Dual role of K_{ATP} channel C-terminal motif in membrane targeting and metabolic regulation

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The coordinated sorting of ion channels to specific plasma membrane domains is necessary for excitable cell physiology. K_{ATP} channels, assembled from pore-forming (Kir6.x) and regulatory sulfonylurea receptor subunits, are critical electrical transducers of the metabolic state of excitable tissues, including skeletal and smooth muscle, heart, brain, kidney, and pancreas. Here we show that the C-terminal domain of Kir6.2 contains a motif conferring membrane targeting in primary excitable cells. Kir6.2 lacking this motif displays aberrant channel targeting due to loss of association with the membrane adapter ankyrin-B (AnkB). Moreover, we demonstrate that this Kir6.2 C-terminal AnkB-binding motif (ABM) serves a dual role in K_{ATP} channel trafficking and membrane metabolic regulation and dysfunction in these pathways results in human excitable cell disease. Thus, the K_{ATP} channel ABM serves as a previously unrecognized bifunctional touch-point for grading K_{ATP} channel gating and membrane targeting and may play a fundamental role in controlling excitable cell metabolic regulation.

ankyrin | cytoskeleton | diabetes | Kir6.2

K_{ATP} channels are critical electrical transducers of the metabolic state of excitable tissues including skeletal and smooth muscle, heart, brain, kidney, and pancreas (1). Mechanistically, decreased metabolism opens K_{ATP} channels, resulting in K^+ efflux, membrane hyperpolarization, and suppression of action potential formation (1). Conversely, increased metabolism closes K_{ATP} channels, resulting in membrane depolarization, stimulation of electrical activity (2), and subsequent triggering of diverse cellular responses, such as release of hormones and neurotransmitters, or muscle contraction.

Given such critical roles in the regulation of electrical excitability in many cell types, it is not surprising that K_{ATP} channel dysfunction results in disease. Human mutations in K_{ATP} channel genes are associated with neonatal diabetes and hyperinsulinemia (3), epilepsy (4), and dilated cardiomyopathy (5). However, despite the clear importance of K_{ATP} function for normal vertebrate physiology, little is resolved regarding the mechanisms responsible for K_{ATP} channel membrane targeting and/or membrane organization.

Here we identify a critical motif in the Kir6.2 C-terminal domain that is essential for normal Kir6.2 membrane targeting. We demonstrate that the Kir6.2 C-terminal motif interacts with the cytoskeletal adapter ankyrin-B (AnkB) and that Kir6.2 displays aberrant membrane trafficking when the motif is disrupted or in cells lacking ankyrin-B expression. We demonstrate that the Kir6.2 C-terminal motif displays an unexpected secondary role in Kir6.2 membrane function (K_{ATP} channel activity) by altering K_{ATP} channel ATP sensitivity. Finally, we demonstrate that a human neonatal diabetes disease mutation located in the Kir6.2 C-terminal motif results in a complex β cell phenotype, likely due to the dual role of the C-terminal motif in both Kir6.2 targeting and K_{ATP} channel membrane activity. Together, our findings define a pathway for K_{ATP} channel targeting in excitable cells, as well as an unexpected mechanism for modulation of K_{ATP} channel metabolic activity.

Results

Kir6.2 Associates with Ankyrin-B. To define molecular determinants of K_{ATP} channel membrane organization, we assessed the Kir6.2 and SUR cytoplasmic domains (Fig. 1*A*) for potential targeting motifs. Interestingly, sequence analysis of Kir6.2 revealed striking similarity between a C-terminal cytoplasmic sequence and a motif recently established to target voltage-gated channels (via ankyrin proteins) to specialized domains of excitable neurons and myocytes (6–10) (Fig. 1 *B* and *C*). Further analysis of this motif revealed high sequence conservation between Kir6.2 orthologs (Fig. 1*C*).

We evaluated potential interaction of Kir6.2 and ankyrin-B (AnkB) by co-immunoprecipitation. AnkB Ig co-immunoprecipitates Kir6.2 from detergent-soluble lysates generated from pancreas and heart (Fig. 1*D* and Fig. S1*A*). We observed no interaction between Kir6.2 and control Ig (Fig. 1*D* and Fig. S1*A*). Moreover, AnkB was unable to co-immunoprecipitate the related Kir6.1 gene product from heart (Fig. S1*B*). We examined a potential direct AnkB/Kir6.2 interaction using purified proteins. In vitro binding assays using radiolabeled full-length Kir6.2 revealed a direct interaction with the AnkB membrane-binding domain (MBD) (Fig. 1*E*), with robust AnkB MBD/Kir6.2 binding in salt concentrations of 500 mM (Fig. S2). Both the AnkB spectrin-binding domain (SBD) and C terminal domain (CTD) displayed minimal binding activities for radiolabeled Kir6.2 similar to GST alone (Fig. 1*E*). Together, our findings indicate that AnkB and Kir6.2 are protein partners in vivo and that this direct interaction is mediated by the AnkB MBD.

AnkB Associates with Kir6.2 C Terminus. Kir6.2 contains a cytosolic N terminus, two transmembrane domains, a P-loop, and a cytosolic C terminus (Fig. 1*F*). We generated Kir6.2 mutants lacking either the N- or C-terminal cytoplasmic domains (Fig. 1*F*) to establish the structural requirements on Kir6.2 for AnkB association. Consistent with the role of a putative AnkB-binding motif (ABM) in the Kir6.2 C terminus, Kir6.2 lacking the C terminus (Kir6.2ΔCT) is devoid of AnkB-binding activity (Fig. 1*F Right*). In contrast, Kir6.2ΔNT binds AnkB MBD (Fig. 1*F Center*), similar to full-length Kir6.2 (Fig. 1*F*). These findings establish the Kir6.2 C-terminal domain as necessary for AnkB association.

A Kir6.2 C-Terminal Motif Is Required for AnkB Association. We evaluated the role of the Kir6.2 C-terminal motif for ankyrin-binding using in vitro binding assays and two additional Kir6.2 mutants (Fig. 1*G*). The first mutant was engineered with a premature stop codon following the putative binding motif (Kir6.2 Δ331;

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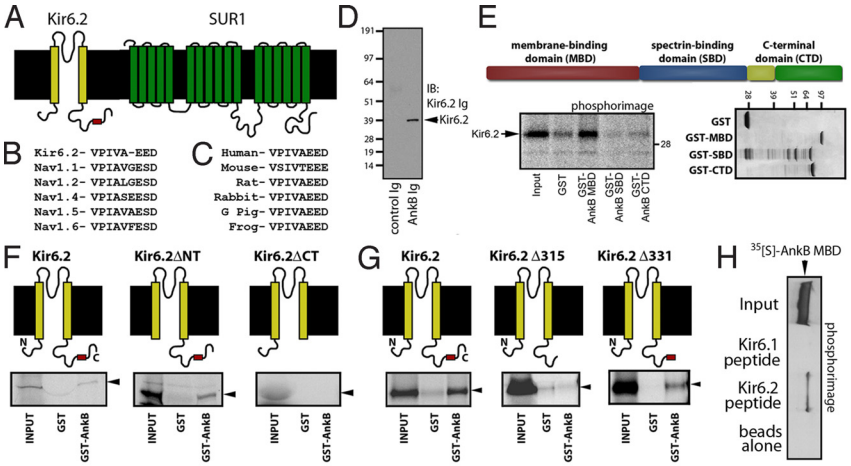

Fig. 1. Kir6.2 C-terminal motif binds AnkB. (*A*) Domain organization of K_{ATP} channel subunits Kir6.2 and SUR. (*B*) Kir6.2 C terminus contains a putative ankyrin-binding motif similar to motif in voltage-gated Na_V channels. (*C*) Kir6.2 motif is highly conserved across vertebrates. (*D*) Kir6.2 associates with AnkB in vivo (pancreas) by co-immunoprecipitation. (*E*) Radiolabeled Kir6.2 directly associates with purified AnkB membrane-binding domain, but not other AnkB domains. Inset shows purified AnkB domains. (*F*) Kir6.2 C-terminal domain containing putative AnkB-binding motif is required for AnkB-binding. (*G*) Kir6.2 C-terminal motif is required for AnkB-binding. Note absence of AnkB-binding for Kir6.2 mutant Δ315 vs. Δ331. (*H*) Kir6.2 C-terminal residues 316–323 are sufficient for AnkB-binding activity (radiolabeled AnkB MBD). In contrast, the corresponding motif in the Kir6.1 C terminus lacks AnkB-binding activity.

Fig. 1*G Right*), while the second mutant was engineered with a premature stop codon preceding the putative binding motif (Kir6.2 Δ315; Fig. 1*G Center*). In vitro binding with immobilized GST-AnkB MBD revealed that only Kir6.2 Δ331 displayed binding activity for AnkB (Fig. 1*G Right*). Binding of Kir6.2 Δ315 for GST-AnkB was similar to GST alone (Fig. 1*G Center*). These data identify an eight-residue motif in Kir6.2 C terminus that is necessary for interaction with AnkB. In fact, Kir6.2 residues 313–325 are necessary and sufficient for the ankyrin interaction, as a biotinylated peptide mimicking the putative Kir6.2 ABM associates with AnkB (Fig. 1*H*). In agreement with co-immunoprecipitation data, the analogous, but nonidentical motif found in Kir6.1 lacked AnkB-binding activity (Fig. 1*H*)

AnkB$^{+/-}$ Mice Display Abnormal Kir6.2 Expression and Localization. We evaluated a role for AnkB in Kir6.2 membrane expression in vivo using mice with reduced AnkB expression (AnkB$^{+/-}$ mice). AnkB is highly expressed in the pancreatic β cell (Fig. 2*A*), with minimal levels observed in other islet cell types or exocrine acinar cells (11). AnkB$^{+/-}$ mice are viable and express 50% reduction in islet AnkB levels (Fig. 2*B*, ≈50% decrease in pancreas by immunoblot, Fig. 2*C*). We observed a striking reduction in expression

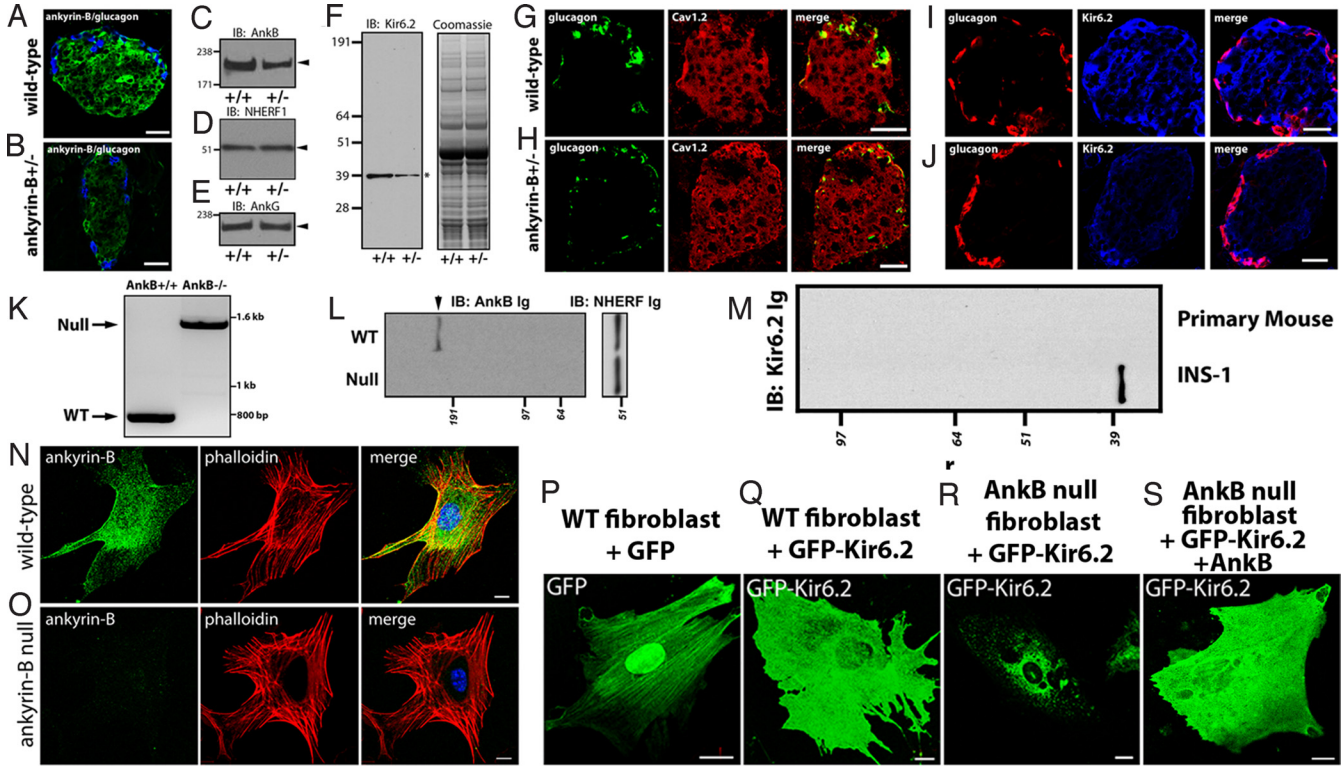

Fig. 2. Kir6.2 membrane expression requires AnkB. (*A* and *B*) AnkB is highly expressed in β cells and this expression is reduced in AnkB$^{+/-}$ islets. (*C–J*) In contrast to other β cell membrane and cytoskeletal proteins, Kir6.2 expression is reduced in cells lacking AnkB by immunoblot and immunostaining. (Scale bar, 10 μm.) (*K–O*) Generation and characterization of primary AnkB$^{-/-}$ fibroblasts. Expanded primary cultures were analyzed for ankyrin expression by PCR genotyping (*K*), immunoblot (*L*), and immunostaining (*N* and *O*; AnkB, green; phalloidin, red; nuclear dye topro-3, blue). (*M*) Primary fibroblasts lack endogenous Kir6.2 expression. (*P–R*) Kir6.2-GFP targeting requires AnkB. (*P*) GFP expression in WT fibroblasts. (*Q*) GFP-Kir6.2 expression in WT fibroblasts. (*R*) GFP-expression in AnkB$^{-/-}$ fibroblasts. (*S*) Expression of exogenous AnkB rescues GFP-Kir6.2 localization in AnkB$^{-/-}$ fibroblasts.

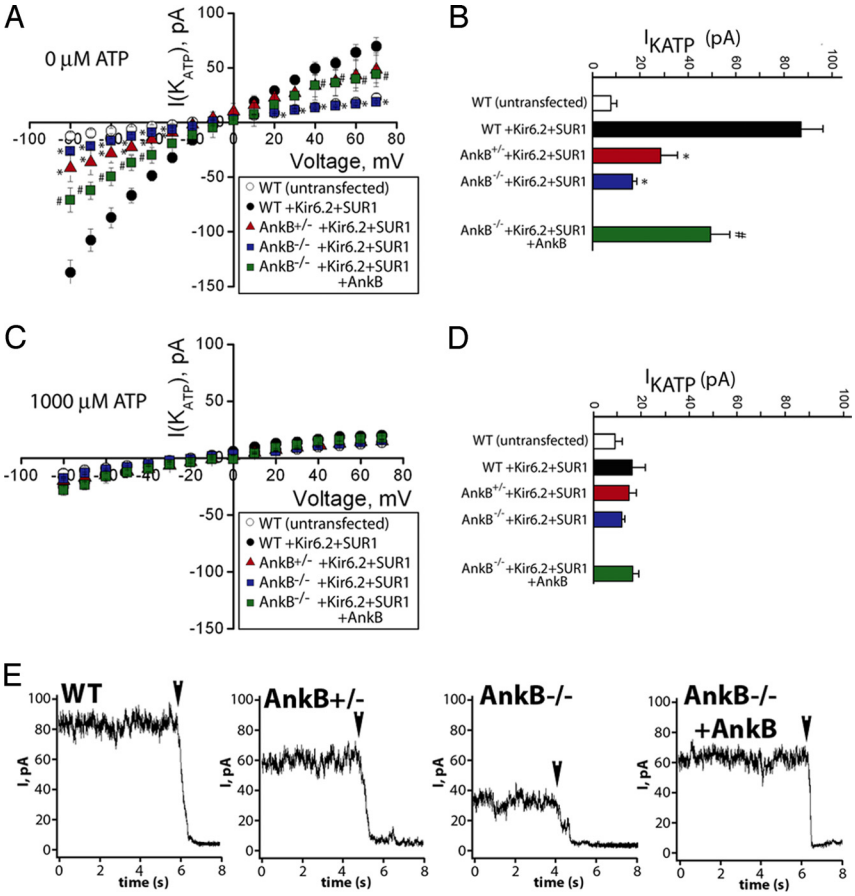

Fig. 3. AnkB is required for Kir6.2 membrane expression. Current-voltage relationship for I_{KATP} recorded from WT, AnkB$^{+/-}$, AnkB$^{-/-}$ fibroblasts transfected with Kir6.2 and SUR1 using the inside-out patch clamp configuration at zero ATP (*A* and *B*) to stimulate the opening of all membrane K_{ATP} channels ($n > 5$, $P < 0.05$), and at 1 mM ATP to block I_{KATP} (*C* and *D*). All pipets displayed similar resistances. (*B* and *D*) Summary data for I_{KATP} current at −60 mV (*B*; 0 ATP; *D*; 1 mM ATP). Note that expression of AnkB cDNA rescues abnormal channel targeting phenotype in AnkB$^{-/-}$ cells. (*E*) I_{KATP} recordings for WT, AnkB$^{+/-}$, AnkB$^{-/-}$ fibroblasts, and AnkB$^{-/-}$ fibroblasts +AnkB cDNA recorded at −60 mV. Arrow denotes change in solution from 0 ATP to 1 mM ATP (channel closure). Note that primary cells displayed a small, endogenous K^+ current (present in nontransfected cells) that was not blocked by 1 mM ATP (ATP insensitive, I_{KATP}-independent).

levels of Kir6.2 in AnkB$^{+/-}$ pancreas by immunoblot and immunostaining (Fig. 2 *F*, *I*, and *J*). In contrast, we observed no difference in expression of $Ca_v1.2$, SUR1, ankyrin-G, or NHERF1 (Fig. 2 *D*, *E*, *G*, and *H* and Fig. S3). Moreover, the levels of two proteins affected by loss of AnkB in heart, Na/Ca exchanger and Na/K ATPase (12), were unchanged in AnkB$^{+/-}$ mouse pancreas (Fig. S3). Real-time PCR revealed no difference in the levels of mRNA encoding Kir6.2 (*KCNJ11*), suggesting that abnormal expression of Kir6.2 in AnkB$^{+/-}$ pancreas is likely due to posttranscriptional events.

AnkB Is Required for Kir6.2 Membrane Expression. As AnkB$^{-/-}$ mice die shortly after birth, we created a primary cell culture system lacking AnkB to directly test the role of AnkB for Kir6.2 membrane targeting. Primary fibroblasts were isolated from wild-type (WT) and AnkB$^{-/-}$ postnatal day 1 mice. Absence of AnkB in primary null cells was tested by genotyping, immunostaining, and immunoblot (Fig. 2 *K*, *L*, *N*, *O*, cells also lack Kir6.2, and Fig. 2*M*) GFP-tagged Kir6.2 (but not GFP) was localized to the membrane of transfected WT fibroblasts (Fig. 2 *P* and *Q*, cells cotransfected with SUR1). In contrast, GFP-Kir6.2 was not targeted to the membrane of AnkB$^{-/-}$ fibroblasts, but instead was localized to a perinuclear region of AnkB$^{-/-}$ cells (Fig. 2*R*).

Finally, we definitively tested the role of AnkB for Kir6.2 membrane expression using inside-out patch clamp measurements of I_{KATP} in WT, AnkB$^{+/-}$, and AnkB$^{-/-}$ fibroblasts transfected with Kir6.2 and SUR1 (Fig. 3). In agreement with qualitative staining experiments, quantitative functional analyses of GFP-Kir6.2 membrane expression revealed a marked reduction of K_{ATP} channel current in AnkB$^{+/-}$ and AnkB$^{-/-}$ primary cells compared with WT fibroblasts (measured at 0 mM ATP to stimulate opening of all membrane associated K_{ATP} channels; Fig. 3, *A*, *B*, and *E*). As expected, I_{KATP} channel currents were blocked by 1 mM ATP (Fig. 3 *C*–*E*). Finally, in both immunostaining and functional electrophysiology experiments, defective Kir6.2 membrane targeting phenotypes in AnkB$^{-/-}$ cells were corrected by re-expression of AnkB cDNA (Fig. S2 and Fig. 3 *A*–*E*). Together, these findings support a requirement of AnkB for efficient Kir6.2 membrane localization.

Human Diabetes Disease Mutation Abolishes AnkB Interaction. We previously identified a human mutation in the ankyrin-binding motif of cardiac $Na_v1.5$ that causes arrhythmias in the Brugada syndrome (8) (Fig. 4*A*). At the cellular level, Brugada syndrome is characterized by decreased $Na_v1.5$ membrane activity. Consistent with this phenotype, the $Na_v1.5$ E1053K mutant lacks ankyrin-binding activity (Fig. S4) and is not targeted to the membrane of primary myocytes (resulting in decreased I_{Na}) (8). Permanent neonatal diabetes mellitus (PNDM) is characterized by the development of diabetes within the first 6 months of birth and life-long insulin dependence (13). In 2004, Vaxillaire identified a Kir6.2 mutation in a 10 year 10 month old insulin-dependent female with PNDM (presented with hyperglycemia at three days old) (14). Strikingly, the disease-causing mutation (E322K) is located in the AnkB-binding motif (ABM) at a homologous position to the $Na_v1.5$ Brugada syndrome disease variant E1053K (8) (Fig. 4*A*). We engineered the human PNDM mutation (E322K) in human Kir6.2 and examined AnkB-binding. Additional lysine and alanine substitutions were generated in this and adjacent negatively charged residues E321 and D323. All charge reversal mutants (E321K, E322K, and D323K) eliminated Kir6.2 AnkB-binding activity (Fig. 4*B*). Consistent with these findings, a peptide against the Kir6.2 AnkB-binding motif engineered with the human E322K mutation displayed minimal binding activity for AnkB (Fig. 4 *C* and *D*). In contrast, neutral alanine substitutions were without effect on

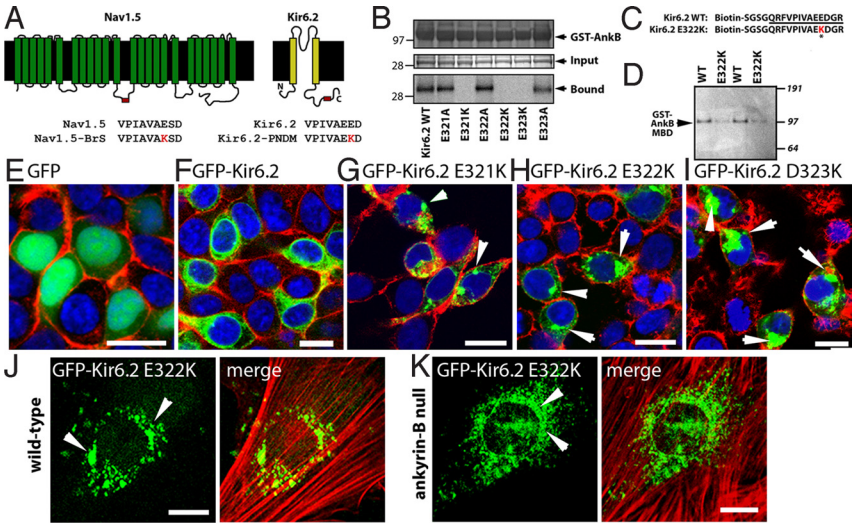

Fig. 4. Kir6.2 C-terminal acidic motif mediates channel targeting and human diabetes mutation in motif abolishes Kir6.2 trafficking. (*A*) Identification of human permanent neonatal diabetes mutation in Kir6.2 C-terminal motif (E322K) and alignment with $Na_v1.5$ human Brugada syndrome arrhythmia mutation (affects ankyrin-binding and channel targeting). (*B*) Human PNDM mutation in Kir6.2 C-terminal motif blocks AnkB-binding. (*C* and *D*) Human PNDM mutation E322K is sufficient to reduce AnkB-binding in context of peptide. (*E* and *F*) In contrast to GFP, GFP-Kir6.2 is expressed primarily at the membrane of HEK293 cells. (*G–K*) GFP-Kir6.2 C-terminal motif mutants that affect AnkB-binding display abnormal membrane targeting in HEK293 cells (*G–I*) and primary WT (*J*) and $AnkB^{-/-}$ fibroblasts (*K*). Note perinuclear expression of mutants (white arrows). Additional staining represents phalloidin (red) and nuclear dye topro-3 (blue). (Scale bar, 10 μm.)

AnkB-binding (Fig. 4*B*). Together, these findings identify a key acidic stretch in the ankyrin-binding motif as critical for AnkB/Kir6.2 interaction and demonstrate that the human Kir6.2 E322K disease mutation disrupts the Kir6.2/AnkB association.

Kir6.2 Disease Mutant Lacking Ankyrin-Binding Activity Is Not Efficiently Targeted. Based on the recognized role of ankyrins for membrane protein targeting (15), we further evaluated the membrane localization of the Kir6.2 human diabetes mutation E322K. In contrast to the WT channel, Kir6.2 E322K (and E321K, D323K) was not efficiently targeted to the membrane of HEK293 cells (Fig. 4 *E–I*) or primary mouse fibroblasts (both cell types express AnkB; Fig. 4 *J* and *K*). Specifically, these mutant subunits were primarily clustered in a perinuclear pattern (Fig. 4 *G–K*). These results demonstrate that the Kir6.2 C-terminal ABM is essential for efficient Kir6.2 membrane targeting, such that disruption of this motif will lead to loss of membrane Kir6.2, an effective Kir6.2 loss-of-function.

Kir6.2 Mutant Lacking AnkB-Binding Activity Displays Decreased ATP Sensitivity. Reduced K_{ATP} channel trafficking due to the E322K mutation should result in a loss of cellular K_{ATP} channel activity, unless the residual membrane channels display strongly augmented K_{ATP} channel opening probability. In fact, the prediction that E322K mutant cells show loss-of-function due to defective membrane insertion is in apparent contrast to the observed mild gain-of-function PNDM phenotype of the human Kir6.2 E322K mutation (14). Based on this discrepancy between the cellular trafficking (Kir6.2 loss-of-function) and mild human (gain-of-function) E322K phenotypes, we analyzed multiple cell lines to identify a mammalian cell system suitable for studying the biophysical gating properties of Kir6.2 trafficking-defective E322K-GFP mutants at the plasma membrane. COSm6 cells lack Kir6.2 but retain AnkB expression (Fig. S5) and have enhanced activity of an AnkB-independent K_{ATP} channel trafficking pathway compared with all other primary and cultured cells examined (see Figs. 2–4). Still, while Kir6.2 E322K trafficking remains significantly impaired in COSm6 cells [≈20% as assessed by qualitative immunostaining (Fig. S6) and quantitative functional $^{86}Rb^+$ efflux experiments performed in "metabolic inhibition" conditions (oligomycin and 2-deoxyglucose) designed to lower cellular [ATP]:[ADP] ratios and fully activate membrane K_{ATP} channels (16) (Fig. S6*B*)], sufficient Kir6.2 E322K-GFP is targeted to the plasma membrane to permit assessment of channel function. Inside-out macropatch recordings of COSm6 cells expressing Kir6.2 E322K revealed a marked difference in ATP sensitivity compared with WT channels (Fig. 5 *A–C*), consistent with previous findings in nonmammalian cells (17). The E322K channel is significantly less sensitive to inhibition by ATP ($K_{1/2}$ for ATP inhibition is shifted from 15 μM in WT to 62 μM in the mutant, Fig. 5*C*) compared to WT channels. In contrast, charge neutralization of E322 (Kir6.2 E322A, retains ankyrin-binding activity; Fig. 4*B*) has no significant effect on Kir6.2 ATP sensitivity or targeting (N.S.; Fig. 5*C*, $n = 21$ WT, $n = 13$ E322A). Consistent with decreased ATP sensitivity (channel gain-of-function), COSm6 cells expressing Kir6.2 E322K displayed increased $^{86}Rb^+$ efflux under basal conditions (simulates physiologic ATP conditions; Fig. S6*C*). These data are consistent with E322K as a Kir6.2 gating gain-of-function mutation and support dual roles for the Kir6.2 C-terminal ABM in both targeting to the membrane and regulation of channel activity by ATP.

Ankyrin/Kir6.2 Binding Regulates I_{KATP}. We next tested whether the Kir6.2 C-terminal ABM directly affects K_{ATP} channel gating using a peptide designed against the Kir6.2 ABM motif to competitively inhibit the ankyrin/Kir6.2 interaction. In agreement with a role of AnkB in regulating K_{ATP} channel biophysical activity, we observed increased K_{ATP} channel currents when the ABM peptide was applied to the cytoplasmic side of the channel in excised macropatches from COSm6 cells (Fig. S7 *A* and *C*). This change in activity was not observed for Kir6.2 E322K channels under identical conditions (Fig. S7 *B* and *C*). Additionally, a control peptide lacking

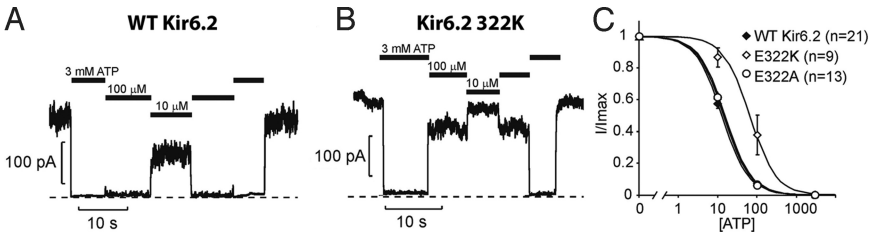

Fig. 5. Kir6.2 C-terminal motif plays role in K_{ATP} channel metabolic regulation. (*A–C*) Membrane-associated K_{ATP} channels composed of E322K Kir6.2 subunits (lack ankyrin-binding) demonstrate a significant reduction in ATP sensitivity. Mutant channels demonstrate a rightward shift in ATP sensitivity (WT Kir6.2 = 10 μM, E322K channels = 62 μM) versus WT channels. (*C*) We observed no difference in the ATP sensitivity of Kir6.2 E322A (displays normal ankyrin-binding activity) compared with WT channels.

ankyrin-binding activity (contains the E322K mutation) had no effect on WT or mutant Kir6.2 E322K channel activity (Fig. S7 *A–C*).

Defects in Kir6.2 E322K Cause Dual Cellular Phenotypes Resulting in Human Disease. Interestingly, the E322K mutation results in two counteracting I_{KATP} phenotypes: decreased membrane localization and reduced ATP sensitivity. A severe hypoinsulinemic/hyperglycemic phenotype can result from even a mild decrease in ATP sensitivity (18). Our data demonstrate a significant reduction in ATP sensitivity (≈50 μM shift in $K_{1/2}$ of ATP binding); however, the E322K phenotype presents with hyperglycemia that is much less severe than in individuals expressing other gain-of-function mutations in Kir6.2 [fasting blood glucose of 342 mg/dL (19 mM) compared with values ranging from 403 mg/dL (22.4 mM) to 1260 mg/dL (70 mM) for other PNDM mutation carriers] (14). Mathematical modeling of our experimental data (Fig. S8 and *SI Appendix*) support the idea that reduced I_{KATP} conductance due to decreased membrane channel expression may protect the β cell from complete elimination of action potential firing (Fig. S8 *E–G*) that would otherwise result if the enhanced opening probability of E322K mutant channels was unopposed by reduced membrane expression. Therefore, our data suggests that the dual molecular phenotype of defects in ATP sensitivity plus abnormal trafficking (i.e., fewer channels with gain-of-function properties) results in the ultimate PNDM clinical phenotype of E322K probands. These findings are consistent with recent findings of other neonatal diabetes mutations that show loss of channel density combined with reduced ATP sensitivity (19). While the mechanism(s) underlying dual phenotypes of other neonatal diabetes mutations are still unclear, our results strongly support dysfunction in ankyrin-based pathways as the mechanism underlying the Kir6.2 E322K human disease phenotype.

Ankyrin Forms a Ternary Complex with Kir6.2 and SUR1. Interaction of Kir6 and SUR subunits is critical for normal K_{ATP} channel regulation (20). In fact, mutations or genetic disruption of either Kir6 or SUR subunits resulting in a loss of subunit association, produce K_{ATP} channel dysfunction (21, 22). We therefore tested whether AnkB-binding affected association of Kir6.2 with SUR1. HEK293 cells (express AnkB, lack Kir6.2, and SUR1) were transfected with combinations of Kir6.2 or Kir6.2 E322K plus or minus SUR1, and the cell lysates were immunoprecipitated using Kir6.2 Ig (Fig. S9*A*). As expected, Kir6.2 Ig co-immunoprecipitated AnkB from cells expressing Kir6.2 alone (Fig. S9*A*). Likewise, Kir6.2 Ig co-immunoprecipitated SUR1 and AnkB from cells expressing both Kir6.2 with SUR1, demonstrating that Kir6.2 associates with both AnkB and SUR1 proteins. Finally, while lacking ankyrin-binding activity, Kir6.2 E322K maintained its association with SUR1, as demonstrated by the ability of Kir6.2 Ig to co-immunoprecipitate SUR1 in cells that express Kir6.2 E322K and SUR1(Fig. S9*A*). We performed similar experiments using AnkB Ig (Fig. S9*B*). Consistent with our prior data, AnkB associated with Kir6.2, but not Kir6.2 E322K(Fig. S9*B*). Additionally, we did not detect interaction of AnkB with SUR1 in cells expressing only SUR1(Fig. S9*B*). However, co-expression of SUR1 with Kir6.2, but not Kir6.2 E322K, resulted in ability of AnkB to co-immunoprecipitate the SUR1 subunit (Fig. S9*B*). Finally, we tested the potential of an ankyrin/Kir6.2/SUR1 ternary complex in vivo. Kir6.2 Ig co-immunoprecipitated both AnkB and SUR1 from mouse pancreas (Fig. S9 *C* and *D*). Similarly, AnkB Ig co-immunoprecipitated both Kir6.2 and SUR1 (Fig. S9 *E* and *F*). Our combined data identify a ternary protein complex required for normal Kir6.2 targeting and regulation in excitable cells. Moreover, our data strongly suggest that AnkB and SUR1 display unique binding sites on the Kir6.2 subunit. In support of these findings, other groups have reported SUR binding regions on the Kir6 N-terminal and first transmembrane domains (23).

Discussion

Here we demonstrate that a Kir6.2 C-terminal motif serves an unexpected dual role for K_{ATP} channel trafficking and membrane metabolic regulation. A Kir6.2 channel mutant associated with human diabetes (E322K) blocks interaction of Kir6.2 with AnkB. Importantly, Kir6.2 E322K lacking AnkB-binding activity is inefficiently targeted and displays abnormal biophysical activity resulting in net gain-of-function phenotype.

For well over a decade, the ion channel field has struggled to identify the molecular link between the K_{ATP} channel and the cytoskeleton (24–26). The presence of an adapter complex was first proposed 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 (25). Specifically, disruption of the actin cytoskeleton has been shown to activate K_{ATP} channel activity in excitable cells by reducing the sensitivity of K_{ATP} channels to ATP-dependent channel closure (24–26). Thus, impairment in ATP sensitivity indicates a transduction pathway of inhibitory gating signals determined by the integrity of the submembrane cytoskeletal network. This suggests that the association of K_{ATP} channels with the cytoskeleton is critical to the modulation of ligand-dependent regulation. Considering that ankyrins link ion channels, such as voltage-gated sodium channels, the Na/K ATPase, anion exchanger, and the Na/Ca exchanger, to the actin-based cytoskeleton (15), our data may finally provide insight on the identity of the link between cytoskeleton and K_{ATP} channel function.

Little is known regarding the mechanisms responsible for Kir6.2 membrane trafficking or local membrane organization. In fact, conflicting data have been published concerning the trafficking of individual K_{ATP} channel subunits. The presence of a putative ER retention signal (RKR) in both Kir6 and SUR subunits led to the postulation that only fully functional K_{ATP} channel assemblies (where retention signals become masked) traffic to the plasma membrane (27). However, studies using Kir6 subunits with mutated RKR sequences demonstrate that regions and mechanisms other than RKR play a role in the trafficking of Kir6.2 (28, 29). Moreover, Kir6 subunits have even been shown to display normal membrane trafficking in heterologous cells in the absence of SUR (29). Our data strongly support a role of ankyrins in Kir6.2 targeting. Consistent with the role of ankyrins in the erythrocyte, AnkB may serve as a static membrane scaffold to tether membrane Kir6.2 with the spectrin/actin-based cytoskeleton. On the other hand, AnkB may play active roles in the posttranslational trafficking of Kir6.2 to specific excitable membrane domains. Alternatively, AnkB may play a dual role in the membrane delivery and retention of Kir6.2. The identification of specific cellular roles for ankyrin polypeptides in protein targeting are an obvious area of future emphasis in the field. Finally, it is important to note that we detected a small, but significant component of membrane I_{KATP} even in the absence of AnkB (I_{KATP} reduced ≈85% in AnkB$^{-/-}$ cells; Fig. 3*B*) in primary cells. Thus, while our data demonstrate a clear and predominant role of AnkB for Kir6.2 targeting in native cells, it is likely that AnkB-independent pathways may also contribute to K_{ATP} channel trafficking and membrane retention. The identification of these pathways is an important key direction for the field.

While structurally similar, Kir6 gene products have diverse functions for human physiology. While Kir6.2 has roles in glucose homeostasis and cardiac ischemic preconditioning (30), Kir6.1 is critical for maintenance of vascular tone (31) and mitochondrial function (32, 33). Our data identify an unanticipated mechanism for the posttranslational regulation of specific Kir6 gene products. Specifically, AnkB directly associates with the C terminus of Kir6.2, but not the structurally similar Kir6.1. We postulate that the absence of a highly conserved proline residue (present in Kir6.2 ABM) in the analogous Kir6.1 motif likely interferes with the required folding of the motif for ankyrin-binding. Thus, our data

suggests that Kir6.1 and Kir6.2 have evolved specialized targeting and membrane regulatory pathways (AnkB-dependent and -independent). While Kir6.1 is not co-expressed with Kir6.2 in the β cell, a number of excitable tissues display both Kir6.1 and Kir6.2, with distinct cell and membrane expression patterns (32). Therefore, the identification of specific cellular cues for Kir6.1 versus Kir6.2 targeting and membrane regulation is an obvious exciting future focus for the field.

How do the molecular phenotypes of the human Kir6.2 E322K mutant ultimately result in PNDM? K_{ATP} channel function is dually regulated by channel membrane expression and open probability. Our findings demonstrate that Kir6.2 lacking AnkB-binding activity results in defects in both regulatory mechanisms. First, Kir6.2 E322K results in reduced membrane expression consistent with a channel loss-of-function phenotype (Figs. 2 and 3). Second, the Kir6.2 E322K channel displays abnormal gating due to reduced ATP sensitivity, producing a channel gain-of-function phenotype (Fig. 5 and Fig. S6*B*). Intuitively, these two phenotypes might be predicted to balance each other (fewer channels with increased function), resulting in normal cellular excitability. However, as shown by many groups, ATP sensitivity is a critical determinant of K_{ATP} channel function. In fact, even small changes in ATP sensitivity may produce large changes in channel activity, β cell excitability, and insulin secretion (18, 34, 35). Importantly, the Kir6.2 E322K mutant results in striking changes in ATP sensitivity (Fig. 5*C*; $K_{1/2}$ for ATP inhibition is shifted from 15 μM in WT to 62 μM in the mutant), consistent with a strong gain-of-function cellular phenotype (17). Thus, as illustrated by our functional data (Figs. 3 and 5, and Fig. S6), modeled by our simulation data (Fig. S8), and consistent with the human phenotype data (14), defects in Kir6.2 E322K membrane trafficking moderate the extreme changes in ATP sensitivity associated with this mutation, effectively shifting the net balance of channel activity toward a mild gain-of-function phenotype (Fig. S6*C* and Fig. S8). In support of our findings, Shyng et al. identified human Kir6.2 mutations with reduced membrane expression that also moderated severe gain-of-function phenotypes due to reduced ATP sensitivity (19). Specifically, Kir6.2 R201C and R201H display striking loss of ATP sensitivity (gain-of-function) compared with another PNDM mutation, Kir6.2 V59M. However, as both R201C and R201H also display reduced membrane expression phenotypes (loss-of-function), the two mutants manifest in much less severe PNDM disease phenotypes compared with V59M (19). Thus, defects in targeting may ameliorate the overall cellular phenotype associated with severe gain-of-function defects in channel gating. Together, these findings illustrate the potentially complex molecular phenotypes underlying human excitable cell disease.

Materials and Methods

Additional procedures can be found in *SI Methods*.

Animals. All mice evaluated were age-matched male littermates and were housed and fed under identical conditions.

Immunoblots and Co-Immunoprecipitations. See *SI Methods* for details.

Binding Experiments. See *SI Methods* for details.

Neonatal Fibroblast Isolation and Immunofluorescence. See *SI Methods* for details.

Transfection. See *SI Methods* for details.

Patch Clamp Electrophysiology. See *SI Methods* for details.

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