LOCAL SIGNALING MICRODOMAINS IN EXCITABLE CELLS: DEFINING NOVEL ROLES FOR ANKYRIN-B IN ION CHANNEL TARGETING AND REGULATION

By

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For Janice Kent Smith

As the original inspiration pushing me into graduate school, the completion of my Ph.D. reminds me of the profound absence I feel since her passing. For someone who struggled with diabetes for nearly 30 years, she always remained optimistic that there would, someday, be a cure. Though humble in comparison to the other discoveries in the field, I dedicate this work to her endless optimism and to her enduring friendship and memory.

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LIST OF ABBREVIATIONS

ABCC	ATP binding cassette, subfamily C
ABCC8	Gene encoding SUR1
ABCC9	Gene encoding SUR2
AChR	Acetylcholine receptor
ADP	Adenosine diphosphate
AEBSF	4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride
AHLT	Adenine, histidine, leucine, tryptophan
ANK	Ankyrin repeat
ANK1	Gene encoding ankyrin-R
ANK2	Gene encoding ankyrin-B
ANK3	Gene encoding ankyrin-G
$AnkB^{+/+}$	Ankyrin-B wild type
$AnkB^{+/-}$	Ankyrin-B heterozygous
$AnkB^{-/-}$	Ankyrin-B null
ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BCA	Bicinchoninic acid
bp	Base pair
Ca^{2+}	Calcium ion
CARP	Carbonic anhydrase-related protein
Ca_v	Voltage-gated Ca^{2+} channel

CD44	Complement of differentiation 44
cDNA	Complementary deoxyribonucleic acid
CFTR	Cystic fibrosis transmembrane conductance regulator
CHAPS	3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate
CHI	Congenital hyperinsulinemia of infancy
C_i	Curie unit of radiation
COP1	Coatamer protein 1
COSm6	*C*V-1 in *O*rigin (African green monkey kidney cells), *S*V40 genetic material
CMRL	Connaught Medical Research Laboratory media
C_T	Comparative threshold
D	Aspartic acid
DAG	Diacylglycerol
DD	Death domain
DEND	Developmental delay, epilepsy, and neonatal diabetes
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's modified eagle medium
DTT	Dithiothreitol
E	Glutamic acid
EDTA	Ethylenediamine tetraacetic acid
EGTA	Ethyleneglycol bis (2-amino ethyl ether)-N,N,N' tetraacetic acid
E_K	Potassium equilibrium constant
EnaC	Epithelial sodium channel
ER/SR	Endoplasmic reticulum/sarcoplasmic reticulum

F	Phenylalanine
FAM	6-fluorescein
FBS	Fetal bovine serum
FCS	Fetal calf serum
g	gravity
G	Glycine
GABA	Gamma-aminobutyric acid
GAPDH	Gene encoding glyceraldehyde-3-phosphate dehydrogenase
GFP	Green fluorescent protein
GIP	Gastric inhibitory peptide
GLP-1	Glucagon-like peptide 1
GST	Glutathione S-transferase
H	Histidine
HA	Hemagglutinin
HAP1A	Huntintin-associated protein 1A
HEK293	Human embryonic kidney cells
HRP	Horseradish peroxidase
I	Isoleucine
IACUC	Institutional Animal Care and Use Committee
iDEND	Intermediate developmental delay, epilepsy, and neonatal diabetes
Ig	Immunoglobulin
I_{KATP}	K_{ATP}-dependent K^+ current
I_{Na}	Sodium current

$InsP_3$	Inositol 1,4,5-trisphosphate
IPTG	Isopropyl 1-thio-β-D-galactopyranoside
K	Lysine
K^+	Potassium ion
$K_{1/2}$	Half maximum velocity
K_{ATP}	ATP-sensitive potassium channel
kb	kilobase
KCNJ8	Gene encoding Kir6.1
KCNJ11	Gene encoding Kir6.2
KCO	K_{ATP} channel opener
kD	Kilodalton
K_{dd}	Mg-ADP dissociation constant for I_{KATP}
kHz	Kilohertz
Kir6.1	Inwardly-rectifying potassium channel 6.1
Kir6.2	Inwardly-rectifying potassium channel 6.2
K_{td}	ADP dissociation constant for K_{ATP} channel
K_{tt}	ATP dissociation constant for K_{ATP} channel
K_v	Voltage-gated potassium channel
L1CAM	Cell adhesion molecule, L1
L	Leucine
LB	Lennox L broth base
LT	Leucine and tryptophan
MBD	Membrane-binding domain

Mg^{2+}	Magnesium ion
mGluR	Metabotropic glutamate receptor
MI	Metabolic inhibitor
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
MRP	Multidrug resistance protein
MUT	Kir6.2 mutant, E322K
N	Asparagine
Na^+	Sodium ion
NaCl	Sodium chloride
Na_v	Voltage-gated sodium channel
NBF	Nucleotide binding fold
NCX	Na^+/Ca^{2+} exchanger
NDM	Neonatal diabetes mellitus
NHA	Na^+/H^+ ATPase
NHERF1	Sodium-hydrogen exchange regulatory factor 1
NKA	Na^+/K^+ ATPase
O_{KATP}	K_{ATP} channel open probability
P	Proline
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PLC	Phospholipase C

P-loop	Pore loop
PMSF	Phenylmethyl sulfonyl fluoride
PNDM	Permanent neonatal diabetes mellitus
PP2A	Protein phosphatase 2A
Q	Glutamine
qRT-PCR	Quantitative real-time polymerase chain reaction
R	Arginine
RACK1	Receptor for activated C-kinase 1
Rb^+	Rubidium ion
Rh	Rhesus factor
RKR	Arginine-lysine-arginine motif
RNA	Ribonucleic acid
S	Serine
SBD	Spectrin-binding domain
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
siRNA	Small interfering ribonucleic acid
SU	Sulfonylurea
SUR1	Sulfonylurea receptor 1
SUR2	Sulfonylurea receptor 2
T	Tryptophan
TBS	Tris buffered saline
TNDM	Transient neonatal diabetes mellitus
TMD	Transmembrane domain

TTBS	Tris buffered saline with Tween-20
T-tubule	Transverse tubule
V	Valine
WT	Wild type
YPD	Yeast peptone dextrose

CHAPTER I

FUNCTIONAL EVOLUTION OF LOCAL SIGNALING DOMAINS IN METAZOAN CELL PHYSIOLOGY

Robert Hooke's first microscopic glimpse into multicellular structure revealed a complex biological organization unimagined before the seventeenth century: microscopic "cells" which he believed were the containers of "noble juices" (Hooke 1665). Since Hooke's original observation, the subcellular organization of vertebrate cells has been defined, revealing a complexity beyond simple compartmentalization (Twyman 1998). While the development of the lipid cell membrane allowed retention of vital cellular components, it produced limited capabilities for communication, as well as decreased access to necessary substrates and the inefficient removal of waste. While early forms of life, such as archaeabacteria, likely expressed simple transporters necessary for salt and water balance (Hille, Armstrong et al. 1999), the evolution of multicellular organisms necessitated the utilization of more complex transmembrane movement. Specifically, the divergence of single-celled life forms into organisms composed of multiple cells types with specific functions required intercellular/intracellular communication, tissue synchronicity, homeostatic measures, and movement. The evolution of specific ion channels, transporters, and pumps became primary mediators of these actions. With the development of the submembranous cytoskeletal network, ion channels were provided with a platform for the micro-compartmentalization of ion channel-mediated activity.

This compartmentalization allowed for more rapid and efficient responses to stimuli, ultimately providing for the highly-choreographed release of hormones, excitation-coupling of the heart, and the firing of synapses. While cells in vertebrate organisms express a distinct 'toolbox' of ion channels, it is the specific subcellular localization and regulation of these ion channels that contribute to the unique function of a particular cell type. For example, the precise localization of voltage-gated sodium channels (Na_v) to the cardiac Z-line (Maier, Westenbroek et al. 2004) provides for the initial upstroke necessary for the heartbeat (Nerbonne and Kass 2005), while these same channels at the nodes of Ranvier (Kazarinova-Noyes and Shrager 2002) and at the axon initial segments (Garrido, Fernandes et al. 2003) allow efficient and rapid propagation of electrical impulses that are necessary for neurotransmitter release, muscle movement, and memory (Vacher, Mohapatra et al. 2008).

Recent work has demonstrated that mutations in ion channel genes which disrupt biophysical activity underlie a number of diseases, including a variety of cardiac arrhythmias (Lehnart, Ackerman et al. 2007), epilepsy (Steinlein 2008), and diabetes (Shield 2007). The identification of numerous 'channelopathies' has been instrumental in defining the roles of various ion channels in physiology; however, most intriguing has been the observation that mutations which disrupt the assembly and regulation of ion channel complexes also result in disease. While the idea of ion channels consisting of only pore-forming subunits persisted in the early days of ion channel research, it is now recognized that most ion channels function within macromolecular complexes, comprised not only of the

pore-forming subunits, but also of associated auxiliary subunits, regulatory enzymes, and targeting proteins (Ashcroft 2006; Grueter, Abiria et al. 2006; Piggott, Bauman et al. 2008). These associated protein partners play vital physiological roles that include feedback ion sensing, enzymatic activity, and the coordination, localization, and targeting of the ion channel assembly. Rapsyn, for example, has been demonstrated to cluster the acetylcholine receptor (AChR) at specific excitatory synapse membranes (Chen, Qian et al. 2007), while the sulfonylurea receptor (SUR) regulatory subunit of the ATP-sensitive potassium channel (K_{ATP}) hydrolyzes adenosine triphosphate (ATP) and stimulates channel opening (Mikhailov, Campbell et al. 2005), in addition to conferring sensitivity to therapeutic drugs (Shindo, Yamada et al. 1998; Ashfield, Gribble et al. 1999). Likewise, MinK and MiRP proteins play critical roles in the modulation of the single channel conductance, gating, and pharmacology of distinct K_v channels (Deschenes and Tomaselli 2002; Pourrier, Schram et al. 2003; Lewis, McCrossan et al. 2004), while the activation/inactivation properties of many voltage-gated channels originate through association with protein kinases and protein phosphatases (Cai, Hernandez et al. 2005; Grueter, Abiria et al. 2006). Considering these necessary roles in ion channel regulation, it is easily appreciated that mutations in accessory subunits can also produce a variety of channelopathies. For example, mutations in rapsyn have been associated with decreases in AChR density, resulting in myasthenic syndrome (Ohno, Engel et al. 2002); loss-of-function mutations in SUR1 causes congenital hyperinsulinemia by decreasing K_{ATP} channel activity (Ashcroft 2005), and loss-of-function

mutations in MinK and MiRP lead to potentially fatal long QT syndrome (Lu, Mahaut-Smith et al. 2003; Krumerman, Gao et al. 2004).

While much work has clearly defined the role of macromolecular complexes in the regulation of ion channel activity, recent advances in the study of channelopathies have also demonstrated that, in addition to proper channel biophysics and regulation by accessory proteins, functional ion channels and transporters require precise expression and localization to specific membrane domains within the cell. A number of human diseases result from disruption of function due to abnormal channel targeting. This has been most clearly defined in such conditions as cystic fibrosis (the cystic fibrosis transmembrane regulator protein, CFTR) (Puchelle, Gaillard et al. 1992), Timothy syndrome (voltage-gated calcium channel, $Ca_v1.2$) (Splawski, Timothy et al. 2005), Liddle syndrome (epithelial sodium channel, ENaC) (Hansson, Schild et al. 1995), and Bartter's syndrome type II (inwardly rectifying potassium channel, Kir1.1) (Derst, Konrad et al. 1997). From work on ion channel trafficking defects, it is now appreciated that ion channels and their accessory entourages are not isolated, functionally discrete islands in the sea of membrane lipids, but rather exist within spatially-defined local membrane domains. Within the past decade, a number of studies have associated dysfunction in the ankyrin family of adaptor polypeptides with ion channel dysfunction in the nervous system and heart (Mohler, Schott et al. 2003; Ango, di Cristo et al. 2004; Mohler, Rivolta et al. 2004; Mohler, Splawski et al. 2004; Lowe, Palygin et al. 2008), specifically in regard to ion channel trafficking and retention at distinct membrane microdomains. Dysfunction in the

cellular mechanisms involved in ion channel trafficking and retention at specialized membrane domains has emerged not only as a new focus in the field of channelopathy research, but has also become an exciting new discovery in the understanding of the complex mechanisms involved in the regulation of ion channel and transporter function and, ultimately, human physiology and pathology.

Summary and Hypothesis

The myriad of diseases associated with impairment of channel function highlight the importance of ion channels in human physiology. To date, over 60 ion channel genes have been associated with human disease (Ashcroft 2006). However, new data from experiments in mice and primary cells demonstrate that the control and regulation of channel function by accessory proteins and the cytoskeleton are equally important for normal physiology. In fact, dysfunction in accessory proteins has been linked with a host of human disorders. These data illustrate that the evolution of multicellular organisms has required the establishment of local signaling domains not only for the regulation of ion channel biophysical properties, but also for efficient targeting. Over the course of my thesis work, I have focused on understanding the role of protein compartmentalization and local signaling for vertebrate physiology. Specifically, I have been most interested in understanding the link between ion channels in excitable cells, the underlying cytoskeleton, and vertebrate physiology. Ankyrins, the family of membrane/cytoskeletal adaptors critical in the trafficking, retention,

and regulation of a number of ion channels, transporters, and pumps, have been identified as critical regulators of ion channel localization to membrane microdomains in excitable cells (reviewed in Chapter II) and are an underlying theme of my work.

In the first part of my thesis, I have focused on the role of ankyrin polypeptides for organizing ion channels on the exterior plasma membrane of excitable cells; namely, the Kir6.2 subunit of the K_{ATP} channel. In the second part of my thesis, I have studied the role of ankyrin polypeptides and underlying cytoskeleton for compartmentalization of ion channels on the interior endoplasmic/sarcoplasmic reticulum (ER/SR) membrane, focusing on the inositol 1,4,5-trisphosphate ($InsP_3$) receptor. Together, my data demonstrate a key role for ankyrin proteins in targeting and regulation of these two key ion channels at diverse membrane domains in multiple excitable cells, as well as a host of critical functions for normal excitable cell function.

The importance of K_{ATP} channel function in the process of insulin secretion has been established for over 20 years, fuelling the search for K_{ATP} channel mutations that result in diabetes (reviewed in Chapter III). These studies have uncovered both heterozygous loss-of-function and gain-of-function mutations that underlie both congenital hyperinsulinemia and neonatal diabetes, respectively (Gloyn, Siddiqui et al. 2006). In addition to the biophysical properties of ion channels, proper K_{ATP} channel localization is critical for function, with loss of K_{ATP} channel surface expression resulting in severe hyperinsulinemia (Partridge, Beech et al. 2001; Taschenberger, Mougey et al. 2002; Dunne, Cosgrove et al.

2004; Yan, Lin et al. 2004). The K_{ATP} channel is also a critical mediator of ischemic preconditioning in the heart (Gumina, Pucar et al. 2003), neural protection from ischemic events (Heron-Milhavet, Xue-Jun et al. 2004), and critical to the regulation of cardiac action potential duration (Janse and Wit 1989; Wilde 1993). Loss of K_{ATP} channel expression in cardiomyocytes and neurons also results in disease, further stressing the need to understand mechanisms underlying the targeting and retention of this ion channel to plasma membrane.

$InsP_3$ receptors, on the other hand, reside on the membrane of the ER/SR and are critical players in the regulation of intracellular Ca^{2+} dynamics (reviewed in Chapter V). Studies in cardiomyocytes, neurons, and beta cells have demonstrated the critical nature of these ion channels to excitation-contraction coupling, synaptic transmission, and insulin release. Likewise, studies have demonstrated significant excitable cell dysfunction resulting from the loss of proper $InsP_3$ receptor expression, localization, and regulation.

Using standard biochemical and electrophysiological techniques, I provide evidence in support of two major findings. First, I present data suggesting that a novel ankyrin-B-dependent mechanism in the trafficking and metabolic regulation of Kir6.2 in excitable cells (presented in Chapter IV). This interaction has not been observed before and represents evidence of a new binding partner for ankyrin-B. Furthermore, the human Kir6.2 mutation, E322K, which is associated with human permanent neonatal diabetes mellitus (PNDM), results in a loss of ankyrin-B-dependent trafficking of Kir6.2 in transfected cells. Moreover, loss of ankyrin-B association with Kir6.2 results in a decrease in K_{ATP} channel ATP

sensitivity. The necessity of the ankyrin-B interaction for K_{ATP} channel sensitivity is a broad-reaching discovery. Not only does it more fully elucidate the mechanisms involved in K_{ATP} channel regulation, but it also represents a novel role for ankyrin polypeptides *in vivo*; a role that supersedes the concept of ankyrins as simple targeting proteins. Additionally, Kir6.2 association with ankyrin-B, a component of the actin/spectrin-based cytoskeleton, may provide the "missing link" between K_{ATP} channel regulation and the cytoskeleton. Based upon these works, I propose that an ankyrin-B-dependent pathway is necessary for K_{ATP} channel localization and function in excitable cells. Moreover, my work implicates a target for pharmacological exploitation in the treatment of insulin secretion disorders.

Second, I present evidence demonstrating the association of ankyrin-B with an N-terminal region of the $InsP_3$ receptor, reversing a previous notion that this interaction occurred with a C-terminal domain of the $InsP_3$ receptor (presented in Chapter VI). The relevance of this finding resolves a long-standing debate regarding the validity and existence of *in vivo* ankyrin-B/$InsP_3$ interactions. The importance of this clarification is essential in understanding the mechanisms underlying $InsP_3$ receptor targeting *in vivo* and provides critical groundwork for elucidating the role of macromolecular signaling complexes at the ER/SR membrane. Considering the importance of $InsP_3$ regulation to excitable cell physiology, determination of the factors which modulate $InsP_3$ receptor targeting and regulation would provide for a critical foundation to the development of pharmacological modification of $InsP_3$ receptor activity. This is of

particular interest with regard to decreased $InsP_3$ receptor expression and aberrant regulation by $InsP_3$.

CHAPTER II

DYSFUNCTION OF ANKYRIN POLYPEPTIDES IN DISEASE

Introduction

Ankyrins are a family of cytosolic proteins whose functions include the organization, targeting, stabilization, and regulation of protein complexes. Specifically, ankyrins tether proteins to the actin/spectrin cytoskeleton, creating distinct functional membrane microdomains. Canonical mammalian ankyrin polypeptides and alternative splice variants are expressed in both tissue-dependent and developmentally-regulated manners, and arise from three separate genes, *ANK1*, *ANK2*, and *ANK3*. *ANK1*, originally identified in erythrocytes, is on human chromosome 8p11 and encodes ankyrin-R (named for its "***r***estricted" distribution in brain and muscle) (Lambert, Yu et al. 1990). *ANK2* is on human chromosome 4q25-27 and encodes ankyrin-B, which demonstrates a ubiquitous expression pattern (hence, "***b***roadly" expressed) (Tse, Menninger et al. 1991). Finally, *ANK3* is on human chromosome 10q21 and encodes ankyrin-G (the largest of the ankyrin polypeptides, "***g***iant" size) (Kapfhamer, Miller et al. 1995). Similar to ankyrin-B, ankyrin-G is ubiquitously-expressed. Ankyrins are also found in other metazoan genomes including *Caenorhabditis elegans* (*unc-44*) (Otsuka, Franco et al. 1995) and *Drosophila* (*Dank1* and *Dank2*) (Dubreuil and Yu 1994; Bouley, Tian et al. 2000). Due to the fact that ankyrin proteins have yet to be found in lower organisms such as *Saccharomyces cerevisiae,*

Arabidopsis thaliana, and *Zea mays*, there is a strong indication that ankyrin polypeptides have evolved with the development of multicellular organisms and represent an important molecular invention in the physiology of metazoan organisms.

Domain Organization of Canonical Ankyrin Polypeptides

Canonical ankyrins have four distinct domains: a membrane-binding domain (MBD) located at the N-terminus, a spectrin-binding domain (SBD), a death domain (DD), and a C-terminal domain (Figure 1). Together with the death domain, the C-terminal domain forms a regulatory domain. All four domains have been associated with distinct protein interactions (Figure 1).

A defining feature of ankyrin polypeptides is the ***membrane-binding domain***, which is composed of 24 consecutive *ANK* repeats folded into four subdomains of six repeats each (Michaely and Bennett 1993) (Figure 2A). An *ANK* repeat is an L-shaped 33 amino acid repeating motif recognized in over 400 proteins in diverse organisms like viruses and humans with functions ranging from transcriptional activation to toxicity (Mosavi, Cammett et al. 2004). Specifically, the *ANK* repeat consists of pairs of antiparallel α-helices stacked side by side and connected by a series of intervening β-hairpin loops (Figure 2A, blowup). It is hypothesized that loop tip residues mediate the specificity for protein interactions with ankyrins (Sedgwick and Smerdon 1999). For example, the $InsP_3$ receptor interacts with the β-hairpin loop tips located within *ANK* repeats 22-24 on ankyrin-B (Mohler, Davis et al. 2004), while the sodium-calcium

exchanger 1 (NCX1) interacts with the β-hairpin loop tips located within *ANK* repeats 16-18 of ankyrin-B (Cunha, Bhasin et al. 2007) (Figure 2B). In the ankyrin family of polypeptides, the 24 *ANK* repeats stack into a solenoid structure (Figure 2A). The role for *ANK* repeat-containing proteins in mediating protein-protein interactions is well-documented, with the interesting characteristic that *ANK* proteins do not bind selectively to a particular class of protein target. This allows for an unparalleled diversity in the unrelated proteins with which they interact (Figure 1). Specifically, protein-protein interactions are achieved by highly-variable residues in the individual β-hairpin loop tips that are able to mediate charge-charge interactions between the ankyrins and their binding partners. As expected, the ankyrin MBD has a number of documented cytosolic, membrane- and cytoskeleton-associated binding partners. Specific binding partners that associate with ankyrins via the MBD include the Na^+/K^+ ATPase (NKA) (Nelson and Veshnock 1987; Morrow, Cianci et al. 1989; Thevananther, Kolli et al. 1998; Zhang, Devarajan et al. 1998; Mohler, Davis et al. 2005), the NCX1 (Li, Burke et al. 1993; Mohler, Davis et al. 2005; Cunha, Bhasin et al. 2007), the anion exchanger (Bennett and Stenbuck 1979; Davis, Otto et al. 1991; Morgans and Kopito 1993; Jons and Drenckhahn 1998), voltage-gated Na^+ channels (Na_v) {Eber, 1996 #18;Srinivasan, 1988 #19;Malhotra, 2000 #20;Lowe, 2008 #2}, the ammonium transporter (Lopez, Metral et al. 2005), $K_v2.1$ and $K_v3.1$ channels (Rasmussen, Frokjaer-Jensen et al. 2007; Xu, Cao et al. 2007), Rhesus (Rh) antigen, and the H^+/K^+ ATPase (HKA) (Festy, Robert et al. 2001). Additionally, ankyrins interact with calcium-induced calcium-release channels of

the ER/SR via the MBD; namely, the $InsP_3$ receptor (Bourguignon, Jin et al. 1993; Joseph and Samanta 1993; Mohler, Davis et al. 2005; Kline, Cunha et al. 2008). Besides ion channels and transporters, the MBD also associates with members of three families of cell adhesion molecules: CD44 polypeptides (Kalomiris and Bourguignon 1988), E-cadherin (Kizhatil, Davis et al. 2007), and L1CAMs (Davis and Bennett 1994; Dubreuil, MacVicar et al. 1996). Additionally, ankyrins have cytosolic binding partners like tubulin (Bennett and Davis 1981; Davis and Bennett 1984; Davis, Otto et al. 1991) and clathrin (Michaely, Kamal et al. 1999). Interestingly, the multivalent MBD is able to interact simultaneously with multiple partners, allowing a single ankyrin polypeptide to facilitate the formation of large protein complexes. (Michaely and Bennett 1995; Michaely and Bennett 1995; Mohler, Davis et al. 2005). In the erythrocyte, for example, ankyrin-R organizes two dimers of the anion exchanger as well as coupling of the anion exchanger with L1CAM (Michaely and Bennett 1995; Michaely and Bennett 1995). In cardiomyocytes, ankyrin-B forms a ternary complex of the NCX1, NKA, and the $InsP_3$ receptor (Mohler, Davis et al. 2005).

The ***spectrin-binding domain*** of ankyrin polypeptides is responsible for interactions between ankyrin and the spectrin/actin cytoskeleton, specifically β-spectrin, both *in vitro* and *in vivo* (Bennett and Stenbuck 1979; Davis and Bennett 1984). These observations led to the notion that the ankyrin/spectrin interaction existed solely to link the plasma membrane to the underlying cytoskeleton. However, more recent work has demonstrated that ankyrins mediate a much more substantial role in membrane domain biogenesis. For example, hereditary

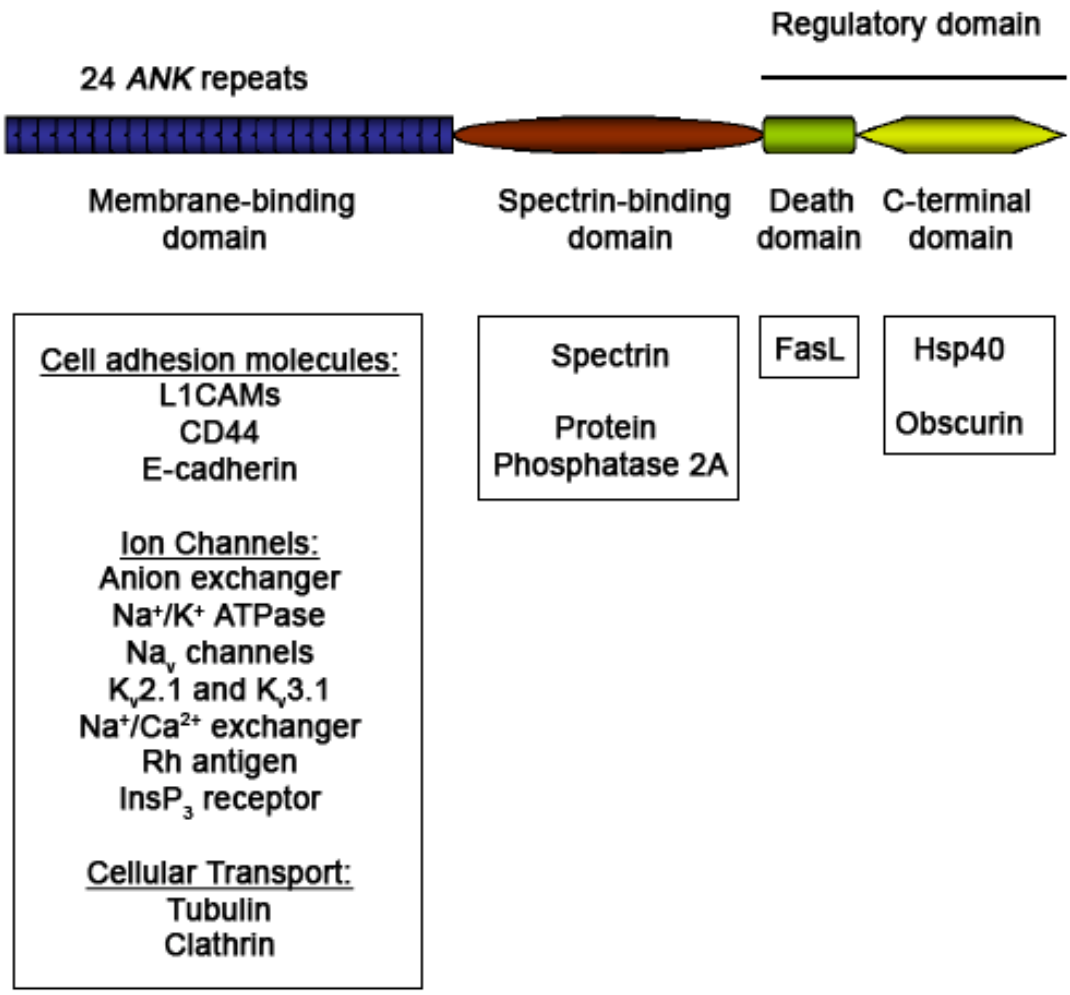

Figure 1. Domain organization and binding partners of canonical ankyrins.

Canonical ankyrins are organized into four domains: an N-terminal membrane-binding domain (blue), containing 24 *ANK* repeats; a spectrin-binding domain (red); a death domain (green); and a C-terminal domain (yellow). Together with the C-terminal domain, the death domain forms a regulatory domain. Ankyrins interact with a number of protein partners, representing a variety of protein families: cell-adhesion molecules, ion channels, cellular transport molecules, cytoskeletal proteins, phosphatases, and heat shock proteins. Adapted from Bennett and Healy, *Trends Mol Med*. 2008 14(1):28-36 (Bennett and Healy 2008).

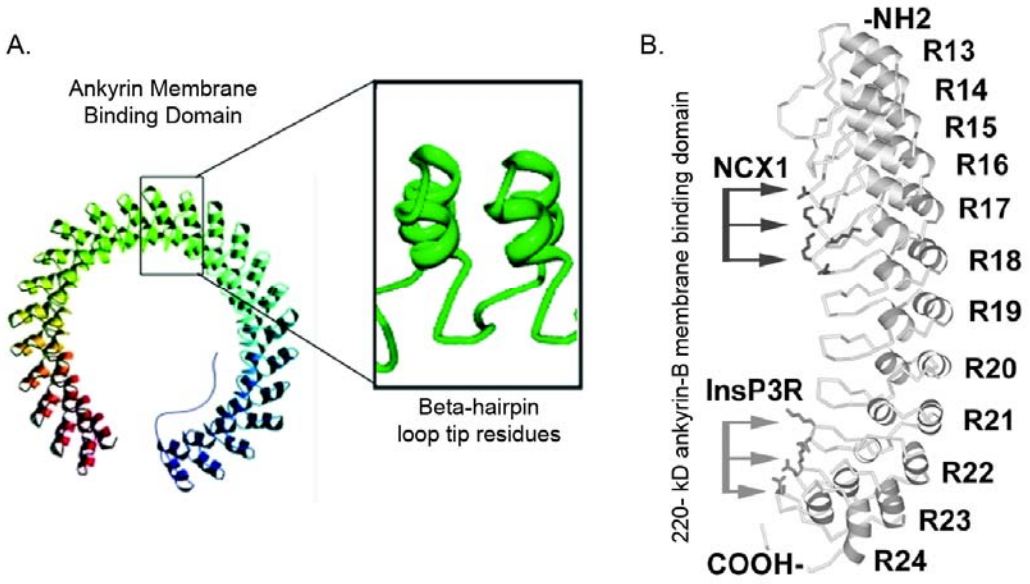

Figure 2. *ANK* repeats mediate interaction with a number of protein binding partners.

(A) 24 *ANK* repeats, divided into four subdomains of six repeats each, are folded into a solenoid structure. Each *ANK* repeat consists of two α-helices separated by a β-hairpin loop (blowup). Unique residues in the β-hairpin loops mediate specific associations with various proteins. (B) Distinct *ANK* repeats mediate interactions with binding partners. The NCX1 associates with ankyrin-B via *ANK* repeats 16-18, while the $InsP_3$ receptor associates with ankyrin-B via *ANK* repeats 22-24. Reprinted with permission from Elsevier Ltd: *Trends in Mol Med* 14(1):28-36, copyright 2008 (Figure 2A) (Bennett and Healy 2008). Reprinted with permission from the American Society for Biochemistry and Molecular Biology: *J Biol Chem* 282(7):4875-83, copyright 2007 (Figure 2B) (Cunha, Bhasin, et al. 2007).

spherocytosis is associated with mutations in ankyrin-R that result in reduced spectrin levels (Bodine, Birkenmeier et al. 1984; Hanspal, Yoon et al. 1991; Eber, Gonzalez et al. 1996). Additionally, there is a significant reduction in β_4-spectrin levels following disruption of ankyrin-G in cerebellar neurons (Jenkins and Bennett 2001), specifically at the axon initial segment. In the cardiomyocyte, it is the interaction between ankyrin-B and β_2-spectrin that is necessary for the precise localization of β_2-spectrin to the domain overlying the M-line (Mohler, Yoon et al. 2004). New and exciting insights into the role of ankyrin polypeptides in membrane biogenesis emerged with the use of small interfering RNA (siRNA) to knock-down ankyrin-G expression in human bronchial epithelial cells. Reduction of ankyrin-G expression results not only in a reduction in β_2-spectrin in the lateral membrane, but also in a complete loss of lateral membrane domains in general (Kizhatil and Bennett 2004), suggesting a necessary role for ankyrins not only in the targeting of ion channels to distinct domains, but also in the formation of entire membrane domains. Similar studies have also demonstrated that ankyrin-G is a critical link between β_2-spectrin and E-cadherin, a component of membranes necessary for lateral membrane formation during early development (Kizhatil, Davis et al. 2007). Specifically, it has been demonstrated that ankyrin-G and β_2-spectrin co-localize with E-cadherin in preimplantation mouse embryos, where knock-down of either ankyrin-G or β_2-spectrin blocks accumulation of E-cadherin at sites of cell-cell contact (Kizhatil, Davis et al. 2007). In addition to spectrin, the spectrin-binding domain associates with the protein phosphatase, PP2A (Bhasin, Cunha et al. 2007). Protein phosphatases

play critical roles in the regulation of a number of ion channels, implying an accessory role for ankyrin polypeptides in the regulation of these proteins via regulation of phosphorylation status. Finally, an ankyrin-dependent pathway is required for sarcolemmal localization of dystrophin and β-dystroglycan, as well as critical for the protection of skeletal muscle against exercise-induced injury and the maintenance of the postnatal neuromuscular junction (Ayalon, Davis et al. 2008). Interestingly, an identified Becker muscular dystrophy mutation (D3335N) was found to reduce ankyrin binding, impairing sarcolemmal localization of dystrophin-Dp71 (Ayalon, Davis et al. 2008). Moreover, ankyrin-B SBD and ankyrin-G SBD were also determined to bind dynactin-4, with loss of a subset of microtubules from sarcolemmal sites in ankyrin-B-depleted muscle (Ayalon, Davis et al. 2008).

The function of the 90 amino acid ankyrin ***death domain*** is currently unknown. While previously published studies have demonstrated a role for death domains in the association of homotypic and heterotypic proteins (Xiao, Towb et al. 1999), there is no evidence to suggest a similar role for the death domain of ankyrins, since ankyrins do not homo-dimerize via their death domains with any significant affinity (Abdi, Mohler et al. 2006). With other death domain-expressing proteins, such as Fas, the death domain is hypothesized to be involved in the apoptotic pathway. Interestingly, the death domain of ankyrin-G has been shown to interact with the death domain of FasL, promoting renal tubule cell death (Del Rio, Imam et al. 2004). Moreover, overexpression of the ankyrin-G death domain results in Fas-mediated apoptosis of renal tubule cells,

suggesting that ankyrins may function in the regulation of apoptosis. Human mutations in the death domain of ankyrin-B, which have been hypothesized to affect structure and/or stability of ankyrin polypeptides, have been identified. Such findings suggest that, while the death domain may be involved in the apoptotic events of certain cells types, it is very likely to be involved in pathways that are yet unknown.

The most divergent domain among ankyrins-R, -G, and –B is the ***C-terminal (regulatory) domain***, which has been shown to mediate the regulation of specific ankyrin functions in cells via intramolecular interactions. More specifically, the C-terminal regulatory domain of ankyrin-B has been demonstrated to interact with the first *ANK* repeat within the ankyrin-B MBD, allowing rescue of normal localization of the $InsP_3$ receptor in ankyrin-$B^{+/-}$ cardiomyocytes, even though this particular *ANK* repeat is not necessary for association with $InsP_3$ receptor (this association is mediated by *ANK* repeats 22-24; Figure 2B) (Abdi, Mohler et al. 2006). In humans, loss-of-function mutations within the C-terminal regulatory domain of ankyrin-B have been associated with cardiac arrhythmias (“ankyrin-B syndrome”), providing compelling evidence for the critical role of this domain in ankyrin polypeptide function. Studies have demonstrated that the C-terminal domain of ankyrin polypeptides is responsible for association with obscurin (Bagnato, Barone et al. 2003; Kontrogianni-Konstantopoulos, Jones et al. 2003; Cunha and Mohler 2008) and the molecular co-chaperone human DnaJ homolog 1, Hdj1/Hsp40 (Mohler, Hoffman et al. 2004).

Ankyrin Dysfunction and Abnormal Physiology

Mutations in ankyrin-R are associated with spherocytosis and hemolytic anemia. Ankyrins were originally identified in the erythrocyte membrane as a critical link between the actin/spectrin cytoskeleton and the integral membrane protein, the anion exchanger (Bennett and Stenbuck 1979). For years, ankyrins were considered simply scaffolding proteins, maintaining a direct connection between the plasma membrane and the underlying cytoskeleton. The spectrin-based lattice of the erythrocyte links the actin cytoskeleton to integral membrane proteins like the anion exchanger. Decreased expression and/or mutated forms of ankyrin-R have been associated with hemolytic anemia in humans (Eber, Gonzalez et al. 1996; Tse and Lux 1999). A spontaneous and similar mutation in mice, *nb/nb*, was originally shown to result in a complete loss of 210 kD ankyrin-R expression in murine erythrocytes, neurons, and striated muscle (White, Birkenmeier et al. 1990), with the phenotype of severe anemia (Bodine, Birkenmeier et al. 1984) and Purkinje neuron degeneration (Peters, Birkenmeier et al. 1991). Based on these findings, researchers were able to show that mutations of the *ANK1* gene in humans were associated with decreased stability of spectrin and the anion exchanger, resulting in spherocytosis and anemia (Peters, Birkenmeier et al. 1991). Interestingly, 50% of Caucasian hereditary spherocytosis cases can be attributed to mutations in ankyrin-R (Delaunay 2002). A much rarer form of anemia resulting from dysfunction in ankyrin-R is due to the absence of the Rh complex (Lopez, Metral et al. 2005). Specifically, the Rh complex requires association with ankyrin-R for

stable expression in the erythrocyte (Lopez, Metral et al. 2005). Loss of association results in features of spherocytosis: decreased erythrocyte membrane stability and anemia (Lopez, Metral et al. 2005).

Ankyrin-G and the biogenesis of membranes in cardiomyocytes and neurons. Ankyrin-G polypeptides are critical players in both the development and maintenance of membrane domains in neurons and cardiomyocytes. In the brain, ankyrin-G co-localizes with both Na_v channels and neurofascin (Kordeli, Lambert et al. 1995; Davis, Lambert et al. 1996). A cerebellar-specific knock-out of ankyrin-G results in a phenotype indicative of cerebellar dysfunction: decreased locomotion, abnormal gait, and significant tremor (Zhou, Lambert et al. 1998). Molecular work with this ankyrin-G mouse model has facilitated the determination of the role of ankyrin-G in the cerebellum: Na_v channel isoforms, neurofascin, and $K_v2.1/K_v3.1$ channels do not properly localize to specific membrane domains when ankyrin-G expression is disrupted (Zhou 1998; Jenkins 2001; Pan 2006). Specifically, loss of ankyrin-G results in reduced Na_v channel localization to the neuromuscular junction as well as a loss of neurofascin and $K_v2.1/K_v3.1$ channel localization to the nodes of Ranvier and the Purkinje cell axon initial segments (Ango, di Cristo et al. 2004; Pan, Kao et al. 2006) The results are abnormalities in cerebellar neuron action potentials, abnormal development of interneuron circuits, and reduced GABAergic synapse formation (Ango, di Cristo et al. 2004).

Likewise, a role for ankyrin-G in $Na_v1.5$ trafficking to excitable domains of the heart has been established. The importance of the $Na_v1.5$/ankyrin-G

interaction was established with the identification and characterization of the human E1053K mutation of $Na_v1.5$, which results in Brugada syndrome (Mohler, Rivolta et al. 2004). Brugada syndrome is characterized as a cardiac arrhythmia associated with an increased risk of sudden cardiac death (Tester and Ackerman 2008). Specifically, the E1053K mutation of $Na_v1.5$ blocks association with ankyrin-G, leading to accumulation of $Na_v1.5$ E1053K in intracellular compartments and ultimately decreased expression of $Na_v1.5$ at the intercalated disc and T-tubule membranes in ventricular cardiomyocytes (Mohler, Rivolta et al. 2004). Interestingly, the cellular phenotype of E1053K is not solely due to retention of mis-folded intermediates in the ER/Golgi. Using HEK293 cells, Lowe and colleagues showed that $Na_v1.5$ E1053K was able to traffic efficiently to the membrane surface; however, these channels demonstrated a decreased threshold for voltage activation (Lowe, Palygin et al. 2008). Therefore, ankyrin-G appears to play roles not only in the targeting of $Na_v1.5$, but also in gating $Na_v1.5$ once at the cell membrane surface (Mohler, Rivolta et al. 2004).

Dysfunction in ankyrin-B and human arrhythmia. Ankyrin-B is necessary for the establishment and maintenance of excitable membrane domains in the brain and striated muscle. Anatomical examinations of mice that are homozygous for a null mutation in ankyrin-B have revealed a number of neurological defects: hypoplasia of the corpus callosum and pyramidal tracts, dilation of the lateral ventricles, and degeneration of long axon tracts (Scotland, Zhou et al. 1998). Since these defects are also associated with a loss of specific cell adhesion molecules throughout the brain, the role of ankyrin-B in normal

vertebrate nervous system function has been firmly established. In addition to the neurological phenotype, ankyrin-B null neonatal mice also display thymic atrophy, pronounced kyphosis, winged scapula, occasional sarcomere disorganization, and elevations in plasma creatinine kinase levels, indicative of muscle damage (Tuvia, Buhusi et al. 1999).

Perhaps the greatest advances in ankyrin research focused on the link between ankyrin-B dysfunction and cardiac arrhythmia. Neonatal cardiomyocytes isolated from both ankyrin-B null and ankyrin-$B^{+/-}$ mice have been reported to display abnormal Ca^{2+} dynamics, abnormal localization of ankyrin-associated proteins (i.e. $InsP_3$ receptor, NCX1, and NKA), and cellular after-depolarizations (Mohler, Gramolini et al. 2002; Mohler, Schott et al. 2003). As a result, ankyrin-$B^{+/-}$ mice exhibit bradycardia, variable heart rate, stress- and exercise-induced polymorphic ventricular arrhythmia, and an increased incidence of sudden cardiac death (Mohler, Schott et al. 2003). In 1995, Schott and colleagues identified a single ankyrin-B variant, E1425G, in a large French kindred that displayed an atypical cardiac arrhythmia: bradycardia, abnormal heart rate, and stress-induced sudden cardiac death (Schott, Charpentier et al. 1995; Mohler, Schott et al. 2003). Using the molecular techniques that had established the basis of cardiac dysfunction in mice, Mohler et al. proved that the E1425G ankyrin-B variant was a loss-of-function mutation that resulted in cardiac arrhythmia and sudden cardiac death (Mohler, Schott et al. 2003). Since the identification of the French E1425G probands, additional probands have been identified (T1404I, V1506G, T1552N, V1777M, R1788W, and E1813K) that

display cardiac arrhythmia, severe sinus bradycardia, idiopathic ventricular fibrillation, polyphasic T waves, and atrial fibrillation (Mohler, Splawski et al. 2004; Mohler, Le Scouarnec et al. 2007; Le Scouarnec, Bhasin et al. 2008) .

CHAPTER III

THE K_{ATP} CHANNEL: THE METABOLIC SENSOR OF EXCITABLE CELLS

Introduction

K_{ATP} channels play critical roles in many cellular functions by coupling cell metabolic status to electrical activity. First discovered in cardiomyocytes (Noma 1983), K_{ATP} channels have since been found in many other tissues and subcellular organelles, including pancreatic beta cells {Ashcroft, 1984 #61;Cook, 1984 #62;Findlay, 1985 #63;Rorsman, 1985 #64}, skeletal muscle (Spruce, Standen et al. 1985), smooth muscle (Standen, Quayle et al. 1989), brain (Ashford, Sturgess et al. 1988), pituitary (Bernardi, De Weille et al. 1993), kidney (Hunter and Giebisch 1988), and in the mitochondria (Inoue, Nagase et al. 1991). By linking cellular metabolic state with membrane potential, K_{ATP} channels are able to regulate a number of cellular functions: hormone secretion, cytoprotection, vascular tone, and the excitability of both neurons and skeletal muscle (Figure 3). Specifically, a reduction in metabolism causes a decrease in the ATP:ADP ratio, opening K_{ATP} channels and allowing K^+ efflux, membrane hyperpolarization, and suppression of electrical activity. Conversely, increased cellular metabolism causes a decrease in the ATP:ADP ratio and results in closure of the K_{ATP} channel, membrane depolarization, and the stimulation of cell electrical activity.

Numerous studies using not only isolated cells and tissues, but also genetically-modified mice or patients with mutations in K_{ATP} channel genes, have demonstrated that K_{ATP} channels play a wide range of physiological roles (Seino and Miki 2004). Their contribution to glucose homeostasis is well-documented with regard to K_{ATP}-dependent regulation of insulin secretion by beta cells (Ashcroft, Harrison et al. 1984), glucagon secretion from pancreatic alpha cells (Gopel, Kanno et al. 2000), somatostatin secretion from pancreatic delta cells (Gopel, Kanno et al. 2000), and glucagon-like peptide 1 (GLP-1) secretion from L-cells (Gribble, Williams et al. 2003). Moreover, K_{ATP} channels in ventromedial hypothalamic neurons mediate the counter-regulatory response to glucose (Miki, Liss et al. 2001), while K_{ATP} channels in the arcuate nucleus are hypothesized to play a role in appetite regulation (Wang, Liu et al. 2004). Interestingly, these glucose-sensing cell types express K_{ATP} channels that are open under resting conditions. In other tissues, however, K_{ATP} channels are closed under physiological conditions and open only in response to ischemia, neurotransmitters, or hormonal stimulation (Figure 3). For example, in cardiac muscle and central neurons, opening of K_{ATP} channels results in the reduction in electrical activity which protects against cardiac stress and seizures (Hernandez-Sanchez, Basile et al. 2001; Yamada, Ji et al. 2001; Suzuki, Sasaki et al. 2002; Zingman, Hodgson et al. 2002; Heron-Milhavet, Xue-Jun et al. 2004). Moreover, K_{ATP} channels are involved in the phenomenon of ischemic preconditioning in the heart (Gumina, Pucar et al. 2003) and in the regulation of vascular smooth muscle tone (Daut, Klieber et al. 1994; Chutkow, Pu et al. 2002; Miki, Suzuki et

al. 2002). Additionally, K_{ATP} channels modulate electrical activity and synaptic neurotransmitter release in the hippocampus, substantia nigra, and the hypothalamus (Amoroso, Schmid-Antomarchi et al. 1990; Schmid-Antomarchi, Amoroso et al. 1990; Liss, Bruns et al. 1999; Zawar, Plant et al. 1999; Griesemer, Zawar et al. 2002; Avshalumov and Rice 2003; Wang, Liu et al. 2004).

Protein Architecture and Regulation of K_{ATP} Channel Function

The K_{ATP} channel is composed of two dissimilar subunits: an inwardly rectifying K^+ channel subunit (Kir6) and a regulatory subunit, SUR. Both subunits are required to form a functional K_{ATP} channel and co-assemble in an obligate 4:4 stoichiometry to form a hetero-octameric channel (Figure 4A) (Aguilar-Bryan and Bryan 1999). Kir6, the pore-forming subunit, belongs to the inwardly rectifying potassium channel family. Inward rectifiers are so named because of their preferential conduction of ions in an inward direction when compared to comparable positive and negative membrane potentials with symmetrical K^+ distribution (Doupnik, Davidson et al. 1995). Beta cells maintain a membrane potential that is positive to the potassium equilibrium potential (E_K) so that K^+ currents are mainly outward, and subject to physiological modulation by rectification (Doupnik, Davidson et al. 1995). Two different Kir6 gene products have been described, Kir6.1 and Kir6.2, which share roughly 70% homology at the amino acid level (Inagaki, Gonoi et al. 1995; Inagaki, Tsuura et al. 1995; Sakura, Ammala et al. 1995). While *KCNJ8* (encoding Kir6.1) is located at

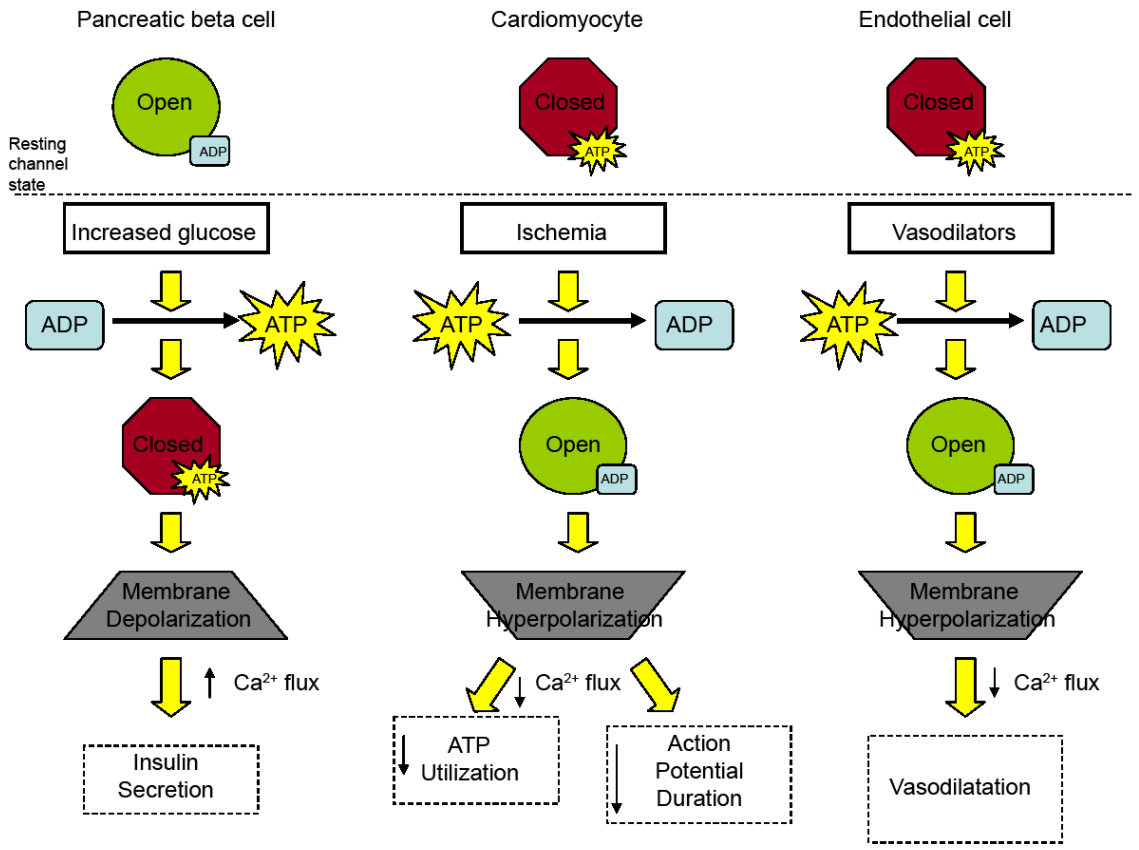

Figure 3. K_{ATP} channels in physiology.

In the resting state, pancreatic beta cell K_{ATP} channels are open, keeping the membrane hyperpolarized. Stimulation via increased plasma glucose results in K_{ATP} channel closure, membrane depolarization, and insulin secretion. In contrast, K_{ATP} channels in cardiomyocytes and endothelial cells are closed in the resting state, opening in response to ischemia and vasodilators, respectively. The opening of the K_{ATP} channels in these cells results in membrane hyperpolarization and either a decrease in the action potential duration of the cardiomyocyte or vasodilatation in the endothelial cell. Adapted from

chromosome position 12p11.23 (Inagaki, Inazawa et al. 1995), *KCNJ11* (encoding Kir6.2) is located at chromosome position 11p15.1 (Inagaki, Gonoi et al. 1995). Both Kir6.1 and Kir6.2 are organized into two transmembrane helical domains, separated by a pore loop (P loop), with cytosolic N- and C-terminal domains (Figure 4B).

The regulatory subunit of the K_{ATP} channel, SUR, is a member of the ATP binding cassette (ABC) transporter superfamily, which also includes the CFTR and the multi-drug resistance proteins (MRPs). SUR proteins were originally identified as components of the K_{ATP} channel as a result of the serendipitous observation that the administration of sulfonamides and sulfonylureas (SUs), such as tolbutamide and glibenclamide, resulted in hypoglycemia (Loubatieres 1957). Two closely related genes encoding the sulfonylurea receptors SUR1 (*ABCC8*) and SUR2 (*ABCC9*) have been cloned and characterized. The SUR1 gene is located immediately upstream of Kir6.2 gene at chromosome 11p15.1 (Thomas, Cote et al. 1995), while the SUR2 gene is located upstream of the Kir6.1 gene at chromosome position 12p12.1 (Chutkow, Simon et al. 1996). SUR2 has two major splice forms, SUR2A and SUR2B. Similar to other members of the ABC subfamily C, SUR1 and SUR2 each contain two six-helix transmembrane domains (TMD1 and TMD2) (Figure 4C). Additionally, SUR polypeptides have an N-terminal TMD0 domain consisting of five transmembrane helices (Figure 4C), unlike other members of the ABC superfamily. SUR also contains two nucleotide binding folds: NBF1, which is between TMD1 and TMD2 and NBF2, which is after TMD2 (Figure 4C). The various Kir6 and SUR

subunits are able to assemble in multiple variations to form K_{ATP} channels with different pharmacological (specifically, glibenclamide, a K_{ATP} channel closer and diazoxide, a K_{ATP} channel closer) and nucleotide sensitivities (Aguilar-Bryan, Nichols et al. 1995; Inagaki, Gonoi et al. 1995; Sakura, Ammala et al. 1995; Chutkow, Simon et al. 1996; Inagaki, Gonoi et al. 1996; Isomoto, Kondo et al. 1996) (Table 1). Comparison of the properties of cloned and native K_{ATP} channels demonstrates that the pancreatic beta cell K_{ATP} channel is composed of Kir6.2 and SUR1, the cardiac type is composed of Kir6.2 and SUR2A, and the smooth muscle type from Kir6.2 and SUR2B, although K_{ATP} channels composed of Kir6.1 and SUR2B have also been identified (Aguilar-Bryan, Nichols et al. 1995; Inagaki, Gonoi et al. 1995; Sakura, Ammala et al. 1995; Chutkow, Simon et al. 1996; Inagaki, Gonoi et al. 1996; Isomoto, Kondo et al. 1996) (Table 1). Kir6.2 and SUR1 are also co-expressed in various brain neurons and are hypothesized to form K_{ATP} channels isolated from these tissues (Karschin, Ecke et al. 1997).

K_{ATP} channels are regulated by numerous cytosolic factors. Perhaps the most studied factors responsible for K_{ATP} channel regulation are adenosine nucleotides, which interact with two different sites on the channel, one inhibitory and the other stimulatory. Increased entry and glycolysis of glucose results in changes in cytosolic concentrations of adenosine nucleotides such as ATP and adenosine diphosphate (ADP). Both sites can be distinguished experimentally, considering that nucleotide binding to the stimulatory site requires Mg^{2+} (Nichols, Shyng et al. 1996). Therefore, in the absence of Mg^{2+}, only the inhibitory effect is

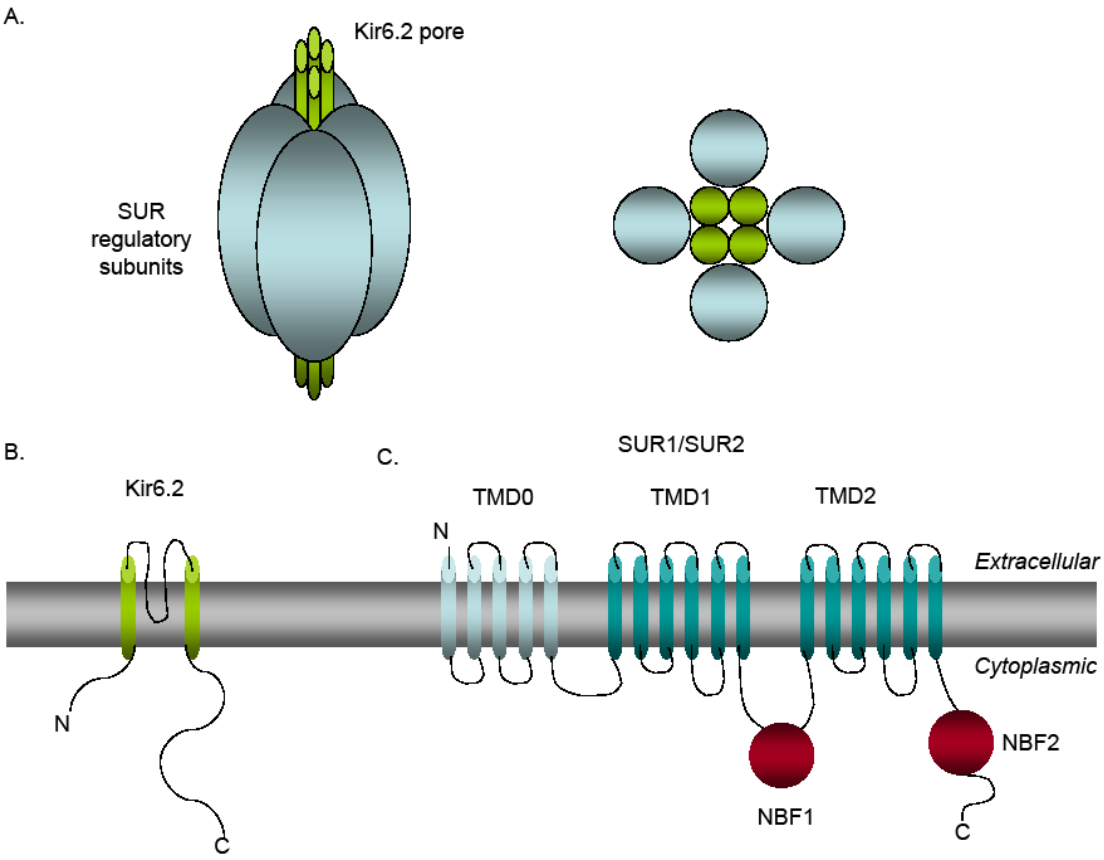

Figure 4. Stoichiometry and domain organization of the K_{ATP} channel.

(A) The K_{ATP} channel is 4:4 hetero-octamer of pore-forming Kir6.2 subunits (green) and regulatory SUR subunits (blue). (B) Kir6.2 is organized into N-terminal and C-terminal cytoplasmic domains and two transmembrane domains (signified in green) with an intervening P-loop. (C) SUR isoforms are comprised of 17 transmembrane domains, organized into three subdomains: TMD0 (light blue; comprised of five transmembrane domains) and TMD1 and TMD2 (dark blue; both of which are comprised of six transmembrane domains). Between TMD1 and TMD2 is the first of two NBFs, NBF1). The second NBF, NBF2, is located after TMD2.

Table 1. Combinations of Kir6 and SUR subunits in distinct tissues with relative sensitivities to ATP, sulfonylureas, and potassium channel openers.

		Properties		
Type of K_{ATP} channel	Subunit combination	ATP sensitivity	Glibenclamide sensitivity	Diazoxide sensitivity
Pancreatic beta cell	SUR1 + Kir6.2	(+) High	(+) High	(+)
Cardiac/skeletal muscle	SUR2A + Kir6.2	(+) Low	(+) Low	(-)
Smooth muscle	SUR2B + Kir6.2	(+) Low	(+)	(+)
Vascular smooth muscle	SUR2B + Kir6.1	(-)	(+)	unknown

observed. Within the cell, however, Mg^{2+} is always present; therefore, channel activity must be determined by the ratio of ATP to ADP. Studies have demonstrated that the binding site for channel inhibition by ATP resides on Kir6.2 (Craig, Ashcroft et al. 2008), where binding of ATP has been shown to stabilize the closed state of the channel (Enkvetchakul and Nichols 2003). On the other hand, in the presence of Mg^{2+}, both MgATP and MgADP stimulate K_{ATP} channel activity via binding to the NBFs of SUR (Nichols, Shyng et al. 1996). It is hypothesized that the balance between activation by the Kir6.2 subunit and inhibition by the SUR subunit in response to nucleotide binding determines the overall level of K_{ATP} channel activity.

K_{ATP} Channel Regulation by the Cytoskeleton

Several elegant experiments have demonstrated a clear role for the actin-based cytoskeleton in the regulation of K_{ATP} channel function (Brady, Alekseev et al. 1996; Furukawa, Yamane et al. 1996; Terzic and Kurachi 1996; Yokoshiki, Katsube et al. 1997). For well over a decade, the K_{ATP} field has struggled to identify the "missing link" between the K_{ATP} channel and the cytoskeleton (Brady, Alekseev et al. 1996; Furukawa, Yamane et al. 1996; Terzic and Kurachi 1996; Yokoshiki, Katsube et al. 1997). The presence of this critical "adapter" was first proposed by Terzic and colleagues (Terzic and Kurachi 1996) in the mid 1990's, based on a series of experiments that demonstrated a striking stimulatory effect of actin filament disrupting agents on K_{ATP} channel opening (Brady, Alekseev et al. 1996), and reproduced by multiple groups. Specifically, it was observed that

disruption of actin cytoskeleton using cytochalasin (or DNAase I) resulted in the activation of K_{ATP} channels in excitable cells (Brady, Alekseev et al. 1996; Furukawa, Yamane et al. 1996; Terzic and Kurachi 1996; Yokoshiki, Katsube et al. 1997). Moreover, work from multiple groups demonstrated that disruption of the actin cytoskeleton resulted in a gain-of-function phenotype by reducing the sensitivity of K_{ATP} channels for ATP-dependent channel closure (Terzic and Kurachi 1996). Additionally, cytoskeletal disruption resulted in reduced K_{ATP} channel sensitivity to closure by SU compounds (Terzic and Kurachi 1996). Thus, the coupled impairment in SU and ATP sensitivities indicates a common transduction pathway of inhibitory gating signals that work in a manner dependent on the integrity of the cytoskeletal network. Based on the role of ankyrins and spectrin in other cell types, it is possible that the ankyrin/spectrin cytoskeletal complex may perform this role for the K_{ATP} channel.

K_{ATP} Channel Trafficking

Considering that changes in channel copy number at the cell plasma membrane can significantly alter the electrical properties of a cell, mechanisms to control channel numbers at the plasma membrane are critical for cellular function (Ma and Jan 2002). Uncontrolled K_{ATP} channel activity, for example, can have deleterious results. Excessive K_{ATP} channel opening in the heart leads to a shortening of the action potential duration and a loss of cellular K^+, which can result in arrhythmia (Janse and Wit 1989; Wilde 1993), while excessive K_{ATP}

channel activity in the brain produces hyperpolarization and reduced excitability, which results in neuron silencing (Yamada and Inagaki 2002). Therefore, regulation of K_{ATP} channel plasma membrane expression under stress is necessary to confer neuronal and cardiac protection without incurring serious damage. As such, Jan and colleagues demonstrated a protein-kinase C-mediated pathway for K_{ATP} channel downregulation via dynamin-dependent endocytosis. The resulting downregulation acts as a brake to lessen the shortening of the action potential duration in cardiomyocytes, as well as the rapid decline of excitability in neurons (Hu, Huang et al. 2003).

Unfortunately, there are little data which fully address the cellular events necessary to regulate K_{ATP} channel targeting and retention. The observation that neither SUR1 nor Kir6.2 subunits are capable of channel activity when expressed alone (Aguilar-Bryan, Nichols et al. 1995; Inagaki, Gonoi et al. 1995; Tucker, Gribble et al. 1997) triggered a number of studies on K_{ATP} channel formation (John, Monck et al. 1998; Makhina and Nichols 1998; Zerangue, Schwappach et al. 1999). Tucker et al. demonstrated that Kir6.2 can be forced to express channel activity independently of SUR1 by truncation of the distal 26-36 residues of Kir6.2 (Tucker, Gribble et al. 1997). Using this truncated construct in combination with a sensitive assay to detect surface expression of K_{ATP} channels in the *Xenopus* oocyte model, an ER localization signal (RKR) was mapped in both the Kir6.2 and SUR1 proteins (Zerangue, Schwappach et al. 1999). In K_{ATP} channels, it is believed that assembly of Kir6.2 and SUR1 subunits in an obligate 4:4 stoichiometry masks the ER localization signals and allows the fully

assembled K_{ATP} channel to exit the ER (Zerangue, Schwappach et al. 1999). However, it has also been demonstrated that Kir6.2 and SUR1 are able to traffic independently of each other, suggesting that other mechanisms may be present that are necessary for K_{ATP} assembly and trafficking (Makhina and Nichols 1998). More recently, the coatamer I (COPI) vesicle coat complex has been shown to specifically recognize the RKR signal present in the Kir6.2 distal tail (Yuan, Michelsen et al. 2003). Additionally, it was revealed that 14-3-3 proteins can also recognize this signal, dependent upon how many copies are in close proximity. Using reporter membrane proteins, a model which implicates dimeric 14-3-3 proteins as sensors for multimeric assembly was proposed (Yuan, Michelsen et al. 2003). Specifically, this model hypothesizes that the COPI vesicle coat recognizes the RKR signal exposed by unassembled subunits or partially assembled complexes, resulting in the retrieval of these proteins to the ER. Once the peptide trafficking signals come into close proximity, 14-3-3 proteins recognize them with high avidity and shield them from recognition by the COPI coat. It remains to be tested whether 14-3-3 proteins are involved in the assembly-dependent trafficking of the K_{ATP} channels. Nevertheless, these studies provide important information that will be useful in any research addressing the role of specific proteins (chaperones, adaptor proteins, or regulatory molecules) in the assembly and trafficking of K_{ATP} channels.

Beta Cell K_{ATP} Channels and Glucose-stimulated Insulin Secretion

The primary physiological function of the beta cell is to sense changes in circulating glucose levels and to respond with an appropriate insulin secretory response. The significance of this function is of profound importance to health because its failure disrupts normal blood glucose homeostasis and results in insulin secretory abnormalities such as diabetes mellitus and hyperinsulinemia (Gloyn, Siddiqui et al. 2006). Although K_{ATP} channels were initially identified in the heart (Noma 1983), their physiological importance was first established in the pancreatic beta cell. In the beta cell, the K_{ATP} channel couples membrane excitability with glucose-stimulated insulin secretion (Ashcroft and Rorsman 1990). According to this paradigm, during conditions of low plasma glucose (fasting conditions), K_{ATP} channels are the dominant beta cell membrane conductance, maintaining the cell in a hyperpolarized state (approximately -70 mV) (Figure 5A). Conversely, following the ingestion of a meal, glucose enters the beta cell and is rapidly converted to ATP. The increased production of ATP gives rise to an increase in the cytoplasmic [ATP]/[ADP] ratio, ATP-dependent closure of the K_{ATP} channel, membrane depolarization, and voltage-dependent Ca^{2+} entry via L-type calcium channels. Calcium influx, in turn, triggers the mobilization, fusion, and exocytosis of insulin granules (Figure 5B). The rise in circulating insulin, in turn, results in increased glucose uptake by the peripheral tissues and a compensatory drop in blood glucose.

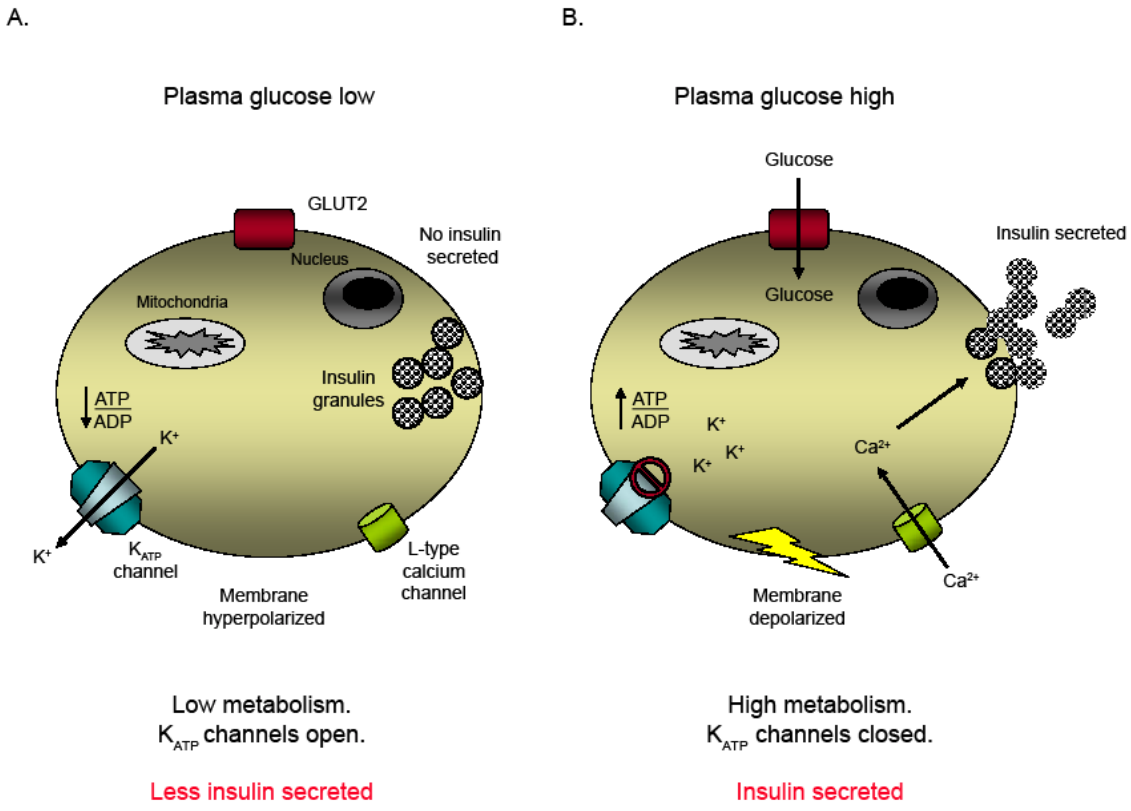

Figure 5. The beta cell K_{ATP} channel couples glucose metabolism to insulin secretion.

(A) Under conditions of low plasma glucose, the K_{ATP} channel is open, keeping the beta cell membrane hyperpolarized, resulting in negligible insulin secretion. (B) Increased cellular metabolism, in response to increased plasma glucose levels, results in an increase in intracellular ATP, ATP-dependent closure of the K_{ATP} channel, membrane depolarization, and the activation of L-type calcium channels. The increase in intracellular Ca^{2+} triggers insulin granule mobilization, fusion, and exocytosis. Adapted from Ashcroft, *J Clin Invest.* 2005 115(8):2047-58 (Ashcroft 2005).

K_{ATP} channels also serve as the main target for pharmacological regulation of insulin secretion. Sulfonylurea drugs (SUs; such as glibenclamide and gliclazide), which are widely used to treat type 2 diabetes, stimulate insulin secretion by closing K_{ATP} channels (Gribble and Reimann 2003). Specifically, SUs bind to unique areas on the regulatory SUR subunits, causing the K_{ATP} channel to close. Thus, SUs by-pass beta cell metabolism, but stimulate the same intracellular events as glucose. In contrast, a structurally diverse group of drugs, the K^+ channel openers (KCOs), open K_{ATP} channels, thereby inhibiting insulin secretion (Ashcroft and Gribble 2000).

K_{ATP} Channel Genes and Insulin Secretion Disorders

Electrical activity of the beta cell membrane is essential for insulin secretion. Specifically, this electrical activity is in the form of Ca^{2+}-dependent action potentials: no insulin secretion in the absence of Ca^{2+}, with the extent of insulin secretion correlated directly to that of electrical activity (Henquin 2000). The K_{ATP} channel not only initiates electrical activity, but also regulates its extent at stimulatory glucose concentrations. This is due to the fact that the K_{ATP} channel controls the resting potential of the pancreatic beta cell (Tarasov, Welters et al. 2006). Considering their critical role in regulating electrical excitability in a number of cell types, it is apparent that disruption of K_{ATP} channel function can lead to disease. Mutations in K_{ATP} channel genes have been associated with neonatal diabetes (Gloyn, Pearson et al. 2004), hyperinsulinemia

(Dunne, Cosgrove et al. 2004), and dilated cardiomyopathy (Bienengraeber, Olson et al. 2004).

Alterations in metabolic signal, in the responsiveness of the K_{ATP} channel to metabolites, or in the number of active K_{ATP} channels at the beta cell membrane surface can lead to altered release of insulin. Specifically, increased metabolic flux, increased sensitivity to inhibitory nucleotides, or reduced density of K_{ATP} channels result in low K_{ATP} activity and, therefore, hyperinsulinemia (a loss-of-function phenotype). On the other hand, decreased metabolic flux, decreased sensitivity to inhibitory nucleotides, or increased density of K_{ATP} channels results in high K_{ATP} channel activity and hypoinsulinemia (a gain-of-function phenotype). These principles underlie the development of congenital hyperinsulinemia of infancy (CHI) and neonatal diabetes mellitus (NDM), respectively (Figure 6).

***Congenital hyperinsulinemia of infancy*.** CHI is characterized by continuous and unregulated insulin secretion in the presence of severe hypoglycemia (Dunne, Cosgrove et al. 2004). Without treatment, persistent hypoglycemia may result in irreversible brain damage. Loss-of-function mutations in SUR1 (*ABCC8*) are the most common cause of CHI, accounting for nearly 50% of diagnosed cases (Glaser, Thornton et al. 2000; Dunne, Cosgrove et al. 2004). There are over 100 CHI mutations in SUR1 (Dunne, Cosgrove et al. 2004; Gloyn, Siddiqui et al. 2006), comprising two functional classes: those that result in no channel protein present at the surface membrane (class I) and those where the channel, though present at the surface membrane, is always closed

(class II). Mechanistically, class I mutations are described by loss of K_{ATP} channels in the plasma membrane, resulting from impaired SUR1 synthesis, abnormal SUR1 maturation, defective channel assembly, or dysfunctional surface membrane trafficking (Partridge, Beech et al. 2001; Taschenberger, Mougey et al. 2002; Dunne, Cosgrove et al. 2004; Yan, Lin et al. 2004) (Figure 6B). On the other hand, class II mutations result in an inability of MgADP to stimulate channel activity {Dunne, 2004 #100;Huopio, 2000 #104;Nichols, 1996 #105}. As such, ATP inhibition becomes dominant and the K_{ATP} channel is closed even at low glucose concentrations. There is no genotype-phenotype correlation regarding K_{ATP} channel mutations with differing degrees of severity presenting in different individuals.

Mutations in Kir6.2 (*KCNJ11*) resulting in CHI are much rarer than SUR1. Similarly, Kir6.2 mutations result in the reduction/abolishment of K_{ATP} channel activity at the plasma membrane. Because the K_{ATP} channel maintains the beta cell membrane potential, decreased channel activity produces a persistent membrane depolarization, resulting in continuous insulin secretion (Figure 6B)

Often CHI is associated with neurological abnormalities; however, it is difficult to ascertain whether such abnormalities result from hypoglycemia or are a direct consequence of the mutation *per se*. Since K_{ATP} channels are normally closed at rest in many extrapancreatic tissues, it is hypothesized that loss-of-function mutations have little effect outside of the beta cell. The majority of individuals with CHI undergo a sub-total pancreatectomy as infants to control the

hyperinsulinemia; whereas, non-surgically treated patients sometimes progress to glucose intolerance or diabetes (Huopio, Otonkoski et al. 2003).

***Neonatal Diabetes Mellitus*.** Neonatal diabetes mellitus is characterized by hyperglycemia developing within the first six months of life with lifelong dependence upon insulin therapy (Edghill, Gloyn et al. 2004). Transgenic mice with overactive K_{ATP} channels are diabetic from birth, suggesting that overactive K_{ATP} channels may be a cause of neonatal diabetes (Koster, Marshall et al. 2000). Such gain-of-function (activating) mutations would make the channel more open under conditions of high plasma glucose (Figure 6A). This would result, hypothetically, in plasma membrane hyperpolarization and impairment of insulin secretion, resulting in neonatal diabetes (Figure 6A). In 2004, the first mutations in Kir6.2 were reported, confirming previous hypotheses regarding the role of Kir6.2 dysfunction in the development of neonatal diabetes (Gloyn, Pearson et al. 2004). To date, over 30 mutations in Kir6.2 are associated with neonatal diabetes (Hattersley and Ashcroft 2005; Flanagan, Patch et al. 2007). An in-depth analysis of these mutations revealed that neonatal diabetes due to mutations in the *KCNJ11* gene represent a rare disease, affecting approximately 1 in 200,000 live births (Edghill, Gloyn et al. 2004). Furthermore, all Kir6.2 mutations identified to date are heterozygous (Babenko, Polak et al. 2006; Ellard, Flanagan et al. 2007) and gain-of-function (Proks, Antcliff et al. 2004; Ashcroft 2005; Hattersley and Ashcroft 2005; Proks, Girard et al. 2005; Girard, Shimomura et al. 2006; Proks, Girard et al. 2006; Shimomura, Girard et al. 2006; Masia, Koster et al. 2007; Shimomura, Horster et al. 2007).

Neonatal diabetes patients with K_{ATP} channel mutations can be categorized into four groups, depending on severity. One group has transient neonatal diabetes mellitus (TNDM) which remits and enables the discontinuance of insulin treatment. Another group has permanent neonatal diabetes mellitus (PNDM) which does not remit and requires insulin therapy throughout life. PNDM and TNDM result from the most common mutations. Some mutations, however, cause more severe phenotypes and are categorized in one of the final two groups. The most severe phenotype is known as DEND syndrome (developmental delay, epilepsy, and neonatal diabetes) (Hattersley and Ashcroft 2005). Some patients, however, have intermediate DEND (iDEND) which manifests with various degrees of motor weakness and also delays in social activities (Hattersley and Ashcroft 2005). The severity of disease is dictated by both the tetrameric organization of the channel as well as the functional consequence of the mutation (Ashcroft 2005) (Figure 7). Mutations that disrupt ATP binding usually cause only neonatal diabetes. This is because binding of just one ATP is sufficient to shut the pore (Markworth, Schwanstecher et al. 2000). Because mutant and wild type subunits associate randomly, in the heterozygous state only one-sixteenth of the channels will have a severe reduction in ATP sensitivity (Figure 7). In contrast, the more severe DEND mutations usually result in a stabilization of the intrinsic open state of the channel, reducing ATP block indirectly (Ashcroft and Rorsman 2004). Because each subunit contributes to gating of the tetrameric pore, heteromeric channels containing both wild type and mutant subunits will be affected (that is 15 out of 16

channels in heterozygotes; Figure 7). Consequently, DEND mutations produce a greater reduction in ATP sensitivity and a more severe disease phenotype. Thus, Kir6.2 mutations illustrate how the tetrameric nature of the K_{ATP} channel pore can result in a spectrum of phenotypes.

Mouse Models of K_{ATP} Channel Mutations Recapitulate Hyperinsulinemia or Neonatal Diabetes

Gain-of-function K_{ATP} channel mutations associated with severe neonatal diabetes in mice (Koster, Marshall et al. 2000) were characterized before it was shown in humans (Gloyn, Pearson et al. 2004). Because the mutations could be targeted to the beta cell, extrapancreatic phenotypes (similar to those seen in human patients) were absent. While no change in insulin content or beta cell number was observed, serum insulin levels were critically low, due to the reduced ATP sensitivity of the mutant K_{ATP} channel introduced into the transgenic mice (Koster, Marshall et al. 2000). Moreover, when this mutant transgene was introduced to the heart, there were no obvious cardiac symptoms (Koster, Knopp et al. 2001), as was seen in patients with gain-of-function K_{ATP} channel mutations (Gloyn, Pearson et al. 2004; Ashcroft 2005). Interestingly, mice that expressed the mutant transgene at lower levels demonstrated impaired glucose tolerance rather than diabetes (Koster, Remedi et al. 2006). Therefore, similar to humans, mice exhibit a spectrum of disease phenotypes which correlate to the extent of K_{ATP} channel activity. A study by Nichols et al. demonstrated that mice heterozygous for Kir6.2 in beta cells demonstrated hyperinsulinemia, in contrast to the prediction that a reduction in Kir6.2 expression would result in a decrease

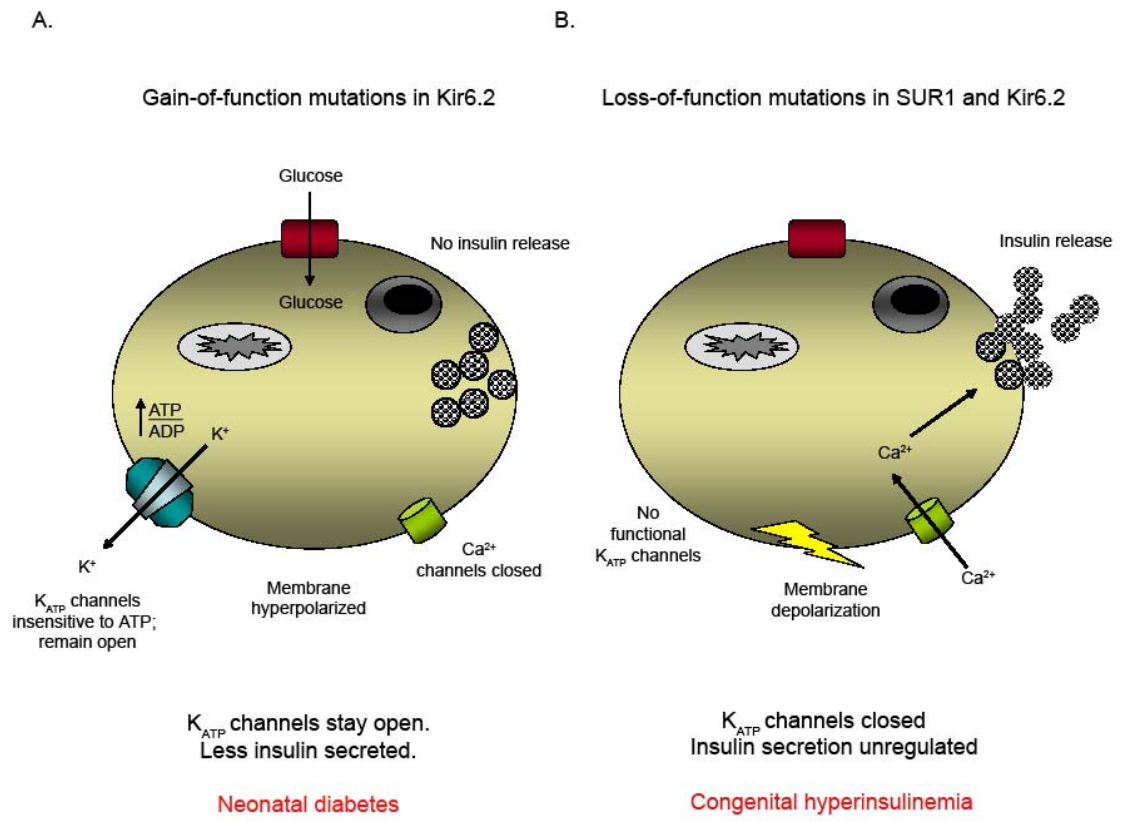

Figure 6. Mutations in K_{ATP} channel genes result in abnormal insulin release.

(A) Gain-of-function mutations in Kir6.2 result in a K_{ATP} channel that is insensitive to ATP; therefore, K_{ATP} channels remain open, even in the presence of high intracellular ATP. The result is hypoinsulinemia and neonatal diabetes. (B) In contrast, loss-of-function mutations in either Kir6.2 or SUR1 result in no functional K_{ATP} channels at the plasma membrane. The result is continual beta cell membrane depolarization and insulin release. This produces congenital hyperinsulinemia. Adapted from Ashcroft, *J Clin Invest*. 2005 115(8):2047-58 (Ashcroft 2005).

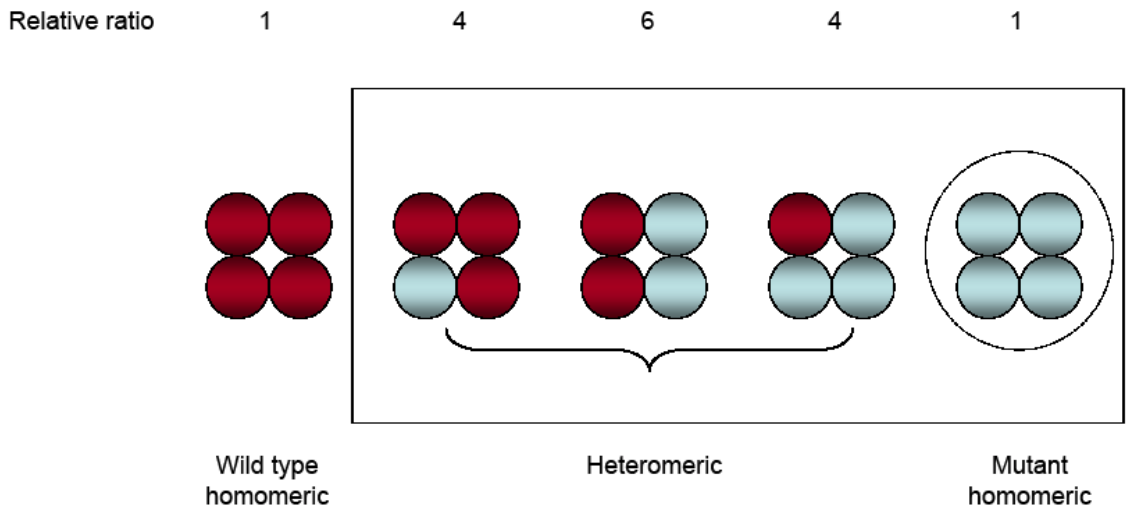

Figure 7. Severity of disease is dictated by both the tetrameric nature of Kir6.2 and the functional consequence of the mutation.

Mutations that disrupt Kir6.2 ATP binding usually cause only neonatal diabetes. This is because binding of just one ATP to the channel is sufficient to close the channel. Because mutant and wild type subunits associate randomly, in the heterozygous state, only one-sixteenth of the channels will have strongly reduced ATP sensitivity (circled). In contrast, the more severe DEND mutations usually stabilize the intrinsic open state of the channel, reducing ATP block indirectly. Because each subunit contributes to gating of the tetrameric pore, heteromeric channels containing both wild type and mutant subunits will be affected (15 out of 16 channels). Consequently, DEND mutations produce a greater reduction in ATP sensitivity and more severe disease. Adapted from Ashcroft, *Nature*. 2006 440(7083):440-7 (Ashcroft 2006).

in insulin secretion (Remedi, Rocheleau et al. 2006). From this study, Nichols proposed an inverse-U model of insulin secretion versus islet excitability. According to Nichols' model, insulin secretion by wild type islets represents the normal secretory responsiveness to glucose. Heterozygous Kir6.2 islets demonstrate hyperinsulinemia, placing this mouse model of decreased channel activity on the "ascending" limb of the curve. Finally, Kir6.2 null islets demonstrate maximally enhanced excitability, demonstrating an undersecretory phenotype, placing them on the "descending" limb of the inverse-U curve (Remedi, Rocheleau et al. 2006).

Mice lacking functional K_{ATP} channels (loss-of-function) had beta cells that were depolarized and had elevated basal $[Ca^{2+}]_i$, as predicted (Miki, Tashiro et al. 1997; Miki, Nagashima et al. 1998; Seghers, Nakazaki et al. 2000; Koster, Remedi et al. 2002). As such, glucose-stimulated insulin secretion from these isolated islets was absent, confirming the necessity of K_{ATP} channels for insulin secretion. Surprisingly, animals expressing a functionally inactivated dominant-negative Kir6.2 mutation (Kir6.2G132S) demonstrated hypoglycemia and hyperinsulinemia as neonates that developed into hyperglycemia due to substantial beta cell loss as adults (Miki, Tashiro et al. 1997). In similar experiments, when either Kir6.2 or SUR1 were genetically deleted, adult mice had reduced insulin secretion, yet were normoglycemic (Miki, Nagashima et al. 1998; Seghers, Nakazaki et al. 2000). Even more surprising was that mice expressing a different dominant-negative mutation in Kir6.2 exhibited hyperinsulinemia as adults and showed no significant beta cell loss (Koster,

Remedi et al. 2002). Based on these studies, it was proposed that this unexpected phenotype was produced by K_{ATP} channel activity that was only partially suppressed (due to the fact that 30% of the beta cells were unaffected).

Concluding Remarks

In the twenty years since their discovery, work with animal models and humans expressing K_{ATP} channel mutations have clearly defined a critical role for K_{ATP} channels in physiology. While some pathways and mechanisms have been identified with regard to K_{ATP} channel activity, there are significant gaps in our knowledge of the molecular mechanisms underlying channel trafficking, regulation, and pharmacological action. For example, the SU family of anti-diabetic drugs have been used since the 1940s to treat diabetes; however, the mechanism of SU activity has not been clearly defined. Likewise, the generation of conflicting data regarding the trafficking and regulation of K_{ATP} channel activity have made understanding the role of K_{ATP} channels in physiology confusing. Considering their vital role in human physiology and as targets of pharmaceutical action, a more complete understanding of K_{ATP} channel activity is necessary to provide the framework for future work regarding K_{ATP} channel regulation and pharmacological modulation.

CHAPTER IV

NOVEL ROLES OF ANKYRIN FOR K_{ATP} CHANNEL TARGETING AND METABOLIC REGULATION

Introduction

The critical function of ankyrin-B in excitable cells, namely the cardiomyocyte, has illuminated the necessary role of ankyrin polypeptides in human physiology. Specifically, ankyrin-B has been demonstrated to participate in the trafficking, retention, and regulation of ion channels to distinct membrane microdomains. The loss of ankyrin-B expression in the cardiomyocyte, for example, results in a significant decrease in the expression and localization of NKA, NCX1, and $InsP_3$ receptor to the transverse tubule, resulting in abnormal Ca^{2+} dynamics and after-depolarizations. The consequence is cardiac arrhythmia and an increased risk for sudden cardiac death (Mohler, Gramolini et al. 2002; Mohler, Schott et al. 2003). Likewise, loss of association of ankyrin-G with $Na_v1.5$ has been determined to be the underlying molecular mechanism of Brugada syndrome, a deadly heart arrhythmia associated with a right bundle branch block (Tester and Ackerman 2008). Ankyrin-G has also been characterized as a necessary component of the axon initial segment and the nodes of Ranvier. In these specialized domains, ankyrin-G functions to target and retain Na_v and K_v channels that are necessary for the generation of action potentials and propagation of neuronal electrical impulses (Kordeli, Lambert et al.

1995; Davis, Lambert et al. 1996). As such, defects in ankyrin-G neuronal pathways of ion channel targeting result in decreased locomotion, abnormal gait and tremor (Zhou, Lambert et al. 1998).

Although ankyrin-B is a ubiquitously-expressed polypeptide, expression in the pancreas has only recently been reported (Mohler, Healy et al. 2007). Specifically, ankyrin-B expression is highly localized to the pancreatic islet, with the pancreatic beta cell expressing the highest levels of ankyrin-B (Mohler, Healy et al. 2007). Like the cardiomyocyte, the electrophysiology of the pancreatic beta cell presides over its function; namely, insulin secretion. A number of ion channels, transporters, and pumps are necessary for the maintenance of beta cell membrane potential. Additionally, many known ankyrin-B binding partners are expressed in the pancreatic beta cells. Based on previous studies describing the roles of ankyrin polypeptides in ion channel trafficking and function in excitable cells, I evaluated the role of ankyrin-B in beta cell physiology. Initial observations that ankyrin-B and Kir6.2 co-immunoprecipitate in both pancreatic and heart lysate led to the exciting *in vitro* observation that ankyrin-B associates with a conserved C-terminal motif in the pore-forming subunit of the K_{ATP} channel, Kir6.2. This interaction is specific to Kir6.2, considering that ankyrin-B is unable to co-immunoprecipitate with a similar isoform, Kir6.1. In addition, I was able to refine the ankyrin-B-binding domain of Kir6.2 to an eight amino acid motif that is both necessary and sufficient for interaction with ankyrin-B. Further analysis revealed that a known human mutation in Kir6.2 associated with PNDM, E322K, is located within this ankyrin-B-binding domain. This mutation results in

loss of the ankyrin-B/Kir6.2 interaction *in vitro* and a loss of Kir6.2 trafficking *in vivo*. Biophysical analysis also revealed that E322K results in a decrease in the ATP sensitivity of the K_{ATP} channel. Finally, preliminary data using a competing peptide imply that association with ankyrin-B is necessary for K_{ATP} channel metabolic regulation *in vivo*. These data suggest a novel role for ankyrin-B in the pancreatic beta cell; namely, the trafficking and regulation of Kir6.2 in excitable tissues.

Methods and Experimental Approach

Animals. Mice used in this study were six month old wild type and ankyrin-B heterozygous (ankyrin-$B^{+/-}$) male littermates. Four month old male Sprague-Dawley rats were also used. Ankyrin-B null animals die before post-natal day 1 (Scotland, Zhou et al. 1998), necessitating the use of ankyrin-$B^{+/-}$ mice for analyses. Ankyrin-$B^{+/-}$ mice (Scotland, Zhou et al. 1998) were generated by backcrossing at least 18 generations (99.5% pure) into C57Bl/6 background (Jackson Laboratories) before experiments. Animals were handled according to approved protocols and animal welfare regulations of the Institutional Animal Care and Use Committee (IACUC). Both ankyrin-$B^{+/-}$ and control mice were housed in the same facility (identical temperature and humidity), consumed the same diet (Lab Diet: 23% protein, 4.5% fat, 6.0% fiber, 8.0% ash, 2.5% minerals 0.95% Ca^{+2}, 0.67% phosphorus, 0.4% non-phytate phosphorus, and 56% complex carbohydrate) from overhead wire feeders and water *ad libitum*, and were kept on identical 12 hour light/dark cycles.

Tissue preparation for sectioning. Pancreatic tissues from wild type and ankyrin-$B^{+/-}$ mice were harvested and immediately placed in 10% formalin overnight, transferred to 0.50 M sucrose for 12 hours, and returned to 10% formalin. Tissues were embedded in paraffin and sectioned to 10 μm thickness by the Vanderbilt University Medical Center Immunohistochemistry Core Laboratory.

Collection and culture of neonatal cardiac fibroblasts. Hearts were dissected from postnatal day 1 or 2 wild type and ankyrin-$B^{-/-}$ mice and placed in 2 ml Ham's F-10 media (Mediatech). Atrial tissue was excised and the ventricular tissue was rinsed to remove residual blood. Rinsed tissue was transferred to 1.5 ml of 0.05% trypsin (Gibco) and 200 μM ethylenediamine tetraacetic acid (EDTA) in Ham's F-10 media. Tissue was minced using forceps and scissors and incubated in trypsin-EDTA at 37 ºC for 15 minutes. Following trituration of minced tissue, the minced ventricular tissue was incubated for an additional 15 minutes at 37 ºC. Two hundred microliters of soybean trypsin inhibitor (2 mg/ml; Worthington) and 200 μl of collagenase (0.2 mg/ml, 1980 U/mg; Sigma) were incubated with the disrupted cells for 50 minutes at 37 ºC. The cell suspension was pelleted, resuspended in complete media (40% Dulbecco's Modified Eagle Medium (DMEM), 40% Ham's F-10 media, and 20% fetal bovine serum (FBS; HyClone)), and plated on culture dishes. After five hours, nonadherent cells (cardiomyocytes) were aspirated from the plate. Isolated fibroblasts were maintained in DMEM + 10% FBS + 0.1% streptomycin/penicillin at 37 ºC with 5% CO_2.

Immunofluorescent staining. Paraffinized tissue slices were deparaffinized by incubation in xylene for 40 minutes followed by 10 minute washes in 100% and 95% ethanol. Deparaffinized slides were pre-incubated in 3% fish gelatin (Sigma) for three hours at 4 ºC. Primary antibody staining proceeded for 12 hours at 4 ºC, followed by extensive washing in 2% fish gelatin + 0.1% Triton X-100 (IF Wash Buffer). Secondary antibody incubation with the appropriate fluorophore-conjugated antibody was carried out for four hours at 4 ºC, followed by extensive washing in IF Wash Buffer. Stained sections were mounted with VectaShield (Vector Laboratories) mounting media and coverslipped (#1 coverslides, Electron Microscopy Sciences). Slides were protected from light and stored at 4 ºC until analysis. Thirty minutes prior to confocal microscopy, the slides were allowed to acclimate to room temperature.

Confocal microscopy. Immunofluorescently-stained images were collected on a Zeiss 510 Meta confocal microscope (40 power-water corrected numerical aperature 1.2 (Zeiss), pinhole equals 1.0 Airy Disc or 63 power-oil 1.5 numerical aperature (Zeiss), pinhole equals 1.0 Airy Disc) using Carl Zeiss Imaging Software. Images were imported into Adobe Photoshop for cropping and linear contrast adjustment.

Tissue collection and preparation for immunoblot analysis. For quantitative immunoblotting and co-immunoprecipitation analysis, pancreatic and heart tissues were flash frozen in liquid nitrogen and ground into a fine powder. The powder was resuspended in three volumes of ice-cold 50 mM Tris HCl (pH 7.35), 10 mM NaCl, 0.32 M sucrose, 5 mM EDTA, 2.5 mM ethyleneglycol bis (2-

amino ethyl ether)-N,N,N' tetraacetic acid (EGTA), 1 mM phenylmethyl sulfonyl fluoride (PMSF), 1 mM 4-(2-aminoethyl) benzenesulfonylfluoride hydrochloride (AEBSF), 10 µg/ml leupeptin, and 10 µg/ml pepstatin (Homogenization Buffer) using a Beckman Polytron homogenizer. The homogenate was centrifuged at 1,000 x *g* at 4 ºC to remove nuclei. Triton X-100 and deoxycholate were added to the post-nuclear supernatant for final concentrations of 1.5% Triton X-100 and 0.75% deoxycholate. The lysate was pelleted at 100,000 x *g* for one hour at 4 ºC. The resulting supernatant was quantitated by bicinchoninic acid assay (BCA Protein Assay Kit, Pierce) followed by spectrophotometric determination at 562 nm. Equal amounts of protein were loaded on gradient gels.

SDS-PAGE and immunoblot analysis. Equal amounts of protein were diluted in reducing agent and loading buffer and boiled for five minutes. Reduced lysates were loaded onto either a 3-8% Tris-acetate gel (NuPAGE, Invitrogen) or a 4-12% bis-tris acrylamide gel (NuPAGE, Invitrogen) and electrophoresed at 100 mV for 15 minutes followed by 150 mV for two hours. Electrophoresed proteins were transferred to nitrocellulose (Hybond ECL Nitrocellulose, 0.45 µm pore size; GE Healthcare) and blocked for three hours in 5% non-fat dry milk (NFDM) in TTBS. Primary antibodies were diluted in 5% NFDM and incubated with nitrocellulose blots for 12 hours at 4 ºC with gentle agitation, followed by three washes in TTBS for 15 minutes at room temperature. Secondary antibodies were diluted in 5% TTBS and incubated with nitrocellulose blots for three hours at 4 ºC, followed by three washes in TTBS for 15 minutes at room temperature. Washed blots were rinsed in chemiluminescent solution

(SuperSignal West Pico Chemiluminescent Substrate, Pierce) for one minute and exposed to autoradiography film (Classic Blue Autoradiography Film, Midamerican Scientific). Films were scanned and band intensity quantitated using QuantityOne software (BioRad).

Antibodies. The following primary antibodies were used for immunofluorescent staining and/or immunoblotting protocols: anti-ankyrin-B (polyclonal and monoclonal) (Mohler, Gramolini et al. 2002), anti-ankyrin-G (polyclonal) (Mohler, Rivolta et al. 2004), anti-Kir6.2 (polyclonal, Alomone labs), anti-Kir6.1 (polyclonal, Alomone labs) anti-SUR1 (polyclonal, Santa Cruz Biotechnology), anti-SUR2A (goat polyclonal, Santa Cruz Biotechnology), anti-$InsP_3$ receptor (polyclonal, Upstate), anti-NHERF1 (sodium-hydrogen exchange regulatory factor 1; polyclonal) (Mohler, Kreda et al. 1999), anti-NCX1 (monoclonal, RDI), anti-NKA (polyclonal, Upstate), anti-$Ca_v1.2$ (polyclonal, Affinity Bioreagents), anti-phalloidin-568 (Molecular Probes), ToPro-3 DNA stain (Molecular Probes), anti-glucagon (monoclonal, Sigma), anti-green fluorescent protein (GFP, polyclonal) (Mohler, Gramolini et al. 2002), and anti-hemagglutinin (HA, monoclonal) (Mohler, Rivolta et al. 2004). Secondary antibodies for immunoblot analysis included horseradish peroxidase (HRP) conjugated donkey anti-rabbit (Jackson Laboratories), donkey anti-mouse (Jackson Laboratories), and donkey anti-goat (Jackson Laboratories). Secondary antibodies for immunofluorescent staining included Alexa-488 donkey anti-rabbit (Molecular Probes), Alexa-568 donkey anti-rabbit (Molecular Probes), Alexa-488 donkey

anti-mouse (Molecular Probes), and Alexa-568 donkey anti-mouse (Molecular Probes).

RNA preparation. Following anesthesia and cervical dislocation, still-beating hearts were excised and flash frozen in liquid nitrogen. Total RNA from pools of heart tissues was isolated and DNase-treated using the RNeasy Fibrous Tissue Micro Kit (Qiagen) per manufacturer's protocols. The quality of total RNA was assessed by polyacrylamide gel microelectrophoresis (Agilent 2100 Bioanalyzer). Genomic DNA contamination was assessed by PCR amplification of total RNA samples without prior cDNA synthesis and no genomic DNA was detected.

TaqMan quantitative RT-PCR. TaqMan Low Density Arrays (TLDA, Applied Biosystems) were completed in a two step RT-PCR process. Briefly, first strand cDNA was synthesized from 200 ng of total RNA by using the High-Capacity cDNA Archive Kit (Applied Biosystems). PCR reactions were carried out in TLDA by using the ABI PRISM 7900HT Sequence Detection System (Applied Biosciences). The 384 wells of each array were preloaded with 96 x 4 pre-designed 6-fluorescein (FAM)-labelled fluorgenic TaqMan Kir6.2 and ankyrin-B probes and primers. The probes were labelled with the fluorescent reporter dye 6-carboxyfluorescein (FAM, Applera Corporation) on the 5' end and with a nonfluorescent quencher on the 3' end. Two nanograms of cDNA from each sample was combined with 1X TaqMan Universal Master Mix (Applied Biosystems) and loaded into each well. The TLDA were cycled at 50 ºC for two minutes and 94.5 ºC for ten minutes, followed by 40 cycles of 97 ºC for 30

seconds and 59.7 ºC for one minute. Data were collected with instrument spectral compensations by using the Applied Biosystems SDS 2.2.1 software and analyzed by using the comparative threshold cycle (C_T) relative quantification method. The *GAPDH* gene was used as an endogenous control to normalize the data. Results in wild type and ankyrin-$B^{+/-}$ animals were expressed as relative mRNA expression level as compared with *GAPDH.*

Statistics. When appropriate, data were analyzed using a two-tailed *t* test with $p < 0.05$ considered significant or ANOVA. Values are expressed as the mean $\pm$ standard deviation. For immunoblot analysis, radiographs were quantitated using QuantityOne software followed by statistical analysis with Sigma Plot software (SigmaPlot).

Co-immunoprecipitation. Protein-A-conjugated agarose beads (Rockland) were incubated with either affinity-purified polyclonal anti-ankyrin-B or control mouse IgG in PBS with 0.1% Triton X-100 and protease inhibitors (Sigma) (Co-IP Binding Buffer) for 12 hours at 4 °C with rotation. Beads were centrifuged and washed three times in ice-cold PBS. Nine milligrams of rat pancreatic or rat heart tissue lysate were added to washed beads, along with protease inhibitor cocktail (Sigma) and 500 µl Co-IP Binding Buffer, and incubated at 4 °C for 12 hours with rotation. Following incubation, the mixtures were centrifuged and washed three times in ice-cold Co-IP Binding Buffer. Proteins were eluted by adding loading buffer plus dithiothreitol (DTT) and boiled for five minutes. Samples were centrifuged and 25 µl of supernatant were loaded onto a 3-8% Tris-acetate gradient gel. Proteins were electrophoresed at 100 mV

for 15 minutes and 150 mV for two hours. Separated proteins were transferred to nitrocellulose and pre-blocked in 5% NFDM in TBS plus Tween-20 (TTBS) for three hours at 4 °C. Anti-Kir6.2 polyclonal antibodies or anti-Kir6.1 polyclonal antibodies were added to 5% NFDM in TTBS and incubated with nitrocellulose blots for 12 hours at 4 °C. Blots were washed three times in TTBS and incubated in 5% NFDM with donkey anti-rabbit HRP-conjugated secondary antibodies for three hours at 4 °C, followed by extensive washing in TTBS. Washed blots were rinsed in chemiluminescent reagent for one minute and exposed to autoradiography film.

Cloning of Kir6.2 cDNA from human GAL4 cardiac cDNA library and subcloning into pBluescript-SK vector. Primers were designed to clone Kir6.2 cDNA from the human GAL4 cardiac cDNA library (BD Biosciences) based on the sequence obtained from GenBank (NM 000525). To facilitate subcloning, primers were designed to incorporate 5' *EcoR*I and 3' *BamH*I restrictions sites. Forward primer: 5' GCC GAA TTC ATG CTG TCC CGC AAG GGC ATC AT 3'; Reverse primer: 5' GGC GGA TCC TCA GGA CAG GGA ATC TGG AGA 3'. PCR products were run out on a 1% agarose gel plus ethidium bromide (0.17 mg/ml) at 100 mV and gel purified (Gel Extraction Kit, Qiagen). Purified PCR products, along with the pBluescript-SK expression vector (Stratagene), were digested with *EcoR*I (20 units; New England Biolabs) and *BamH*I (20 units; New England Biolabs) for five hours at 37 °C. Digested vector was further incubated with calf intestinal alkaline phosphatase (0.01 µg/µl, Promega) for two hours at 37 ºC. Digested products were separated on a 1% agarose plus ethidium

bromide gel and purified. Ligation of digested Kir6.2 and pBluescript-SK vector was performed using T4 DNA ligase (LigaFast, Promega) overnight at 4 ºC. Ligated products were transformed into DH5α *Escherichia coli* cells using a standard heat-shock protocol, plated on Lennox L Broth Base (LB) agar containing ampicillin (0.05 µg/µl), and incubated at 37 °C overnight. Positive colonies were expanded and plasmids purified. Restriction digestion was performed to identify potential clones and sequences were verified.

Production of Kir6.2 truncated and mutated constructs. A series of mutated and truncated Kir6.2 constructs in the context of the pBluescript-SK vector (Table 2) were generated by either PCR-based or site-directed mutagenesis protocols. Site-directed mutants (E321K, E321A, E322K, E322A, D323K, and D323A) were generated using the full-length Kir6.2 (in pBluescript-SK) as a template, along with distinct primers (Table 3) and the QuikChange II mutagenesis kit (Stratagene) to incorporate the appropriate mutation (Table 3). Kir6.2-NT (amino acids 74-390), Kir6.2-CT (amino acids 1-159), Kir6.2truncA (amino acids 1-315), and Kir6.2truncB (amino acids 1-330) were constructed using specially-designed primers in a PCR-based reaction to generate the insert (flanked by 5' *EcoR*I and 3' *BamH*I restriction sites), followed by ligation into the pBluescript-SK vector using T4 DNA ligase (Promega). Specifically, Kir6.2-NT was designed to be translated beginning with amino acid 74. Kir6.2-CT was generated by inserting a premature stop codon at amino acid position 160. Kir6.2truncA introduced a premature stop codon at amino acid 316 (prior to the putative ankyrin-B binding site), while Kir6.2truncB introduced a premature stop

codon at amino acid 331 (following the putative ankyrin-B binding site). Site-directed mutants were constructed to introduce either an alanine (A) or lysine (K) in place of amino acids E321, E322, or D323. All sequences were verified by sequencing prior to experimentation.

Production and purification of ankyrin-B GST-conjugated and ankyrin-G GST-conjugated proteins. cDNAs for the ankyrin-B membrane-binding domain plus the proximal portion of the spectrin-binding domain (amino acids 1-959), the membrane-binding domain (amino acids 1-861), spectrin-binding domain (amino acids 862-1444), and C-terminal domain (amino acids 1556-1840), as well as the membrane-binding domain of ankyrin-G (amino acids 1-886) were PCR-amplified, cloned into pGEX6P-1 (Amersham Biosciences), and sequenced to confirm correct sequences. To facilitate cloning, all constructs were engineered to contain 5' *EcoR*I and 3' *Xho*I restriction sites. BL21(DE3)pLysS cells (Invitrogen) were transformed with the ankyrin-B pGEX6p-1 constructs and grown overnight at 37 ºC in LB supplemented with 0.05 µg/µl ampicillin. The overnight cultures were sub-cultured for large-scale expression. Cells were grown to an optical density of 0.6-0.8 and induced with 1 mM isopropyl 1-thio-β-D-galactopyranoside (IPTG) for two hours at 37 ºC. Cells were centrifuged for 10 minutes at 8,000 x *g*, resuspended in PBS, and frozen at -80 ºC following resuspension. Cells were lysed by thawing. The crude extract was suspended in a solution of PBS, 1 mM DTT, 1 mM EDTA, 40 µg/ml AEBSF, 10 µg/ml leupeptin, 40 µg/ml benzamidine, 10 µg/ml peptstatin (Lysis Buffer) and

Table 2. Kir6.2 constructs used in *in vitro* binding and/or transfection experiments.

Construct ID	Description
FLKir6.2	Wild type Kir6.2
Kir6.2-NT	Kir6.2 lacking first 73 amino acids
Kir6.2-CT	Kir6.2 lacking last 230 amino acids
Kir6.2truncA	Kir6.2 prematurely truncated prior to VPIVAEED
Kir6.2truncB	Kir6.2 prematurely truncated after VPIVAEED
Kir6.2 E321K	Site-directed mutant, glutamic acid to lysine substitution
Kir6.2 E322K	Site-directed mutant, glutamic acid to lysine substitution
Kir6.2 D323K	Site-directed mutant, aspartic acid to lysine substitution
Kir6.2 E321A	Site-directed mutant, glutamic acid to alanine substitution
Kir6.2 E322A	Site-directed mutant, glutamic acid to alanine substitution
Kir6.2 D323A	Site-directed mutant, aspartic acid to alanine substitution

Table 3. Primers used to engineer unique Kir6.2 constructs.

Construct Name	Primers
Kir6.2-NT	FW: 5' CGG AAT TCA TGT TCA CCA TGT CCT TCC TGT GC 3'
	REV: 5' GGC GGA TCC TCA GGA CAG GGA ATC TGG AGA GAT GCT G 3'
Kir6.2-CT	FW: 5' GCC GAA TTC ATG CTG TCC CGC AAG GGC ATC ATC CCC G 3'
	REV: 5' GGC GGA TCC TCA GTT GAT CAT GAG CCC CAC GAT 3'
Kir6.2truncA	FW: 5' GCC GAA TTC ATG CTG TCC CGC AAG GGC ATC ATC CCC G3'
	REV: 5' GCG CGC GGA TCC TCA AAA GCG CTG GCC CCA CAG GAT CTC 3'
Kir6.2truncB	FW: 5' GCC GAA TTC ATG CTG TCC CGC AAG GGC ATC ATC CCC G 3'
	REV: 5' GCG CGC GGA TCC TCA GTA GTC CAC AGA GTA ACG TCC GTC 3'
Kir6.2 E321K	FW: 5' GTG CCC ATT GTA GCT AAG GAG GAC GGA CGT TAC 3'
	REV: 5' GTA ACG TCC GTC CTC CTT AGC TAC AAT GGG CAC 3'
Kir6.2 E321A	FW: 5' GTG CCC ATT GTA GCT GCG GAG GAC GGA CGT 3'
	REV: 5' ACG TCC GTC CTC CGC AGC TAC AAT GGG CAC 3'
Kir6.2 E322K	FW: 5' ATT GTA GCT GAG AAG GAC GGA CGT TAC TCT 3'
	REV: 5' AGA GTA ACG TCC GTC CTT CTC AGC TAC AAT 3'
Kir6.2 E322A	FW: 5' CCC ATT GTA GCT GAG GCG GAC GGA CGT TAC 3'
	REV: 5' GTA ACG TCC GTC CGC CTC AGC TAC AAT GGG 3'
Kir6.2 D323K	FW: 5' GTA GCT GAG GAG AAG GGA CGT TAC TCT GTG 3'
	REV: 5' CAC AGA GTA ACG TCC CTT CTC CTC AGC TAC 3'
Kir6.2 D323A	FW: 5' ATT GTA GCT GAG GAG GCC GGA CGT TAC TCT 3'
	REV: 5' AGA GTA ACG TCC GGC CTC CTC AGC TAC AAT 3'

was pushed through a 22-gauge syringe to shear bacterial DNA. Cell debris was removed by centrifugation at 11,000 x *g* for one hour at 4 ºC. Supernatants were added to 2 ml of equilibrated glutathione-agarose (Amersham Biosciences) in small batches for four hours at 4 ºC. The glutathione-agarose was washed with PBS containing 0.65 M NaCl and eluted using 50 mM Tris-HCl, 10 mM glutathione, 1 mM EDTA, 1 mM DTT, pH 8.0 (Elution Buffer). Protein sizes were verified by SDS-PAGE and Coomassie staining. Purified protein was quantitated using the BCA method.

In vitro *transcription/translation and binding experiments.* Kir6.2 constructs (Table 2) were *in vitro* transcribed/translated using rabbit reticulocyte lysate, [^{35}S]-methionine (20 µCi of Redivue L-[^{35}S]-methionine (GE Healthcare), T7 polymerase, and 0.75 µg plasmid DNA (TNT Coupled Rabbit Reticulocyte Lysate System, Promega). For binding experiments, 20 µg of purified glutathione S-transferase (GST) or GST-ankyrin-B membrane-binding domain (MBD) were coupled to glutathione sepharose for two hours at 4 ºC in 50 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100 (IV Binding Buffer). Following extensive washes in 50 mM Tris-HCl pH 7.3, 1 mM EDTA, 1 mM EGTA, 500 mM NaCl, 1% Triton X-100 (IV Wash Buffer), conjugated beads were incubated with Kir6.2 *in vitro* translation products for four hours at 4 ºC in IV Binding Buffer plus protease inhibitor cocktail. Following incubation, binding reactions were washed five times in IV Wash Buffer, eluted by boiling in loading buffer and reducing agent, and analyzed by SDS-PAGE. Gels were stained with Coomassie to show presence of *in vitro* translated and GST proteins (and to

confirm equal loading of proteins into the binding reactions) prior to drying the gel using a gel dryer (Biorad). Radiolabelled proteins were detected by phosphorimaging.

***Pull-down analysis*.** Two biotinylated Kir6.2 oligopeptides (WT: GQRFVPIVAEEDGR and MUT: GQRFVPIVAEKDGR; Biosynthesis, Inc.) were constructed with an SGSG linker between the biotin and the Kir6.2 sequence. Twenty micrograms of oligopeptide were conjugated to 40 µl bead volume of strepavidin beads (Thermo Scientific) for four hours at 4 ºC in IV Binding Buffer. The beads were washed extensively in IV Binding Buffer and 10 µg of purified ankyrin-B MBD-GST were added to the washed beads, along with 500 µl of IV Binding Buffer. The reactions were incubated overnight at 4 ºC with gentle rotation. The reactions were centrifuged and washed five times in IV Binding Buffer. Elution was performed by boiling the beads in loading buffer and reducing agent for five minutes. Eluted solutions were centrifuged and separated by SDS-PAGE. Separated proteins were transferred to nitrocellulose and incubated overnight at 4 ºC in a solution of 5% TTBS plus anti-ankyrin-B antibodies. Blots were washed three times (15 minutes each) in TTBS before incubation with donkey anti-rabbit antibodies diluted in 5% TTBS for three hours at 4 ºC. Blots were washed three times (15 minutes each) in TTBS and incubated for one minute in chemiluminescent reagent and exposed to autoradiography film.

Subcloning of Kir6.2 constructs into a GFP-fusion vector. In order to generate Kir6.2 constructs engineered with a C-terminal GFP fusion, a PCR-

based approach to produce full-length and mutant Kir6.2 fragments. Specifically, primers were designed to incorporate 5' *EcoR*I and 3' *BamH*I restriction sites to facilitate ligation. Additionally, the termination codon was removed from each construct in order to allow the expression of the C-terminal GFP gene. The PCR products were ligated into the pAcGFP1N2 expression vector (Clontech). Positive clones were identified by restriction digestion and the sequences verified prior to experimentation.

Cell culture. HEK293 cells were maintained DMEM (Gibco) supplemented with 10% FBS (HyClone) and 0.1% penicillin/streptomycin. Cells were kept at 37° C in 5% CO_2. COSm6 cells (a gift from Dr. Colin Nichols at Washington University, St. Louis) were cultured in DMEM supplemented with 10% FBS and 0.1% penicillin/streptomycin. Cells were kept at 37° C in 5% CO_2.

Transfection of HEK293, COSm6, and cardiac fibroblast. Cultured cells and fibroblasts were split 24 hours prior to transfection (30% confluence at time of transfection). Qiagen Effectene reagent and corresponding manufacturer's instructions were used to co-transfect cells with SUR1 and Kir6.2-GFP, Kir6.2-E322K-GFP, or empty pAcGFP1N2 cDNA constructs (5:1 SUR1 cDNA to GFP construct cDNA). Transfection was carried out for nine hours at 37 °C and the cells were allowed to recover overnight. Transfected cells were plated on Mattek plates (Mattek Cultureware) for further immunolabeling, confocal analysis, and electrophysiological analysis.

Isolation of pancreatic islets. Mice were anesthetized with 2-bromo-2-chloro-1,1,1-trifluoroethane (Sigma) in an anesthetizing chamber and killed by

cervical dislocation, in accordance with IACUC standards. Pancreata were cannulated and injected with Hank's balanced salt solution (Sigma) containing type XI collagenase (Sigma; 0.3 mg/ml, pH 7.4). The pancreata were digested for five minutes at 37 ºC, hand-shaken and washed three times in Hank's solution. Islets were manually isolated under a dissecting microscope and maintained overnight in CMRL medium (Gibco BRL; containing 5.6 mM glucose) in a humidified 37 ºC incubator. Trypsin-treated islets were dispersed into isolated cells by resuspending gently in complete CMRL medium (supplemented with 10% FCS), penicillin (100 U/ml) and streptomycin (100 µg/ml). Isolated cells were plated on glass coverslips.

Rubidium flux experiments. Cells were pre-incubated for one hour with $^{86}Rb^{+}$ (in the form of rubidium chloride, 1.5 mCi/ml; Amersham Biosciences). Loaded cells were washed twice with RPMI-1640 medium (Sigma) at 37 ºC. $^{86}Rb^{+}$ efflux was assayed by replacing the bath solution with Ringer's solution containing metabolic inhibitor (MI), with or without 1 µM glibenclamide. MI solution contained 2.5 mg/ml oligomycin, 1 mM 2-deoxyglucose, along with 10 mM tetraethylammonium to inhibit voltage-gated K^{+} channels and 10 µM nifedipine to block Ca^{2+} entry. The bath solution was replaced with fresh solution every five minutes over a 40 minute time period and counted in a scintillation counter. $^{86}Rb^{+}$ efflux in the presence or absence of glibenclamide was fitted by a single exponential. The reciprocal of the exponential time constant (rate constant) for each efflux experiment is then proportional to the K^{+} (Rb^{+}) conductance in the cell membranes.

Electrophysiological analysis. Macroscopic currents from dispersed cells were recorded using standard whole-cell voltage-clamp configuration (Koster, Remedi et al. 2002). Electrodes were filled with K-INT (140 mM KCl, 10 mM K-HEPES, 1 mM K-EGTA, pH 7.4) plus 1 mM $MgCl_2$ and 1 mM ATP (potassium salt). Experiments were digitized into a microcomputer using Pclamp8.0 (Axon Instruments, La Jolla, CA) software. Off-line analysis was performed in Microsoft Excel.

Mathematical Modeling. Pancreatic beta cell electrical activity was simulated using the mathematical model developed by Fridlyand et al. (Fridlyand, Tamarina et al. 2003). This model has been used with to generate insight into molecular mechanisms underlying the oscillations in transmembrane potential that trigger insulin release from pancreatic beta cells. Importantly, this model accounts for dynamic changes in intracellular ATP levels that regulate cell excitability through I_{KATP}. The model includes a detailed formulation for the fraction of open K_{ATP} channels {Fridlyand, 2003 #1775; Magnus, 1998 #1776}:

$$O_{KATP,m} = \frac{0.08\left(1+\frac{2[ADP]_i}{K_{dd}}\right)+0.89\left(\frac{[ADP]_i}{K_{dd}}\right)^2}{\left(1+\frac{[ADP]_i}{K_{dd}}\right)^2\left(1+\frac{[ADP]_i}{K_{td}}+\left(\frac{[ATP]_i}{K_{tt}}\right)^h\right)} \qquad [1]$$

K_{td} and K_{dd} values of 57 μM and 31 μM, respectively, were used as measured by Hopkins et al. in excised, inside-out patches from adult mouse pancreatic beta cells (Hopkins, Fatherazi et al. 1992). Values for h and K_{tt} (1.32 μM and 15 μM,

respectively) were chosen to agree with observed measurements in COSm6 cells. The E322K mutation is in the ankyrin-B binding domain of Kir6.2. The data in this study show that the mutation affects channel trafficking as well as ATP sensitivity. The following assumptions in this model were made of the E322K heterozygous mutant: 1) E322K and WT subunits are incorporated with equal probability into tetrameric channels, resulting in five functionally distinct populations (Figure 7): 4 WT, 3 WT:1 mutant, 2 WT:2 mutant, 1 WT:3 mutant, 4 mutant; 2) Kir6.2 subunits exert an additive effect on the open state probability of the tetrameric channel (Craig, Ashcroft et al. 2008), such that given the ATP sensitivity of each individual subunit, the sensitivity of the tetramer can be determined as $1/K_{tt,tetr} = 1/K_{tt,1} + 1/K_{tt,2} + 1/K_{tt,3} + 1/K_{tt,4}$; 3) K_{ATP} tetrameric channels will traffic differently depending on their stoichiometry. For example, K_{ATP} channels with four mutant subunits will display the most severe trafficking defect due to loss of ankyrin-B binding at all four subunits, while channels with four WT subunits will traffic normally.

ATP sensitivity and peptide competition assays. COSm6 cells were transfected with cDNAs encoding Kir6.2 (with mutations as described, and a C-terminal GFP tag), and SUR1, using the Fugene 6 transfection reagent (Roche). Patch-clamp experiments were made at room temperature, using the Dynaflow microfluidic capillary chip-based platform (Cellectricon, Inc.) and DF-16ProII chips. This device generates laminar flow at solution outlets to prevent mixing between solutions, and a computer-controlled stepper motor is used to move the chip relative to the patch pipette, allowing for rapid solution exchange around the

membrane patch. This device also minimizes the amount of peptide reagents necessary for the experiment. Data were normally filtered at 0.5-2 kHz, signals were digitized at 5 kHz and stored directly on computer hard drive using Clampex software (Axon, Inc.). The standard pipette (extracellular) and bath (cytoplasmic) solutions had the following composition: 140 mM KCl, 1 mM K-EGTA, 1 mM K-EDTA, 4 mM K_2HPO_4, pH 7.3. Peptides were stored as 500 μM stocks (made up in standard recording solution) at -20 ºC, and diluted to working concentrations on each experimental day. ATP was stored as a 100 mM stock (dipotassium salt), with the pH previously adjusted to 7.3, and diluted for each experiment.

Results

Ankyrin-B$^{+/-}$ mice display abnormal Kir6.2 expression and localization

Ankyrin-B is highly expressed in pancreatic beta cells (Figure 8A), with minimal levels observed in other pancreatic islet cell types (Figure 8A, C) {Mohler, 2007 #1368}. Moreover, ankyrin-B is not significantly expressed in pancreatic exocrine acinar cells (Figure 8B, D). Mice heterozygous for an ankyrin-B null mutation (ankyrin-B$^{+/-}$ mice) are viable and express significantly less islet ankyrin-B levels (Figure 8C, D) (Mohler, Healy et al. 2007).

Ankyrins are required for the targeting of ion channels, transporters, and pumps in a host of tissues (Bennett and Baines 2001). To identify the role of ankyrin-B in the beta cell, expression and localization of a number of key beta cell ion channels, transporters, and cytoskeletal proteins in ankyrin-B$^{+/-}$ pancreas were evaluated. There was a striking reduction in expression levels of Kir6.2

(Figure 9G and Figure 10B, >50%, n=10, $p<0.05$), the α-subunit of the K_{ATP} channel in ankyrin-$B^{+/-}$ pancreas and a complete loss of Kir6.2 expression in ankyrin-B null pancreatic lysates (Figure 12A). In contrast, there were no differences in expression of the L-type calcium channel $Ca_v1.2$, SUR1, ankyrin-G, or NHERF1 by immunoblot (Figure 9B, C, F, I, and J and Figure 10D, E, G). Moreover, the levels of two membrane proteins affected by loss of ankyrin-B in heart, NCX1 and NKA (Mohler, Schott et al. 2003; Mohler, Davis et al. 2005; Cunha, Bhasin et al. 2007), were unchanged in ankyrin-$B^{+/-}$ pancreatic tissue (Figure 9D, E and Figure 10C, F). As expected, there was a significant reduction in ankyrin-B expression in ankyrin-$B^{+/-}$ pancreas as compared with wild type (Figure 9I and Figure 10A) and a complete loss of ankyrin-B expression in pancreatic lysates from ankyrin-B null mice (Figure 12B).

Reduction in Kir6.2 levels as analyzed by immunoblot was paralleled by Kir6.2 immunostaining in ankyrin-$B^{+/-}$ pancreatic islets. Specifically, there was a striking reduction in the expression of Kir6.2 in ankyrin-$B^{+/-}$ beta cells (Figure 9K, L), while the expression of other key beta cell proteins was unaffected (see $Ca_v1.2$ in Figure 9I, J). Additionally, real-time PCR revealed no significant difference in the levels of mRNA encoding Kir6.2 (*KCNJ11*), suggesting that the abnormal expression of Kir6.2 in ankyrin-$B^{+/-}$ pancreas is likely due to post-transcriptional events (Figure 11). Together, these findings support a potential role for ankyrin-B in the expression of Kir6.2 in excitable cells.

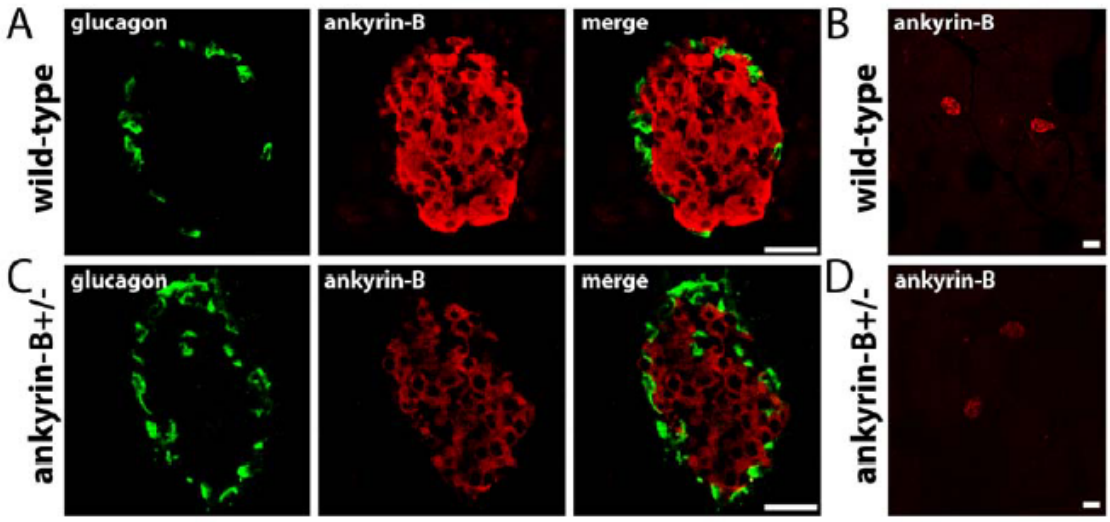

Figure 8. Ankyrin-B is highly expressed in beta cells of pancreatic islets.

(A) High magnification of wild type pancreatic islets demonstrates significant beta cell expression of ankyrin-B. Additionally, ankyrin-B expression is limited to the pancreatic beta cells, with negligible expression in the exocrine tissue (B and D; low magnification view of the pancreatic islets) or alpha cells (anti-glucagon staining, panels A and C). As anticipated, the expression of ankyrin-B in ankyrin-$B^{+/-}$ islets (C and D) is significantly reduced as compared to wild type (A and B). Scale bar equals 10 microns.

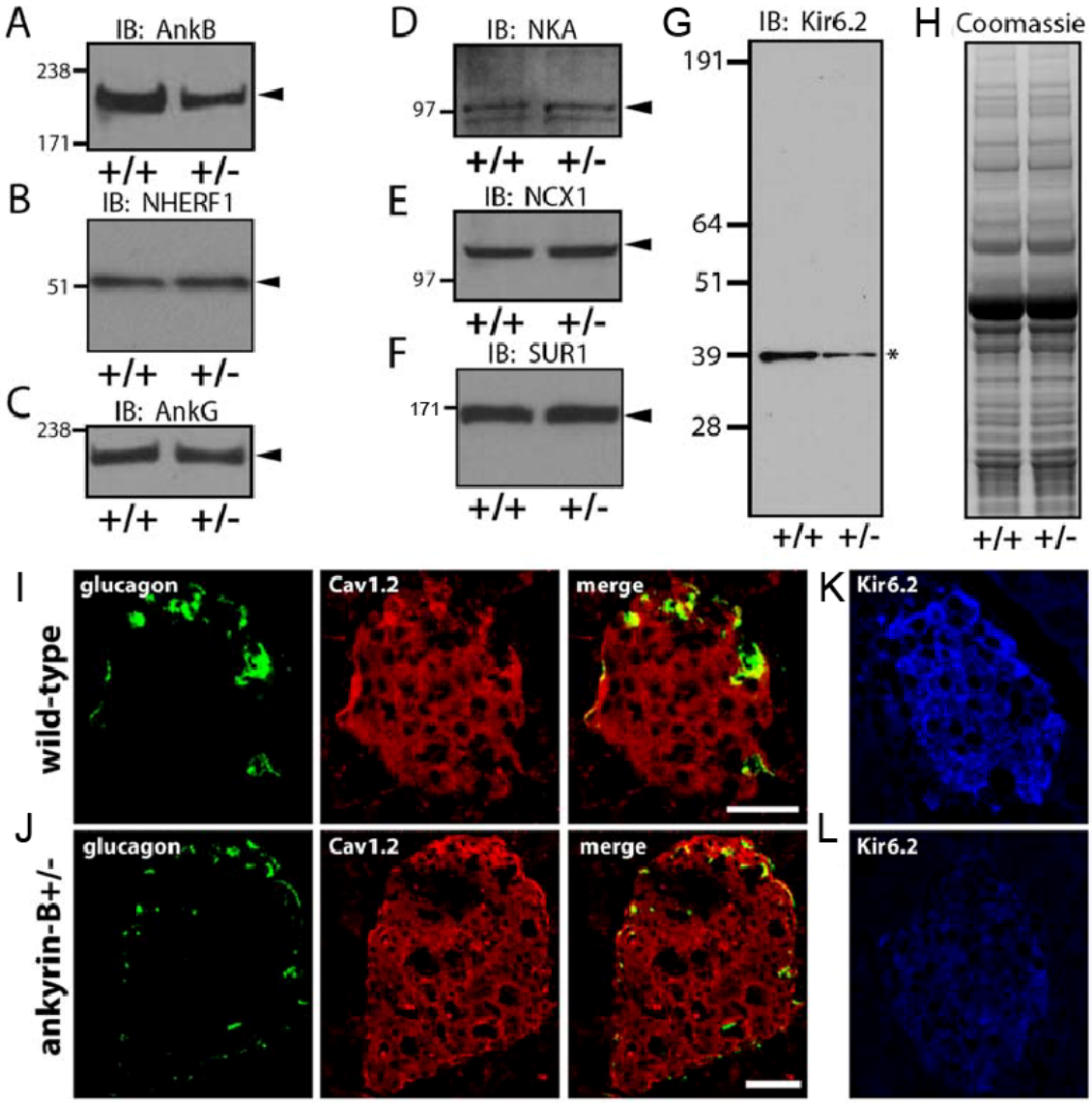

Figure 9. Expression and localization of the pore-forming subunit of the K_{ATP} channel, Kir6.2, is reduced in pancreatic tissue from ankyrin-B$^{+/-}$ mice.

(A) Ankyrin-B$^{+/-}$ pancreatic lysates express significantly less ankyrin-B than wild type while there are no significant differences in the expression of (B) NHERF1, (C) Ankyrin-G, (D) NKA, (E) NCXI, (F) SUR1, and (I) and (J) $Ca_v1.2$. The expression of Kir6.2 is significantly reduced in pancreatic lysates from ankyrin-B$^{+/-}$ mice as analyzed by immunoblot (G) and in pancreatic islets as analyzed by immunofluorescent staining (K and L). (H) Coomassie staining and (B) NHERF1 staining confirm equal loading of protein. Scale bar equals 10 microns.

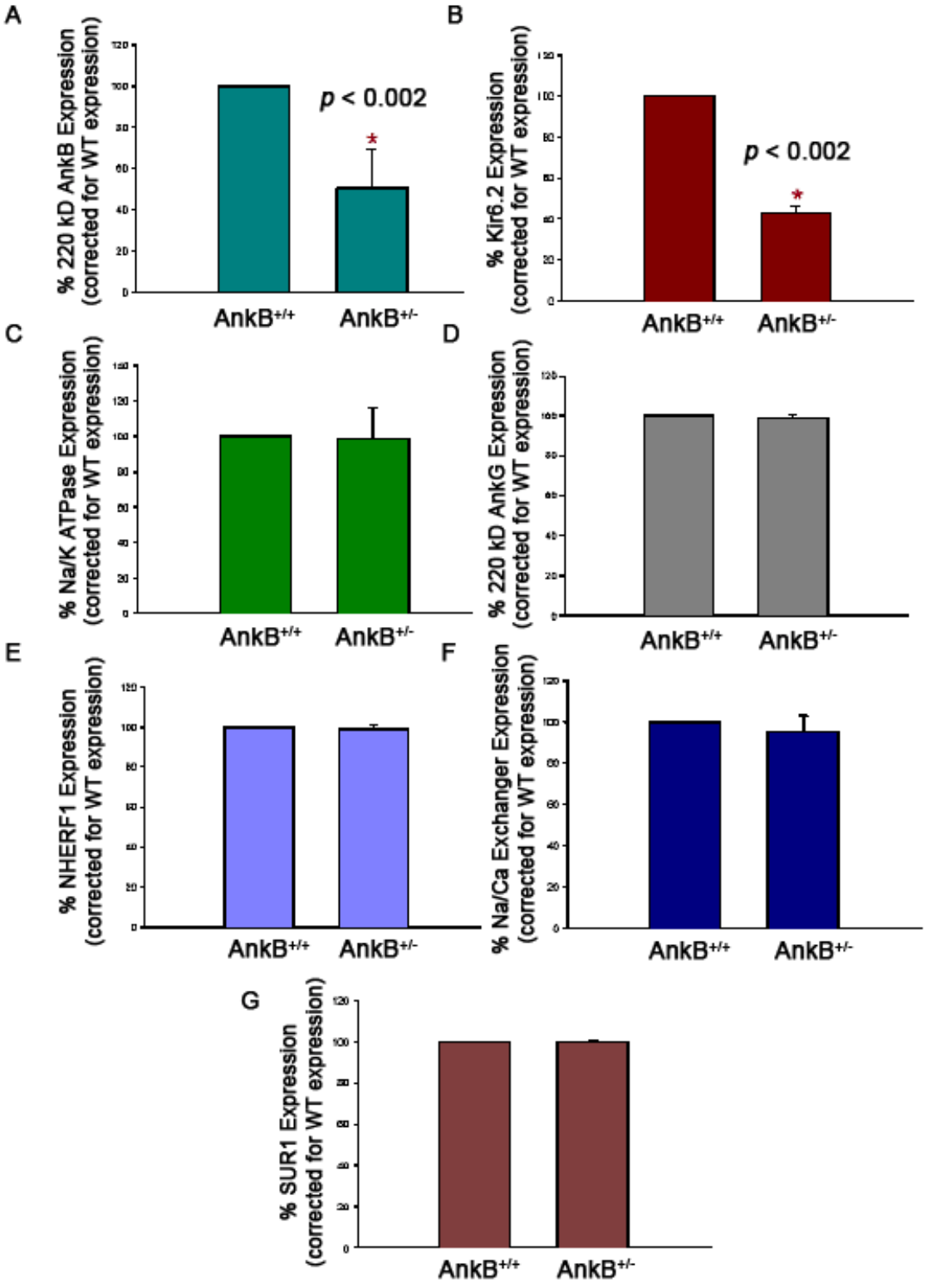
A
% 220 kD AnkB Expression (corrected for WT expression)
p < 0.002
*
AnkB+/+
AnkB+/-
B
% Kir6.2 Expression (corrected for WT expression)
p < 0.002
*
AnkB+/+
AnkB+/-
C
% Na/K ATPase Expression (corrected for WT expression)
AnkB+/+
AnkB+/-
D
% 220 kD AnkG Expression (corrected for WT expression)
AnkB+/+
AnkB+/-
E
% NHERF1 Expression (corrected for WT expression)
AnkB+/+
AnkB+/-
F
% Na/Ca Exchanger Expression (corrected for WT expression)
AnkB+/+
AnkB+/-
G
% SUR1 Expression (corrected for WT expression)
AnkB+/+
AnkB+/-

Figure 10. Differences in ankyrin-B and Kir6.2 expression from wild type and and ankyrin-B$^{+/-}$ pancreatic lysates is statistically significant.

(A and B) A two-tailed *t*-test demonstrated that differences in ankyrin-B and Kir6.2 expression between wild type and ankyrin-B$^{+/-}$ pancreatic lysates are significantly different (p <0.002), while there are no statistical differences in the expression levels of (C) NKA, (D) ankyrin-G, (E) NHERF1, (F) NCX1, and (G) SUR1 between wild type and ankyrin-B$^{+/-}$ pancreatic lysates.

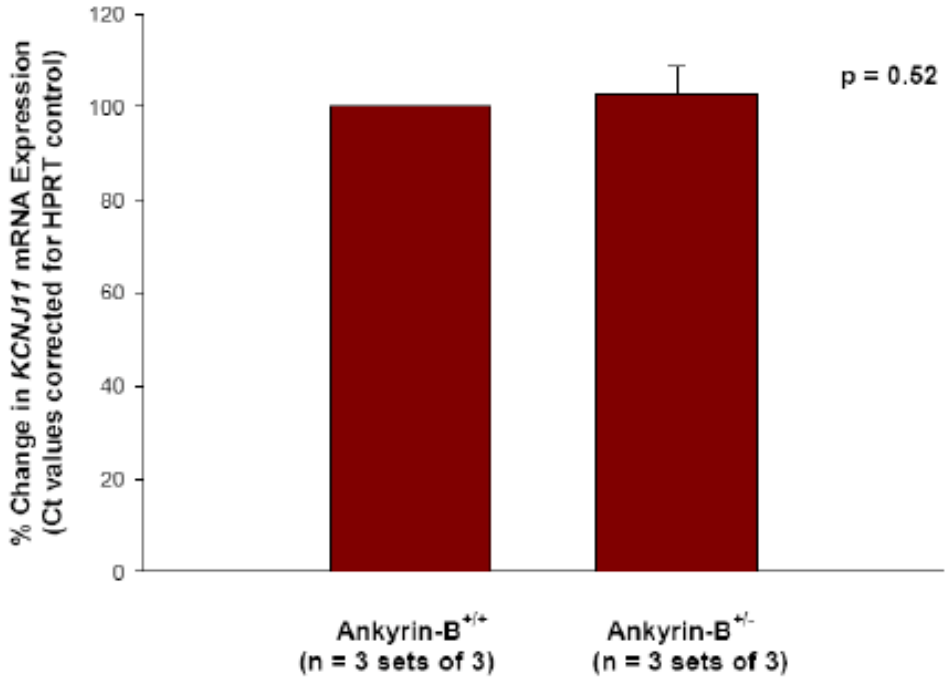

Figure 11. There is no significant difference in Kir6.2 mRNA levels between wild type and ankyrin-B$^{+/-}$ tissues.

Quantitative real-time polymerase chain reaction (qRT-PCR) demonstrates no significant difference in the mRNA levels of Kir6.2 between wild type and ankyrin-B$^{+/-}$ tissue, suggesting that differences in protein levels are due to a post-transcriptional event.

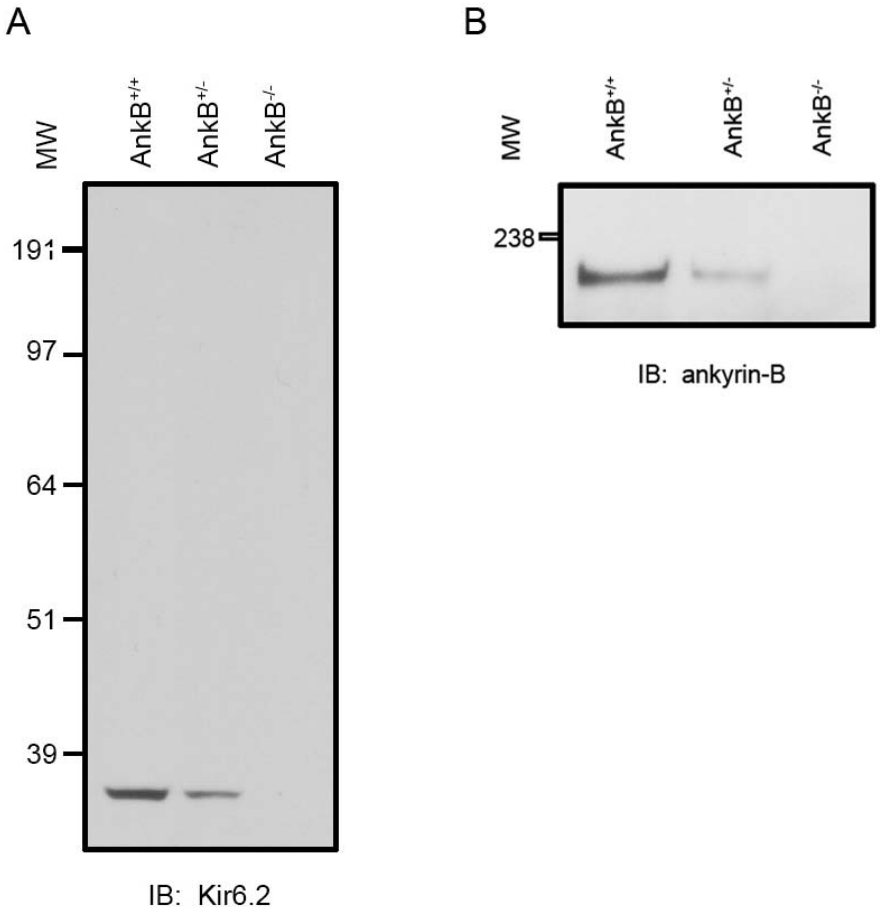

Figure 12. Ankyrin-B null pancreatic lysates do not express discernible levels of Kir6.2 or ankyrin-B.

Pooled pancreata from post-natal day 1 wild type, ankyrin-$B^{+/-}$, and ankyrin-B null mice were analyzed by immunoblot. While ankyrin-$B^{+/-}$ pancreatic lysates express significantly less Kir6.2 (A) and ankyrin-B (B) than wild type, there is no discernable expression of either Kir6.2 or ankyrin-B in ankyrin-B null pancreatic lysates.

Islets isolated from ankyrin-B$^{+/-}$ mice demonstrate decreased insulin secretion

To demonstrate an *in vivo* response to decreased ankyrin-B expression levels on islet activity, islets were isolated from six month old wild type and ankyrin-B$^{+/-}$ mice. These islets were analyzed for the ability to secrete insulin in response to 23 mM glucose (stimulatory concentration used in insulin secretion assays). Ankyrin-B$^{+/-}$ islets demonstrate a subtle, but statistically significant, decrease in insulin secretion compared to wild type islets (Figure 13A). In order to demonstrate that the decrease in insulin secretion is not due to any differences in islet insulin content, the insulin content of five isolated islets from both wild type and ankyrin-B$^{+/-}$ pancreata was measured. According to Figure 13B, there is no significant difference in the insulin content of wild type and ankyrin-B$^{+/-}$ islets, suggesting that any differences in insulin secretion is not due to differences in islet insulin content. Since decreased insulin secretion is associated with hyperglycemia, fasting blood glucose levels were measured. Ankyrin-B$^{+/-}$ mice are significantly more hyperglycemic than wild type, suggesting an abnormality in insulin secretion in these mice.

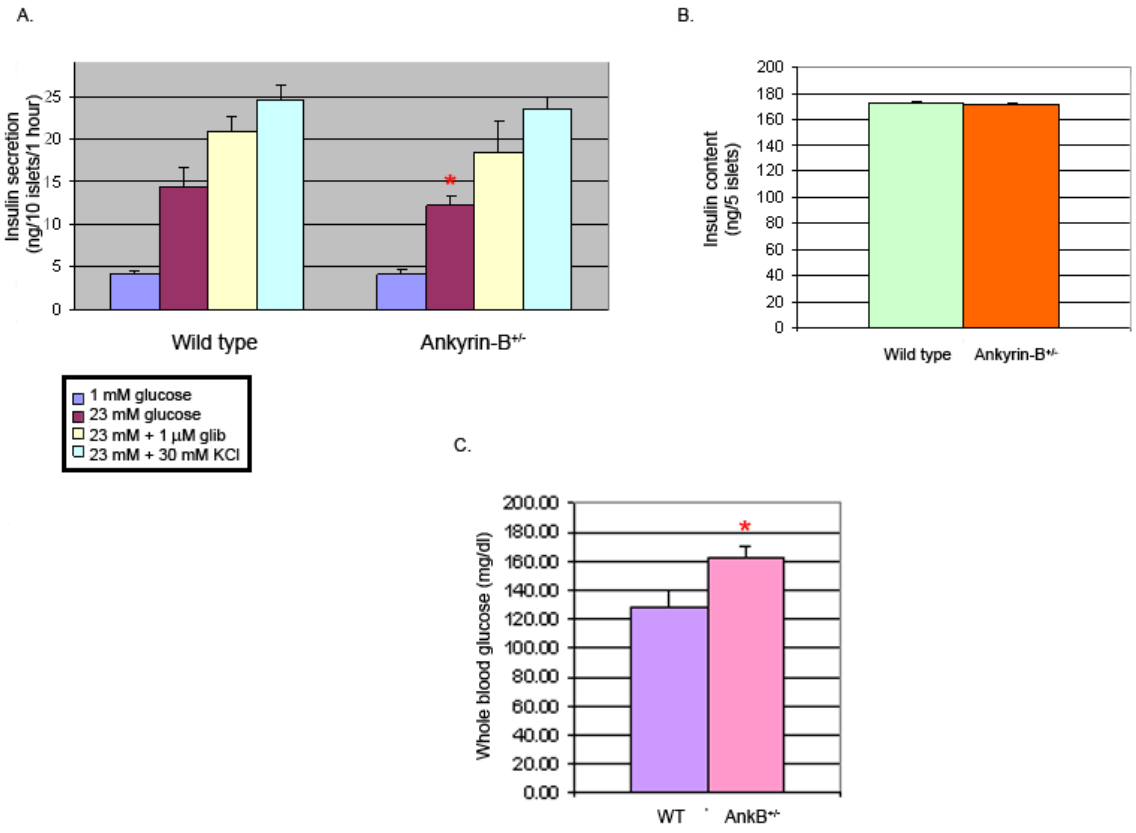

Figure 13. Islets from ankyrin-B$^{+/-}$ mice secrete less insulin than wild type islets under stimulatory conditions.

(A) Pancreatic islets isolated from wild type and ankyrin-B$^{+/-}$ mice were stimulated with 23 mM glucose and insulin secretion measured after one hour. Islets from ankyrin-B$^{+/-}$ mice secrete significantly less insulin in response to 23 mM glucose than wild type. (B) Islets were analyzed for insulin content, demonstrating no significant differences in insulin content between the isolated islets from wild type and ankyrin-B$^{+/-}$ mice. (C) Ankyrin-B$^{+/-}$ mice exhibit hyperglycemia with fasting blood glucose levels significantly higher than wild type.

Ankyrin-B co-immunoprecipitates with Kir6.2 in the pancreas and heart

Co-immunoprecipitation experiments were performed to evaluate a potential *in vivo* ankyrin-B/Kir6.2 interaction. Affinity-purified ankyrin-B Ig co-immunoprecipitates Kir6.2 from detergent-soluble lysates generated from rat pancreas and rat heart (Figure 14A). No interaction between Kir6.2 and control Ig was observed (Figure 14A). Moreover, ankyrin-B was unable to co-immunoprecipitate the Kir6.1 isoform from cardiac tissue (Figure 14B), suggesting that ankyrin-B interaction is specific for Kir6.2.

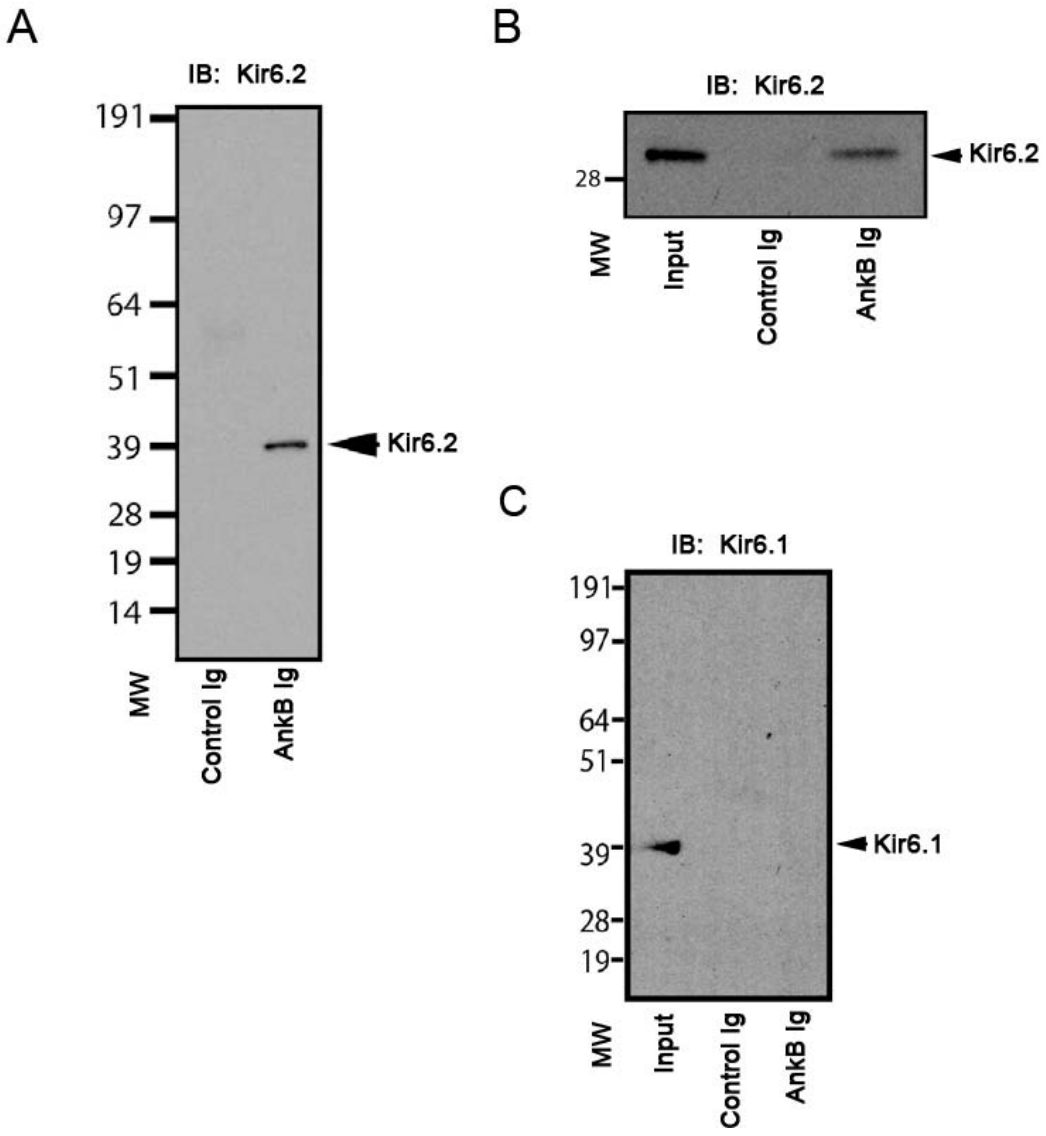

Figure 14. Ankyrin-B associates *in vivo* with Kir6.2 in both pancreas and heart.

(A) Kir6.2 co-immunoprecipitates with ankyrin-B from rat pancreatic lysate using affinity-purified anti-ankyrin-B antibodies, while control antibodies show no non-specific association. (B) Ankyrin-B and Kir6.2 also co-immunoprecipitate from rat heart lysate, while (C) ankyrin-B and Kir6.1 do not. Control antibodies show no non-specific association in heart lysate.

Ankyrin-B MBD directly interacts with *in vitro* translated full-length Kir6.2

A potential direct ankyrin-B/Kir6.2 interaction was explored using purified proteins. GST-fusion proteins corresponding to the three major domains of ankyrin-B (MBD, SBD, and CTD regulatory domains, Figure 15A) were generated and purified from bacteria (Figure 15C). *In vitro* binding assays using radiolabeled full-length Kir6.2 revealed a direct interaction of the ankyrin-B MBD (Figure 15B). Both the ankyrin-B SBD and CTD displayed binding activity below background (Figure 15B). Since the ankyrin-B MBD fusion protein used for binding experiments in Figure 14B contains 180 residues of the SBD N-terminus (Mohler, Davis et al. 2004), a new fusion protein lacking these SBD residues was generated to verify that binding occurred solely within the MBD. As shown in Figure 15D, residues within the MBD are sufficient for Kir6.2 interaction. In fact, significant ankyrin-B MBD/Kir6.2 binding was detected even in salt concentrations as high as 500 mM (Figure 15E). Together, these new findings support that ankyrin-B and Kir6.2 are protein partners *in vivo* and that this direct interaction is mediated by the ankyrin-B MBD.

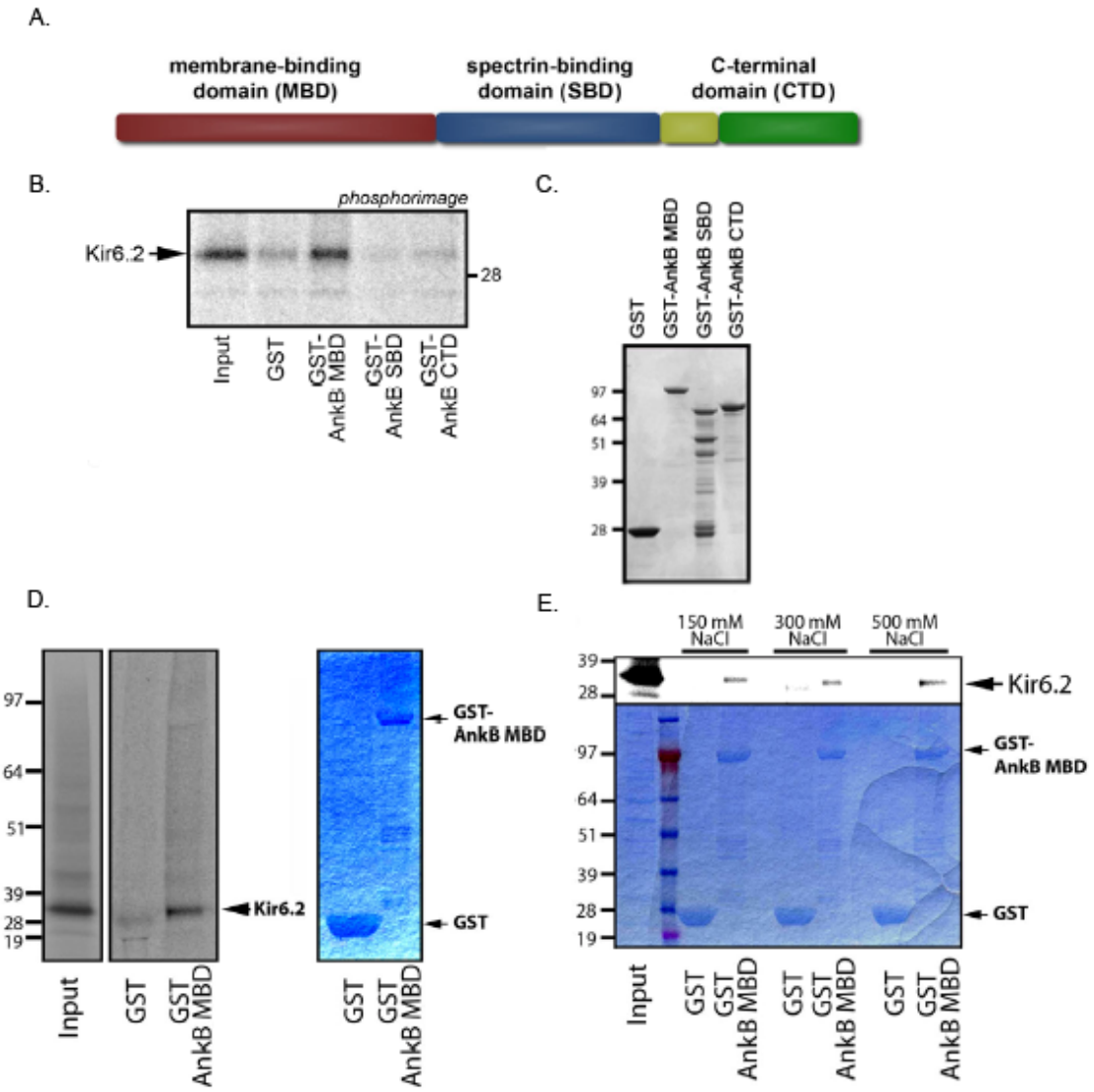

Figure 15. Ankyrin-B associates with Kir6.2 *in vitro*.

(A and B) *In vitro* translated full-length Kir6.2 associates with the MBD containing a portion of the SBD of ankyrin-B, but shows no significant association with the SBD or CTD of ankyrin-B. (C) Coomassie staining demonstrating equal amounts of GST-ankyrin-B MBD, SBD, and CTD were used in the *in vitro* binding assay. (D) *In vitro* translated Kir6.2 associates with the ankyrin-B MBD (note loading of GST-ankyrin-B MBD and GST alone) and this interaction persists at salt concentrations as high as 500 mM NaCl (top panel, E; bottom panel demonstrates equal loading of purified GST proteins).

Ankyrin-B MBD interacts with the C-terminal cytoplasmic domain of Kir6.2

Kir6.2 has two transmembrane domains: a cytosolic N-terminus (residues 1-73) and a cytosolic C-terminus (residues 161-390) (Figure 16A). Ankyrin is a strictly cytosolic protein; therefore, association with Kir6.2 should be limited to cytosolic domains. To establish the structural requirements on Kir6.2 for ankyrin-B association, Kir6.2 mutant polypeptides were generated that lacked either the N- or C-terminal cytoplasmic domains (Kir6.2-NT and Kir6.2–CT, respectively; Figure 16B, C). Radiolabeled full-length and mutant Kir6.2 proteins were incubated with immobilized GST-ankyrin-B MBD or GST alone. Similar to full-length Kir6.2, there was significant binding of Kir6.2-NT with GST-ankyrin-B MBD (Figure 16D, E). In contrast, Kir6.2 lacking the cytoplasmic C-terminus lacked ankyrin-binding activity (Figure 16F), even though the ankyrin-B polypeptide was present at equivalent levels (Figure 16G). These findings establish the Kir6.2 C-terminal cytoplasmic domain as the site of ankyrin-B/Kir6.2 interaction.

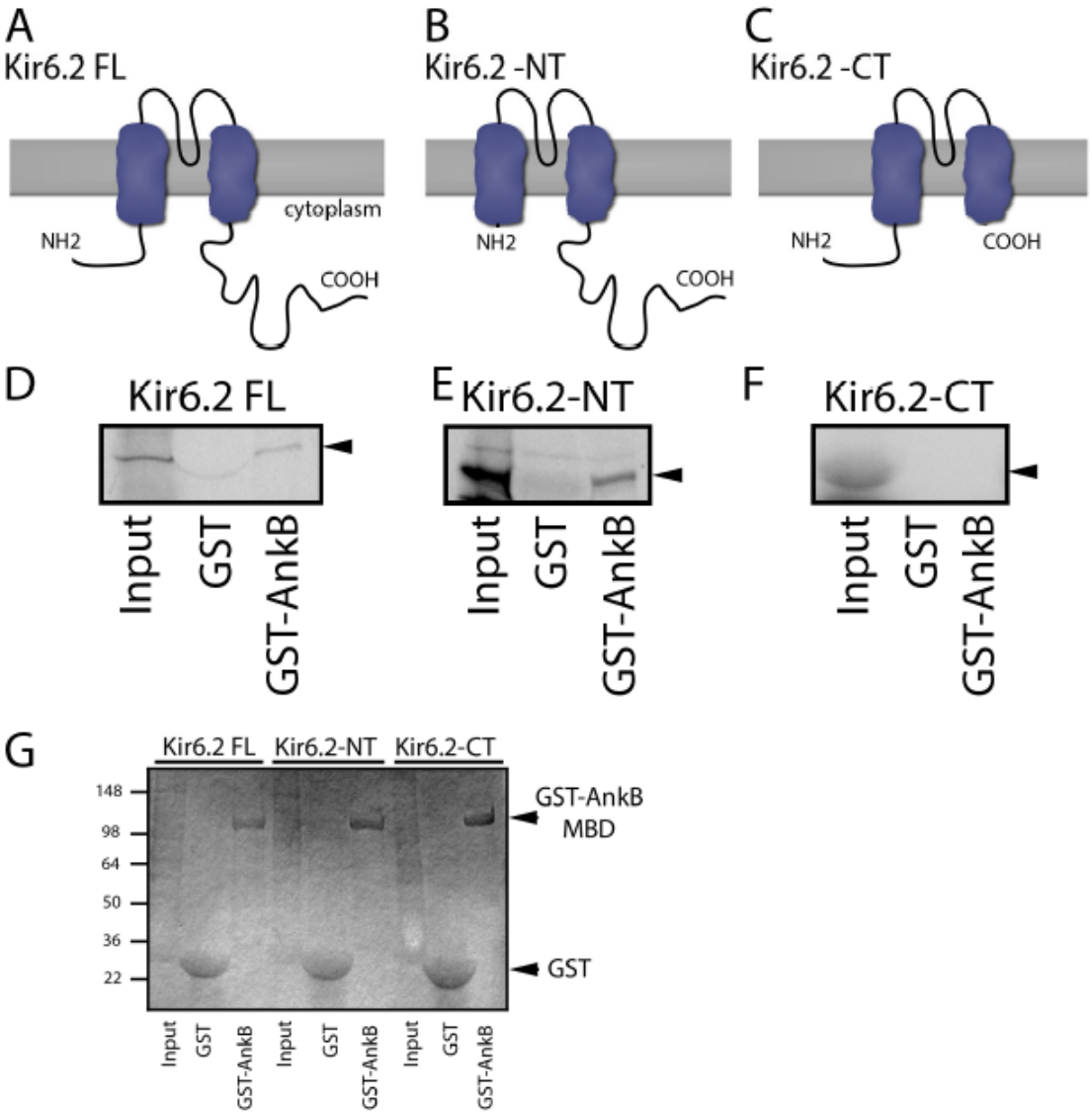

Figure 16. Ankyrin-B associates with the C-terminal cytoplasmic domain of Kir6.2.

(A) *In vitro* translated full-length Kir6.2 and Kir6.2 engineered to remove the (B) N-terminal cytoplasmic portion (residues 1-73) are able to associate with the membrane-binding domain of ankyrin-B (D and E), while Kir6.2 engineered to remove the (C) C-terminal cytoplasmic domain (residues 160-390) loses interaction with ankyrin-B (F). (G) Coomassie stain demonstrating equal amounts of GST-ankyrin-B MBD or GST alone were added to each reaction.

An eight amino acid motif in the C-terminal domain of Kir6.2 is both necessary and sufficient for interaction with ankyrin-B

Ankyrin-G interacts with voltage-gated Na_v channel isoforms via a conserved cytoplasmic motif (Garrido 2003; Lemaillet 2003; Mohler 2004) (Figure 17A). A similar motif has also been recently described in $K_v2.1$ and $K_v3.1$ channels (Chung 2006; Pan 2006; Rasmussen 2007). Analysis of the primary sequence of the Kir6.2 C-terminus revealed an eight amino acid motif strikingly similar to the ankyrin-binding motif found on Na_v and K_v channels (Figure 17B). This motif is conserved across species (Figure 17B) and is not represented in human Kir6.1 (Figure 17B). We evaluated the role of this motif for ankyrin-binding using *in vitro* binding assays and two Kir6.2 mutants. The first mutant was engineered with a premature stop codon following the putative ankyrin-binding motif (Kir6.2 1-330 (Kir6.2truncB), Figure 17D), while the second mutant was engineered with a premature stop codon preceding the putative ankyrin-binding motif (Kir6.2 1-314 (Kir6.2truncA), Figure 17E). *In vitro* binding with immobilized GST-ankyrin-B MBD revealed that only the Kir6.2 1-330 mutant displayed binding activity for ankyrin-B (Figure 18G), similar to that of full-length Kir6.2 (Figure 17C, F). Binding of Kir6.2 1-314 for GST-ankyrin-B MBD was similar to GST alone (Figure 17H). These data suggest that an eight residue motif found in other ankyrin-binding partners is necessary for the interaction of Kir6.2 with ankyrin-B.

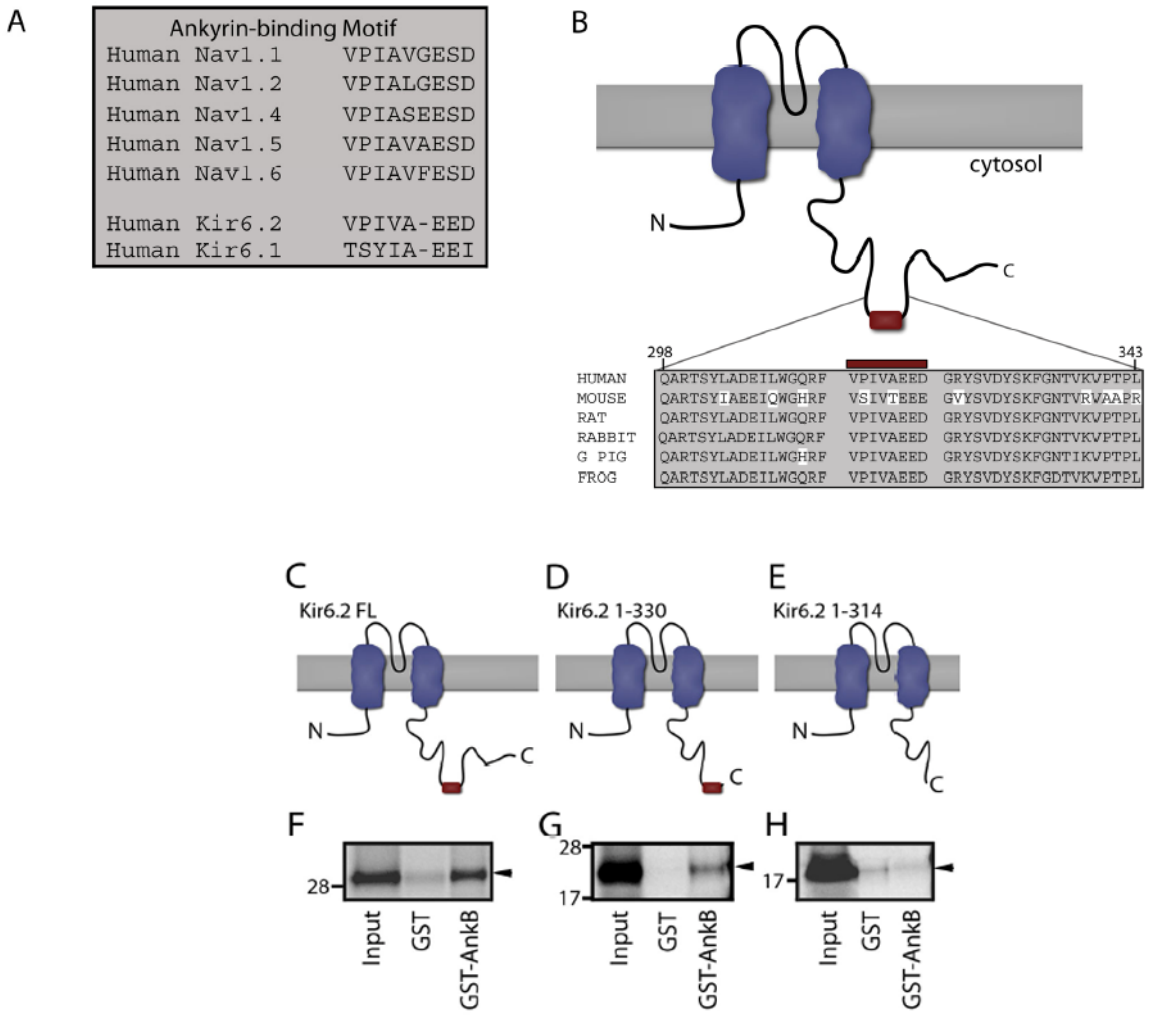

Figure 17. A C-terminal eight amino acid motif in Kir6.2 is necessary for interaction with ankyrin-B MBD.

(A) Evaluation of the primary sequence of the C-terminal cytoplasmic domain of Kir6.2 revealed an eight amino acid motif that is not only conserved among Kir6.2 peptides from a number of species, including rat, mouse, and human (B), but also shares striking similarity to a motif in the Na_v family of proteins required for interaction with ankyrin-G (A). Kir6.2 peptides were engineered to prematurely truncate before (E), or after (D) the Kir6.2 VPIVAEED motif (red box). (H) *In vitro* translated peptide lacking this motif was unable to associate with ankyrin-B MBD, while (F) *in vitro* translated full-length Kir6.2 or (G) Kir6.2 truncated immediately after VPIVAEED retained the ankyrin-B interaction.

To determine if Kir6.2 residues 316-323 are necessary *and* sufficient for ankyrin-B interaction, a biotinylated peptide against the putative Kir6.2 C-terminal ankyrin-binding motif was generated (Figure 18A). The peptide was engineered with an N-terminal biotin and an S-G-S-G linker between the biotin and the peptide. The peptide was coupled to strepavidin beads and incubated with purified ankyrin-B MBD. As displayed in Figure 18B, Kir6.2 residues 316-323 are sufficient for ankyrin-B interaction. Consistent with data using full-length channel, a pure peptide generated against the Kir6.2 E322K disease mutant displayed significantly reduced ankyrin-binding activity (Figure 18B). No binding was observed with GST alone (or control beads) with either peptide. Together, these results demonstrate that Kir6.2 C-terminal residues 316-323 are necessary *and* sufficient for association with ankyrin-B.

Figure 18. A C-terminal motif in Kir6.2 is sufficient for interaction with ankyrin-B.

(A) Two biotinylated peptides (Kir6.2 WT and Kir6.2 E322K) were synthesized for use in an in vitro pulldown experiment. (B) While the WT peptide showed significant association with GST-ankyrin-B MBD, the E322K mutant peptide showed decreased association. There was no discernable association between the peptide and GST alone or with beads alone.

A known human PNDM mutation in Kir6.2, E322K, results in loss of interaction with ankyrin-B

A previously identified human variant in the ankyrin-binding motif of $Na_v1.5$ is associated with human Brugada syndrome arrhythmia (Mohler, Rivolta et al. 2004) (Figure 19A). At the cellular level, human Brugada syndrome is characterized by decreased $Na_v1.5$ current (Priori, Napolitano et al. 2002). Consistent with this phenotype, the $Na_v1.5$ E1053K mutant lacks ankyrin-binding activity (Figure 19B) and is not targeted to the membrane of primary adult cardiomyocytes (resulting in decreased I_{Na}) (Mohler, Rivolta et al. 2004).

A human Kir6.2 disease gene mutation that blocks the ankyrin-B/Kir6.2 interaction was identified. In 2004, Vaxillaire and colleagues identified a Kir6.2 gene variant in a three-day-old female with hyperglycemia. This child was insulin-dependent at 10 years, 10 months and characterized with the PNDM phenotype (Vaxillaire, Populaire et al. 2004). Interestingly, this Kir6.2 mutant (964G>A leading to E322K) is located in the Kir6.2 ankyrin-binding motif (Figure 19A). Moreover, this variant is located in an acidic motif that correlates with the $Na_v1.5$ Brugada syndrome disease variant E1053K (also negative to positive charge reversal, see Figure 19A).

To determine whether the Kir6.2 PNDM disease variant E322K affects ankyrin-binding, the human PNDM mutation was generated in the context of full-length human Kir6.2. Additionally, Kir6.2 E321K (analogous residue affected in $Na_v1.5$ Brugada E1053K mutation) and Kir6.2 D323K were generated (Figure 19C). Finally, acidic charges were changed to non-charged alanine residues (Kir6.2 E321A, E322A, D323A) in three additional mutants. Kir6.2 mutants

E321K, E322K (PNDM mutation), and D323K nearly abolish ankyrin-B-binding activity (Figure 19C). In contrast, neutral amino acids substitution mutations in the Kir6.2 ankyrin-binding motif (E321A, E322A, D323A) had no effect on ankyrin-binding (Figure 19C). These findings identify a key acidic stretch in the ankyrin-binding motif as critical for ankyrin-B/Kir6.2 interaction. More significantly, these findings suggest that the human Kir6.2 E322K disease mutation disrupts the ankyrin-B/Kir6.2 interaction.

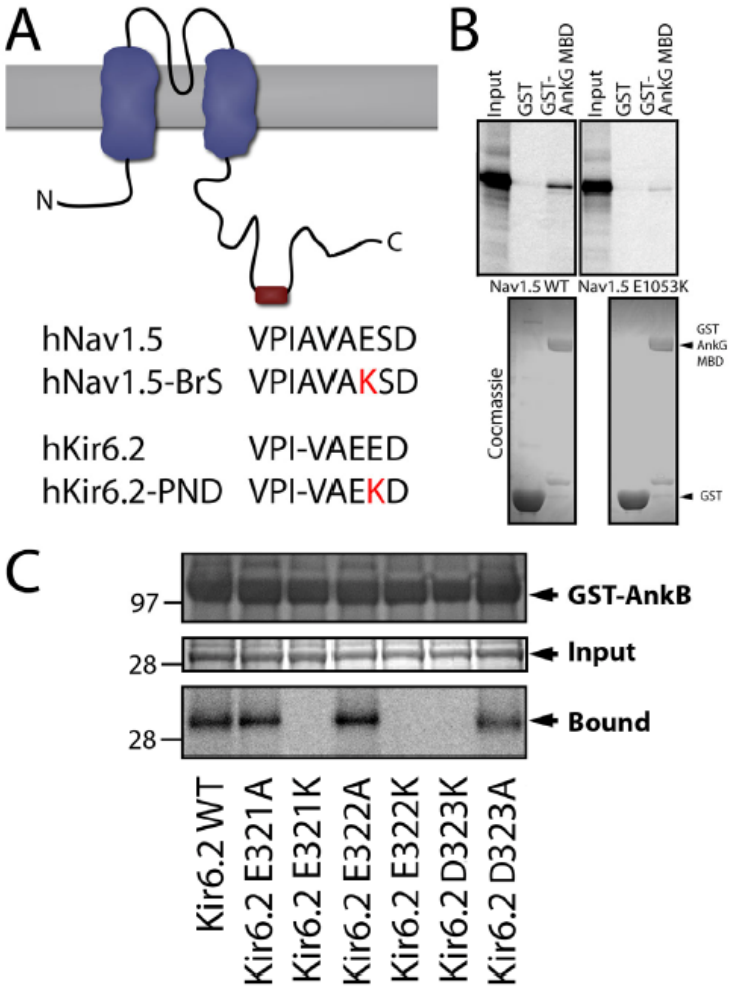

Figure 19. Three charged amino acids within the VPIVAEED motif are necessary for interaction with ankyrin-B.

(A) A known human mutation within the putative ankyrin-B binding motif, E322K, is associated with human permanent neonatal diabetes mellitus (PNDM). Similarly, a mutation in the Na_v family of proteins within the corresponding motif results in loss of interaction with ankyrin-G. (B) *In vitro* translated $Na_v1.5$ E1053K, the mutation which disrupts the $Na_v1.5$/ankyrin-G interaction, is unable to associate with ankyrin-G MBD, while wild type $Na_v1.5$ can associate. (C) Kir6.2 constructs containing site-directed mutagenesis of three key charged residues (EED) show that interaction with ankyrin-B is lost when any of these residues are mutated to the opposite charge, but not when mutated to an alanine (note equal loading of Kir6.2 peptides and GST-ankyrin-B MBD).

Kir6.2 association is specific to ankyrin-B and not ankyrin-G

In order to show that the association of Kir6.2 is specific to ankyrin-B and not another member of the ankyrin family, ankyrin-G, *in vitro* binding analysis was used. Specifically, *in vitro* translated, radiolabeled Kir6.2 was incubated with immobilized GST-tagged ankyrin-B MBD, ankyrin-G MBD, or GST alone. While Kir6.2 shows *in vitro* association with ankyrin-B MBD, the association with ankyrin-G MBD is below that of control (GST alone) (Figure 20). This is consistent with previously published reports which demonstrate that ankyrin gene products show specificity for binding partners.

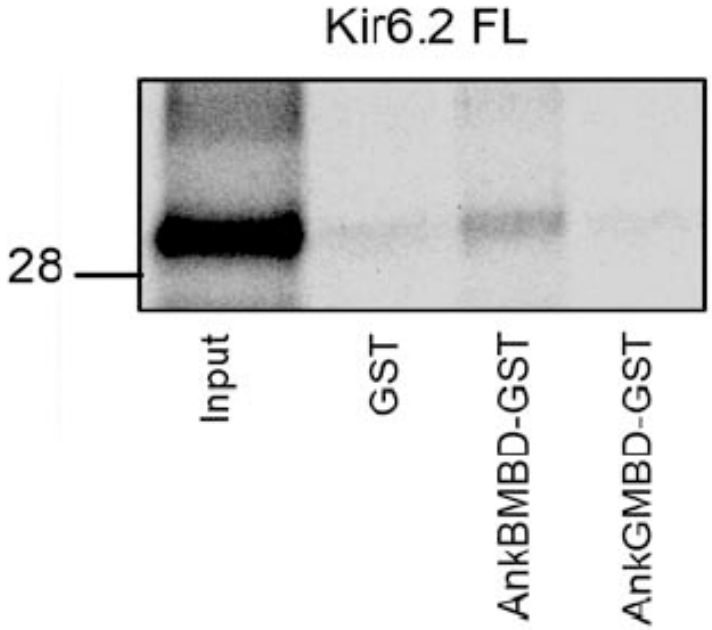

Figure 20. Kir6.2 association is specific to ankyrin-B and not ankyrin-G.

In vitro translated Kir6.2 is able to associate with immobilized GST-ankyrin-B-MBD, but not GST-ankyrin-G-MBD or GST alone.

Ankyrin-B is required for Kir6.2 membrane expression

Ankyrin-B is ubiquitously expressed in vertebrate tissues. Moreover, all tested cultured vertebrate heterologous cell lines express ankyrin-B. Therefore, a primary cell culture system lacking ankyrin-B needed to be developed in order to test the role of ankyrin-B expression for Kir6.2 membrane targeting. Primary cardiac fibroblasts were isolated from ankyrin-$B^{-/-}$ post-natal day 1 mice (ankyrin-$B^{-/-}$ mice die shortly after birth (Scotland, Zhou et al. 1998)). Fibroblasts were expanded and the ankyrin-$B^{-/-}$ genotype of the resulting cell culture was confirmed by genotyping, immunoblot, and immunostaining (Figure 21A-D). Wild-type and ankyrin-$B^{-/-}$ fibroblasts displayed similar properties in culture with respect to cell division, growth, size, and morphology. Immunoblotting of fibroblasts also revealed lack of Kir6.2 expression (Figure 21E). Thus, a primary cell system lacking ankyrin-B expression was established.

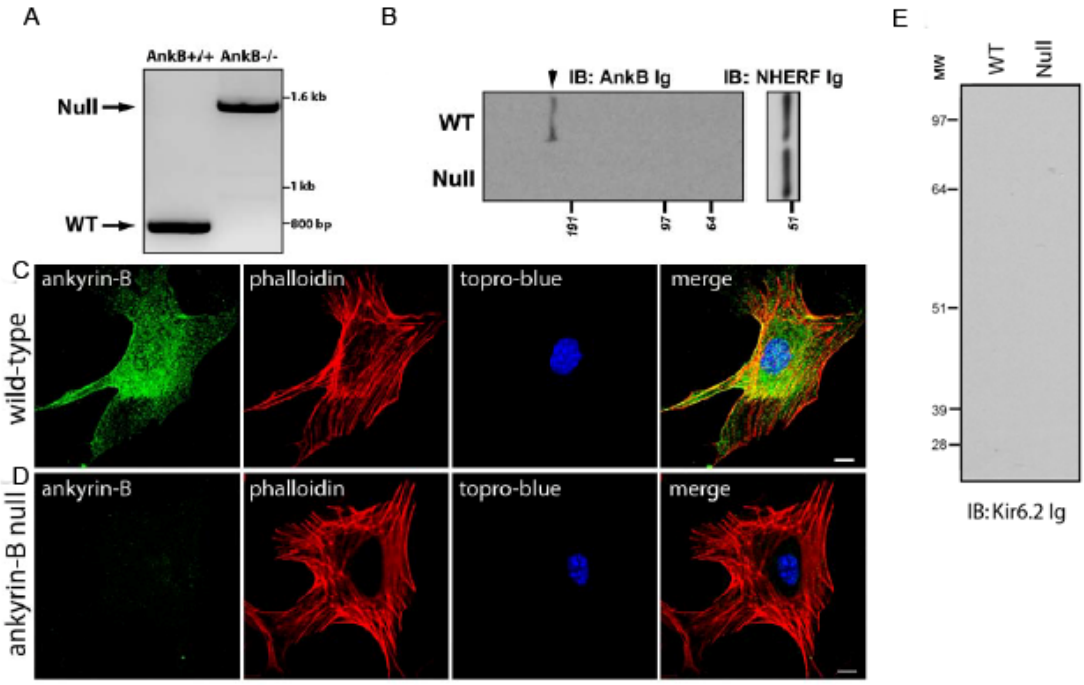

Figure 21. Ankyrin-B wild type and null cardiac fibroblasts can be isolated and expanded in culture.

Cardiac fibroblasts isolated from postnatal day 1 wild type and ankyrin-B null mice were verified by (A) Genotyping (the null mice were engineered with an 800 bp neocassette disrupting the *ANK2* gene), (B) immunoblotting (note equal loading of protein as assayed by NHERF1 expression), and (C) immunofluorescence (red staining indicates phalloidin staining, while blue staining denotes ToPro staining of genetic material). Scale bar equals 10 microns.

A Kir6.2 cDNA tagged with GFP was created to monitor the subcellular localization of Kir6.2 in wild type and ankyrin-B$^{-/-}$ fibroblasts. Kir6.2 was cloned from human heart cDNA library and engineered with a C-terminal GFP tag. Previously published work demonstrates no significant changes in Kir6.2 expression or function following the addition of a C-terminal GFP fusion {Makhina, 1998 #161; Makhina, 1998 #1619}. Co-transfection of GFP-Kir6.2 with SUR (at 5:1 SUR:Kir6.2 ratio) into wild type fibroblasts revealed GFP-Kir6.2 fluorescence present at the plasma membrane (Figure 22A, C). In contrast, GFP-Kir6.2 was not efficiently targeted to the membrane of ankyrin-B$^{-/-}$ fibroblasts, but instead localized to a perinuclear compartment (Figure 22B, D). Together, these findings support a requirement of ankyrin-B expression for Kir6.2 membrane targeting.

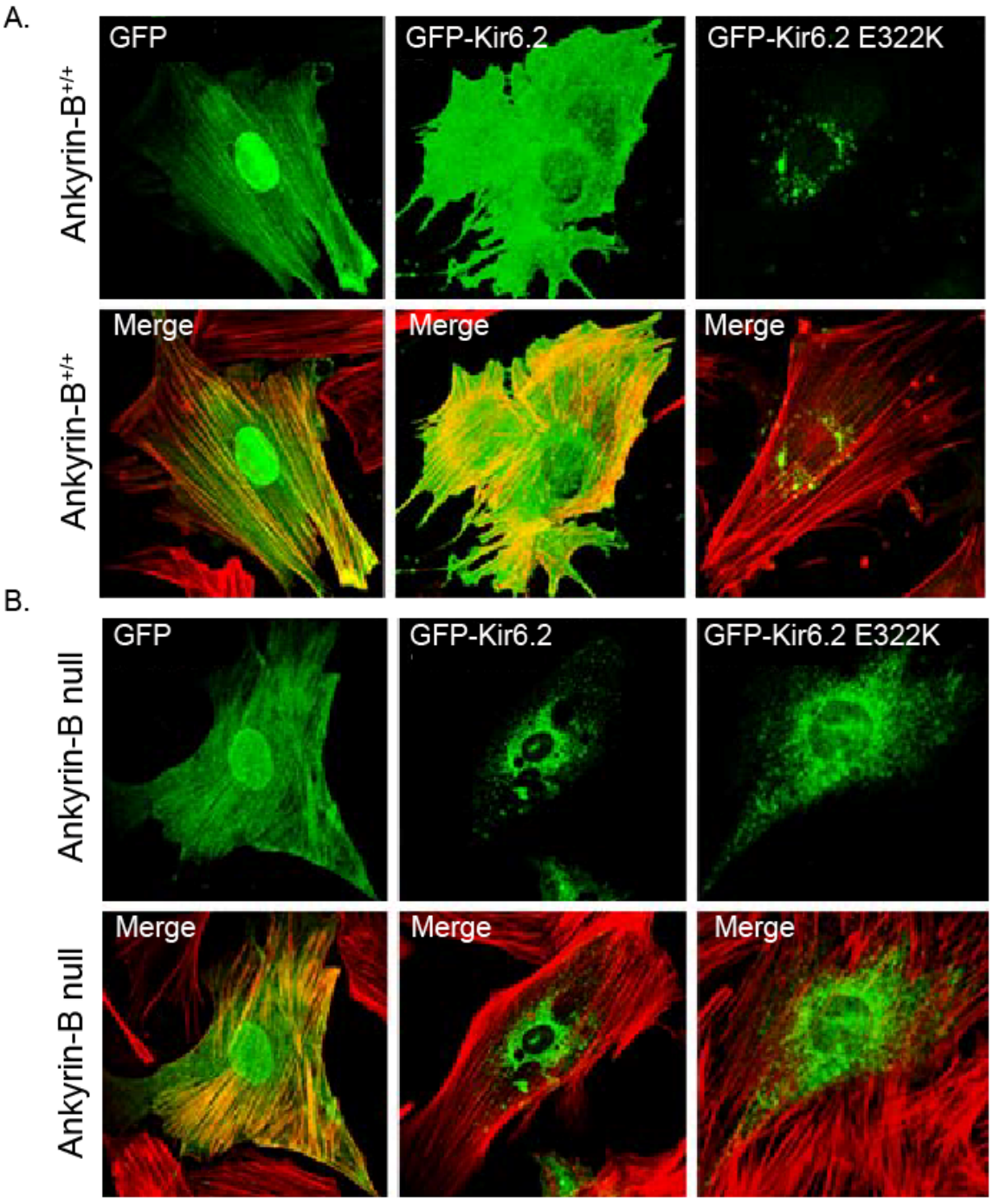
A.
GFP
GFP-Kir6.2
GFP-Kir6.2 E322K
Ankyrin-B+/+
Merge
Merge
Merge
Ankyrin-B+/+
B.
GFP
GFP-Kir6.2
GFP-Kir6.2 E322K
Ankyrin-B null
Merge
Merge
Merge
Ankyrin-B null

Figure 22. Ankyrin-B is required for Kir6.2 localization in cardiac fibroblasts.

(A) Wild type fibroblasts co-transfected with SUR1 and GFP-Kir6.2 cDNAs show diffuse localization of GFP-Kir6.2 (middle panel), distinct from the localization pattern of GFP alone (left panel). Co-transfection with SUR1 and GFP-Kir6.2 E322K cDNAs shows a perinuclear localization (right panel). (B) Ankyrin-B null fibroblasts co-transfected with SUR1 and GFP-Kir6.2 show abnormal localization of GFP-Kir6.2 (middle panel), which is similar to what is observed with ankyrin-B null fibroblasts co-transfected with SUR1 and GFP-Kir6.2 E322K (right panel), suggesting a necessary role for ankyrin-B in *in vivo* localization of Kir6.2. Red corresponds to phalloidin-568 staining.

Kir6.2 disease mutant lacking ankyrin-binding activity is not efficiently targeted

Based on the abnormal membrane trafficking of $Na_v1.5$ E1053K disease mutation lacking ankyrin-binding activity (Mohler, Rivolta et al. 2004), the membrane targeting of the PNDM Kir6.2 disease mutation, E322K, was evaluated. Site-directed mutagenesis was used to generate full-length GFP-tagged wild type Kir6.2, E321K, E322K, and D323K. Wild-type and mutant channels were co-transfected with SUR cDNA into HEK293 cells. Immunoblots of co-transfected cells confirmed approximately equal protein expression between wild type and mutant cell lines. As expected, wild type GFP-Kir6.2 was primarily localized to the plasma membrane of transfected HEK293 cells (compare Figure 23B). In contrast, Kir6.2 mutants E321K, E322K, and D323K were not as efficiently targeted to the plasma membrane (Figure 23C-E). While residual low levels of plasma membrane staining for all of the mutants was observed, a significant fraction of the overall GFP-Kir6.2 mutant localization was clustered in a peri-nuclear pattern (arrows in Figure 23C-E). These results demonstrate that direct ankyrin-B/Kir6.2 interactions are required for efficient Kir6.2 membrane targeting in heterologous HEK293 cells (equivalent to Kir6.2 loss-of-function). However, these findings are in stark contrast to the observed gain-of-function PNDM phenotype of the human Kir6.2 E322K mutation *in vivo* (Vaxillaire, Populaire et al. 2004).

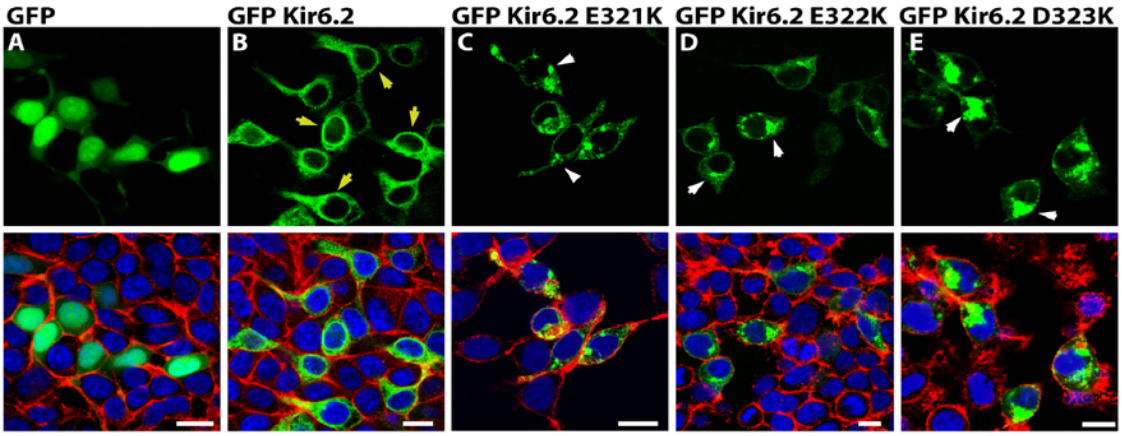

Figure 23. Interaction with ankyrin-B is necessary for efficient targeting of Kir6.2 in HEK293 cells.

(A-E) HEK293 cells co-transfected with SUR1 and wild type or mutant GFP-tagged Kir6.2 demonstrates that while wild type GFP-Kir6.2 shows a distinct plasma membrane localization (yellow arrows), Kir6.2 mutants that disrupt the ankyrin-B/Kir6.2 interaction (E321K, E322K (the human PNDM mutation), and D323K) show perinuclear localization (white arrowheads) indicative of inefficient targeting. Red corresponds to phalloidin-568 staining and blue corresponds to ToPro staining of nuclear material. Scale bar equals 10 microns.

Kir6.2 mutant lacking ankyrin-binding activity displays decreased ATP sensitivity

In addition to key roles in protein membrane targeting, recent work identified an unexpected role for ankyrin polypeptides in regulating ion channel biophysical activity {Shirahata, 2006 #1071; Mohler, 2004 #938}. Moreover, the dependence of ankyrin-pathways for ion channel targeting in cultured heterologous cell systems and vertebrate tissues are cell-type specific. In fact, these differential trafficking systems (ankyrin-dependent vs. ankyrin-independent) have enabled the study of the effect of ankyrin activity on channel biophysics in the absence of competing membrane trafficking defects {Shirahata, 2006 #1071; Mohler, 2004 #938}. Using confocal imaging of transfected heterologous cell lines, a number of cell lines were tested to identify a system to analyze the biophysical function of GFP-Kir6.2 E322K at the plasma membrane. After testing a number of lines (HEK293, CHO, NIH3T3), COSm6 cells were determined to most efficiently target Kir6.2 E322K to the plasma membrane (although trafficking is still impaired by ~20%, see Figure 24A-C). Interestingly, these same cells have been widely used to study Kir6.2 mutant function as they lack endogenous Kir6.2 expression (Pinney, MacMullen et al. 2008) (Figure 24E), yet they do express endogenous ankyrin-B (Figure 24D). In agreement with the previous imaging studies, COSm6 cells expressing GFP-Kir6.2 and GFP-Kir6.2 E322K demonstrated only a mild reduction in Kir6.2 membrane levels (under metabolic inhibition conditions; cells stably express SUR1; as assessed by Rb^+ flux assays (Figure 24B, C). Thus, the COSm6 cell line provides a suitable system for the

analysis of Kir6.2 membrane function without the interfering defects associated with ankyrin-mediated targeting.

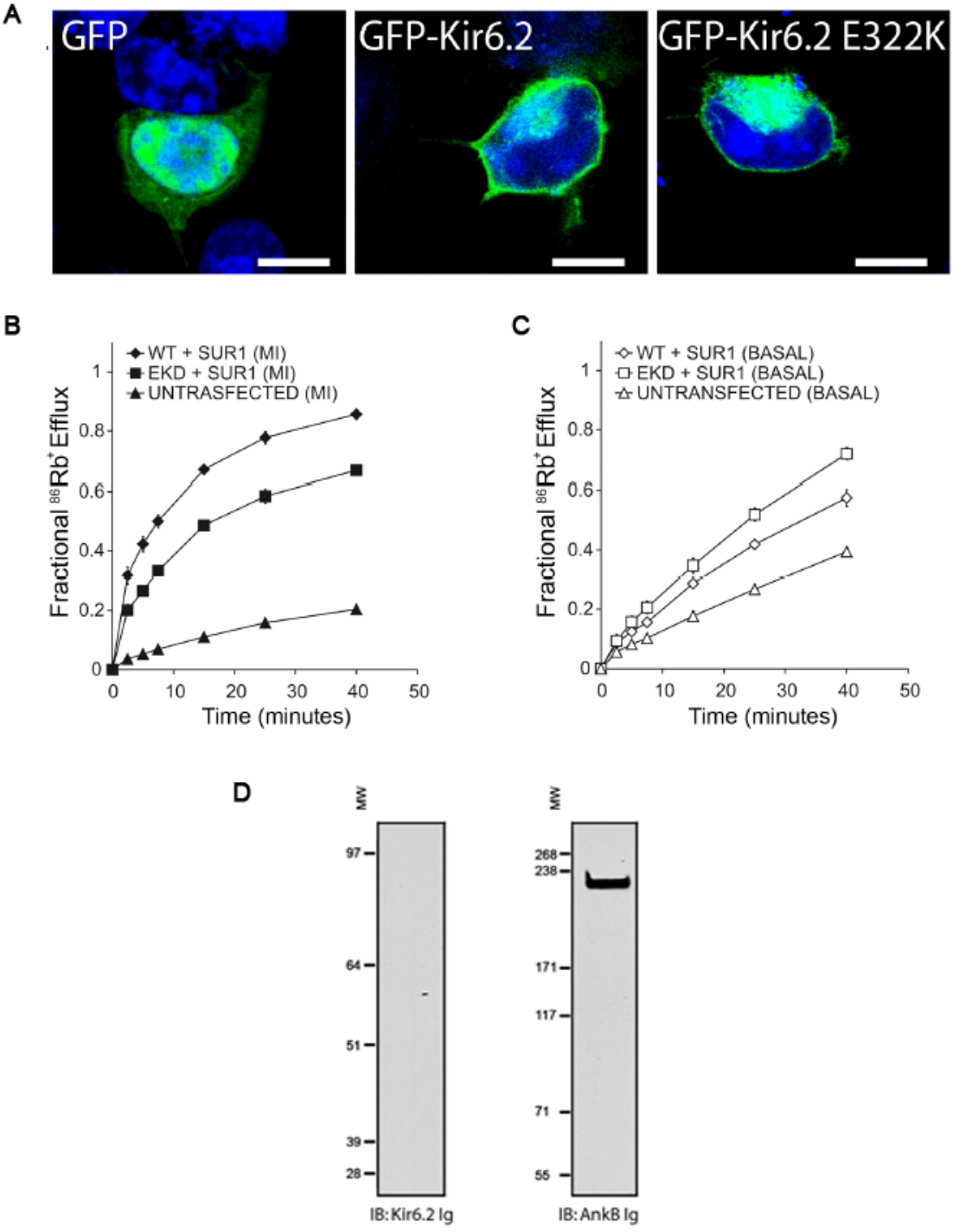
A
GFP
GFP-Kir6.2
GFP-Kir6.2 E322K
B
WT + SUR1 (MI)
EKD + SUR1 (MI)
UNTRASFECTED (MI)
Fractional 86Rb+ Efflux
Time (minutes)
C
WT + SUR1 (BASAL)
EKD + SUR1 (BASAL)
UNTRANSFECTED (BASAL)
Fractional 86Rb+ Efflux
Time (minutes)
D
MW
97
64
51
39
28
IB: Kir6.2 Ig
MW
268
238
171
117
71
55
IB: AnkB Ig

Figure 24. COSm6 cells demonstrate significant plasma membrane expression of E322K Kir6.2-GFP.

(A) While GFP-Kir6.2 shows significant plasma membrane localization in COSm6 cells, the GFP-Kir6.2 E322K mutant also demonstrates plasma membrane localization, though reduced. Rubidium flux analysis (under metabolic inhibition with (C) or without (B) glibenclamide administration) demonstrated that the surface expression of GFP-Kir6.2 E322K is reduced by ~15% as compared with wild type. Thus, the COSm6 cell line provides a cell model system to evaluate Kir6.2 E322K function independently of defects in ankyrin-B-dependent targeting. Nuclei are stained blue. (D) COSm6 cells express endogenous ankyrin-B, but no endogenous Kir6.2. Blue corresponds to ToPro staining of nuclear material. Scale bar equals 10 microns.

Transfected COSm6 cells were utilized to test the potential role of ankyrin-B/Kir6.2 membrane function independent of a targeting phenotype. Single-channel ATP sensitivity experiments were performed for the wild type and mutant channel. As shown in Figure 25B, the Kir6.2 E322K channel displays a significant reduction in ATP sensitivity compared with the wild type channel (Figure 25A). Specifically, the mutant channel that lacks ankyrin-B-binding activity is much less sensitive to ATP, as indicated by a shift in the $K_{1/2}$ for ATP binding from 15 μM in wild type to 62 μM in the mutant (Figure 25C). These data are consistent with E322K as a Kir6.2 gain-of-function mutation, and are consistent with the insulin-dependent gain-of-function clinical phenotype of this channel mutation in the human population.

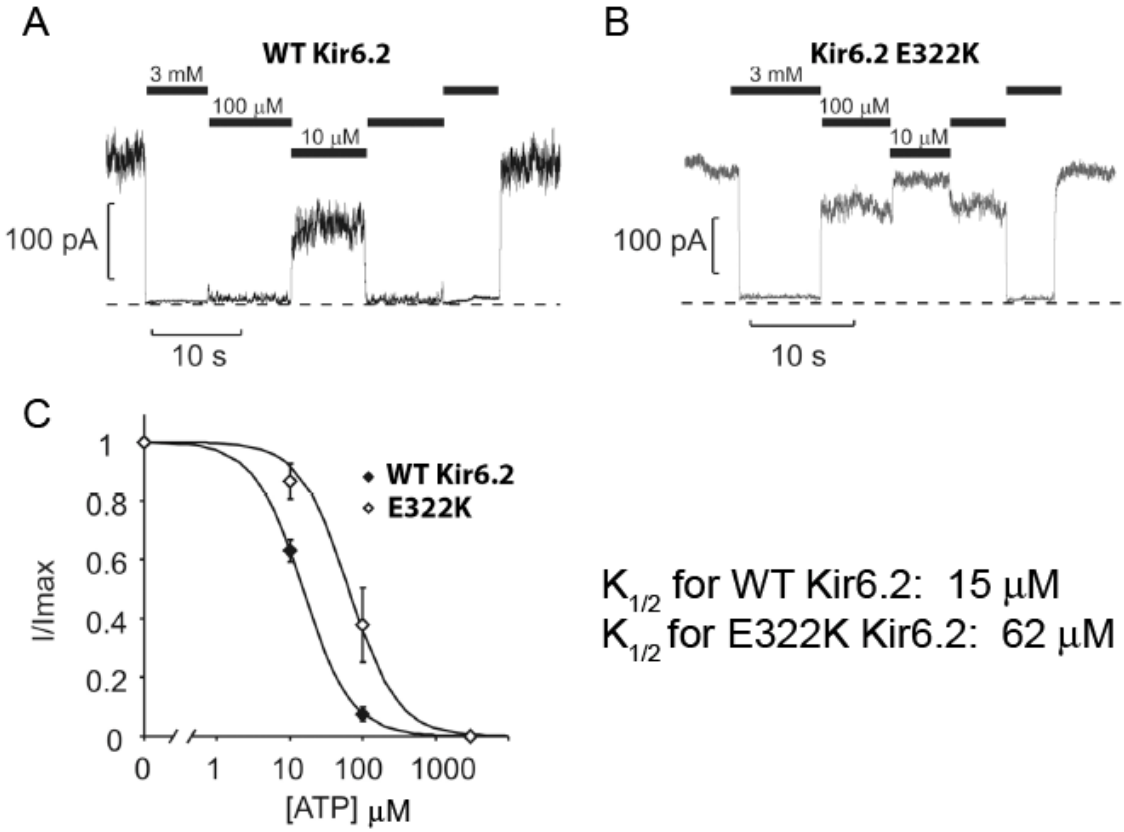

Figure 25. The Kir6.2 E322K mutant demonstrates reduced ATP sensitivity.

(A) Wild type Kir6.2 demonstrates ATP sensitivity where most channels are closed at 100 μM ATP, whereas, (B) Kir6.2 E322K demonstrates a significant reduction in ATP sensitivity, indicated by most channels remaining open at 100 μM ATP. (C) When $K_{1/2}$ values are calculated, Kir6.2 E322K demonstrates a rightward shift in ATP sensitivity (62 μM) as compared with wild type ATP sensitivity (15 μM).

ATP sensitivity of Kir6.2 can be modulated using a competing peptide

In order to more fully explore the role of ankyrin-B in the regulation of Kir6.2 metabolic regulation, two unique peptides were designed for a competition assay. Specifically, one peptide (WT peptide) corresponds to the ankyrin-B binding motif of Kir6.2 (VPIVAEED) while the second contains the PNDM mutation (VPIVAEKD). The WT peptide, in theory, should compete off the endogenous ankyrin-B/Kir6.2 interaction. COSm6 cells co-transfected with SUR1 and wild type Kir6.2-GFP were assayed for ATP sensitivity in the presence and absence of each peptide. Figure 26A demonstrates that the addition of the WT peptide reduces the ATP sensitivity of wild type Kir6.2, while the mutant peptide had no effect. As a control, these two peptides were used in an ATP sensitivity assay with COSm6 cells co-transfected with SUR1 and E322K Kir6.2-GFP. These results demonstrate no effect of either peptide (Figure 26B). Together, these data suggest a role for ankyrin-B in the metabolic regulation of the Kir6.2 subunit of the K_{ATP} channel.

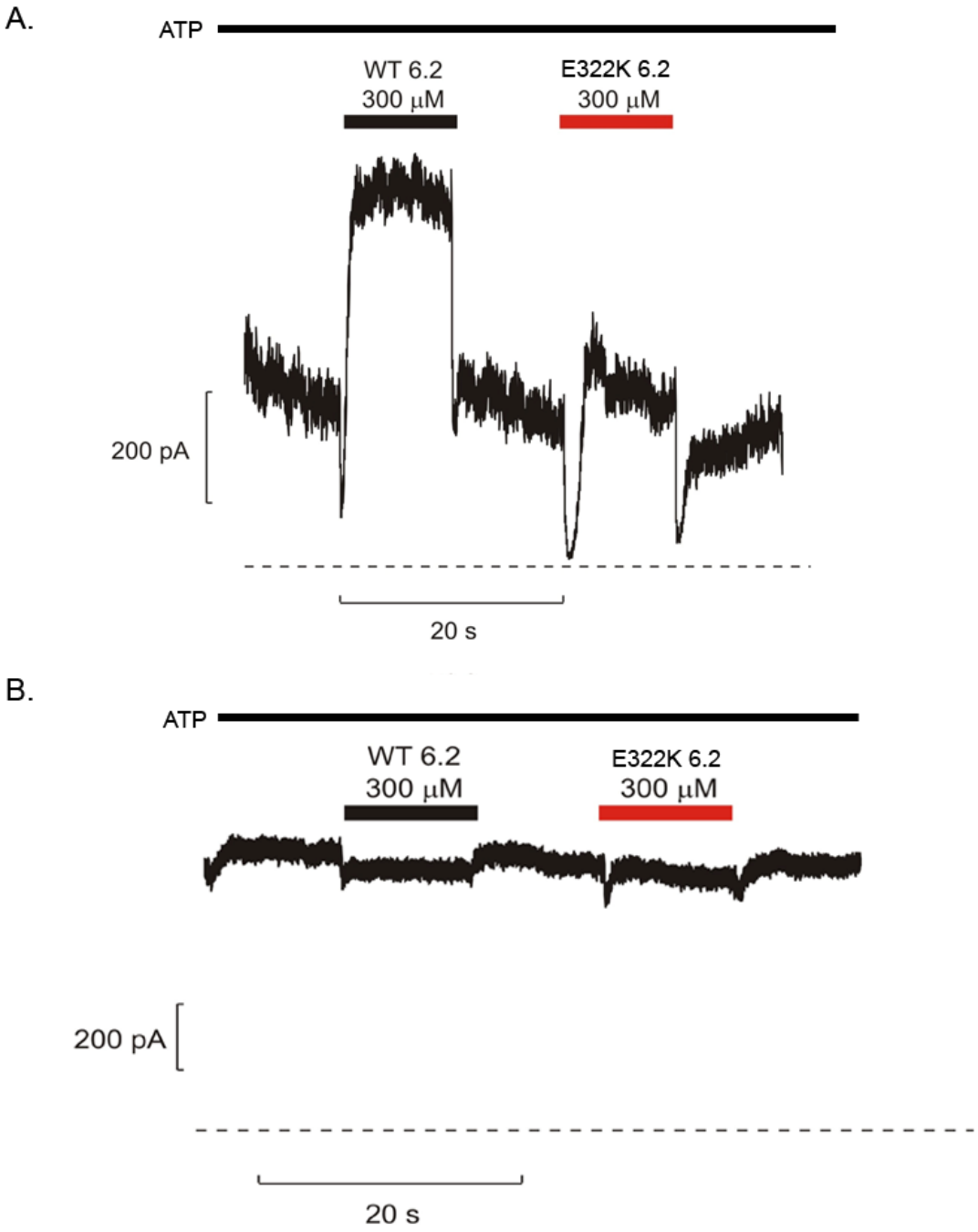

Figure 26. ATP sensitivity can be modulated using a competing peptide.

(A) Administration of WT peptide, corresponding to the ankyrin-B-binding motif of Kir6.2, results in a significant decrease in K_{ATP} channel ATP sensitivity, while administration of the MUT peptide has no effect. (B) Neither peptide has an effect on E322K-containing K_{ATP} channel ATP sensitivity.

Discussion

Ankyrin-B is necessary for the trafficking of a number of ion channels, transporters, and pumps to distinct microdomains of cellular membranes. Numerous studies have demonstrated the necessity of this targeting pathway, with significant alterations in cellular function resulting when ankyrin-B is removed or mutated (Mohler, Schott et al. 2003; Mohler, Splawski et al. 2004; Mohler, Davis et al. 2005; Mohler, Healy et al. 2007). The role of ankyrin-B-mediated pathways in excitable cells, like neurons and cardiomyocytes, has been well-established and can be applicable to an excitable cell like the pancreatic beta cell. In addition, a number of known ankyrin-B binding partners are present in the pancreatic beta cell; namely, the NKA, NCX1 exchanger, and the $InsP_3$ receptor. The discovery of ankyrin-B expression in the pancreas lays the foundation for studies as to the role of ankyrin polypeptides in beta cell function and, possibly, human disease.

The K_{ATP} channel is a critical component of the beta cell membrane, with severe dysfunction resulting from the loss of K_{ATP} channel subunits or mutations in K_{ATP} channel genes. The unexpected observation that Kir6.2 expression is significantly decreased in heart and pancreatic lysates from ankyrin-$B^{+/-}$ mice led to *in vitro* binding analyses which confirmed a direct interaction between ankyrin-B MBD and the C-terminal cytosolic domain of Kir6.2. Referring to previously published data regarding the ankyrin-G binding domain of $Na_v1.5$, a conserved eight amino acid motif was found to be both necessary and sufficient for ankyrin-B association with Kir6.2. Perhaps most exciting about these observations is the

realization that a known human PNDM mutation, E322K, resides in this eight amino acid motif. My data further implicate three charged amino acids, E321, E322, D323 as necessary for interaction with ankyrin-B MBD. Furthermore, mutation of these residues to an opposite charge results in significant loss of plasma membrane localization of Kir6.2 in HEK293 cells, indicating a necessary role for ankyrin-B in Kir6.2 targeting *in vivo*. Finally, the E322K mutation demonstrates a significant reduction in ATP sensitivity. Preliminary data suggest that it may be the association with ankyrin-B which confers ATP sensitivity to the Kir6.2 subunit of the K_{ATP} channel. However, future work is required to validate the role of ankyrin-B in K_{ATP} channel metabolic regulation.

Three-dimensional evaluation of the Kir6.2 protein reveals that the ankyrin-B binding motif protrudes from the protein in a finger-like projection. Such presentation may be necessary for ankyrin-B association (Figure 27). Close evaluation of a similar site on Kir6.1, which does not demonstrate an *in vivo* association with ankyrin-B (Figure 14) reveals that Kir6.1 lacks a central proline residue that is present in the ankyrin-B-binding motif of Kir6.2 (VPIVAEED versus TSYIAEEI). Perhaps this proline provides for the three-dimensional structure of the binding motif, allowing presentation of this region of Kir6.2 to ankyrin-B. Likewise, the absence of the third negatively-charged amino acid may play a key role in the absence of Kir6.1/ankyrin-B association. It will be interesting to evaluate the crystal structure of Kir6.1, which is currently unknown, in order to determine whether this protrusion is present.

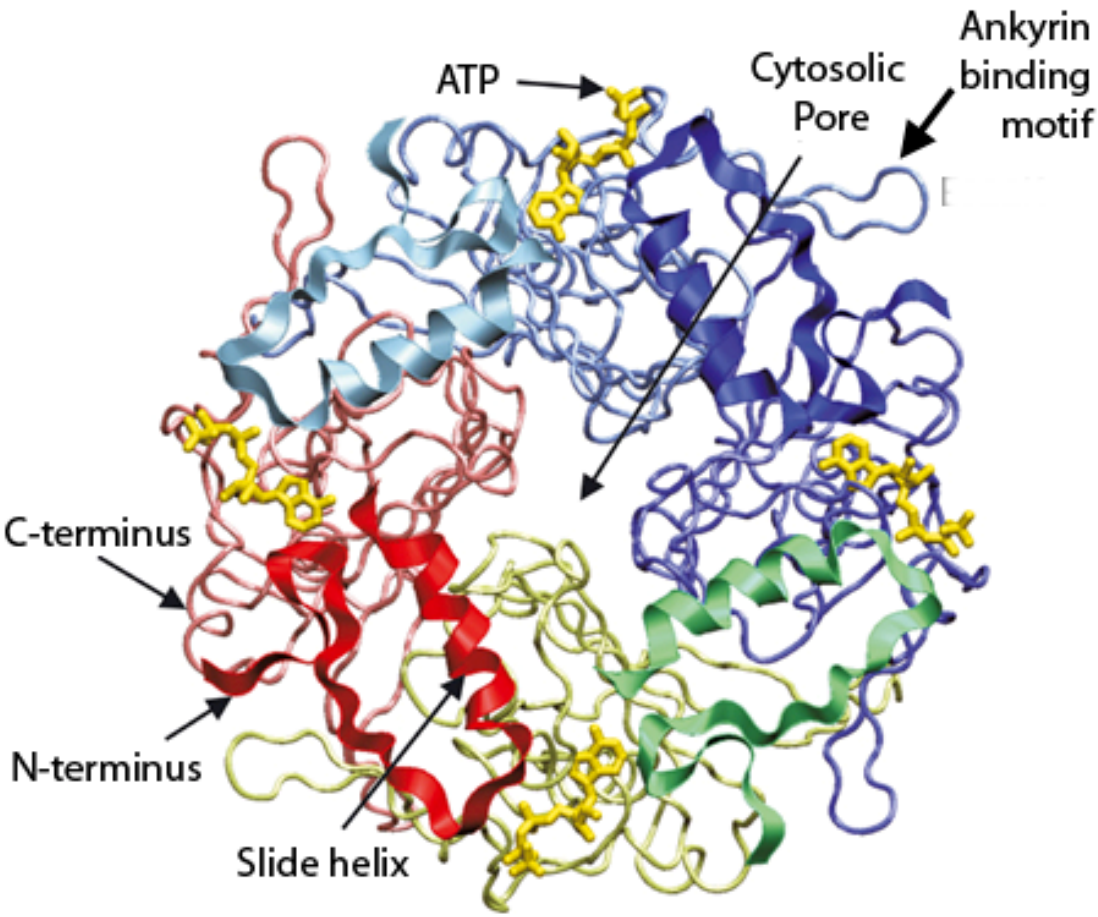

Figure 27. The ankyrin-B-binding motif of Kir6.2 resides on the outside of the Kir6.2 subunit.

The ankyrin-B-binding motif of Kir6.2 protrudes out from the Kir6.2 polypeptide, providing an easily accessible region to the cytosolic ankyrin-B. Reprinted by permission from Macmillan Publishers Ltd: *EMBO Rep* 6(5):470-5, copyright 2005 (Proks, Girard et al. 2005).

Dual targeting and ATP sensitivity cellular phenotypes and human disease. The current findings of this study illustrate that the Kir6.2 E322K mutation is associated with two molecular phenotypes: 1) reduced channel membrane targeting (loss-of-function) and 2) decreased ATP sensitivity (gain-of-function). Interestingly, these two defects would be expected to have opposite effects on I_{KATP} and therefore beta cell excitability. Based on the link between the Kir6.2 E322K and human permanent neonatal diabetes (typically I_{KATP} gain-of-function), I hypothesized that decreased ATP sensitivity represented the primary cellular phenotype resulting in disease. To test this prediction, I used mathematical modeling and computer simulation to determine the effect of altered ATP sensitivity associated with the Kir6.2 E322K mutation on beta cell excitability. An established model of the pancreatic beta cell was modified (Fridlyand, Tamarina et al. 2003) to investigate the effect of reduced ATP sensitivity on beta cell firing rate (Figure 28A). ATP sensitivity is accounted for in the model as the fraction of open ATP channels, O_{KATP} (See Equation 1 in Methods and Experimental Approach). O_{KATP} for the control K_{ATP} channel (WT, *black line*) and the E322K mutant (*blue line*) was fit to my measured data in COSm6 cells (Figure 28B). O_{KATP} for the heterozygous tetrameric channel (WT/E322K to mimic human phenotype, *red line*) was calculated to be 25 μM based on the fact that Kir6.2 subunits exert an additive effect on the open state probability of the tetrameric channel (Craig, Ashcroft et al. 2008), as described in the Methods section. Surprisingly, introducing the effect of the heterozygous E322K mutation on O_{KATP} into the beta cell model completely eliminates

spontaneous membrane depolarization (Figure 28C). Unexpectedly, these electrical findings are inconsistent (significantly too severe) with the observed human E322K phenotype, which requires a much lower dose of insulin to maintain glucose homeostasis as compared with individuals harboring other gain-of-function mutations in Kir6.2, suggesting a much more moderate effect of the E322K mutation on beta cell activity (Vaxillaire, Populaire et al. 2004).

Next, the effect of abnormal Kir6.2 E322K trafficking on beta cell excitability and membrane depolarization was analyzed. I_{KATP} conductance was reduced to 85% or 60% of its control value, to simulate changes observed in COSm6 and HEK293 cells, respectively (Figure 28D). Surprisingly, reducing I_{KATP} conductance even to 60% of its control value only slightly increased the rate of spontaneous membrane depolarization (Figure 28D). Again, these phenotypes were inconsistent with the observed human Kir6.2 E322K phenotype.

Finally, the dual phenotypes observed in the study were modeled. The revised model, including both trafficking defects along with reduced ATP sensitivity, produces a net decrease in cell excitability more consistent with the observed human E322K phenotype (moderate insulin dependence) due to a net increase in I_{KATP} (Figure 28E). Specifically, there was a 29% decrease in the bursting period of the E322K heterozygous mutant compared to WT (76 ms and 98 ms, respectively) (Figure 28E). These results suggest that, while the ATP sensitivity represents the dominant cellular phenotype resulting in PNDM, the reduction in I_{KATP} conductance due to reduced membrane channels (abnormal

trafficking) acts to protect the beta cell from the elimination of action potential firing.

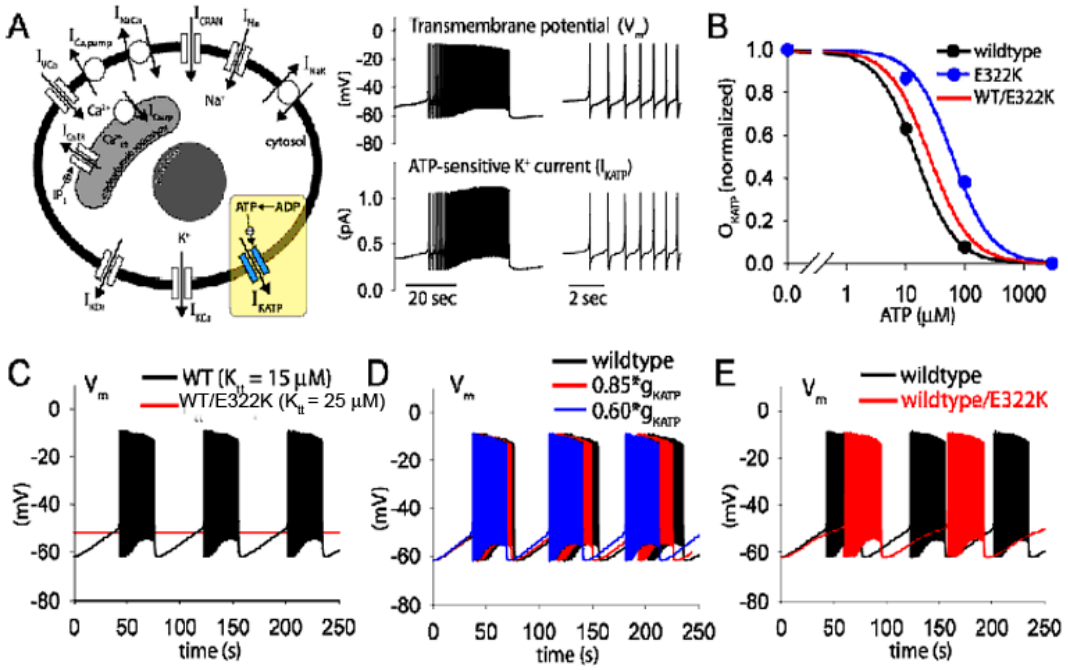

Figure 28. Dual targeting and ATP sensitivity cellular phenotypes and human disease.

(A) Schematic of the mouse pancreatic beta cell mathematical model and simulated spontaneous oscillations in transmembrane potential (right, top) and I_{KATP} (right, bottom). (B) ATP dependence of the open probability (O_{KATP}) for control (WT, black line) and E322K mutant (blue line) K_{ATP} channels fit to my measured data in COSm6 cells (closed circles; data presented in Figure 26). O_{KATP} for simulated E322K heterozygous mutant channel (WT/E322K, red line) is also shown). (C) Steady-state transmembrane potential in control model (black line) and with O_{KATP} corresponding to the heterozygous E322K K_{ATP} channel (red line). While the control model shows spontaneous oscillations in transmembrane potential, the heterozygous model is completely inexcitable. (D) Spontaneous oscillations in transmembrane potential are shown for the control model (WT, black line), and with I_{KATP} conductance reduced to 85% (red line) or 60% (blue line) of control value to simulate changes observed in COSm6 and HEK293 cells, respectively (original data presented in Figures). Reducing I_{KATP} conductance produces a slight increase in the rate of spontaneous membrane depolarization due to a loss of repolarizing current. (E) Simulated spontaneous oscillations in transmembrane potential in the control (WT, black line) and E322K heterozygous mutant (red line), including both defects in K_{ATP} channel trafficking and ATP sensitivity. Notice that while the spontaneous firing rate of the heterozygous mutant cell is dramatically slower than WT, the cell remains excitable.

CHAPTER V

THE INOSITOL 1,4,5-TRISPHOSPHATE RECEPTOR AND LOCAL CALCIUM SIGNALING

Introduction

A very steep gradient of Ca^{2+} (roughly four orders of magnitude) exists across the plasma membrane of all eukaryotic cells; specifically, cytosolic free calcium is maintained at 100 nM, while the extracellular milieu generally has a Ca^{2+} concentration over 1 mM (Mikoshiba 2007). This gradient is necessary for cellular survival, considering the cytotoxic consequences of high concentrations of intracellular Ca^{2+}. In contrast, the highly-choreographed release of Ca^{2+} from the ER/SR into distinct cellular microdomains allows for an array of cellular functions: fertilization, embryonic pattern formation, cell differentiation and proliferation, transcription factor activation, synaptic function, cardiac contractility, and apoptosis (Streb, Irvine et al. 1983; Furuichi, Yoshikawa et al. 1989; Berridge 1993; Furuichi, Kohda et al. 1994). This versatility in function lies in the speed, amplitude, and spatio-temporal patterning of Ca^{2+} signaling. In order to generate this versatility, the cell has several calcium-handling ion channels, transporters, and pumps at its disposal.

$InsP_3$ receptors are calcium-release channels found in the ER/SR membrane of diverse cell types. Specifically, calcium release from these intracellular stores is activated by $InsP_3$, a second messenger produced via the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2). $InsP_3$ is produced in

response to a number of extracellular stimuli, including hormones, growth factors, and neurotransmitters. Binding of $InsP_3$ to $InsP_3$ receptors increases $InsP_3$ receptor calcium sensitivity and, in a biphasic manner, induces a stimulatory effect at low calcium concentrations and an inhibitory effect at higher calcium concentrations (Taylor and Laude 2002). $InsP_3$ receptor-induced release of calcium from intracellular stores provides positive feedback whereby calcium amplifies its own release.

$InsP_3$ Receptor Structure and Regulation

The functional $InsP_3$ receptor complex exists as a homo- or hetero-tetramer of 260 kD $InsP_3$ receptor subunits. Three distinct isoforms (types I, II, and III) have been cloned (Furuichi, Yoshikawa et al. 1989; Mignery, Sudhof et al. 1989; Sudhof, Newton et al. 1991; Blondel, Takeda et al. 1993; Maranto 1994; Morgan, De Smedt et al. 1996) with isoform expression varying with cell type and with isoform-specific functions (Perez, Ramos-Franco et al. 1997). Cerebellar Purkinje cells, for example, express $InsP_3$ receptor type I almost exclusively (Wojcikiewicz 1995), while pancreatic acinar cells express types II and III (Bush, Stuart et al. 1994), and many epithelia express all three isoforms (Bush, Stuart et al. 1994; Nathanson, Fallon et al. 1994; Wojcikiewicz 1995). The formation of hetero-tetramers, due to the association of different isoform subunits, allows an additional level of receptor diversity (Joseph, Lin et al. 1995; Monkawa, Miyawaki et al. 1995; Onoue, Tanaka et al. 2000).

The three isoforms share a similar domain structure. Types II and III $InsP_3$ receptors have an overall sequence homology with type I $InsP_3$ receptor of 69% and 64%, respectively (Sudhof, Newton et al. 1991; Blondel, Takeda et al. 1993). However, the three isoforms possess different affinities for $InsP_3$ (type II > type I > type III) (Sudhof, Newton et al. 1991; Maranto 1994; Newton, Mignery et al. 1994). Each isoform contains a large regulatory domain between the N-terminal $InsP_3$ binding site and the C-terminal pore-forming region (Figure 29A). The pore-forming region contains six transmembrane domains (Figure 29B), which is where many intracellular compounds exert their modulatory effects (Patel, Joseph et al. 1999). The regulatory domain has several Ca^{2+} binding sites, as well as specific binding sites for ATP (Maeda, Kawasaki et al. 1991). Additional regulatory control of the $InsP_3$ receptor is via associated proteins (Thrower, Lea et al. 1998; MacKrill 1999), which permit a wide range of responses that are observed for $InsP_3$ receptor-induced Ca^{2+} release. Finally, phosphorylation status of the $InsP_3$ receptor also affects the properties of Ca^{2+} signaling (LeBeau, Yule et al. 1999). Pancreatic cells, for example, display different Ca^{2+} responses depending on the agonist. Specifically, cholecystokinin causes rapid phosphorylation of $InsP_3$ receptors, while acetylcholine does not produce such a response (LeBeau, Yule et al. 1999).

$InsP_3$ Receptors Form Macromolecular Signaling Complexes

The expression, localization, and function of the $InsP_3$ receptors are regulated by various cellular proteins (Figure 30). Cytochrome *c* (Boehning,

Patterson et al. 2003) and huntingtin (Htt)-associated protein-1A (HAP1A) (Tang, Tu et al. 2003; Bezprozvanny and Hayden 2004) were found to bind to the C-terminal region. Interestingly, $InsP_3$ receptor I activation by $InsP_3$ is sensitized by the polyglutamine (polyQ) expansion of Htt caused by Huntington disease. Protein phosphatases PPI and PP2A (DeSouza, Reiken et al. 2002; Tang, Tu et al. 2003), receptor for activated C-kinase 1 (RACK1) (Patterson, van Rossum et al. 2004), and chromogramin (Yoo 2000) have also been reported to bind to the channel region and regulate channel activity. Carbonic anhydrase-related protein (CARP) has been found to bind to a central part between the $InsP_3$ binding core and the channel region to regulate channel activity (Hirota, Ando et al. 2003). Homer binds to the N-terminal suppressor region (Tu, Xiao et al. 1998), possibly orchestrating the association of $InsP_3$ receptors to group 1 metabotropic glutamate receptors (mGluR1), forming mutimeric, junctional Ca^{2+} signaling networks in cerebellar Purkinje cells (Sandona, Scolari et al. 2003).

The discovery of numerous binding partners suggests that the $InsP_3$ receptor forms a macromolecular signaling complex and functions as the center of multiple signaling cascades. The diversity of Ca^{2+} signaling patterns, as well as the subcellular distribution of $InsP_3$ receptors, is very likely influenced by the components of the $InsP_3$ receptor signaling complex, which can differ between cell types and even between distinct subcellular spaces.

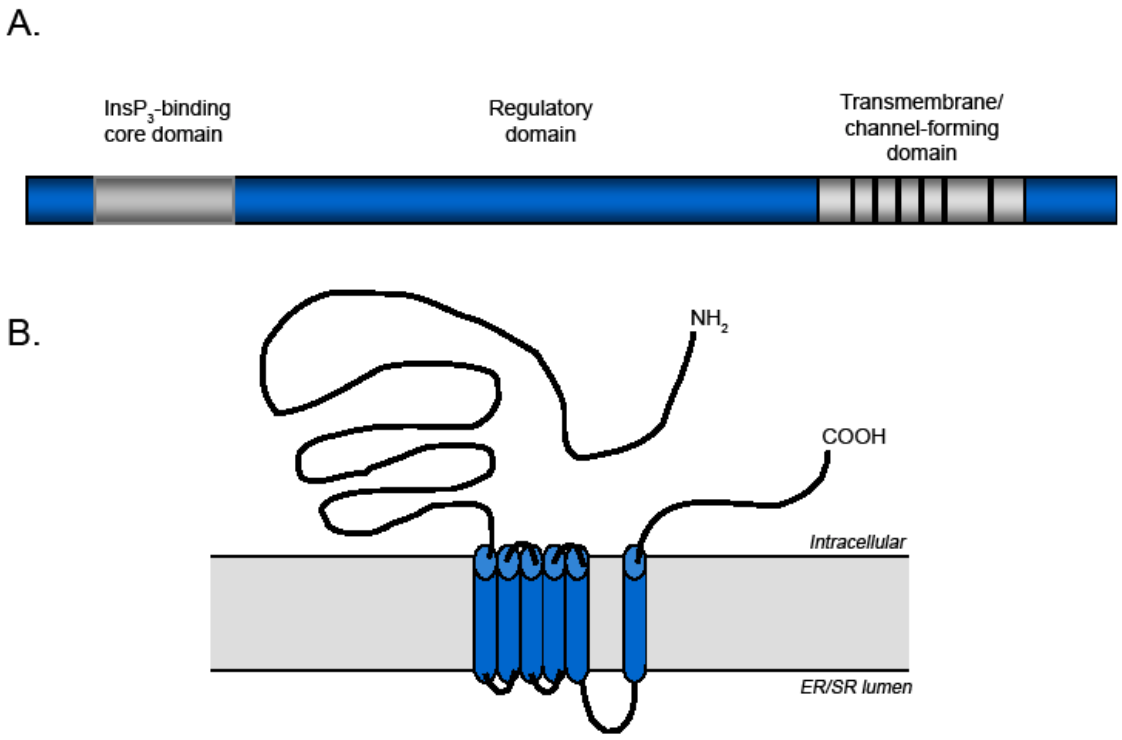

Figure 29. Domain organization of the $InsP_3$ receptor

(A) $InsP_3$ receptor isoforms have three general domains: an N-terminal $InsP_3$-binding domain, a central regulatory domain, and a C-terminal transmembrane/channel-forming domain. (B) The $InsP_3$ receptor localizes in the ER/SR membrane via six transmembrane domains. Both the N- and C-termini reside in the cytoplasm.

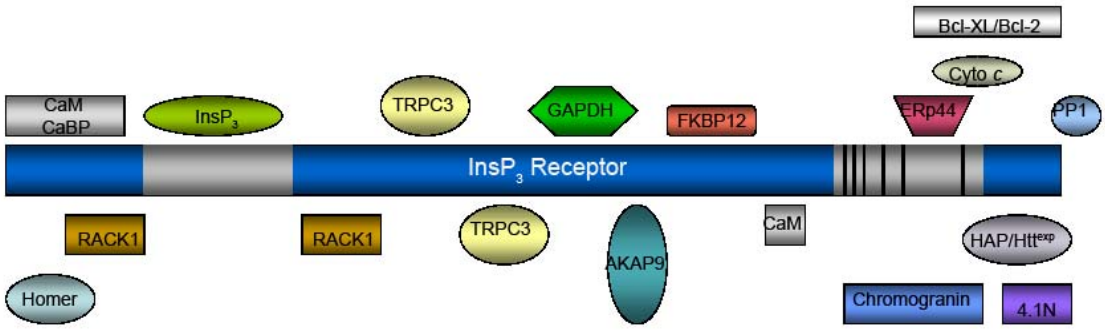

Figure 30. A representative collection of $InsP_3$ receptor binding partners.

The $InsP_3$ receptor has a host of protein binding partners that are involved in the targeting and regulation of channel activity.

$InsP_3$ Receptor Function in Excitable Cells

Beta cells. Stimulation by several neurotransmitters, growth factors, metabolites, and hormones have been reported to result in an increase in intracellular Ca^{2+} concentration by enhancing the turnover of phosphoinositides (Berridge, Heslop et al. 1984). $InsP_3$ opens the $InsP_3$ receptor on the ER, releases Ca^{2+} from the lumenal store, and produces an increase in cytosolic $Ca^{2+.}$ Exposure of isolated rat pancreatic islets to high levels of glucose has three important effects with regard to $InsP_3$ receptor-mediated activity. First, exposure to glucose rapidly increases the transcription and expression of the type III $InsP_3$ receptor in beta cells (Lee, Jonas et al. 1999). Second, the exposure to glucose enhances the incorporation of palmitate into phospholipids (Berne 1975). Glucose and other secretagogues not only stimulate phospholipid biosynthesis directly, but also supply one of the metabolic precursors for phospholipid biosynthesis: glycerol-3-phosphate. Enhanced phospholipid synthesis results in the generation of PIP_2, which can be cleaved by phospholipase C (PLC) into diacylglycerol (DAG) and $InsP_3$. Finally, rapid increases in $InsP_3$ occur following PIP_2 hydrolysis in islets in response to glucose (Biden, Peter-Riesch et al. 1987). Therefore, glucose increases the expression of type III $InsP_3$ receptor, the $InsP_3$-generating capability of beta cells, and also the production of $InsP_3$ by the beta cell, each occurring in a time-specific manner. Ultimately, these effects promote insulin secretion by increasing the intracellular Ca^{2+} concentration.

Considering that modulators of insulin secretion, especially second messengers like $InsP_3$, can affect intracellular Ca^{2+} directly, it can easily be appreciated that alterations in these secondary messengers can lead to altered insulin secretion. While the production of ATP via glycolysis and the citric acid cycle results in stimulus-secretion coupling by the beta cell, high concentrations of ATP can inhibit $InsP_3$-induced Ca^{2+} release by the type I $InsP_3$ receptor (Bezprozvanny and Ehrlich 1993), producing significant alterations in intracellular Ca^{2+}, and possibly defects in insulin secretion. Likewise, Ca^{2+} can regulate its own release by the type I $InsP_3$ receptor (Bezprozvanny, Watras et al. 1991). As a result, both ATP and Ca^{2+} play vital roles with regards to $InsP_3$ receptor-mediated Ca^{2+} release and insulin secretion by the beta cell.

Cardiomyocytes. $InsP_3$ receptors play crucial roles in regulating a diverse array of cardiac functions. Strategic localization of $InsP_3$ receptors in cytoplasmic compartments and the nucleus enables them to participate in subsarcolemmal, bulk cytoplasmic, and nuclear Ca^{2+} signaling in embryonic stem cell-derived and neonatal cardiomyocytes, and in adult cardiac myocytes from the atria and ventricles. Specifically, atrial $InsP_3$ receptors are involved in the increase in contractility due to elevated Ca^{2+} transients (Vites and Pappano 1995), while the dyadic localization of $InsP_3$ receptors, where excitation-contraction coupling is initiated, in the ventricular myocytes boosts the amplitude of systolic Ca^{2+} transients (Domeier, Zima et al. 2008). Cardiac nuclear $InsP_3$ receptors are involved in the activation of nuclear Ca^{2+}/calmodulin-dependent

protein kinase II ($CaMKII\delta_B$) to promote phosphorylation of histone deacetylase (HDACs) and, therefore, transcription (Wu, Zhang, et al. 2006).

Interestingly, expression of $InsP_3$ receptors is altered in cardiac disease such as atrial fibrillation (Kaplan, Jurkovicova et al. 2007), arrhythmias (Woodcock, Arthur et al. 2000; Mohler, Schott et al. 2003), diabetic cardiomyopathy (Guner, Arioglu et al. 2004; Zhou, Hu et al. 2006), hypertrophy (Berridge 2003) and heart failure (Go, Moschella et al. 1995), suggesting the involvement of $InsP_3$ signaling in the pathology of these diseases. Thus, $InsP_3$ receptors play important physiological and pathological functions in the heart, ranging from the regulation of pacemaking, excitation-contraction, and excitation-transcription coupling to the initiation and/or progression of arrhythmias, hypertrophy, and heart failure.

Diseases Associated with $InsP_3$ Receptors and $InsP_3$ Receptor-mediated Signaling

$InsP_3$ is an important second messenger that mediates the effects of many neurotransmitters and hormones. Consequently, changes in the level of $InsP_3$, either as a result of altered synthesis or abnormal metabolism, will influence $InsP_3$ receptor gating and may be expected to have profound physiological effects. Manipulation of $InsP_3$ levels may also provide a therapeutic target for disease in which neurotransmitters that elevate $InsP_3$, or the receptors for these transmitters, are affected.

The opisthotonos mouse. No human disease associated with mutations in any of the genes encoding $InsP_3$ receptor have been identified, but a number

of naturally-occurring mutations in mice produce disease. The *opisthotonos* (*opt*) mouse, which has a deletion in the $InsP_3$ receptor I gene, displays epileptic-like activity (Street, Bosma et al. 1997). A less severe, but similar, ataxic phenotype is observed in the mutant mice *staggerer*, *nervous*, and *Purkinje-cell degeneration*, which express low levels of $InsP_3$ receptor I. In addition, mice in which the $InsP_3$ receptor I gene has been knocked-out either die *in utero* or exhibit ataxia and epileptic seizures at about nine days after birth and die before weaning (Matsumoto, Nakagawa et al. 1996). Thus, $InsP_3$ receptor I is essential for normal brain function, which may explain why this type of mutation is not seen in the human population, as such individuals would probably not survive *in utero*.

Manic depression and lithium treatment. Manic depression is characterized by dramatic mood swings between mania and depression and can be controlled effectively with the carefully-monitored administration of lithium. It has been argued that the therapeutic effects of lithium result from its potent inhibitory effect on inositol phosphate metabolism (Berridge, Downes et al. 1989). Inhibition of inositol monophosphate phosphatase by lithium will cause depletion of inositol and reduce the synthesis of inositol lipids required for formation of $InsP_3$. Inositol depletion will be particularly significant in those cells which are unable to replenish inositol by uptake from external sources; this is the case for brain neurons where the inositol supply is limited by the blood-brain barrier. By blocking the supply of inositol, lithium will impair $InsP_3$ generation in response to neurotransmitters and dampen the effects of receptor hyperactivity, which is thought to be responsible for the mood swings found in manic depression.

Lowe's oculocerebrorenal syndrome. Lowe's oculocerebrorenal syndrome is an X-linked disease which affects the kidneys, the brain, and the lens of the eye (Attree, Olivos et al. 1992). Patients exhibit profound mental retardation, blindness as a result of cataracts, glaucoma, and microphthalmos (small eyes) (Attree, Olivos et al. 1992). The renal defect manifests principally as proteinuria and rickets. Lowe's oculocerebrorenal syndrome is not caused by mutations in the $InsP_3$ receptor itself, but instead results from defective regulation of Ca^{2+} release due to impairment of $InsP_3$ metabolism. The gene responsible for this disease is located on the long arm of chromosome X and encodes a 112 kD protein with 71% similarity to the human inositol polyphosphate-5-phosphatase (Attree, Olivos et al. 1992). This suggests that the protein may be a related phosphatase and thus also involved in the metabolism of $InsP_3$. It is expressed in the brain, skeletal muscle, heart, kidney, lung, and placenta. The loss of the functional protein is expected to lead to the elevation of cytosolic $InsP_3$, and therefore an increase in intracellular Ca^{2+}, which could account for the profoundly deleterious effects associated with the loss of this protein phosphatase.

Summary

The $InsP_3$ receptor is an ER/SR signaling center that forms a macromolecular signaling complex based on its scaffolding properties. Biochemical and biophysical evaluation of the molecular properties of $InsP_3$ receptors have led to the discovery of various mechanisms that contribute to the fine-tuning of $InsP_3$ receptor-mediated Ca^{2+} signals. These mechanisms underlie

the assembly of $InsP_3$ receptor-associated molecules in addition to the subcellular localization of the signal. The discovery of numerous binding partners suggests that the $InsP_3$ receptor forms a macromolecular signaling complex that can function as a center of multiple local signaling cascades. The diversity of Ca^{2+} signaling patterns, as well as the subcellular distribution mechanism of $InsP_3$ receptors, is most likely a product of the members of the $InsP_3$ receptor-signaling complex, which differs from cell to cell and subcellular space to subcellular space.

While no known human mutations in any of the $InsP_3$ receptor genes have been reported, modulation of the molecules which regulate $InsP_3$ receptor localization and activity result in significant alterations in cellular physiology. Loss of $InsP_3$ receptor localization in the cardiomyocyte SR as a result of loss of ankyrin-B expression, contributes to severe dysfunction in intracellular Ca^{2+} dynamics, resulting in an increased risk of sudden cardiac death (Mohler, Davis et al. 2003). Likewise, a reduction in the generation of intracellular $InsP_3$ has been associated with mental retardation, blindness, glaucoma, microphthalmos, and proteinuria. Therapeutic modulation of $InsP_3$ production via the administration of lithium has been used as an effective treatment for manic-depression. Future studies to determine the roles of other $InsP_3$ receptor binding partners will likely provide vital clues to the modulation and targeting of $InsP_3$ receptors *in vivo*.

CHAPTER VI

ANKYRIN-B INTERACTS WITH A CYTOPLASMIC N-TERMINAL REGION OF THE $InsP_3$ RECEPTOR

Introduction

In 1993, two independent groups identified an interaction between the $InsP_3$ receptor and the adaptor protein ankyrin (Bourguignon, Jin et al. 1993; Joseph and Samanta 1993). Specifically, Joseph and colleagues demonstrated that the $InsP_3$ receptor co-immunoprecipitated with ankyrin-R from brain (Joseph and Samanta 1993). Moreover, Bourguignon and colleagues showed that ankyrin-R and $InsP_3$ receptor co-purified from murine T-lymphoma cells (Bourguignon and Jin 1995), interacted directly with high affinity, and that ankyrin-R inhibited $InsP_3$-binding and $InsP_3$-dependent radiolabeled calcium flux (Bourguignon, Iida et al. 1993; Bourguignon, Jin et al. 1993). Over the past fifteen years, a number of groups have replicated ankyrin/$InsP_3$ receptor interactions using purified proteins, co-immunoprecipitation assays, ^{3}[H]-$InsP_3$ binding experiments, and pulse-chase biosynthesis experiments (Feng and Kraus-Friedmann 1993; Tuvia, Buhusi et al. 1999; Hayashi, Maurice et al. 2000; Hayashi and Su 2001; Mohler, Schott et al. 2003; Mohler, Davis et al. 2004; Mohler, Splawski et al. 2004; Mohler, Davis et al. 2005; Liu, Miyakawa et al. 2007). Additional evidence for the relevance of the ankyrin/$InsP_3$ receptor interaction comes from mouse models with reduced ankyrin-B expression that display significant defects in $InsP_3$ receptor expression, subcellular localization,

and decreased $InsP_3$ receptor post-translational stability (Mohler, Davis et al. 2004).

Multiple independent studies have documented *in vitro* and *in vivo* interactions between ankyrin polypeptides and the $InsP_3$ receptor. Moreover, loss of ankyrin-B leads to loss of $InsP_3$ receptor membrane expression and stability in cardiomyocytes. Despite extensive biochemical and functional data, the validity of *in vivo* ankyrin/$InsP_3$ receptor interactions remains controversial. This controversy is based on inconsistencies between a previously-identified ankyrin-binding region on the $InsP_3$ receptor and $InsP_3$ receptor topology data that demonstrate the inaccessibility of this lumenal binding site on the $InsP_3$ receptor to cytosolic ankyrin polypeptides. Despite overwhelming evidence supporting the ankyrin/$InsP_3$ receptor interaction, questions remain as to the validity of the interaction based on $InsP_3$ receptor structural mapping data performed nearly a dozen years ago (Bourguignon and Jin 1995). In 1995, an 11 residue motif in the $InsP_3$ receptor (GGVGDVLRKPS, Figure 31A-B) was identified as the binding site for ankyrin (Bourguignon and Jin 1995). This site (2548-2558 on rat $InsP_3$ receptor I) corresponded to a site of amino acid similarity to the ankyrin-binding site on CD44 ((Bourguignon and Jin 1995), see Figure 31B). Furthermore, a peptide corresponding to this sequence competed ankyrin/$InsP_3$ receptor interactions and blocked ankyrin-induced inhibitory effects on $InsP_3$ binding and $InsP_3$-dependent calcium release events (Bourguignon and Jin 1995). These data were reported before the topology of the $InsP_3$ receptor was resolved, and the authors speculated that the predicted binding site resided

in the cytosol, consistent with the localization of cytoplasmic ankyrin (Bourguignon and Jin 1995). Several groups have since determined the membrane topology of the $InsP_3$ receptor, indicating that residues 2548-2558 reside in the ER lumen (see arrow in Figure 31A) and are, therefore, unable to interact with cytosolic ankyrin polypeptides. Subsequently, despite compelling biochemical and animal findings, both ankyrin and $InsP_3$ receptor fields remain unclear regarding the feasibility of *in vivo* ankyrin/$InsP_3$ receptor interactions (see comments in (Patel, Joseph et al. 1999; Roderick and Bootman 2003; Mohler, Davis et al. 2004; Patterson, Boehning et al. 2004; Vermassen, Parys et al. 2004; Foskett, White et al. 2007)).

Here, two approaches were used to revisit the structural requirements for ankyrin-binding on the $InsP_3$ receptor. Using yeast two-hybrid and *in vitro* binding experiments, I was able to map the ankyrin-binding region on the $InsP_3$ receptor type I to residues 955-991 (Kline, Cunha et al. 2008). Based on the membrane topology of the $InsP_3$ receptor, these data demonstrate that the ankyrin-binding region is located on the cytoplasmic face of the receptor, validating the feasibility of the *in vivo* ankyrin/$InsP_3$ receptor interactions.

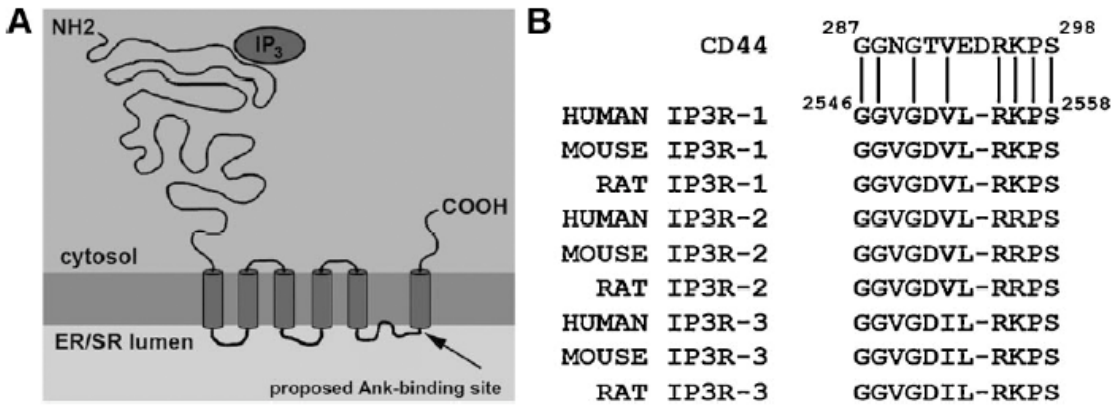

Figure 31. Putative ankyrin-B-binding site on the $InsP_3$ receptor.

(A) Domain organization of the $InsP_3$ receptor monomer, depicting membrane topology, cytoplasmic and lumenal protein orientation, and the previously proposed ankyrin-binding motif in the C-terminal domain. (B) Putative ankyrin-binding site in $InsP_3$ receptor identified by sequence similarity with CD44 ankyrin-binding motif. Minimal ankyrin-binding residues on CD44 (Bourguignon and Jin 1995) are presented. Amino acid sequences below CD44 denote sequence homology of residues in $InsP_3$ receptor C-terminus and the CD44 sequence. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

Methods and Experimental Approach

Design of $InsP_3$ receptor constructs. $InsP_3$ receptor constructs were engineered into pACT2 (Clontech) for yeast two-hybrid assays using standard molecular techniques and full-length rat $InsP_3$ receptor I as the template. $InsP_3$ receptor constructs were also engineered into pcDNA3.1(+) for *in vitro* translation experiments. Positive clones were confirmed by restriction digestion and sequencing. For all *in vitro* translated constructs, an initiator methionine was introduced at the beginning of the coding sequence to facilitate translation. $InsP_3$ receptor constructs were designed based on trypsin proteolysis mapping (Yoshikawa, Iwasaki et al. 1999) to preserve major folding domains in the binding assays. Due to the large size of the cytoplasmic N-terminus, this region was subdivided into seven fragments.

Design of ankyrin-B constructs. The MBD, plus residues of the SBD, of human 220 kD ankyrin-B (amino acids 1-958) were cloned into pGEX-6P1 (Amersham Biosciences) for expression as a GST-fusion protein (C-terminal GST). Protein expression was carried out in BL21(DE3)pLysS (Promega) competent cells and purified using glutathione-sepharose.

Full-length $InsP_3$ receptor production. $InsP_3$ receptor constructs lacking amino acids 924-991 were generated using baculovirus. Full-length wild type or mutant cDNAs were co-expressed in a standard transfection vector (Clontech) with *Bsu*36I-digested BacPAK6 viral DNA into *Spondoptera frugiperda* cells (*Sf*21 cells, Clontech) using the BacPAK Baculovirus Expression System (Clontech). *Sf*21 cells were infected in monolayer cultures with a MOI of ten for

72 hours at 27 °C. Cells were harvested and washed in PBS and cell pellets were flash frozen and stored at -80 °C. All of the following procedures were performed at 4 °C in the presence of protease inhibitors (100 µg/ml AEBSF, 100 µg/ml benzamidine, 30 µg/ml leupeptin, and 10 µg/ml pepstatin). Cells were syringed and sonicated in cell homogenization buffer (PBS, 1 mM EDTA, 1 mM DTT, and 1 mM sodium azide) then centrifuged at 100,000 x *g* for 30 minutes to collect membranes. Cell membranes were pre-extracted with 20 mM 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS; pH 12) for 30 minutes followed by 20 mM PBS (pH 7.3), 0.5 M NaCl, 0.5 M urea, 0.5% Triton X-100, and 0.5 mM β-mercaptoethanol. Cell residue was resuspended in extraction buffer (50 mM PBS (pH 8.0), 0.3 M NaCl, 10 mM imidazole, 0.2% Triton X-100, 1 mM β-mercaptoethanol, 1 mM sodium azide, and 2% Sarkosyl) for 20 minutes. The extract was centrifuged at 100,000 x *g* for 1 hour and the supernatant collected and diluted 10 fold in extraction buffer lacking Sarkosyl. Recombinant full-length $InsP_3$ receptor was solubilized from membranes as described (Mohler, Davis et al. 2005). Full-length protein expression was confirmed by SDS-PAGE/immunoblot on cell lysates using affinity-purified anti-$InsP_3$ receptor antibodies generated against the C-terminal of the $InsP_3$ receptor (Mohler, Davis et al. 2004). Equal quantities of cell lysate were added to binding reactions.

Yeast two-hybrid. One round of transformation was performed to ensure that none of the plasmids induced autoactivation. pACT2 DNA (0.05 µg) carrying the $InsP_3$ receptor insert and 0.05 µg of pAS2-1 DNA carrying the ankyrin-B MBD

were co-transformed into AH109 yeast (using herring sperm DNA as a carrier) utilizing a standard lithium acetate protocol. Co-transformed yeast were cultured at 30 °C on yeast peptone dextrose (YPD) media lacking leucine (-L), lacking tryptophan (-T), and lacking both leucine and tryptophan (-LT). Colonies capable of growth on –LT media (referred to as double transformants) were further selected on media lacking adenine, histidine, leucine, and tryptophan (-AHLT). The positive control was a co-transformation of 0.05 µg TD1-1 and 0.05 µg pVA3 and the negative control was a co-transformation of 0.05 µg TD1-1 and 0.05 µg pLAM5. Transformants growing on –AHLT media following 3-5 days of 30 °C incubation were considered positive interactions.

In vitro *transcription/translation and binding*. $InsP_3$ receptor constructs were transcribed and translated using the TNT Coupled Reticulocyte Lysate System (Promega) with 20 µCi of Redivue L-[^{35}S]-methionine (GE Healthcare) and 0.75 µg plasmid DNA. Twenty micrograms of purified GST-ankyrin-B MBD or purified GST were coupled to glutathione sepharose for two hours at 4 °C in binding buffer (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 150 mM NaCl, 0.1% Triton X-100). The beads were washed extensively in Buffer A (50 mM Tris pH 7.4, 1 mM EDTA, 1 mM EGTA, 500 mM NaCl, 1% Triton X-100) and incubated with $InsP_3$ receptor *in vitro* translated products representing $InsP_3$ receptor amino acids 924-1582, 924-1550, 924-1454, 924-1398, 924-1352, 924-1297, 924-1226, 924-1164, 924-1125, 924-991, 785-954, 955-1143, and 2403-2575 or full-length $InsP_3$ receptor products generated by baculoviral expression for four hours at 4 °C in binding buffer plus protease

inhibitor cocktail (Sigma). Binding reactions were washed extensively in Buffer A, eluted, and analyzed by SDS-PAGE. Radiolabelled proteins were detected by phosphorimaging. Full-length $InsP_3$ receptor products were detected by immunoblot with affinity purified anti-$InsP_3$ receptor antibodies.

Results

Ankyrin-B interacts with the $InsP_3$ receptor cytoplasmic N-terminal domain

Using a yeast two-hybrid approach to evaluate the interaction of ankyrin-B with $InsP_3$ receptor, the ankyrin-B MBD was expressed as a fusion with the GAL4 DNA-binding domain ("bait"). Likewise, $InsP_3$ receptor constructs were fused to the GAL4 activation domain ("prey"). Due to the large size of the $InsP_3$ receptor cytoplasmic N-terminus, this region was subdivided into seven fragments (Figure 32A, B).

No interaction between the ankyrin-B MBD and an $InsP_3$ receptor fusion protein containing the proposed ankyrin-binding site (Bourguignon and Jin 1995) at residues 2548-2558 was observed (Figure 32C, D). Instead, ankyrin-B MBD interacted with $InsP_3$ receptor fusion protein containing residues 924-1582 (Figure 32C, D). No interactions were detected between ankyrin-B MBD and $InsP_3$ receptor fusion proteins using $InsP_3$ receptor 1-346, 1-923, 347-923, 1581-1931, 1581-2210, and 1932-2210 (Figure 32C, D). These data demonstrate that a large N-terminal region of rat $InsP_3$ receptor I contains the ankyrin-B binding motif. Overall, these data demonstrate that the ankyrin-B binding region resides on the cytoplasmic, and not the lumenal, face of the $InsP_3$ receptor.

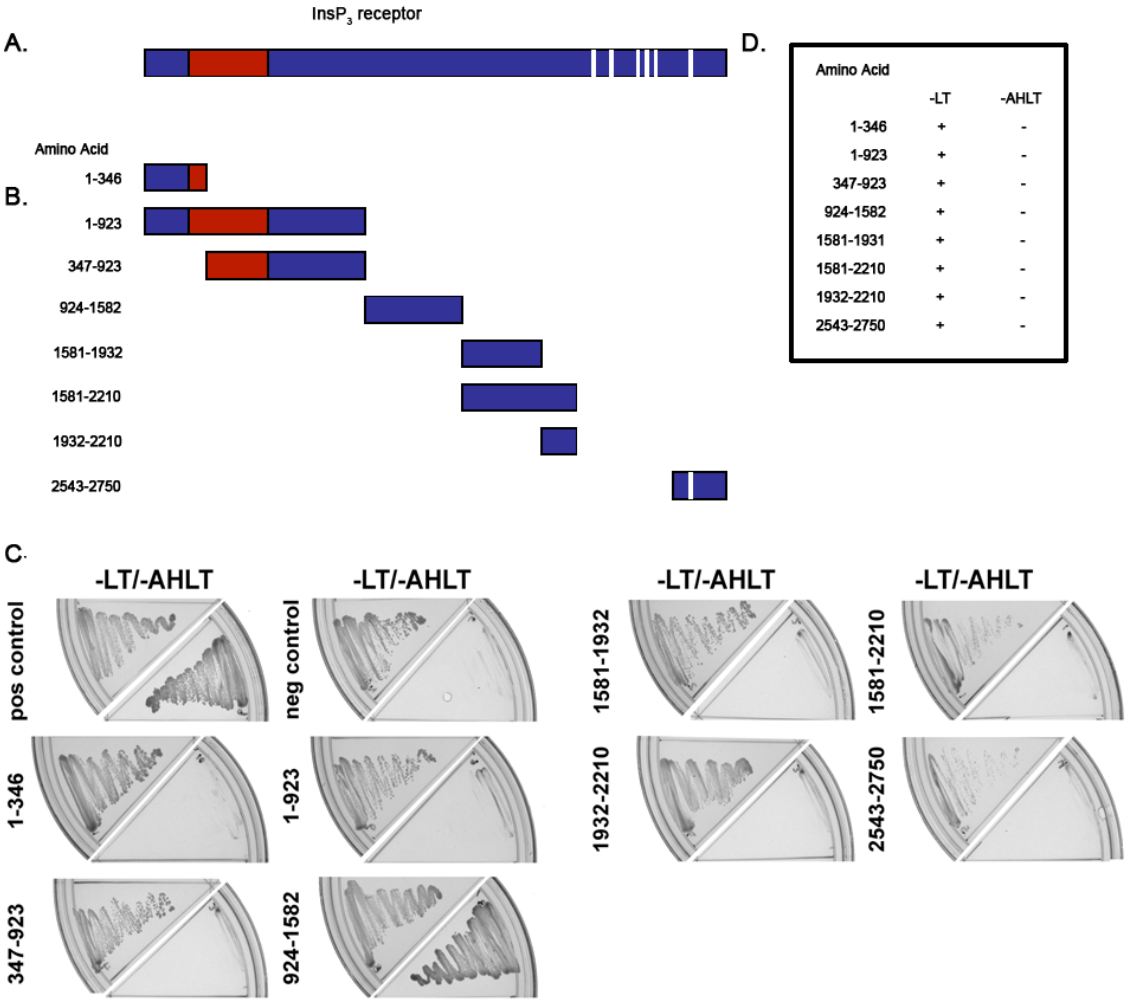
A.
InsP3 receptor
B.
Amino Acid
1-346
1-923
347-923
924-1582
1581-1932
1581-2210
1932-2210
2543-2750
D.
Amino Acid
-LT
-AHLT
1-346 + -
1-923 + -
347-923 + -
924-1582 + -
1581-1931 + -
1581-2210 + -
1932-2210 + -
2543-2750 + -
C.
-LT/-AHLT
-LT/-AHLT
-LT/-AHLT
-LT/-AHLT
pos control
neg control
1581-1932
1581-2210
1-346
1-923
1932-2210
2543-2750
347-923
924-1582

Figure 32. The ankyrin-B binding motif resides within amino acids 924-1582 of the $InsP_3$ receptor.

(A) Diagram of the $InsP_3$ receptor monomer (da Fonseca, Morris et al. 2003). (B) $InsP_3$ receptor yeast two-hybrid constructs. Fragments of the cytoplasmic regions of the $InsP_3$ receptor monomer used in the initial assessment of the structural requirements of $InsP_3$ receptor for interaction with ankyrin-B. $InsP_3$ receptor construct 2543-2750 contains the previously predicted ankyrin-binding domain (Bourguignon and Jin 1995). Constructs were designed based on identified structural folding boundaries defined by limited trypsin digestion (Yoshikawa, Iwasaki et al. 1999). (C) AH109 yeast cultures were co-transformed with ankyrin-B MBD (bait) and a unique $InsP_3$ receptor construct (prey). Co-expression of bait and prey proteins was confirmed by inoculating onto –LT media. Positive interactions were determined by growth on -AHLT media after five days incubation. TD1-1 served as bait for both the positive and negative controls, while pVA3 and pLAM5 were used as prey for the positive and negative controls, respectively. (D) Summary data for ankyrin-B MBD/$InsP_3$ receptor construct interactions. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

Defining the minimal binding region on $InsP_3$ receptor for ankyrin-B

Yeast two-hybrid. Initial binding assays identified a domain within the N-terminal region of the $InsP_3$ receptor (residues 924-1582) that is required for ankyrin binding. Due to the large size of the $InsP_3$ receptor N-terminus, the ankyrin-binding region was further narrowed. Using standard molecular biology techniques, additional $InsP_3$ receptor prey plasmids based on residues 924-1582 were created. Ten additional mutants were engineered (Figure 33) and screened for interaction in yeast against the ankyrin-B MBD. All ten $InsP_3$ receptor truncated constructs bound to the ankyrin-B MBD (Figure 34A). Even the smallest construct, corresponding to $InsP_3$ receptor I residues 924-991 displayed significant ankyrin-B binding activity (Figure 34A).

Additionally, yeast two-hybrid analyses were performed with an $InsP_3$ receptor construct lacking residues 924-977 to determine whether these residues were necessary for the ankyrin-B/$InsP_3$ receptor interaction (designated IP3R 978-1582). While co-expression of $InsP_3$ receptor construct 978-1582 with ankyrin-B in AH109 cells was observed, there was no detectable binding of this GAL4-fusion protein with the ankyrin-B MBD (Figure 34B). Therefore, these data demonstrate that $InsP_3$ receptor residues within 924-991 are necessary, and sufficient, for interaction with ankyrin-B in the context of yeast.

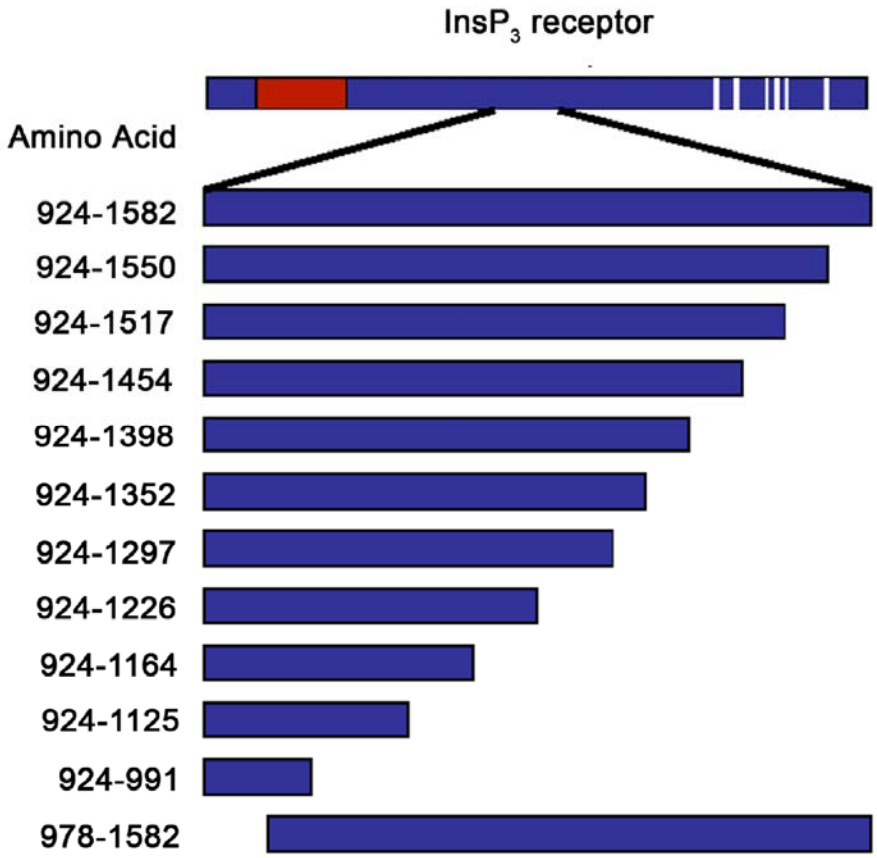

Figure 33. Identification of minimal requirements on $InsP_3$ receptor for ankyrin binding.

$InsP_3$ receptor truncation constructs were generated to define the minimal structural requirements on $InsP_3$ receptor for ankyrin binding. Constructs represent truncations based on rat $InsP_3$ receptor I residues 924-1582. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

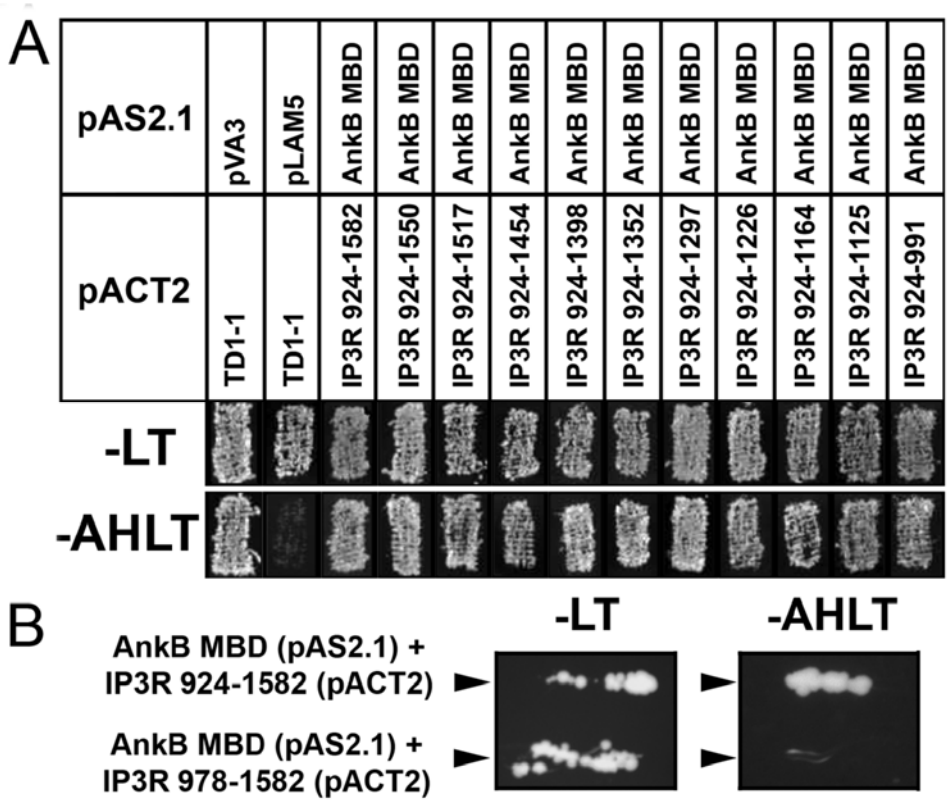

Figure 34. Defining the minimal ankyrin binding site on the $InsP_3$ receptor N-terminus.

(A) Ankyrin-B MBD (bait) and a series of $InsP_3$ receptor constructs (prey, Figure 34) were co-transformed into AH109 yeast. Positive interaction is seen with all constructs, with the smallest interacting region of the $InsP_3$ receptor corresponding to amino acids 924-991. Note that this region is located on the cytoplasmic N-terminus of the $InsP_3$ receptor (refer to Figure 30). (B) $InsP_3$ receptor lacking residues 978-1582 (designated 978-1582) fails to interact with ankyrin-B MBD in yeast. Yeast two-hybrid analysis with the 924-1582 prey construct was used as a positive control. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

In vitro *binding analysis*. The yeast two-hybrid mapping data was confirmed using *in vitro* binding assays. $InsP_3$ receptor truncation constructs were subcloned into the pcDNA3.1(+) expression plasmid under the control of the T7 promotor for *in vitro* transcription/translation in the presence of ^{35}[S]-methionine. The purified, radiolabelled translation products were incubated with GST alone or GST-ankyrin-B MBD. Following incubation and extensive washing, bound radiolabelled $InsP_3$ receptor products were eluted and analyzed by SDS-PAGE and phosphorimaging. Consistent with prior experiments performed in yeast, *in vitro* binding experiments demonstrated binding of all $InsP_3$ receptor truncation constructs (including $InsP_3$ receptor 924-991) (Figure 35A, B) with GST-ankyrin-B MBD. No binding of control GST with any $InsP_3$ receptor product was observed (Figure 35A, B).

To validate the binding results in the context of a full-length $InsP_3$ receptor, baculoviral expression (in *Sf*21 cells) was used to generate full-length $InsP_3$ receptor and an $InsP_3$ receptor mutant lacking residues 924-991 ($InsP_3$ receptor Δ924-991, Figure 36C). Approximately equal amounts of $InsP_3$ receptor proteins were incubated to immobilized GST or GST-ankyrin-B MBD. Following extensive high salt washes, bound protein was eluted and analyzed by SDS-PAGE and immunoblot using affinity-purified $InsP_3$ receptor Ig. While association of wild type full-length $InsP_3$ receptor with ankyrin-B MBD was observed, no association was detected between ankyrin and $InsP_3$ receptor Δ924-991 (Figure 35C). Together, my binding data demonstrate that $InsP_3$ receptor residues 924-991 are necessary and sufficient for ankyrin-B/$InsP_3$ receptor interaction.

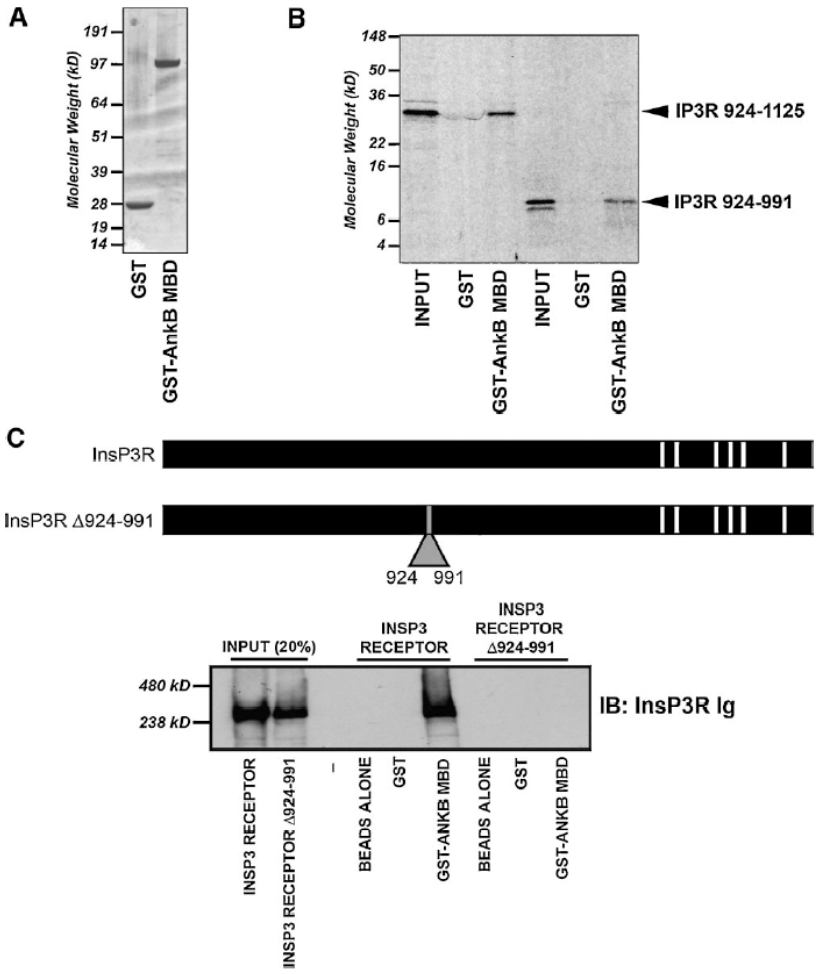

Figure 35. $InsP_3$ receptor residues 924-991 are required and sufficient for ankyrin-B association.

(A) Coomassie blue stained gel depicting purified GST and GST-ankyrin-B MBD. (B) Representative *in vitro* binding reactions using radiolabeled $InsP_3$ receptor constructs 924-991 and 924-1125. Both constructs are able to associate with GST-ankyrin-B MBD. (C) Full-length $InsP_3$ receptor and $InsP_3$ receptor Δ924-991 were incubated with GST-ankyrin-B MBD. While full-length $InsP_3$ receptor associates with ankyrin-B MBD, the $InsP_3$ receptor lacking residues 924-991 cannot. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

To further narrow the ankyrin-binding domain of the $InsP_3$ receptor, *in vitro* binding reactions were performed using GST-ankyrin-B MBD and *in vitro* translated products of each half of the identified ankyrin-binding domain. Specifically, $InsP_3$ receptor products consisting of residues 785-954 (contains amino acids 924-954) and 955-1143 (contains amino acids 955-991) were generated (Figure 36A). Larger polypeptides were used in order to incorporate more radiolabelled methionine residues and to increase peptide size for SDS-PAGE analysis. $InsP_3$ receptor construct 955-1143 associated with ankyrin-B MBD (Figure 36B, C); whereas, $InsP_3$ receptor residues 785-954 did not (Figure 36B, C), suggesting that the ankyrin-binding region resides in amino acids 955-991 of the $InsP_3$ receptor. Additionally, the $InsP_3$ receptor C-terminal construct 2403-2575, which contains a previously predicted ankyrin-binding motif, showed no detectable association with ankyrin-B (Figure 36B, C), consistent with my yeast two-hybrid data.

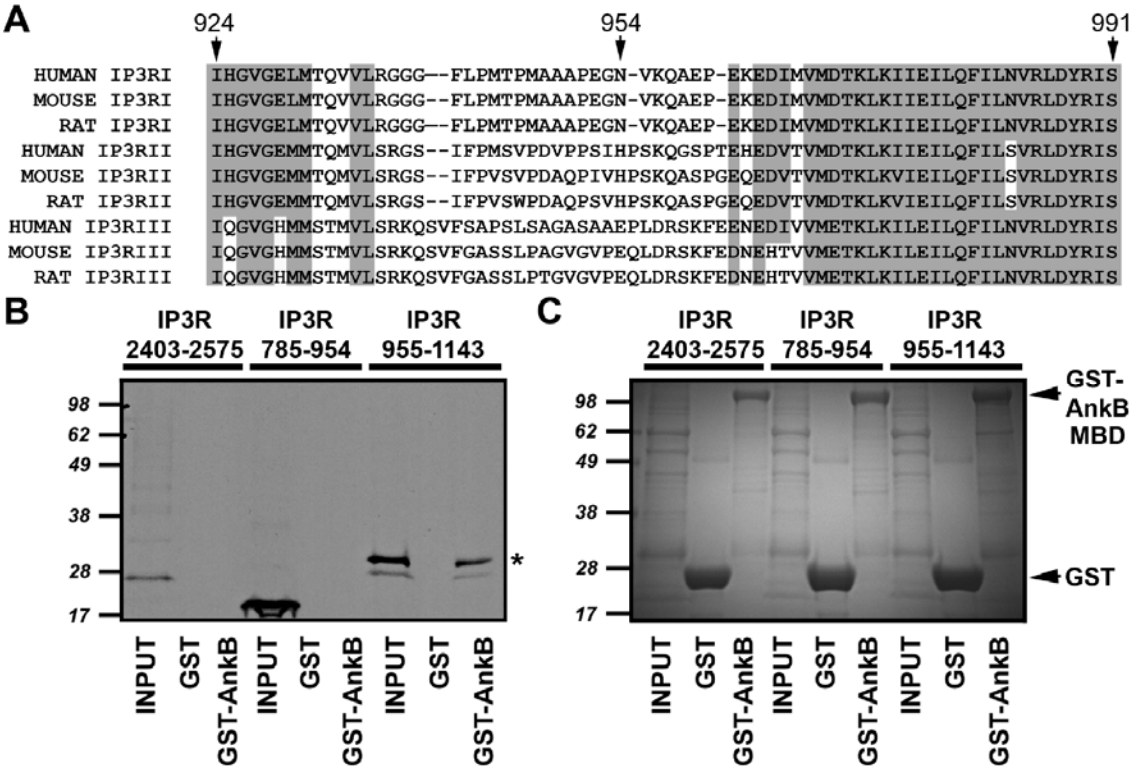

Figure 36. Ankyrin-B associates with $InsP_3$ receptor residues 955-991.

(A) Alignment of the ankyrin-B binding domain of the $InsP_3$ receptor. Note high sequence conservation in N- and C-terminal regions of the domain. (B) $InsP_3$ receptor ankyrin-binding domain (924-991) was split into two constructs and *in vitro* translated. An $InsP_3$ receptor C-terminal domain construct (residues 2403-2575) containing the previously predicted ankyrin-binding domain was also *in vitro* translated. While $InsP_3$ receptor protein 955-1143 showed *in vitro* association with ankyrin-B MBD, $InsP_3$ receptor proteins 785-954 and 2403-2575 did not. Reprinted with permission of Wiley-Liss, Inc. a subsidiary of John Wiley & Sons, Inc. Kline, et al. "Revisiting Ankyrin-$InsP_3$ Receptor Interactions: Ankyrin-B Associates with the Cytoplasmic N-Terminus of the $InsP_3$ Receptor." *J Cell Biochem* 104(4):1244-53 copyright 2008 by John Wiley & Sons, Inc. (Kline, et al. 2008).

Discussion

The current study was designed to evaluate the amino acid requirements on $InsP_3$ receptor for ankyrin-B interaction. Consistent with prior studies, I observed interaction between ankyrin-B and the $InsP_3$ receptor (Bourguignon, Iida et al. 1993; Bourguignon, Jin et al. 1993; Feng and Kraus-Friedmann 1993; Joseph and Samanta 1993; Tuvia, Buhusi et al. 1999; Hayashi, Maurice et al. 2000; Hayashi and Su 2001; Mohler, Schott et al. 2003; Mohler, Davis et al. 2004; Mohler, Splawski et al. 2004; Mohler, Davis et al. 2005; Liu, Miyakawa et al. 2007). However, my data establish that ankyrin interacts with the cytoplasmic and not the lumenal face of the $InsP_3$ receptor. Specifically, ankyrin-B interacts with the cytoplasmic N-terminal domain of the $InsP_3$ receptor within residues 955-991. The new $InsP_3$ receptor cytosolic ankyrin-binding region is consistent with an *in vivo* ankyrin/$InsP_3$ receptor interaction. These new results resolve the ability of these two proteins to interact in an *in vivo* cellular environment.

Ankyrins likely play multiple roles in normal $InsP_3$ receptor function. Based on work from multiple labs, ankyrin has a central role in the targeting of $InsP_3$ receptor to ER/SR membrane domains in both non-excitable and excitable tissues (Bourguignon, Iida et al. 1993; Bourguignon, Jin et al. 1993; Feng and Kraus-Friedmann 1993; Joseph and Samanta 1993; Tuvia, Buhusi et al. 1999; Hayashi, Maurice et al. 2000; Hayashi and Su 2001; Mohler, Schott et al. 2003; Mohler, Davis et al. 2004; Mohler, Splawski et al. 2004; Singleton and Bourguignon 2004; Mohler, Davis et al. 2005; Liu, Miyakawa et al. 2007). Moreover, ankyrin isoforms likely stabilize the receptor in close proximity with

effector and regulatory proteins (Lencesova, O'Neill et al. 2004; Mohler, Davis et al. 2005; Bhasin, Cunha et al. 2007; Liu, Miyakawa et al. 2007).

In summary, my new data identify the binding region on the ER/SR $InsP_3$ receptor for ankyrin-B. Consistent with an *in vivo* association of cytosolic ankyrin with $InsP_3$ receptor, my data demonstrate that ankyrin-B directly associates within amino acids 955-991 of the cytoplasmic N-terminal domain of $InsP_3$ receptor.

CHAPTER VII

CONCLUSIONS AND FUTURE DIRECTIONS

My findings present evidence of a critical role for ankyrin-B in the targeting and regulation of two important channels in the excitable cell: the K_{ATP} channel and the $InsP_3$ receptor. Specifically, I have presented data which demonstrate that ankyrin-B associates *in vitro* with Kir6.2, the pore-forming subunit of the K_{ATP} channel, and that this association is necessary for Kir6.2 targeting *in vivo*. Ankyrin-$B^{+/-}$ mice demonstrate significant hyperglycemia and isolated islets from ankyrin-$B^{+/-}$ mice exhibit a significant reduction in insulin secretion, suggesting that loss of ankyrin-B expression results in a diabetic phenotype. Furthermore, I presented evidence that a known human mutation associated with PNDM, E322K, results in two distinct phenotypes: loss of ankyrin-B-mediated Kir6.2 targeting *in vivo* and a significant reduction in the ATP sensitivity of Kir6.2. I also presented preliminary data supporting a role for ankyrin-B in the modulation of Kir6.2 ATP sensitivity. These two phenotypes (a loss-of-function and a gain-of-function, respectively) results in neonatal diabetes. The ankyrin-B/Kir6.2 association is a novel finding and suggests a possible target for therapeutic modulation of K_{ATP} channel activity. Finally, my data demonstrates that ankyrin-B associates with the $InsP_3$ receptor via a cytoplasmic N-terminal domain on the $InsP_3$ receptor. While numerous studies have demonstrated *in vitro* and *in vivo* interactions between ankyrin-B and the $InsP_3$ receptor, there has been much

confusion about the validity of this interaction. This confusion arose from a published study demonstrating that ankyrin-B associated *in vitro* with an 11 amino acid motif in the C-terminal domain of the $InsP_3$ receptor. Structural studies later demonstrated that this motif resides in the lumen of the ER/SR and theoretically inaccessible to ankyrin-B. My work conclusively demonstrates that this 11 amino acid motif is not the ankyrin-B-binding site *in vivo* and provides further evidence that the binding motif resides in the cytoplasmic N-terminus of the $InsP_3$ receptor, nearly 2000 amino acids upstream of the originally reported site.

While my work has demonstrated a key role of ankyrins in ion channel targeting and biophysical regulation, I have found that with each single observation I have made over the last four years there were three or four new and more exciting questions to be asked. In this chapter, I will focus on a number of promising future directions that I view as critical next steps for the field.

Ankyrin Objectives

***Ankyrin targeting and specificity*.** While there is much circumstantial evidence to support a role for ankyrins in ion channel targeting and biophysical regulation, the precise nature of how ankyrin polypeptides are targeted remains a primary focus of research. While a number of cell adhesion molecules (L1CAMs and CD44) (Kalomiris 1988; Ango 2004) and cytoskeletal elements (titin, tubulin, obscurin, and β-spectrin) (Kontrogianni-Konstantopoulos 2003; Davis 1984;

Bagnato 2003) have been implicated in the membrane localization of ankyrins, there are still questions regarding cell-type specific mechanisms associated with that targeting. While cell adhesion molecules have been shown to target ankyrins in the central nervous system, it remains to be determined if these same molecules are required for ankyrin localization in other tissues. Likewise, cytoskeletal elements have been implicated in the targeting of ankyrins in the cardiomyocyte and erythrocyte; however, the role of cytoskeletal elements in the targeting of ankyrins in other cell types has yet to be elucidated. Along that same vein, identification of the intracellular trafficking systems (myosins, GTPases, GEFs, etc.) that are necessary for ankyrin targeting, as well as for ankyrin-assisted targeting, are still a major focus of on-going research.

While ankyrins have been demonstrated to be necessary for proper ion channel localization, they have also been demonstrated to increase the stability of certain proteins at the plasma membrane (Cunha 2007; Mohler 2004). Whether this increased stability is a characteristic of all ankyrin-binding partners or whether the increased stability occurs in a cell-specific/ion channel-specific manner remain to be answered. Ankyrins are reported to show cell-specific function; namely, association with distinct ion channels occurs in a cell-type specific manner. Ankyrin-B, for example is responsible for the targeting of the NKA in cardiomyocytes (Mohler 2005); however, ankyrin-G targets the NKA in bronchial epithelial cells Kizhatil 2007), even though both cell types express ankyrin-B and ankyrin-G. The focus of future studies is the determination of cell-

specific pathways responsible for the cell-specific function of ankyrin polypeptides.

K_{ATP} Channel and $InsP_3$ Receptor Objectives

Further clarification of the ankyrin-B/Kir6.2 interaction. While my data elucidate the ankyrin-B binding motif of Kir6.2, the region of ankyrin-B necessary for Kir6.2 interaction remains to be determined. Likewise, the determination of half-life/stability of Kir6.2 and the K_{ATP} channel in the presence/absence of endogenous ankyrin-B could clarify a role for ankyrin-B in K_{ATP} channel expression at the plasma membrane. Though many studies have demonstrated that Kir6.2 associates with SUR subunits, the nature of that association has not been clarified. Future studies should focus on determining the definitive binding site on Kir6.2 for SUR subunits. Determination of this site would provide vital insight into the mechanisms by which this channel is formed, trafficked, and regulated.

Role of ankyrin-B in the modulation of Kir6.2 ATP sensitivity. While my preliminary data suggest a role for ankyrin-B in the regulation of K_{ATP} channel ATP sensitivity, further work must be performed to more clearly define this role. Specifically, the production of an endogenous peptide to compete association of ankyrin-B with Kir6.2 can be used not only to evaluate the effects on Kir6.2 targeting and ATP sensitivity, but also to determine functional consequences of disrupting this association; namely, insulin secretion. This can be performed either by transfection of the competing peptide into a cell line of insulin secretion (INS-1 cells, for instance) or generation of a mouse model expressing an

inducible (and beta cell-specific) competing peptide that disrupts the ankyrin-B/Kir6.2 interaction.

The role of ankyrin-B in beta cell physiology and glucose homeostasis. The effect of loss of ankyrin-B expression can be analyzed via the generation of an inducible beta cell-specific knock-out of ankyrin-B. Evaluation of glucose homeostasis in the ankyrin-$B^{+/-}$ mouse model is not without complications. Since this model represents a global reduction in ankyrin-B, it would be difficult to draw conclusions as to the role of ankyrin-B in beta cell insulin secretion. The generation of a beta cell-specific knock-out of ankyrin-B would allow for the evaluation of ankyrin-B for beta cell activity, without the complicating factors associated with extrapancreatic tissues involved in glucose homeostasis, most of which also express ankyrin-B. Also, it was surprising that no discernible differences in the expression of NKA and NCX were found in pancreatic lysates from ankyrin-$B^{+/-}$ mice. Ankyrin-$B^{+/-}$ cardiac tissue demonstrates a significant decrease in the expression and localization of both NCX and NKA (Mohler, 2003), demonstrating a role for ankyrin-B in the targeting of these ion channels in cardiomyocytes. As mentioned previously, ankyrin polypeptides demonstrate cell-specific activity with regard to the targeting of ion channels. While ankyrin-B is responsible for the targeting of NKA in cardiomyocytes, ankyrin-G is required for the targeting of NKA in bronchial epithelial cells, even though both cells types express both ankyrin-B and ankyrin-G. Since pancreatic beta cells express both ankyrin-B and ankyrin-G, it would be

interesting to investigate the role of ankyrin-G in the targeting of NCX and NKA in pancreatic beta cells.

K_{ATP} channels also play critical roles in glucose homeostasis outside of the beta cell. K_{ATP} channels in the skeletal muscle and adipose tissue are involved in insulin-stimulated glucose uptake. K_{ATP} channels in ventromedial hypothalamic neurons mediate the counter-regulatory response to glucose (Miki, Liss et al. 2001), while K_{ATP} channels in the arcuate nucleus are hypothesized to play a role in appetite regulation (Wang, Liu et al. 2004). It will be interesting to determine whether ankyrin-B-based pathways for K_{ATP} channel targeting and metabolic regulation exists within these cells types.

Ankyrin-B and role of the cytoskeleton in K_{ATP} channel modulation. While numerous studies have demonstrated a definitive role of the cytoskeleton in Kir6.2 metabolic regulation (outlined in Chapter III) (Brady, Alekseev et al. 1996; Furukawa, Yamane et al. 1996; Terzic and Kurachi 1996; Yokoshiki, Katsube et al. 1997), my results suggest that the association with ankyrin-B, as a linker between Kir6.2 and the cytoskeleton, may resolve underlying questions regarding those previously-published observations. Specifically, the use of a competing peptide to remove ankyrin-B association from the actin-spectrin cytoskeleton, while still maintaining the ankyrin-B/Kir6.2 association, could be critical in confirming that hypothesis.

Ankyrin-B modulation of the Kir6.2/SUR association. Further studies regarding the role of ankyrin-B in the regulation of SUR/Kir6.2 association are also warranted. Many studies have demonstrated a loss of sulfonylurea-induced

K_{ATP} channel closure in the presence of cytoskeletal disrupting drugs (Terzic and Kurachi 1996). Ankyrin-B may play a role in either the sensitivity of SUR subunits for sulfonylurea action or may be involved in the transduction of sulfonylurea binding to the SUR subunit to conformational changes in the Kir6.2 pore. Considering the reports demonstrating a correction of SUR trafficking defects following the administration of sulfonylureas (Yan 2004), future work with regard to ankyrin-B and SUR subunits is also vital in understanding the role of ankyrin-B in K_{ATP} channel targeting and modulation.

The role of ankyrin-B in the targeting of K_{ATP} channels to non-insulin containing granules. While K_{ATP} channel expression at the beta cell plasma membrane is critical for stimulus-secretion coupling, much published data demonstrate that the majority of K_{ATP} channel expression is on non-insulin containing granules (Yang and Wenna et al. 2007). While the role of those channels has yet to be determined, it is of interest to evaluate whether ankyrin-B is also involved in the targeting and metabolic regulation of granule K_{ATP} channels as well. Knock-down of ankyrin-B expression in a cell model of insulin secretion, like the INS-1 cell line, could be used to determine the effect on both plasma membrane and granule K_{ATP} channel expression, as well as evaluation of the functional effects of loss of ankyrin-B; namely, insulin secretion.

The role of ankyrin-B in beta cell PP2A targeting. Protein phosphorylation has been demonstrated to play a vital role in insulin secretion. The overall state of protein phosphorylation is a function of both the phosphorylation by various kinases and the dephosphorylation by protein

phosphatases. Therefore, net phosphorylation levels can be elevated by either activation of protein kinases or inhibition of protein phosphatases. Recent work has established a role for ankyrin-B in the targeting of the protein phosphatase PP2A in heart via association with the B56α subunit of PP2A (Bhasin 2007). PP2A has been shown to play a vital role in beta cell physiology via its regulation of insulin secretion. Specifically, inhibition of PP2A by okadaic acid results in almost complete suppression of insulin secretion (Krautheim 1999), as well as reduced stimulation of acetyl-CoA carboxylase (Kowluru 2001). Acetyl Co-A carboxylase catalyzes the formation of malonyl-CoA, a precursor in the biosynthesis of long-chain fatty acids, which have been implicated in insulin secretion. Future work focusing on the role of ankyrin-B in the phosphorylation status of critical beta cell proteins, via the formation of macromolecular signaling complexes, will be critical in determining the function of ankyrin-B in the macromolecular complexes necessary for efficient beta cell function.

The effect of human ankyrin-B mutations on K_{ATP} channel targeting and regulation: A number of human ankyrin-B mutations have been identified which result in a loss of ankyrin-B-dependent targeting of a number of ion channels (Lehnart 2007). Clinical evaluation of these individuals with regard to glucose homeostasis need to be performed, as well as *in vivo* and *in vitro* work to determine the role of these mutations in the targeting/regulation of K_{ATP} channel activity, as well as K_{ATP} channel stability. This is of clinical relevance to individuals who have been identified as carriers of these mutations, so that

appropriate therapeutic measures can be implemented early, specifically, in regard to lifestyle changes and the administration of anti-diabetic medication.

$InsP_3$ receptor expression in the pancreatic beta cell. While the K_{ATP} channel represents a critical regulator of insulin secretion, it is not the only ankyrin-B binding partner present in the pancreatic beta cell. The role of ankyrin-B in the targeting and regulation of the $InsP_3$ receptor in the pancreatic beta cell has not been evaluated. Activation of muscarinic receptors on the beta cell results in release of Ca^{2+} from the ER, via the activation of PLC and the generation of $InsP_3$. The $InsP_3$ receptor-mediated pathway for insulin secretion can bypass the K_{ATP}-dependent glucose-stimulated pathway and stimulate insulin secretion in response to acetylcholine, GLP-1, and gastric inhibitory peptide (GIP). Considering that some of the pharmacological agents used to treat type 2 diabetes are GLP-1 mimetics (exenatide/Byetta™) (Heine 2005) or inhibitors of the breakdown of endogenous GLP-1 and/or GIP (vildagliptin/Galvus™ and sitagliptin/Januvia™) (Herman 2005; Ahren 2004), further evaluation of the role of the $InsP_3$ receptors in beta cell physiology is an important focus for future work.

Ankyrin-B in extrapancreatic K_{ATP} channel function. The K_{ATP} channel is a critical component in the physiology of cardiomyocytes and neurons, providing cytoprotective functions as well as coordination of electrical activity. I have generated preliminary data demonstrating that ankyrin-B and Kir6.2 co-immunoprecipitate from rat pancreatic heart lysate (Figure 14), suggesting that ankyrin-B may be involved in the targeting and/or regulation of Kir6.2 in cardiac

tissue. Evaluation for defects in action potential duration, as well as defects in ischemic preconditioning, in ankyrin-$B^{+/-}$ mice would be a critical first step in the evaluation of ankyrin-B-dependent pathways for K_{ATP} channel targeting and function in cardiomyocytes.

Further definition of the ankyrin-B-binding region of the $InsP_3$ receptor. All three major $InsP_3$ receptor isoforms associate with ankyrin-B (Mohler, Davis et al. 2004). Furthermore, the expression of the three $InsP_3$ receptor isoforms is reduced in tissues derived from ankyrin-$B^{+/-}$ or ankyrin-B null mice (Mohler, Davis et al. 2004). Therefore, the precise binding site on the $InsP_3$ receptor is likely a conserved motif within the receptor subtypes (Figure 36A). While a previous study proposed that ankyrin directly associated with $InsP_3$ receptor at a site based on limited sequence similarity to an ankyrin-binding region of CD44 (Bourguignon and Jin 1995), it is now clear that most ankyrin-binding proteins do not have conserved sequence binding motifs. Instead, ankyrin-binding proteins associate with unstructured and non-conserved protein motifs (*reviewed in* (Bennett and Baines 2001)). Our group previously identified a series of positively-charged residues on the ankyrin-B membrane-binding domain that are essential for $InsP_3$ receptor binding. Based on these data, we predict that ankyrin-B will interact with a conserved, and likely negatively-charged, site within the 955-991 region of the $InsP_3$ receptor. Future goals will be to further refine the binding site for ankyrin and to analyze the cellular targeting of an $InsP_3$ receptor that lacks ankyrin-binding. Unfortunately, due to the homo- and hetero-trimeric organization of the $InsP_3$ receptor protein complex,

these experiments will not likely be straightforward. Additionally, using current viral technologies, it is difficult to express full-length $InsP_3$ receptor mutants in primary cells.

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