construct, though associated with somatodendritic membrane, failed to restore GABAergic synapse formation (Figure 14). Moreover, C70A 480 kDa ankyrin-G MP exists in different somatodendritic microdomains which are no longer colocalized with neurofascin (Figure 14), indicating that lipid modification restricts C70A 480 kDa ankyrin-G in membrane domains distinct from endogenous ankyrin-G. Collectively, these results demonstrated that ankyrin-G requires both its giant exon as well as its association with the plasma membrane through C70-dependent palmitoylation to form functional microdomains for promoting surface accumulation of GABAergic synapses.
Figure 12: The giant insert in 480 kDa ankyrin-G is critical in rescuing the formation of GABAergic innervations in total ankyrin-G KO neurons.

Rescue of somatodendritic GABA\(_A\) receptor, gephyrin, and vGAT in ankyrin-G K/O hippocampal neurons by transfection with cDNA encoding 480 kDa ankyrin-G but not 190 kDa ankyrin-G. Arrow heads indicate GABA\(_A\) receptor, gephyrin, or vGAT clusters. Scale bars represent 5 \(\mu\)m in all axes.
Figure 13: Quantification of $\text{GABA}_A$ receptor, gephyrin, and vGAT density in ankyrin-G KO and rescued neurons.

Density of GABAergic synaptic components. **$P<0.005$. ***$P<0.0005$. ns = nonsignificant (One-way ANOVA $p<0.0001$ followed by Tukey post-hoc test, $n=10-15$ cells per group). Error bar, SEM.
Figure 14: 480 kDa ankyrin-G requires proper membrane association through palmitoylation to promote GABAergic synaptogenesis in ankyrin-G KO neurons.

(A), C70A 480 kDa ankyrin-G and MP 480 kDa ankyrin-G C70A are unable to rescue the clustering of GABA\(_A\) receptor, gephyrin, or vGAT. Scale bars represent 5 \(\mu\)m in all axes. (B), quantification of (A) *\(P<0.05\) **\(P<0.005\) ***\(P<0.0005\) (unpaired t-test, n = 5-15 cells per group). Error bar, SEM. (C), MP 480 kDa ankyrin-G C70A exists in different membrane microdomains through lipid modification as illustrated by the loss of colocalization with neurofascin. Scale bars represent 1 \(\mu\)m in all axes.
Given that AIS is essential for the firing of action potential, we verified whether the lack of an AIS in ankyrin-G KO may lead to a loss of activity in neurons and a subsequent homeostatic downregulation of GABAergic synapses. We observed GABAergic synapse formation even with prolonged tetrodotoxin treatment (Figure 15), confirming that the absence of GABAergic synapses was not due to the lack of neuronal activity in ankyrin-G KO neurons.

![Figure 15: Prolonged treatment of TTX does not affect the formation of GABAergic synapses in cultured hippocampal neurons.](image)

(A), GABA<sub>A</sub> receptor, gephyrin, and vGAT still accumulate on the somatodendritic membrane and the AIS of neurons treated with TTX for 18 days. Scale bars represent 5 µm in all axes. (B), the quantification of GABAergic cluster density in (A) shows no significant difference with or without TTX treatment. ns = nonsignificant (unpaired t-test, n = 10-15 cells per group). Error bar, SEM.
In order to address the functional significance of the observed histochemical difference, we collaborated with Masashi Tanaka from Richard Mooney’s lab for the whole-cell patch clamp to measure miniature inhibitory postsynaptic currents (mIPSC) in cultured neurons. Inhibitors to sodium channel, AMPA receptor, NMDA receptor, and glycine receptor (TTX, DNQX, D-AP5, and strychnine, respectively) were applied to isolate GABA_\alpha receptor-mediated spontaneous activity. The loss of GABA_\alpha receptor staining on the membrane of ankyrin-G KO pyramidal neurons unsurprisingly accounted for the significantly decreased mIPSC frequency and amplitude (Figure 16). Given that the frequency of GABA_\alpha receptor spontaneous firing results from the stimulation of presynaptic GABA release and the amplitude depends on the cumulative number of receptor molecules on the surface, this finding further reinforced the observation of a deficit in the clustering of both presynaptic vGAT and postsynaptic GABA_\alpha receptor immunochemically.
Figure 16: Total ankyrin-G KO hippocampal neurons display deficits in amplitude and frequency of GABA\_ receptor-related spontaneous firing activity.

Loss of GABA\_ receptor-related mIPSC frequency and amplitude in cultured hippocampal ankyrin-G KO neurons. Upper panel shows a representative raw trace of each phenotype. **P<0.005 (t test, n = 10-15 cells per group). Error bar, SEM.
2.3.5 GABARAP binds to the insert region of 480 kDa ankyrin-G both in situ and in vitro.

We then addressed the mechanism of how the insert in ankyrin-G associates with GABAergic synapses. Using yeast two-hybrid assays (Y2H), Paul Jenkins and Chirag Vasavda first identified GABA\(_A\) receptor-associated protein (GABARAP) as a potential interacting protein of the insert region in 480 kDa ankyrin-G (Figure 18). To verify whether endogenous ankyrin-G and GABARAP interact with each other at specific location, I then performed proximity ligation assay in cultured neurons (Figure 19). Briefly, this method depends on the dual proximal binding by pairs of detection reagents to generate amplifiable DNA loops which serve as surrogate markers for detection of adjacent proteins at single-molecule resolution in live cells (Soderberg et al., 2006). Antibody pair ankyrin-G and GABARAP yielded numerous proximity ligation puncta on the surface of somatodendritic membrane and inside of the AIS in wild-type neurons. The in situ ligation signals were completely lost in neurons lacking ankyrin-G. Changing the antibody pair to antibodies that recognize molecules outside of the detection limit such as GFP and ankyrin-G also showed no localized amplification signal. This result proved the specific in situ association between endogenous ankyrin-G and GABARAP on the somatodendritic membrane and AIS.
Figure 17: 480 kDa ankyrin-G contains a potential LIR motif with critical tryptophan residue within residue 1479-2337 of yeast two-hybrid binding region (Y2H).

Figure 18: Full length GABARAP interacts with the WT but not W1989R mutated insert region of 480 kDa ankyrin-G in yeast two-hybrid assay.

A representative yeast two-hybrid assay demonstrates the binding between full length GABARAP and the residues 1479-2337 in 480 kDa ankyrin-G (see Figure 17). The point mutation W1989R in residue 1479-2337 abolishes the interaction with GABARAP. Positive (RanBPM + pGAD) and negative control (T7 + pGAD) are shown on the right. Top panel: minus AHLT plate. Bottom panel: minus LT plate.
Figure 19: Endogenous ankyrin-G interacts with GABARAP in situ on the somatic membrane and dendritic shaft through proximity ligation.

Proximity ligation assay reveals in situ interaction between ankyrin-G and GABARAP that is lost in ankyrin-G KO neurons and does not occur with antibody against GFP. Arrows: spines devoid of ligation signal. Scale bars represent 5 µm in all axes.
We then moved to *in vitro* approach to test the binding between purified 480 kDa insert region and full length GABARAP by Isothermal Titration Calorimetry (ITC) (Figure 20, 21). Using concentrated GABARAP as syringe ligand to titrate into cell containing unstructured region of insert in 480 kDa ankyrin-G, we obtained the binding affinity of around 20 nM $K_d$ from the highly-exothermic reaction (the tighter interaction between two molecule has more exothermic enthalpy change). GABARAP and other members of LC3 family bind to the LC3-interacting region (LIR) which consists of a common W-x-x-L motif in target proteins (Alemu et al., 2012; Birgisdottir et al., 2013). After examining the peptide sequence of the insert region we found a potential LIR motif with the requisite tryptophan residue followed by a hydrophobic phenylalanine in the +2 position and N-terminal acidic residues such as aspartic acid (Figure 17). Search of the Human Exome variant database (http://evs.gs.washington.edu/EVS/) revealed an uncharacterized variant of 480 kDa ankyrin-G in which residue 1989 is an arginine instead of a tryptophan. We thus mutated tryptophan 1989 into arginine in the unstructured region of 480 kDa ankyrin-G and evaluated the effect on binding with GABARAP using ITC. The consequence was dramatic, as point mutation W1989R decreased the binding affinity by 200 fold of wild-type insert region (Figure 21). Collectively, we not only identified GABA receptors-associated protein GABARAP as novel binding partner to the insert region of 480 kDa ankyrin-G *in situ* and *in vitro* but
also mapped the primary binding site on the insert region to the putative LIR motif selective for LC3 family.
Figure 20: Coomassie Blue staining of protein gel from purification of full length GABARAP (17kDa) and AnkG insert residue 1819-2335 (65 kDa).
Figure 21: WT insert region in 480 kDa ankyrin-G binds directly to full length GABARAP at the LIR motif including W1989.

Left panel, direct binding between GABARAP and WT ankyrin-G insert with K_d around 20 nM. Right panel, loss of binding of GABARAP to W1989R ankyrin-G with 200 fold less K_d. Values were determined by Isothermal Titration Calorimetry using GABARAP (syringe) and WT or W1989R 480 kDa ankyrin-G (residues 1819-2335) (cell).
2.3.6 The W1989R mutation in LIR motif of ankyrin-G fails to rescue GABAergic synapse clustering.

We then studied if W1989R point mutation of 480 kDa ankyrin-G, by losing the binding affinity to GABARAP, still retained the ability to localize GABAergic synapses. Strikingly, compared to the transfection of wild-type 480 kDa ankyrin-G plasmid which restored GABAergic innervations in ankyrin-G KO neurons, W1989R mutation of 480 kDa ankyrin-G failed to rescue the formation of GABAergic synapses as exemplified by the absence of GABA\(_A\) receptor, gephyrin, and vGAT on both somatodendritic membrane and the AIS (Figure 22). Nevertheless, this mutated construct fully clustered VGSC and neurofascin on the somatodendritic membrane with an AIS positive of neurofascin, VGSC, and beta-4 spectrin (Figure 24). Consistent with our prolonged tetrodotoxin treatment result, this further confirmed that the loss of GABAergic synapses was not due to the lack of neuronal activity or functional AIS (Figure 15), but to the diminished interaction with GABARAP. We then collaborated with Masa to translate the deficit from staining level into functional output. In comparison with WT 480 kDa ankyrin-G which restored mIPSC frequency and amplitude in ankyrin-G KO neurons, he found that W1989R 480 kDa ankyrin-G caused reduced mIPSC frequency and amplitude similar to neurons lacking ankyrin-G (Figure 23). Together, these results demonstrated that the association between 480 kDa ankyrin-G and GABARAP is essential for GABAergic synaptogenesis in cultured neurons.
Figure 22: W1989R 480 kDa ankyrin-G fails to rescue GABAergic synaptogenesis in total ankyrin-G KO neurons.

In contrast to the restoration of GABAergic innervations with WT 480 kDa ankyrin-G, W1989R 480 kDa ankyrin-G fails to accumulate GABAA receptor, gephyrin, or vGAT on the somatodendritic membrane and the AIS of ankyrin-G KO neurons. Scale bars represent 5 µm in all axes. Quantification of GABAergic component density in rescue experiment. ***P<0.0005 (unpaired t-test, n = 5-10 cells per group). Error bar, SEM.
W1989R mutation of ankyrin-G prevents restoration of mIPSCs in ankyrin-G KO neurons. Upper panel shows a representative raw trace of each phenotype. Scale bars represent 5 µm in all axes. *P<0.05. **P<0.005. ***P<0.0005. ns = nonsignificant (One-way ANOVA p<0.0001 followed by Tukey post-hoc test, n= 5-10 cells per group). Error bar, SEM.
Figure 24: W1989R 480 kDa ankyrin-G still forms functional axon initial segment in total ankyrin-G KO neurons.

W1989R 480 kDa ankyrin-G accumulates VGSC, neurofascin, and beta-4 spectrin on the AIS as well as VSGC and neurofascin on the somatodendritic membrane. Arrow heads denote proteins concentrated on the AIS as marked by ankyrin-G. Arrows indicate clusters on the somatodendritic membrane colocalized with ankyrin-G. Scale bars represent 5 µm in all axes.
2.3.7 Ankyrin-G stabilizes GABA$_A$ receptors on the somatodendritic membrane through impeding receptor endocytosis.

The surface level of functional GABA$_A$ receptors has been shown to be determined by substantial endocytosis at extrasynaptic sites for regulated synaptic inhibition (Kittler et al., 2005; Kittler et al., 2000). In order to understand whether endocytosis played a role in the phenotype we observed in ankyrin-G KO neurons, we applied dynamin inhibitor dynasore to suppress receptor internalization (Chung et al., 2010; Macia et al., 2006). Remarkably, dynasore fully restored GABA$_A$ receptor surface localization of ankyrin-G KO neurons (Figure 25). This result was in line with our finding that the protein level of major GABAergic components remained the same in Exon 37 KO mice (Figure 26), emphasizing that GABAergic inhibitory complexes are mislocalized from the membrane rather than down-regulated in ankyrin-G KO neurons.
Figure 25: Dynasore treatment of total ankyrin-G KO neurons rescues the membrane accumulation of GABA$_A$ receptors.

Dynasore (80 nM) restores GABA$_A$ receptors in ankyrin-G KO neurons. Scale bars represent 5 µm in all axes. **P<0.005 (t test, n = 10-15 cells per treatment). Error bar, SEM.
Figure 26: GABAergic components remain the same in Exon 37 KO mice brain lysates.

(A), in WT brain, there are 3 ankyrin-G isoforms: 480, 270, and 190 kDa ankyrin-G. In exon 37 KO brain, both 480 and 270 kDa ankyrin-G are excised out, leaving upregulated 190 kDa ankyrin-G from in-frame splicing. (B), the protein levels of GABA\textsubscript{A} receptor, GABARAP, vGAT, and gephyrin are not altered in exon 37 KO brain. (C), the quantification of band intensity shown in (B) normalized to GAPDH, with WT value set as 1. No significant difference exists between WT and exon 37 KO brain (n=2).
To further investigate the effect on endocytosis of ankyrin-G KO, we utilized a receptor internalization assay to distinguish the amount of internalized receptors at given time points (Goodkin et al., 2005). We first recapitulated dynasore outcome in ankyrin-G KO neurons by inhibiting endocytosis using cold temperature (Figure 27). At both 15 min and 30 min time points, we observed significantly higher amounts of internalized receptor in ankyrin-G KO neurons than nearby wild-type neurons (Figure 27). Hence we concluded that the loss of ankyrin-G on the somatodendritic membrane exposed surface receptors to active endocytotic machinery and subsequently more accumulative internalized receptor inside the cell.
Figure 27: GABA\(_A\) receptors undergo faster internalization rate in total ankyrin-G KO neurons.

GABA\(_A\) receptors undergo more rapid internalization in ankyrin-G KO neurons. See Material and Method. Dotted lines delineate individual cell. Scale bar represents 5 µm. ***P<0.0005 (One-way ANOVA p<0.0001 followed by Tukey post-hoc test, n= 15-30 cells per group). Error bar, SEM.
2.3.8 Ankyrin-G associates with extrasynaptic receptors but not synaptic complex.

To further study the micro-scale organization of different proteins on neuronal surface, the somatodendritic membrane was cropped, turned to the side, and deconvolved to visualize microscopic immunochemical signals (Figure 5A). Ankyrin-G exhibited substantial colocalization with GABARAP and GABA\textsubscript{A} receptor (Pearson correlation coefficient = 0.6 and 0.5, respectively); however, it was excluded from areas containing synaptic markers vGAT and gephyrin (Pearson correlation coefficient = 0.2) (Figure 28). GABARAP has been shown to directly bind to the intracellular loop of GABA\textsubscript{A} receptor \(\gamma\)2 subunit (Wang et al., 1999). Taken together, these results imply that 480 kDa ankyrin-G interacts with extrasynaptic GABA\textsubscript{A} receptors through the association with GABARAP on the somatodendritic membrane, perhaps by inhibiting receptor uptake from endocytosis-active extrasynaptic sites thus enabling the lateral movement of receptor in and out of the synapses during the development and maturation of GABAergic synaptogenesis (Figure 29). In the absence of 480 kDa ankyrin-G or the case of mutated 480 kDa ankyrin-G that no longer binds to GABARAP, GABA\textsubscript{A} receptors are subjected to constitutive endocytosis and removed from the membrane thereby the proper formation of GABAergic synapses is largely impaired.
Figure 28: Ankyrin-G colocalizes with GABARAP and extrasynaptic GABA\(_A\) receptor but is excluded from synaptic region.

Ankyrin-G overlaps with GABA\(_A\) receptor and GABARAP but is excluded from GABAergic synapses marked by gephyrin or vGAT in 3D-rendered high resolution images. Scale bars represent 1 \(\mu\)m in all axes.
Figure 29: Ankyrin-G stabilizes extrasynaptic GABA_A receptors through GABARAP and prevents receptor internalization.
2.4 Discussion

In contrast to excitatory synapses, inhibitory synapses remain poorly understood especially in terms of their functional organization and membrane assembly. A growing body of evidence indicates that synaptic adhesion molecules which interact in a homo- or heterophilic fashion across the synaptic cleft mediate the formation, maturation, and the plasticity of synaptic connections (Dalva et al., 2007; Levinson and El-Husseini, 2005). As a member of L1 family, cell adhesion molecule neurofascin is membrane-spanning protein with conserved cytoplasmic domains that are believed to associate with ankyrin-G and play important roles in neuronal development (Davis and Bennett, 1994). Previous studies proposed that the diffuse expression of neurofascin at the soma regulates gephyrin cluster formation and localization (Burkarth et al., 2007). The same group proposed that the amount of neurofascin at the AIS determines the number and size of gephyrin, GABA\(A\) receptor, and presynaptic GAD clusters (Kriebel et al., 2011). However, the W1989R 480kDa ankyrin-G construct is fully capable of restoring the accumulation of neurofascin in both the somatodendritic membrane and the AIS while GABAergic components remain diffuse. This implies that neurofascin itself is not sufficient to localize GABAergic synapses. In Purkinje neurons, an ankyrin-G-dependent subcellular gradient of neurofascin, that is expressed at low levels in the cell body and very high levels at the AIS, has been shown to direct GABAergic innervations at the AIS (Ango et al., 2004). Our observation that Purkinje neurons lack somatodendritic ankyrin-
G indicates that ankyrin-G-mediated GABAergic synaptogenesis is neuron-type specific in cortical pyramidal neurons and the possibility that ankyrin-G-mediated neurofascin localization might directly modulate GABAergic synapses in different types of neurons.

Synapses possess many of the same features as intercellular junctions in non-neuronal tissues but differ in properties such as asymmetrical alignment, speed of signal transmission, and use-dependent plasticity (Sudhof, 2008). Synaptically localized cell adhesion molecules interact and induce the formation of new synapses or modulate the function of existing synapses through signaling cascades or secondary protein–protein interactions (Dalva et al., 2007). Numerous synaptic adhesion molecules have therefore been well-characterized as bidirectionally coordinating synapse differentiation including neuroligins and neurexins, EphBs and ephrin-Bs, cadherin and immunoglobulin-containing adhesion molecules (Dalva et al., 2007; Waites et al., 2005). Among them, neuroligin 2 preferentially localizes to inhibitory synapses and induces the formation of inhibitory contacts (Chih et al., 2005; Poulopoulos et al., 2009; Varoqueaux et al., 2004), although the effect of neuroligin 2 deletion on GABAergic innervation needs further elucidation. Several neuroligin 2 interacting proteins have been identified as components of the synapse, including presynaptic neurexin and postsynaptic gephyrin (Graf et al., 2004; Poulopoulos et al., 2009; Sugita et al., 2001). It has been reported that synaptic scaffolding molecule (S-SCAM)/membrane-associated guanylate kinase with inverted organization (MAGI)-2 (S-SCAM/MAGI-2) can interact with neuroligin 2 and β-
dystroglycan, providing a link between the neurexin-neuroligin adhesion system and the dystrophin-glycoprotein complex (DGC) (Sassoe-Pognetto et al., 2011; Sumita et al., 2007). Moreover, presynaptic neurexin interacts with dystroglycan, suggesting a potential role of DGC in mediating trans-synaptic interaction between pre- and postsynaptic complexes (Sassoe-Pognetto et al., 2011; Sugita et al., 2001). DGC is a large, membrane-spanning protein complex that connects the cytoskeleton to the extracellular matrix (Ervasti and Campbell, 1991). Interestingly, in skeletal muscles ankyrin-G binds directly to dystrophin and β-dystroglycan and maintains them at costameres (Ayalon et al., 2008). Regardless of the specific presence of DGC in inhibitory synapses (Grady et al., 2006; Knuesel et al., 1999; Levi et al., 2002), the role of this complex for inhibitory synaptogenesis as well as its association with ankyrin-G in non-muscle tissues remains unexplained. From our study of the ankyrin-G outpost it is very likely that the DGC may have possible synaptic-specific functions and ankyrin-G may further stabilize multiple synaptic cell adhesion molecules through interaction with the DGC, including the perisomatic neurexin-neuroligin complex.

Our results demonstrate the specific in situ interaction between GABARAP and ankyrin-G on both the somatodendritic membrane and the AIS. This is consistent with the finding from a recent study wherein endogenously expressed GFP-GABARAP is enriched in the AIS of pyramidal neurons and Purkinje neurons, in addition to cell bodies and dendrites (Koike et al., 2013). We also demonstrate the requirement for
GABARAP in the formation of GABAergic innervations by using the W1989R mutant 480 kDa ankyrin-G which impairs GABARAP binding and fails to rescue GABAergic clustering. However, GABARAP-knockdown mice appeared unaffected in terms of synaptic strength, which likely reflects functional redundancy from other GABARAP isoforms such as GABARAPL1/GEC-1 that can also interact with the GABA\textsubscript{A} receptor (Mansuy-Schlick et al., 2006; O'Sullivan et al., 2005).

Subtype-specific properties of GABA\textsubscript{A} receptors have been shown to be involved in receptor subcellular localization (Brunig et al., 2002; Nusser et al., 1998). Generally, receptors comprised of \( \alpha 1-3 \) are largely synaptically located for phasic inhibition while those comprised of \( \alpha 4-6 \) serve as a predominantly extrasynaptic population for tonic inhibition (Farrant and Nusser, 2005; Glykys et al., 2008; Kasugai et al., 2010; Thomson and Jovanovic, 2010). On the other hand, \( \beta 2 \) and \( \beta 3 \) subunits-containing GABA\textsubscript{A} receptors have been shown to concentrate in both synaptic and extrasynaptic sites (Brunig et al., 2002; Glykys et al., 2008; Kasugai et al., 2010; Luscher et al., 2011). Hence in our study, the use of the antibody against receptor \( \beta 2/3 \) subunits would reveal total receptor populations including both synaptic and extrasynaptic membrane areas on the whole surface. \( \gamma 2 \) subunits have drawn interest due to their incorporation in receptors at both synaptic and extrasynaptic sites (Brickley and Mody, 2012; Kasugai et al., 2010). GABARAP interacts with the intracellular domain of GABA\textsubscript{A} receptor \( \gamma 2 \) subunits both in vitro and in vivo (Nymann-Andersen et al., 2002; Wang et al., 1999) but it is not
concentrated at GABAergic synapses (Kneussel et al., 2000). This may result from the competition with other molecules such as gephyrin binding to γ2 subunit at synapses (Essrich et al., 1998; Kneussel et al., 1999; Schweizer et al., 2003). Analyzing receptor kinetics at distinct subcellular locations disclosed that extrasynaptic sites undergo extensive clathrin-mediated endocytosis even as they serve as primary sites for newly inserted receptor (Bogdanov et al., 2006; Gonzalez et al., 2012). Given the presence of γ2 subunits in both synaptic and extrasynaptic GABA_A receptors and the preferential extrasynaptic localization of newly-inserted receptors (Bogdanov et al., 2006), we hypothesize that before reaching steady state, receptors with γ subunits first insert at extrasynaptic sites where they associate with 480 kDa ankyrin-G through GABARAP. This association protects receptors from endocytosis, allowing them to undergo lateral movement into synaptic sites, which further modulates the efficacy of synaptic inhibition. A study using GABARAP siRNA showed that NMDA-induced GABA_A receptor clustering and spontaneous firing require GABARAP, glutamate receptor interacting protein (GRIP), and N-ethylmaleimide-sensitive factor (NSF) in a CaMKII-mediated fashion, demonstrating that GABARAP-dependent potentiation of inhibitory synapses involves increased receptor exocytosis rather than reduced endocytosis (Marsden et al., 2007). We found that the loss of GABAergic synapses in ankyrin-G KO cells was not due to the lack of neuronal activity as illustrated by GABAergic synapse formation under prolonged tetrodotoxin treatment (Figure 15). Consequently, we
described the activity-independent inhibition of receptor endocytosis by the GABARAP-ankyrin-G complex, though the detailed mechanism of how GABARAP-ankyrin-G impedes receptor endocytosis needs further clarification.

\( \text{GABA}_\Lambda \) receptor is the essential postsynaptic component of inhibitory GABAergic synapses in the CNS (Farrant and Kaila, 2007). A study using human embryonic kidney 293 (HEK293) cells co-cultured with hippocampal neurons demonstrated that heterogeneously-expressed \( \text{GABA}_\Lambda \) receptor was sufficient to promote axon adhesion and the formation of functional synapses (Fuchs et al., 2013). Our report is the first to show that \textit{in vivo} brain slices as well as \textit{in vitro} dissociated cultured neurons, the \( \text{GABA}_\Lambda \) receptor itself is adequate to drive GABAergic synaptogenesis as evidenced by the requirement of GABARAP-ankyrin-G association in forming full GABAergic complexes containing gephyrin and vGAT. Nevertheless, though first identified as a glycine receptor interacting protein, postsynaptic scaffold protein gephyrin has been strongly implicated in regulating \( \text{GABA}_\Lambda \) receptor clustering through direct binding to the intracellular loops of multiple subunits (Essrich et al., 1998; Jacob et al., 2005). Genetic inactivation of gephyrin led to disrupted accumulation of certain receptor subunits known to be enriched in synapses while minimal changes occurred to receptor subtypes involved in extrasynaptic targeting (Jacob et al., 2005; Kneussel et al., 2001; Kneussel et al., 1999; Levi et al., 2004). On the other hand, the loss of the receptor \( \gamma_2 \) subunit resulted in defective \( \text{GABA}_\Lambda \) receptors and gephyrin
localization at postsynaptic sites (Essrich et al., 1998; Schweizer et al., 2003). Taken together, these data show mutual dependence between gephyrin and the receptor, with gephyrin promoting receptor aggregation specifically at synaptic sites, while the receptor also supports gephyrin clustering through direct protein interaction. To explain the significance of the GABA$_A$ receptor in driving GABAergic synapse formation, we propose that the absence of ankyrin-G causes extensive receptor endocytosis. Impaired surface GABA$_A$ receptor accumulation then destabilizes gephyrin-containing postsynaptic complexes, ultimately leading to the diffusion of presynaptic complex from postsynaptic sites.
Chapter 3. Conclusion and future direction

This discovery of somatodendritic 480 kDa ankyrin-G provides a novel mechanism for somatodendritic GABAergic synapse formation. Several mutations of ANK3 (encodes ankyrin-G) have been consistently linked to schizophrenia and bipolar disorder in Genome wide association studies (GWAS) (Ferreira et al., 2008; Iqbal et al., 2013; Lee et al., 2012), revealing altered genetic features of ankyrin-G underlying disease susceptibility. Our study emphasizes the significance of surface GABA_\text{A} receptors in directing GABAergic synaptogenesis by ankyrin-G/GABARAP-mediated inhibition of receptor endocytosis, thus sheds light on how compromised cellular localization of inhibitory synaptic receptors contributes to the mechanisms of psychological disease.

In addition to GABAergic synapses, somatodendritic membrane also harbors distinct types of ion channels, receptors, and transmembrane proteins involved in cell adhesion or intracellular signaling pathways (Lai and Jan, 2006). One of the future directions will be to determine whether ankyrin-G also mediates the accumulation of other somatodendritic-clustered membrane proteins such as Cav1.2, Kv2.1, and neuroligins through the association with GABARAP or other adaptor proteins (Graf et al., 2004; Hell et al., 1993; Misonou et al., 2004). As a member of LC3 family involved in intracellular trafficking, GABARAP may have functions other than mediating GABA_\text{A} receptor localization or alternative binding partners yet to be discovered (Chen and Olsen, 2007; Mohrluder et al., 2009). Therefore, it would be compelling to explore if
ankyrin-G stabilizes multiple membrane proteins on the somatodendritic compartment through the same interaction with GABARAP, or distinct adaptor proteins with roles in regulating or impeding endocytosis.

Our data using W1989R 480 kDa ankyrin-G suggested the strong connection between GABAergic synapse formation and the association of GABARAP and 480 kDa ankyrin-G. As a result, it will be interesting to make a W1989R knock-in mouse and address whether it phenocopies the specific in vitro loss of GABAergic innervations or possesses other deficit, if ankyrin-G/GABARAP association is responsible for the stabilization of other membrane proteins. Since single mutation W1989R 480 kDa ankyrin-G builds fully functional AIS by the normal recruitment of other AIS proteins, which allows us to investigate the selective KO of somatodendritic ankyrin-G on GABAergic synaptogenesis without compromising the integrity of the AIS. This will also resolve the complexity of potential compensation effect from various GABARAP isoforms by demolishing the interaction between the insert of 480 kDa ankyrin-G and all GABARAP variants that possibly bind to the LIR motif. Collectively, this study introduces a novel mechanism of how proteins localize and retain on the somatodendritic membrane of neurons, and more work should be conducted to describe the detailed network with endocytosis machinery and other potential proteins that might be involved in orchestrating and preserving these functional domains of somatodendritic membrane.
Appendix. Identification of full length ankyrin-G alternative spliced isoforms

1. Background and Overview

As a multi-domain modular peptide, ankyrin-G is subjected to extensive splicing from the use of alternative domains which gives rise to functional diversity in various transcripts. Previously Northern blot and Western blot have confirmed the existence of multiple ankyrin-G isoforms widely distributed in different mice tissues (Peters et al., 1995). Several reports have thus been focused on tissue-specific splicing of ankyrin-G peptide in nervous system, skeletal muscle, macrophages, and kidney. The neuronal specific ankyrin-G isoforms were discussed in the previous part. In skeletal muscle, totally five novel ankyrin-G isoforms (AnkG_{197}, AnkG_{217}, AnkG_{109}, AnkG_{128}, AnkG_{130}) were discovered from screening of rat skeletal muscle cDNA clones in addition to AnkG_{107} reported previously (Gagelin et al., 2002; Hopitzan et al., 2005) (Figure 30). When expressed in muscle fibers in vivo, small ankyrins (AnkG_{109}, AnkG_{128}, AnkG_{130}, and AnkG_{107}) revealed the targeting to sarcolemma costameres, a structural-functional component that links peripheral sarcomeres to the extracellular matrix (Hopitzan et al., 2005). Three variably spliced insert regions with size as 17/18 residues, 195 residues, and 76 residues are present in muscle tissues, among which 76 residues in the C-terminus is specific to muscle ankyrins (Hopitzan et al., 2005; Peters et al., 1995). The 76 residues contain a conserved domain which interacts with cytoskeletal proteins plectin and filamin C thus confers ankyrin-G with a gain of function in muscle through alternative
splicing (Maiweilidan et al., 2011). In mouse macrophages, ankyrin-G is expressed exclusively as isoforms lacking membrane binding domain (Hoock et al., 1997). Alternative splicing leads to the association of these smaller isoforms with intracellular lysosomes instead of plasma membrane, which adds functional diversity to ankyrin-G for intracellular membrane biology. Another alternative transcript of ankyrin-G, AnkG119, lacks part of repeats in membrane binding domain while possesses spectrin binding motif and a truncated C-terminus (Devarajan et al., 1996). AnkG119 binds to beta-1 sigma spectrin within dynactin complex and forms organized microdomains on Golgi-associated membrane which contributes to polarized vesicular transport from endoplasmic reticulum to Golgi in kidney cells and muscle (Devarajan et al., 1997; Devarajan et al., 1996). In kidney, Western blot revealed four distinct ankyrin-G isoforms, AnkG215, AnkG200, AnkG170, and AnkG120, among which AnkG170 and AnkG120 are lacking ankyrin repeats (Doctor et al., 1998). Distal tubule displayed abundance of AnkG215 and AnkG200, while proximal tubule showed preference toward AnkG170 and AnkG120 with limited amount of bigger ankyrin-G isoforms. This proximal vs distal difference in ankyrin-G isoforms was consistent in proximal tubule-originated Pig kidney epithelial cells (LLC-PK1) and distal tubule-derived Madin-Darby Canine Kidney epithelial cells (MDCK) (Doctor et al., 1998; Madin and Darby, 1958; Nielsen et al., 1998). Collectively, the examples and significance of ankyrin-G alternative splicing were addressed in certain tissues and cell lines, while potential functions of most
existing spliced variants are yet to be discovered. However, due to the large size, low abundance, and inadequate annotation of ankyrin-G transcripts, a systematic search for all full length ankyrin-G isoforms with information of actual exon usage has not been developed. Therefore in this study, we utilized the knowledge of 5’ starting and 3’ ending sequences from known annotated ankyrin-G constructs in database for the attempt of recovering full length transcripts using nested PCR and cDNA reverse-transcribed from tissue RNA.

Among membrane binding domain, spectrin binding domain, UPA, death domain, and a C-terminus, alternative splicing events have been reported to be concentrated on the C-terminal regulatory domain. Compared to other modular and folded domains, C-terminus is relatively unstructured and extended, thus is able to harbor alternative spliced insertions or deletions without affecting the folding of the peptide. Interestingly, ankyrin repeats in membrane binding domain are encoded by exons which begin and end at the same amino acid residue within the repeat sequence (Cai and Zhang, 2006; Cunha et al., 2008). As a result, it is possible to generate isoforms containing different numbers and linear combinations of ankyrin repeats that would still fold into extended but shorter solenoids (Bennett and Lorenzo, 2013). Such alternatively spliced variants lacking part of internal ankyrin repeats have been described for ankyrin-B (Cunha et al., 2008). In our attempt to recover full length alternatively spliced ankyrin-G isoforms, variants with modified repeats number and arrangement would
also be prime targets to obtain comprehensive understanding of actual exon usage of ankyrin-G gene.

Figure 30: Schematic representation of rat skeletal muscle ankyrin-G isoforms.

(From Hopitzan et al., 2005)
2. Results

2.1 Selected newly-discovered “organic” ankyrin-G full-length isoforms contain an alternatively spliced insert C.

The canonical 190 kDa ankyrin-G construct we currently used was screened and isolated from a rat kidney cDNA library (Thevananther et al., 1998). In order to address what are other “organic” ankyrin-G isoforms in the genome, we recovered full length ankyrin-G variants from RNA transcripts of heart and kidney by RT-PCR, subcloned them into vector, and completely sequenced them in both directions. The result demonstrated various domain compositions of ankyrin-G isoforms ranging from 210 kDa to 40 kDa (Figure 31). Among them one was a canonical 190 kDa ankyrin-G which consists of membrane binding domain, spectrin binding domain, ZU5, UPA, death domain, and a C-terminus. Alternatively, some 190 kDa ankyrin-G isoforms contain one or two insertion sequence in the linker between membrane binding domain and spectrin binding domain (insert A and B) and/or a 20 kDa insertion region within the C-terminus (insert C) which makes it around 210 kDa (Peters et al., 1995). Moreover, we recovered smaller ankyrin-G isoforms around 100 kDa (120 kDa with insert C) that lacked membrane binding repeats and began with spectrin binding domain, confirming that smaller ankyrin-G isoforms were synthesized in cell (Hoock et al., 1997; Morrow et al., 1989; Peters et al., 1995). In addition to ankyrin-G isoforms which were previously characterized and cloned, we discovered even smaller ankyrin-G isoforms starting with the second ZU5 or UPA domain with corresponding molecular weight 75kDa and 60
kDa, respectively. The smallest one with intact 5’ START and 3’ STOP codon we recovered contained only the death domain and a C-terminal tail, making a peptide around 40 kDa. Interestingly, we obtained several ankyrin-G isoforms with internal truncation of various lengths in the repeats region of membrane binding domain from heart tissue. One isoform encoded a truncation of two third of repeats and the first ZU5, while another lacked one third of repeats. These variants would be predicted to lose interactions (loss-of-function) as well as gain new partners (gain-of-function) due to the juxtaposition of otherwise separated ankyrin repeats (Bennett and Lorenzo, 2013).

In our collection of different ankyrin-G variants, two smaller insert regions (insert A and B) were associated with isoforms containing repeats, while insert C was found in isoforms with or without repeats as well as the one with internal truncation. Comprehensively, this would explain the existence of multiple peptide bands detected in tissue samples blotting with antibody against C-terminus of ankyrin-G, which resulted from alternative splicing of ankyrin-G gene. Future work focused on expressing distinct ankyrin-G isoforms in ankyrin-G knockdown cardiomyocyte would gain novel insight into the specific localization and function of each isoform and the role of alternative splicing in increasing ankyrin-G peptide functional properties.
Figure 31: RT-PCR from heart and kidney tissues reveals multiple ankyrin-G variants with various internal insertions and deletions.

The collection of full length ankyrin-G isoforms from RT-PCR containing 5′ and 3′ end sequences. The name of tissue and number indicate where the defined isoform is pulled out from how many independent experiments. The calculated molecular weight of each isoform is labeled on the far right.
2.2 Insert C is an unstructured but divergent peptide predicted to be phosphorylated.

Given the presence of the insertion region C at the C-terminus of multiple ankyrin-G isoforms varied in length and domain composition, we decided to characterize the biochemical nature and potential biological function of this insertion. The whole insert C peptide was 195 amino acids in length and acidic with the isoelectric point (pI) calculated by the sequence around 4 (Figure 32, 33). The protein disorder tendency predicted by amino acid sequence revealed the intrinsic unstructured nature throughout the whole peptide, except for the middle part that may possess some degree of secondary structure yet to be defined (Figure 34). To address more biophysical features of insert C, we cloned the full length rat insert C peptide from an expression plasmid (a kind gift from Dr. Kordeli) into pMAL vector, expressed the peptide in bacteria, and purified the peptide with tags removed. Using Circular Dichroism (CD) spectroscopy, we confirmed the extended, random coil trait of insert C as indicated by minimum structural change during temperature meltdown (Figure 35). Interestingly, CD result also suggested a small beta sheet in insert C from the degree of ellipticity around 228 nm, validating the observation of a probable secondary structure from disorder tendency predicted by amino acid sequence.
Figure 32: Insert C localizes within the C-terminus of ankyrin-G peptides.

Figure 33: Primary sequence of 195 amino acids of insert C.

Figure 34: Insert C peptide is predicted to be mostly unstructured.

The prediction plot is calculated by http://iupred.enzim.hu/ using full length insert C sequence as input.
Figure 35: Circular Dichroism during temperature meltdown confirms the unstructured nature of insert C.

Purified insert C peptide is used in Circular Dichroism assay to determine the potential secondary structure. Curves from 5°C and 85°C are plotted on Excel to compare the effect of temperature meltdown on insert C intrinsic structure.
The sequence of insert C, compared to other domains of ankyrin-G, was relatively divergent in evolution. The sequences from human, monkey, and dog are similar (over 90% identical), while those from mouse, chicken, and frog are 50-70% identical to human and the sequence of fish is only 40% to human (Figure 36). This suggested that the insert C region may be the source of evolutionary diversity which granted distinct function in certain species.
Figure 36: Insert C sequence is divergent between different species.
The primary peptide sequence also provided prediction of potential phosphorylation sites by kinases with known consensus sequence. The full length ankyrin-G contained multiple protein phosphorylation “hot spots” including the linker between membrane binding domain and spectrin binding domain, insert C, and most regions of the C-terminus, while highly-structured repeats region and spectrin binding region had limited predicted phosphorylation sites (Figure 37).
Figure 37: The whole ankyrin-G peptide is predicted to be highly phosphorylated especially on the C-terminus region.

Each vertical line denotes one potential phosphorylation site with consensus sequence from each kinase. Kinases listed here include CK1, CK2, IKK, PDK, PKB, PKC, PAK1, PLK1, Aurora, GSK-3, MAPK, and CaM. Red dots indicate the number of potential phosphorylation events on single site.
In 2012, a paper revealed a non-conservative amino acid change from aspartic acid to tyrosine in ANK3 gene of bipolar disorder patients, which resided within the sequence of insert C (Figure 38) (Dedman et al., 2012). This aspartic acid was conserved in all vertebrates except for fish, and was part of the consensus “SXXE/D” motif of casein kinase II (CKII) (Figure 39) (Meggio and Pinna, 2003). Thus we generated insert C D794Y construct and purified the mutated peptide along with WT insert C peptide for performing \textit{in vitro} kinase assay. Though WT insert C showed significant phosphorylation signal by purified CKII, we also detected decent phosphorylation of D794Y insert C by CKII (Figure 40). This result indicated the probable multi-phosphorylation sites from CKII in full length insert C, as mutating one site was not sufficient to obliterate the all phosphorylation signals \textit{in vitro}. Nevertheless, the kinase assay further confirmed the sequence prediction that insert C was highly phosphorylated, which may suggest additional level of regulation for the function of ankyrin-G isoforms containing insert C.
Figure 38: Insert C amino acid sequence with D794Y residue highlighted in red.

Figure 39: D794 is conserved in all vertebrates except for fish.
Figure 40: Insert C has multiple CKII phosphorylation sites.

In vitro kinase assay demonstrate that purified insert C D794Y is still phosphorylated by CKII as in WT insert C. Phosphorylated insert C peptide is shown at around 30 kDa on the blot. Three types of controls are used to verify the specificity of kinase reaction with no kinase, no radioactive ATP, or no substrate.
2.3 Isoforms containing insert C are the major transcripts in most non-neuronal tissues.

In order to detect ankyrin-G isoforms with insert C, we used purified insert C peptide to generate polyclonal antibody against insert C. Surprisingly, the Western Blot using affinity column-purified insert C antibody in various tissue lysates revealed that ankyrin-G isoforms containing insert C were major transcripts in heart, while they were alternatively-spliced variants in kidney and fibroblast (Figure 41). Remarkably, in MDCK cells, the 210 kDa ankyrin-G with insert C was the only form which also contained the membrane binding domain. This corrected the molecular weight of the largest peptide in MDCK cell lysates previously detected by antibody raised against ankyrin-G C-terminus to be 210 kDa, instead of 190 kDa. Given that 190 kDa ankyrin-G was able to promote de novo basolateral membrane biogenesis and interact with beta-2 spectrin and E-cadherin (Kizhatil and Bennett, 2004; Kizhatil et al., 2007), the effect of adding insert C to existing or novel ankyrin-G functions in epithelial cells thus needed to be further determined.
Figure 41: Insert C is the major transcript in heart and MDCK cells and the alternatively spliced form in brain, kidney, and NIH3T3 cells.

Affinity-purified insert C antibody is used in comparison to the antibody against total ankyrin-G in brain, heart, kidney, MDCK, and NIH3T3 lysates. In addition to 210 kDa peptide, insert C antibody also recognizes several smaller isoforms in various tissues.
2.4 Both 210 and 190 kDa ankyrin-G but not smaller ankyrin-G can rescue the height of basolateral membrane in insert C KO and membrane binding domain KO cells.

Previous study showed that 190 kDa ankyrin-G was sufficient to rescue the loss of basolateral membrane human bronchial epithelial cells transfected with siRNA against 190 kDa ankyrin-G (Kizhatil and Bennett, 2004). With various organic ankyrin-G isoforms discovered, we then addressed the role of ankyrins with different lengths and domain compositions in basolateral membrane biogenesis. We thus cloned the insert C into rat 190/100 kDa ankyrin-G to generate rat 210/120 kDa ankyrin-G counterparts of mouse sequence recovered in previous RT-PCR screen. We designed the inducible shRNA against insert C and spectrin binding domain (SBD) in Tet-pLKO-2A-mcherry vector (He et al., 2012) and generated lentivirus to infect MDCK cells. The knock down efficiency of each hairpin was verified using Western Blot (Figure 42). Both insert C KO and membrane binding domain (MBD) KO showed the knock down of the big ankyrin-G isoform. Moreover, the knock down of the duplet ankyrin-G bands around 150 kDa in insert C KO MDCK cells suggested that both bands contain insert C. MDCK cells stably expressing shRNA against insert C, when induced with doxycycline (insert C KO MDCK), phenocopied the loss of basolateral membrane height as from the siRNA against 190 kDa ankyrin-G (Kizhatil and Bennett, 2004) (Figure 42), further confirming that the big ankyrin-G isoform in MDCK cells required for basolateral membrane biogenesis also contained insert C. Nevertheless, both 210 kDa and 190 kDa localized to
the basolateral membrane compartment and rescued the height of the membrane, defined as the distance between the bottom of tight junction protein ZO-1 and the end of the lateral membrane, with no significant difference. Interestingly, two smaller ankyrin-G isoforms, 120 kDa (with insert C) and 100 kDa (without insert C), also accumulated at some degree on the basolateral membrane of insert C-KO MDCK cells (Figure 42). This could be explained by the interaction with spectrin which was tethered to the membrane from pleckstrin homology (PH) domain by spectrin binding domain in those small ankyrin-G isoforms. However, without membrane binding domain, small ankyrin-G failed to rescue the loss of basolateral membrane height though remained on the membrane, indicating that the membrane association of ankyrin-G alone was insufficient for de novo biogenesis and the maintenance of basolateral membrane. Among four defined ankyrin-G isoforms used in the rescue experiment, we did not observe any significant difference between each pair which only differed in the presence of insert C (Figure 42). This suggested that insert C was dispensable in directing the biogenesis of basolateral membrane, while it might play other roles in yet to be found functions of epithelial ankyrin-G.
Figure 42: Both 210 and 190 kDa ankyrin-G rescued the height of basolateral membrane in ankyrin-G KO MDCK cells.

XZ planes of lateral membrane under different conditions (green, GFP-ankyrin-G isoform; red, mCherry-PLKO construct of shRNA; white, ZO-1). Untransfected ankyrin-G KO cells show defect in lateral membrane height. Ankyrin-G KO cells transfected with GFP-190 or 210 kDa ankyrin-G but not GFP-120 or 100 kDa ankyrin-G show the rescue of basolateral membrane height. The result is quantified on the right. n=5-10 cells per group. Error bar, SEM. Bottom right, two days doxycycline induction could selectively knock down 90% ~200kDa ankyrin-G in MBD-shRNA and all ankyrin-G bands in C-shRNA and SBD-shRNA.
2.5 210 kDa ankyrin-G has the same mobility as 190 kDa ankyrin-G on the membrane of MDCK cells.

One hypothesis was that the addition of insert C might affect ankyrin-G’s mobility in the membrane-associated domains. We thus transfected GFP-tagged 190 or 210 kDa ankyrin-G into insert C-KO MDCK stable cells and imagined live cell fluorescence recovery after photobleaching (FRAP). After photobleaching, GFP-190 kDa ankyrin-G exhibited rapid recovery to around 40% of original fluorescence intensity but little to none further recovery in the remaining time, demonstrating around 60% immobile ankyrin-G fraction in the time scale of this experiment. GFP-210 kDa ankyrin-G showed no difference with GFP-190 kDa ankyrin-G in the whole time course of FRAP, with similar recovery rate and population (Figure 43). As a consequence, we concluded that the presence of insert C and the resulting interaction unknown partners did not affect the dynamic of ankyrin-G within basolateral membrane domains, though it did not exclude the possibility that insert C might bind to distinct proteins for functions other than membrane biogenesis and restriction in membrane-associated domains.
Figure 43: Both 210 and 190 kDa ankyrin-G displayed similar membrane mobility.

FRAP experiment of transfected GFP-tagged 190 or 210 kDa ankyrin-G on the basolateral membrane of MDCK cells reveals the similar dynamic of two ankyrin-G isoforms on the membrane. Fluorescence readings were normalized and plotted according to pre-bleached value as 1.0. n= 15 cells per group.
2.6 In fibroblast, both 190 and 210 kDa ankyrin-G localize predominantly on the membrane, while 210 kDa ankyrin-G is concentrated at the cleavage furrow of dividing cells.

Fibroblast is one of the model cell lines in studying cell division and migration. Based on the Western Blot result of NIH3T3 fibroblast cell lysates, ankyrin-G isoforms with membrane binding domain existed as duplet with two bands around one to one ratio, one 210 kDa with insert C and one 190 kDa without insert C. Therefore, unlike MDCK cells which only had one 210 kDa isoform, NIH3T3 allowed us to specifically knock down 210 kDa ankyrin-G and investigate potential distinct function of 210 kDa ankyrin-G from remaining 190 kDa ankyrin-G.

Using inducible shRNA and lentiviral transfection, we generated NIH3T3 fibroblast stable cell line expressing either shRNA raised against insert C (insert C-KO) or against spectrin binding domain (total KO), which eliminated both “big” and “small” ankyrin-G isoforms beyond 100 kDa. The knock down efficiency was confirmed by antibodies against insert C or C-terminus of ankyrin-G in Western Blot (Figure 44) and in immunofluorescence staining (Figure 45). In shRNA-insert C NIH3T3 cells, no specific immunolabeling from insert C antibody was detected while the membrane staining by total ankyrin-G antibody was decreased by half, indicating the knock down of 210 kDa ankyrin-G. In shRNA-SBD total KO cells, all specific ankyrin-G staining from both antibodies were gone.
In order to relate the observed immunofluorescence signal to specific ankyrin-G variant, we used defined ankyrin-G constructs in total KO fibroblast (shRNA-SBD). Transfected 190 kDa ankyrin-G in total KO fibroblast accumulated predominantly on the plasma membrane, while transfected 210 kDa ankyrin-G showed similar membrane association (Figure 46). Interestingly, immunolabeling of ankyrin-G variants with insert C from insert C specific antibody accumulated not only on the plasma membrane, but also in the mid body of dividing cells (Figure 47). This mid body staining from insert C antibody was confirmed with total antibody, indicating that a certain population of ankyrin-G isoforms containing insert C localized to the mid body of cleavage furrow and thus 210 kDa insert C may have additional role in regulating cell division especially at telophase.
Figure 44: shRNA against insert C targeted only 210 kDa ankyrin-G isoforms.

Western Blot shows the knock down effect of each stable shRNA-transfected NIH3T3 cells upon 48 hours of doxycycline treatment. Only 210 kDa but not 190 kDa ankyrin-G was eliminated from shRNA-insert C. Transfected GFP-190 kDa ankyrin-G in shRNA-insert C cells appears as a band around 210 kDa (190 kDa ankyrin-G + 20 kDa GFP), while transfected GFP-210 kDa ankyrin-G appears as a band around 230 kDa (210 kDa ankyrin-G + 20 kDa GFP).
Figure 45: shRNA against insert C removed insert C immunolabeling while shRNA against SBD eliminated all staining from both antibodies.

Compared to shRNA-Luciferase which immunolabeling of ankyrin-G peptides from both antibodies accumulates on the plasma membrane, shRNA-insert C results in the loss of all insert C antibody staining and half of membrane signal by total ankyrin-G antibody. No immunolabeling from either antibody was detected in shRNA-SBD total KO cells.
Figure 46: Both 190 and 210 kDa ankyrin-G localize to the plasma membrane of NIH3T3 cells.

Figure 47: Ankyrin-G isoforms containing insert C is enriched in cleavage furrow.

Both insert C and total ankyrin-G antibodies recognize mid body structure of telophasic NIH3T3 cells, while total ankyrin-G antibody also stains population without insert C on the plasma membrane.
2.7 Stable insert C KO NIH3T3 cells have deficit in cell migration and division.

To address the role of insert C containing ankyrin-G in cell migration and/or division, we applied wound healing migration assay to monitor live cell activity as a population. Control fibroblasts containing shRNA against luciferase migrated and almost closed the 500 µm wide gap 16 hours after the spacer was removed. In contrast, the live imaging of insert C-KO fibroblasts showed fewer cells undergoing cytokinesis, and the final coverage of only about 10% of the gap (Figure 48). Unlike control cells which displayed numerous cellular extensions as they were moving toward the center of the gap, insert C-KO fibroblasts tended to wiggle randomly and moved without direction. Previous finding demonstrated that epithelial cells depleted of 190 kDa ankyrin-G progressed to late telophase stage as determined by the formation of mid body but failed to elongate the lateral membrane (Kizhatil and Bennett, 2004; Kizhatil et al., 2007). Together with the immunofluorescence staining result of insert C-containing ankyrin-G at the mid body and the cell division/migration phenotype of insert C-KO fibroblasts, we speculated that the biological function of insert C might be mediating cytokinesis at the cleavage furrow through the telophase, though the detailed mechanism and the indication to cell migration needed to be further investigated.
Figure 48: Insert C KO NIH3T3 cells display deficiency in migration and division.

Wound healing live-imaging experiment demonstrates the defect in cell migration and division of shRNA-insert C NIH3T3 cells. After 16 hours, shRNA-Luc cells actively divide and migrate to cover 70% of the gap, while shRNA-insert C cells only cover 10% of the gap and rarely divide. Yellow dotted line indicates the front of migrating cell population.
2.8 Insert C containing ankyrin-G in brain is developmentally regulated and associated with postsynaptic density protein PSD95.

Based on the previous Western Blot result, alternatively-spliced insert C peptide was present only in a small fraction of brain ankyrin-G, including 210 kDa and higher molecular weight isoforms (Figure 41). To further address the potential change of insert C-containing ankyrin-G abundance in brain, we collected brain lysates of different developmental stages and blotted for insert C and compared with the blot from total ankyrin-G antibody (Figure 49). Interestingly, before postnatal day (PND) 10, ankyrin-G appeared as duplet at around 480 kDa together with bands around 270 kDa and 190 kDa. Both bands in ankyrin-G duplet around 480 kDa contained insert C before PND 10. After PND 10, the duplet reduced to only one band around 480 kDa, whose amount was up-regulated at PND 15 and stayed steady afterwards. However, this peptide did not contain insert C. On the other hand, bands around 270 kDa and 190 kDa were also up-regulated after PND 10, and the 210 kDa band with insert C became evident after PND 15. The bands at 480 kDa and 270 kDa were previously reported as neuronal specific ankyrin-G, which localized to nodes of Ranvier and axon initial segment (Kordeli et al., 1995). These results indicated several significant findings. First, insert C-containing ankyrin-G variants, including 210 kDa and duplet at 480 kDa, were developmentally regulated. Second, between PND 5 and 10, a developmental switch occurred that several ankyrin-G isoforms were down-regulated while some were up-regulated. Third, brain-specific 270 kDa but not 480 kDa ankyrin-G also encoded insert C. Collectively, this
suggested that insert C might play unique roles in neuronal system, which related to the window of mouse brain development between PND 5 and 10.

Figure 49: Ankyrin-G isoforms containing insert C are developmentally regulated.

Antibodies against insert C and total ankyrin-G are used to blot brain lysates from postnatal day 1, 5, 10, 15, 30, and 35. Multiple ankyrin-G isoforms are developmentally regulated in the brain at time points examined here.
Using antibody against insert C in adult mouse brain section, we found that insert C-containing ankyrin-G peptides localized distinctly to dendritic field in hippocampal CA1 region. In comparison, antibody against total ankyrin-G and 270 kDa ankyrin-G labeled predominantly axon initial segments and nodes of Ranvier (Figure 50). In higher magnification, ankyrin-G with insert C exhibited dendritic association with MAP2 positive dendrites (Figure 50), while postsynaptic markers working in perfused brain sample section will be needed to investigate the fine neuronal or even astrocytic localization of insert C-ankyrin-G. We then used isolated cultured hippocampal neurons to address the pattern of insert C containing ankyrin-G relative to dendritic marker MAP2. Surprisingly, ankyrin-G with insert C associated with postsynaptic density complex PSD95 and clustered on the neck of individual spine (Figure 51). In addition to the spine, insert C immunolabeling was also observed throughout the dendritic shaft and somatic region. However, instead of a defined structural compartment, the staining of insert C was relatively dynamic, varying in size and number in each neuron. Therefore based on the location of insert C peptide, we suspected that neuronal ankyrin-G with insert C may be associated with specific cisternae organelle at the spine and regulated by neuronal activity, though the possibility of astrocytic insert C-ankyrin-G population could not be ruled out using this system.
Figure 50: ankyrin-G isoforms containing insert C localize distinctly to dendritic field in CA1 region of hippocampus.

Upper panel, in contrast to antibody against neuronal specific isoform 270/480 kDa ankyrin-G which is enriched in the axon initial segment, antibody against insert C labels dendritic field of CA1 region in hippocampus. Lower panel, the magnified picture illustrates the dendritic association of insert C-containing ankyrin-G.
Figure 51: Insert C immunolabeling associate with PSD95 in dendrites and spines.

In cultured neurons, insert C-containing ankyrin-G localizes to the dendritic shaft and the neck of individual spines. The boxes in upper panel indicate magnified areas shown in the middle and lower panels.
2.9 190 kDa ankyrin-G localizes at the neuropile region and unmyelinated axons in the brain.

From the Western blot of brain lysates at different stages, we also discovered that not 190 kDa ankyrin-G was a dominant isoform together with two neuronal-specific ankyrin-G, 270 kDa and 480 kDa (Kordeli et al., 1995). Although the role of 190 kDa ankyrin-G was addressed in epithelial cells, the localization and function of 190 kDa ankyrin-G in neuronal system remained unknown. First, to investigate whether 190 kDa ankyrin-G was neuronal or associated with astrocytes or oligodendrocytes, we used cultured rat neurons which contained little to none astrocytes in the system and cultured glial cells/astrocytes as our lysates in Western blot. The result by antibody recognized all ankyrin-G showed that cultured neurons samples contained similar ratio and composition of ankyrin-G peptides as seen in brain lysates, while glial samples, confirmed by Glial Fibrillary Acidic Protein antibody, expressed different patterns of immunochemical bands with much less intensity (Figure 52). This indicated that even though our neuronal lysates might still contain few astrocytes, 190 kDa ankyrin-G was likely to be associated with neurons instead of astrocytes.

Next, we used the antibody raised against the neuronal specific insert for the immunofluorescence staining of brain section, and compared the pattern with the section stained with antibody against total ankyrin-G (Figure 53). Given that 3 dominant populations of ankyrin-G peptides in brain lysates encoded the insert, theoretically anything not labeled with insert antibody but with total antibody should be indicative to
the localization of 190 kDa ankyrin-G. The tiled whole brain staining confirmed that ankyrin-G containing neuronal specific insert accumulated in the initial segment and the nodal region, which were also labeled by antibody against total ankyrin-G. Remarkably, the antibody against total ankyrin-G also recognized neuropile region in the cortex, and unmyelinated axon bundles throughout the brain, which were devoid of signals from neuronal insert antibody (Figure 53). This demonstrated for the first time the potential localization of prominent 190 kDa ankyrin-G in neuronal system, though more tools such as antibody specific to 190 kDa or GFP-190 kDa transgenic mice are needed for fully characterizing the physiological distribution and function of 190 kDa ankyrin-G that is distinct from the initial segment / nodal ankyrin-G.
Neuronal and glial lysates are both from DIV 21 cultures blotted with two antibodies.

Tiled whole brain immunofluorescence staining using antibody against 270/480 kDa ankyrin-G or total ankyrin-G reveals the differential localization of 190 kDa ankyrin-G (red without green) and 270/480 kDa ankyrin-G (yellow).
3. Discussion

In this study we recovered full length ankyrin-G isoforms with actual exon usage by RT-PCR in heart and kidney tissues. We then focused on an alternatively spliced insert C on the C-terminus of multiple ankyrin-G isoforms and characterized the potential function of insert C in various cell lines. Using cellular assays developed in the lab such as FRAP and basolateral membrane height rescue in ankyrin-G KO MDCK cells, we found that 210 kDa ankyrin-G with insert C is indistinguishable from 190 kDa ankyrin-G in membrane motility and basolateral membrane biogenesis, regardless of the fact that the 210 kDa ankyrin-G is the only isoform that also encodes membrane binding domain in MDCK cell lysates. These results suggest that insert C may not contribute to the known functions of ankyrin-G in MDCK cells. However, in mouse fibroblasts, knock down of insert C-containing isoforms led to the deficits in cell division and migration, which cannot be compensated by the presence of 190 kDa ankyrin-G isoform. Taken together the specific cellular localization of isoforms containing insert C in fibroblasts in the cleavage furrow, these indicate that insert C might be involved in the process of cytokinesis. Moreover, in MDCK cells, knocking down of ankyrin-G isoform with membrane binding domain also showed cell division deficits in the late telophase stage (Kizhatil and Bennett, 2004). Given that the only ankyrin-G isoform with membrane binding domain in MDCK cells also encodes insert C, it is probable that the function of
insert C is indispensable in completing cell division thus 190 kDa ankyrin-G without insert C would not be able to rescue cell division and migration deficits in MDCK cells.

Another potential role of C-insert that yet to be addressed could be auto-inhibiting or regulating the function of the rest ankyrin-G molecule via intramolecular interaction. The widespread use of autoinhibition as another level for regulation is well documented, as inhibitions of DNA-protein interaction and protein-protein interaction being frequently observed in different classes of proteins (Pufall and Graves, 2002). However, the common thread in the mechanism of autoinhibition is intramolecular interaction that either directly or allosterically interferes with the function of other targeted domain. In the case of ankyrin-B in human bronchial epithelial cells, the presence of the linker between membrane binding domain and spectrin binding domain directly interacts with ankyrin repeats and inhibits the basolateral membrane targeting of the membrane binding domain, rendering ankyrin-B to be associated with intracellular vesicles (He et al., 2013). This model suggests an autoinhibitory mechanism where an unstructured linker region of ankyrin-B represses the activity of membrane binding domain through direct association, thus competing with membrane partners such as E-cadherin (He et al., 2013). Similar intramolecular interaction and regulatory effect have not been reported in ankyrin-G peptide, although the unstructured C-terminus provides a suitable platform for binding to other domains of ankyrin-G. The
extended insert C peptide within the C-terminus would thus be the candidate of evaluating modular organization and autoinhibitory regulation in ankyrin-G protein.

**4. Conclusion and future direction**

To fully understand the potential biological function of insert C, several cellular assays should be developed for addressing cell division and migration process in both epithelial cells and fibroblasts. For epithelial cells, the knockdown of adhesion molecule E-cadherin or the activation of other signaling pathways such as TGF-beta could be applied to induce epithelial-mesenchymal transition (EMT) (Cano et al., 2000; Xu et al., 2009). EMT processes in WT MDCK cells and insert C KO MDCK cells could be compared to reveal the role of insert C in cell migration. To further investigate the involvement of insert C in fibroblast cytokinesis and division using wound healing assay, stable cell line expressing shRNA-resistant GFP-tagged insert C isoforms under insert C KO background is needed to generate uniform cellular population and address the rescue effect by specific insert C isoform.

To determine whether or not insert C performs autoinhibitory effect on other parts of ankyrin-G peptide, each individual domain could be purified and used in immunoprecipitation assay. If direct association is observed between insert C and any given domain, it is likely that the existence of insert C renders the activity of ankyrin-G peptide and thus regulates the specific function or localization of ankyrin-G isoforms containing insert C.
In conclusion, our study recovered full length alternatively spliced ankyrin-G isoforms with actual exon usage in heart and kidney tissues. We obtained several distinct isoforms with internal deletions of ankyrin repeats and first ZU5 domain, and others with insertions between membrane binding domain and first ZU5 domain as well as within the C-terminus region. The characterization of the alternatively spliced insert C from C-terminus revealed the unstructured nature of the peptide, and the prevalence in various tissues as ankyrin-G variants encoding insert C being the major isoforms. The targeting of insert C by shRNA in epithelial MDCK cells resulted in the loss of basolateral membrane which was rescued by 190 kDa ankyrin-G isoform without insert C, while it led to deficits in cytokinesis and cell migration in NIH3T3 fibroblast in the presence of 190 kDa ankyrin-G. Future studies will be focused on addressing the potential autoinhibitory role of insert C in ankyrin-G function, and the involvement of ankyrin-G isoforms containing insert C in coordinating cytokinesis and induced cellular migration.

5. Material and Methods

RNA extraction and cDNA reverse transcription.

Mice heart or kidney was dissected and flash froze in liquid nitrogen before homogenized into fine powder using mortar and pestle pre-chilled on dry ice. The tissue powder was transferred to Eppendorf tubes and 1mL Trizol (Sigma-Aldrich) was added per 100 mg tissue. Homogenized sample was incubated for 5 minutes at room
temperature before 0.2 mL chloroform per 1 mL of Trizol was added, followed by vigorous shaking and incubation at room temperature for another 10 minutes. Samples were then centrifuged at 4°C at 12,000 x g for 15 minutes. Following centrifugation, the upper colorless aqueous layer was transferred carefully into a new tube without disturbing the interphase and lower phenol-chloroform phase. 0.5 mL isopropanol was added to every 1mL of initial Trizol used for homogenization, and the tube was incubated at -20°C for no less than 2 hours before spun down again at 4°C at 12,000 x g for 10 minutes. The resulting pellet was washed with 75% ethanol and re-precipitated at -20°C for 30 minutes, followed by centrifugation at 4°C at 7,500 x g for 5 minutes and air dry at room temperature. Lastly, 40-80 µL DEPC-ddH₂O was used to dissolve the RNA pellet before storing at -20°C or proceeding to reverse transcription.

For reverse transcription, 2 µL dT oligos were added to each tube containing 2 µg RNA and DEPC-ddH₂O up to 12 µL for annealing reaction at 75°C for 5-10 minutes. Then the tube was chilled on ice, followed by adding of elongation mix containing 1 µL 10 mM dNTP, 2 µL 10X elongation buffer, 1 µL M-MLV reverse transcriptase (all from Ambion), and 4 µL DEPC-ddH₂O. The reaction was carried at 43°C for 2 hours prior to the heat inactivation at 90°C for 10 minutes and storage at -20°C.

**Nested PCR, subcloning, and reconstitution of isoforms**

Given that ankyrin-G messenger in tissues were rare and lengthy, nested PCR was applied to further enrich selected transcripts. Based on genomic sequence data, 4
alternative 5’ end starting sequences and only one 3’ end tail sequence of annotated ankyrin-G variants were selected. The first pair of PCR primers was designed from the UTR sequence right before the starting methionine (5’ primer) and after the STOP codon (3’ primer). The second pair of primers for the nested PCR was designed starting at the methionine codon (5’ primer) and ending at the STOP codon (3’ primer). There were totally 4 different 5’ primers of each round for covering 4 unique 5’ starting sequence of all known ankyrin-G variants, together with only one 3’ primer of each round. Both rounds of PCR were performed using SuperTaq Plus polymerase chain reaction kit (Ambion) which added a single deoxyadenosine at the 3’ end of products.

PCR products were separated on the gel by DNA electrophoresis, and bands beyond 3 kilo bases were purified from the gel and eluted in water. 4 µL of eluate was used to in TOPO TA cloning (Life Technologies) together with 1 µL of vector and 1 µL of salt solution per reaction, followed by ligation at room temperature for 3-5 hours and transformation. Colonies were selected and screened for possible inserts at various sizes using enzyme digestion. Positive clones with inserts bigger than 3 kilo bases were sent for full length sequencing using primers from subcloning vector and internal primers.

DNA construct and antibodies

Plasmid containing rat sequence of insert C was a generous gift from Dr. Kordeli. Insert C was cloned into GFP-190 kDa and GFP-100 kDa ankyrin-G to generate GFP-210 kDa and GFP-120 kDa ankyrin-G, respectively. Full length insert C was also cloned into
expression vector pGEX/MAL for the generation of purified peptide. D794Y mutation was obtained using Quikchange II XL mutagenesis kit (Agilent) from insert C construct in expression vector pGEX/MAL.

To generate shRNA constructs into PLKO-BFP lentiviral vector (He et al., 2012) for genetic knockdown, both sense and antisense strains were designed using siRNA Selection Program from Massachusetts Institute of Technology and three 21 nucleotides-long oligos were selected for each target sequence with loop sequence of CTCGAG. For insert C knockdown in MDCK cells, dog sequence was used to design oligos while for insert C knockdown in NIH3T3 cells, mouse sequence was used instead. Two second generation packaging plasmids for virus generations, pMD2.G and psPAX2, were from Addgene.

Rabbit polyclonal anti-insert C antibody was generated as described below. Rabbit polyclonal antibody against shared insert region of 270/480 kDa ankyrin-G was a generous gift from Dr. Jenkins. Mouse anti-PSD95 antibody was from Millipore. Mouse anti-ZO-1 antibody was from Life Technologies.

**Circular Dichroism**

Purified 195 amino acids insert C peptide was dialyzed in buffer containing 5 mM phosphate buffer pH=7.4 and 25 mM NaF and the final concentration 10 µM of the peptide was used. 5 different temperatures for temperature melt were 5°C, 25°C, 37°C, 60°C, and 85°C. Buffer containing no peptide was used as negative control. Starting
wavelength was 350 nm and ending wavelength was 190 nm with 1nm as each wavelength step. Results from each temperature melt were plotted using Excel with wavelength as x-axis and molar ellipticity in deg cm² dmol⁻¹ as y-axis.

In vitro phosphorylation kinase assay

Purified WT insert C or insert C peptide with D794Y mutation was used as 1 mM for kinase assay. 10 µM CDK2 (NEB lab), 200 µM ATP (NEB lab), and 10 µCi ATP³² (Perkin Elmer) were added to the reaction at 30°C, which was terminated after 1 hour by adding of 25 µL 5X PAGE buffer. 10 µL sample was added to each lane on the gel, which was transferred at room temperature at 600 mA for 3 hours prior to the exposure to the phosphor screen overnight for the detection of radioactivity.

Generation of insert C antibody

Purified insert C peptide was expressed and generated with protocol described in previous section. 3 mg purified peptide was incubated with 1mg activated glutaraldehyde-RSA as one to one molar ratio at 4°C overnight, followed by the adding of 2 volume of Freud’s complete adjuvant (Sigma Aldrich) and submission to the lab in Division of Laboratory Animal Resource. Three immunizations and boosting for three rabbits were scheduled over the course of 60 days. Bleed was collected and spun down at 2,000 xg at 4°C for 10 minutes, prior to the adding of one to one volume ratio of Blot buffer and PMSF (Sigma Aldrich) to the final concentration of 100 µg/mL. The resulting serum was then incubated at 55°C for 15 minutes and stored at -20°C.
For generating affinity column for further purification of antibody, 1.5 mg antigen (insert C peptide) was dialyzed overnight in coupling buffer containing 0.5 M NaCl, 50 mM phosphate buffer pH=7.4 and 1mM EDTA. On the next day, 5 g activated CNBr powder (GE Healthcare) was measured in the sintered glass funnel on vacuum flask, washed and stirred briefly with cold 1 mM HCl before drained with vacuum. This washing step was repeated three times and the last wash with 1 mM HCl was stirred occasionally for 5 minutes at room temperature. The beads were then washed with coupling buffer briefly once and immediately transferred to 50 mL tube containing dialyzed antigen in coupling buffer and tumbled with antigen solution at 4°C overnight. On the next day, beads were spun down briefly (no more than 500 g) at 4°C and washed with the following sequence: 50 mL washing buffer containing 1 M NaCl, 50 mM phosphate buffer pH=7.4, and 1 mM EDTA twice, 50 mL blot buffer twice, 20 mL blot buffer 2 containing 2 M urea, 0.1 M glycine, and 1 % Triton X-100 once, 50 mL blot buffer once. After the last wash, beads were resuspended with 10 mL blot buffer, loaded onto column, and washed again with 10 mL blot buffer. Column was then capped and stored at 4°C.

To regenerate RSA and MBP columns for affinity purification of antibody, the following washing sequence was applied: 200mL/ea high salt buffer containing 1M NaCl, 10 mM phosphate buffer pH=7.4, and 1 mM EDTA, blot buffer, blot buffer 2, and blot buffer without Triton X-100. The column was eluted with 4 M MgCl$_2$ until the
absorbance at 280 nm of the eluate became 0 and then washed extensively with 400 mL blot buffer. To set sequential columns, RSA column was connected with MBP column followed by the affinity column. All serum was pooled and loaded onto the first RSA column at the speed of 0.5 mL/min at 4°C and the flow through was collected in a beaker after affinity column. After all serum was depleted, affinity column was disconnected and a wash adaptor was placed on top of the column for the same washing sequence from regenerating RSA/MBP column. The column was eluted with 4 M MgCl2, washed with and stored in blot buffer without Triton X-100 at 4°C. Absorbance at 280 nm of eluate was measured and fractions containing antibody were pooled and dialyzed in buffer containing 150 mM NaCl, 10 mM phosphate buffer pH=7.4, 10 mM EDTA, 1 mM NaN3, 10% sucrose at 4°C overnight. The antibody was then dialyzed in buffer with 150 mM NaCl, 10 mM phosphate buffer pH=7.4, 5 mM EDTA, 1 mM NaN3 followed by buffer with 150 mM NaCl, 10 mM phosphate buffer pH=7.4, 1 mM EDTA, 1 mM NaN3 at least 6 hours of each at 4°C. Lastly, antibody was dialyzed in storage buffer containing 150 mM NaCl, 10 mM phosphate buffer pH=7.4, 1 mM EDTA, 1 mM NaN3, and 50% glycerol at 4°C overnight. On the next day, antibody solution was quantified by absorbance at 280 nm, aliquoted, and stored at -20°C.

**Generation of doxycycline-inducible shRNA stable cell line**

The protocol of generating stable MDCK cell line with doxycycline-inducible shRNA was reported earlier (He et al., 2012). 13.5µL of 100 µM sense and antisense oligo
as each pair were added to 3 µL 10X annealing buffer (1 M NaCl and 100 mM Tris-HCl pH=7.4) for annealing reaction which started at 95°C for 5 minutes, 70°C for 10 minutes, and decreased to 4°C at a rate of 0.2°C per minute. 1 µL of annealed hairpin was then ligated to digested PLKO vector prior to the transformation to Stab3 competent cells. Positive clone containing the hairpin was selected, amplified, and transfected along with second generation packaging vectors pMD2.G and psPAX2 for making virus in HEK293T cells using calcium phosphate protocol (Clontech). Briefly, HEK293T cells cultured in 10 cm dish were fed with 6 mL whole growth media prior to transfection. For each dish, 20 µg shRNA constructs, 15 µg psPAX2, and 6 µg pMD2.G in 500 µL ddH2O were mixed first then added to 500 µL of 2X HBS. 50 µL 2.5M CaCl2 was then added dropwise to the mixture followed by gentle pipetting. The complex was incubated at room temperature in the dark for 20 minutes before added dropwise to each dish. After 6 hours, the media was replaced with 10 mL fresh growth media. The media were changed 24, 48, and 72 hours after transfection with fresh growth media while old media were collected, filtered with 0.45 µm cell filter, and stored at 4°C. The viral supernatant was centrifuged at 20,000 x g for 4 hours at 4°C and the pellet was dissolved and incubated in 500 µL cold PBS at 4°C overnight. The resulting viral supernatant was used for infection of MDCK or NIH3T3 cells.

1 million MDCK or NIH3T3 cells were plated in each well of 6-wells plate (Genesee). 150 µL virus and the final concentration of 8 µg/mL polybrene (Santa Cruz)
were added to each well while cells were still in suspension followed by gentle shaking. On the next day, media containing virus were replaced by fresh growth media. Two days after virus infection, cells were trypsinized and sent to cell sorting facility for the selection of top 5% brightest cells expressing BFP-tagged lentiviral vector. Sorted cells were amplified and frozen down for storage or used as the stable cell line expressing inducible shRNA upon the addition of 10 µg/mL doxycycline (Sigma Aldrich) for 48 hours, or subject to another round of cell sorting for brighter cells if needed.

**Transfection**

For transient transfection of plasmid into MDCK or NIH3T3 cells, lipofectamine 2000 reagent (Life Technologies) was used according to manufacturer’s protocol. Generally, for cells grown on the center of each Mat-Tek plates prepared for the immunofluorescence staining, 100 ng DNA was added to 25 µL Opti-MEM (Life Technologies). The mixture was then added dropwise to 25 µL Opti-MEM containing 1 µL lipofectamine 2000, and the complex was incubated at room temperature for 15-20 minutes. 50 µL complex were added dropwise to cells in 150 µL growth media on the center of Mat-Tek plates and incubated at 37°C for 6-8 hours. Cells were then fed with 2 mL fresh growth media per each Mat-Tek plate and proceeded to immunofluorescence staining after 24-48 hours. For FRAP experiments, 300 ng DNA and 2 µL lipofectamine 2000 were used for the complex.

**Immunofluorescence staining**
For immunofluorescence staining of non-neuronal cells, live cells cultured in Mat-Tek plates were fixed with 4% PFA/PBS at room temperature for 15 minutes and permeabilized with 0.05% Triton X-100 in PBS before blocked with 5% BSA/PBST. Primary antibodies were used with proper dilution in 5% BSA/PBST at 4°C overnight. On the next day, cells were washed and incubated with Alexa Fluor secondary antibody in 5 % BSA/PBST at room temperature for 2 hours prior to the final washing and mounting with Vectashield (Vectorlabs).

**Fluorescence Recovery After Photobleaching (FRAP)**

MDCK cells were grown on Mat-Tek plates and transfected with either 300 ng GFP-190 kDa ankyrin-G or GFP-210 kDa ankyrin-G using protocol described in the earlier section. For FRAP experiment, a humidified, temperature-controller chamber was used to maintain the cell cultures at 37°C and 5% CO₂ in Zeiss LSM 780 laser-scanning confocal microscope. Selected membrane region was bleached by 100% laser power for 5 seconds, and images were captured using 1% laser every 2 seconds for the next 500 seconds. An unbleached region with the same size as the bleached region was also imaged to normalize the FRAP results. GraphPad Prism version 5 was used to analyze the data.

**Live cell migration assay**

NIH3T3 cells were grown inside of wells of cell culture-insert (ibidi) on the poly-d-lysine coated Mat-Tek plates. Upon reaching confluency, the insert was removed with
care not to disturb the edges of cell layer and 2.5 mL growth media with 10 mM HEPES was added to fill the whole plate. For live imaging experiments, a humidified, temperature-controller chamber was used to maintain the cell cultures at 37°C and 5% CO₂ in Zeiss LSM 780 laser-scanning confocal microscope. Images were taken using 10X objective and 488 nm laser was used for PMT channel with minimum power. Each image was taken every 180 seconds for total of 16 hours. Video was generated using ZEN software (Zeiss).
References


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Biography

Wei Chou Tseng was born in the city of Taipei, Taiwan on May 26th, 1985. She received her Bachelor of Science degree from the Department of Biochemistry and Biotechnology at National Taiwan University in June, 2007. She then decided to pursue a PhD degree in biomedical science, and was admitted to the Department of Pharmacology and Cancer Biology at Duke University in the same year. She finished her PhD training with Dr. Vann Bennett in 2014.

Membership and award

- American Society for Cell Biology, 2013-2014
- Duke Scholars in Molecular Medicine- Neuroscience, 2013-2014

Publications


