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Research Paper

Regulation of K~ATP~ Channel Expression and Activity by the SUR1 Nucleotide Binding Fold 1

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KEY WORDS

ACKNOWLEDGEMENTS

ABSTRACT

ATP-sensitive K$^+$ (K~ATP~) channels are oligomeric complexes of pore-forming Kir6 subunits and regulatory Sulfonylurea Receptor (SUR) subunits. SUR, an ATP-Binding Cassette (ABC) transporter, confers Mg-nucleotide stimulation to the channel via nucleotide interactions with its two cytoplasmic domains (Nucleotide Binding Folds 1 and 2; NBF1 and NBF2). Regulation of K~ATP~ channel expression is a complex process involving subunit assembly in the ER, SUR glycosylation in the Golgi, and trafficking to the plasma membrane. Dysregulation can occur at different steps of the pathway, as revealed by disease-causing mutations. Here, we have addressed the role of SUR1 NBF1 in gating and expression of reconstituted channels. Deletion of NBF1 severely impairs channel expression and abolishes MgADP stimulation. Total SUR1 protein levels are decreased, suggestive of increased protein degradation, but they are not rescued by treatment with sulfonylureas or the proteasomal inhibitor MG-132. Similar effects of NBF1 deletion are observed in recombinant K~ATP~ channels obtained by "splitting" SUR1 into two separate polypeptides (a N-terminal "half" and a C-terminal "half"). Interestingly, the location of the "splitting point" in the vicinity of NBF1 has marked effects on the MgADP stimulation of resulting channels. Finally, ablation of the ER retention motif upstream of NBF1 (in either "split" or full-length SUR1) does not rescue expression of channels lacking NBF1. These results indicate that, in addition to NBF1 being required for MgADP stimulation of the channel, it plays an important role in the regulation of channel expression that is independent of the ER retention checkpoint and the proteasomal degradation pathway.

INTRODUCTION

In responding to changes in cellular [ATP]/[ADP], ATP-sensitive potassium (K~ATP~) channels link cellular metabolism to membrane excitability. They are heterooctamers of two subunits[1-3]: four pore-forming Kir6.2 potassium channel subunits plus four regulatory sulfonylurea receptor (SUR) subunits. SUR, like all other ATP-Binding Cassette (ABC) transporters, contains two transmembrane domains (TMD1 and TMD2) and two cytoplasmic domains (Nucleotide Binding Folds 1 and 2, NBF1 and NBF2); in addition, it possesses a N-terminal transmembrane domain (TMD0), which mediates SUR-Kir6 interactions.[4,5] While ATP inhibits the K~ATP~ channel by direct binding to the cytoplasmic domains of Kir6,[6,7] SUR is responsible for channel regulation by other ligands, including high-affinity inhibition by sulfonylurea drugs,[8] stimulation by potassium channel openers (KCO's),[9] and stimulation by Mg-nucleotides.[10,11] Crystallographic and biochemical studies on prokaryotic NBFs[12-15] suggest that Mg-nucleotide binding at the NBFs of SUR induces formation of a NBF1-NBF2 heterodimer, which mediates ATP hydrolysis. Consistent with this model, mutations in the predicted binding sites of either NBF significantly impair or completely abolish Mg-nucleotide stimulation of K~ATP~ channels.[16,17]

K~ATP~ channels are assembled in the Endoplasmic Reticulum (ER) from Kir6 and SUR subunits. Each subunit possesses an ER retention motif, RKR, which prevents trafficking of mismatched subunits to the membrane: in Kir6.2, it is located in the C-terminus, whereas in SUR1 it is located in the cytoplasmic loop between TMD1 and NBF1 (residues 648–650).[6,18,19] In the fully-assembled K~ATP~ channel complex, each retention signal is apparently masked by the other subunit, and the channel can exit the ER.[19] Ablation of this motif by mutation to AAA, or by truncation of the C-terminus in the case of Kir6.2,[6] facilitates trafficking of either subunit alone. Trafficking of SUR1 to the membrane is also dependent on N-linked glycosylation at N10 and N1050.[20] Because of this complex

glycosylation, SUR1 migrates as multiple molecular weight species on electrophoresis from cell lysates: the lower molecular weight species corresponds to the immature, core-glycosylated ER fraction, and the higher molecular weight species correspond to the mature, fully-glycosylated plasma membrane fraction.[21,22]

Dysregulation of SUR assembly and trafficking has important physiological consequences, as evidenced by mutations that cause retention of SUR1 in the ER[23] or in the Golgi,[24] leading to reduced channel expression and causing Persistent Hyperinsulinemic Hypoglycemia of Infancy (PHHI) in humans. Some mutations can be fully or partially rescued by ablation of the RKR motif.[23,25] Others can be rescued by sulfonylurea treatment,[26] which reduces degradation of the mutant protein via the ubiquitination-proteasomal degradation pathway.[27]

In this study, we have examined the role of NBF1 in the regulation of K$_{ATP}$ channel expression and gating by engineering recombinant channels lacking NBF1. Deletion of NBF1 from SUR1 has drastically deleterious effects on channel formation and on channel stimulation by MgADP. Total SUR1 protein levels are decreased by NBF1 deletion, suggestive of increased protein degradation, and they are not rescued by treatment with sulfonylureas or the proteasomal inhibitor MG-132. Deletion of NBF1 has equivalent effects when K$_{ATP}$ channels are generated by coexpression of Kir6.2 with "split" SUR1 constructs (an N-terminal fragment and a C-terminal fragment[4]), demonstrating that the effects of NBF1 deletion are not a result of the artificial connection of TMD1 to TMD2. Interestingly, the MgADP stimulation of K$_{ATP}$ channels formed by "split" SUR1 constructs is differentially impaired depending on the location of the "splitting" point. Finally, ablation of the ER retention motif in either the full-length or the "split" SUR1 background fails to rescue the expression deficit caused by deletion of NBF1. Thus, the requirement for NBF1 for channel expression is independent of the control of subunit association in the ER and of protein degradation by the proteasomal pathway, and is likely related to misfolding of the SUR1ΔNBF1 protein.

MATERIALS AND METHODS

Transfection of COSm6 cells with cloned cDNA. cDNA was transfected into COSm6 cells using FuGENE 6 Transfection Reagent (Roche Diagnostics, Indianapolis, IN). Typically, 1.1 μg of total DNA (0.3 μg mouse Kir6.2[28] plus 0.5 μg hamster SUR1[29] plus 0.3 μg GFP as a marker for transfection) was mixed with 3 μL FuGENE 6; cells were incubated in the presence of the transfection mixture for 12-24 hrs, and then plated on sterile glass coverslips for growth prior to patch-clamp experiments. All transfections were done in parallel.

Split SUR1 constructs (FLAG-NSUR1 and His-CSUR1) were engineered by PCR from full-length SUR1 (M1-K1580) using epitope-containing 5' primers (FLAG: DYKDDDDK, His: HHHHHH). FLAG-NSUR1 constructs begin at M1 and end at L614, N647, A674, D936, A1000, or S1045; His-CSUR1 constructs end at K1580 and begin at S615, D675, Q937, C1001, or P1046. When cotransfected into COS cells, 0.3 μg DNA of each was used in place of full-length SUR1. In constructs indicated as [AAA], the RKR motif (R648-K649-R650) was mutated to AAA. ΔNBF1 constructs, lacking D675-D936, were also engineered by two-step PCR from full-length SUR1. All PCR-generated constructs were confirmed by direct DNA sequencing.

Electrophysiological methods. Inside-out membranes were patch-clamped in a chamber mounted on the stage of an inverted microscope (Nikon, Garden City, NY). The chamber consists of four lanes (each containing different solutions) that run into the same end-pool, where the ground electrode is placed.[30] A float connected to a tension transducer senses the solution level in the end-pool and controls the solution level by varying the outflow. COS cells (2–5 days post-transfection) that fluoresced green under UV illumination were selected for patch-clamping. Membrane patches were voltage-clamped using a CV-4 headstage and an Axopatch 1-D amplifier, and currents were digitized with a Digidata 1322A board (all from Axon Instruments, Union City, CA). Bath and pipette control solutions (K$_{INT}$) contained, in mM: 150 KCl, 10 HEPES, and 1 EGTA (pH 7.4). ATP and ADP were added to the bathing solution as dipotassium salts. Where indicated, MgCl$_2$ was added to the bathing solution to a calculated [Mg$^{2+}_{free}$] of 0.5 mM.

Data were analyzed offline with the pClamp 8.2 software suite (Axon Instruments, Union City, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA). Results are presented as mean ± SEM (standard error of the mean). Statistical tests and p-values are noted in figure legends where appropriate.

Immunoblotting of COS cell lysates. Transfected COS cells were grown in 6-well plates for 2 days (transfections were done in parallel). Cells were washed twice with 4°C PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na$_2$HPO4, 2 mM KH$_2$PO4) and lysed in 250 μL lysis buffer (150 mM NaCl, 20 mM HEPES, 10 mM EDTA, 1% NP-40, one "Complete Mini" protease inhibitor tablet [Roche Diagnostics, Indianapolis, IN] per 10 mL, pH 7) for 30 min at 4°C under mild rocking. Crude lysates were collected by pipetting, centrifuged for 5 min at 13,000 rpm at 4°C in a microcentrifuge, and transferred to clean microcentrifuge tubes. Where indicated, 10 μM glibenclamide was added to the culture medium 24 hours prior to lysis, and 10 μM MG-132 was added to the medium 6 hours prior to lysis (control cells were exposed to the vehicle, 1% DMSO).

Samples (6 μg total protein/lane) were resolved by SDS-PAGE (7.5% acrylamide) and electrophoretically transferred to nitrocellulose filters (Hybond-ECL, Amersham Biosciences). Filters were blocked in TBS buffer (200 mM NaCl, 20 mM Tris-HCl, pH 7.4) plus 5% nonfat dry milk at 4°C overnight, and then bathed in a 1:1000 dilution of the primary antibody (mouse monoclonal anti-FLAG antibody, Sigma, St. Louis, MO) in TBS plus 5% milk at room temperature for 1 hr. Filters were washed with TBS plus 5% milk for 30 min at room temperature, and then bathed in a 1:1000 dilution of the secondary antibody (sheep anti-mouse IgG, horseradish peroxidase linked, Amersham Biosciences) in TBS plus 5% milk at room temperature for 1 hr. Filters were then sequentially washed with TBS plus 5% milk, TBS plus 0.1% Tween, and TBS, each for 30 min at room temperature. Finally, an enhanced chemiluminescence system (Supersignal West Femto Maximum Sensitivity Substrate, Pierce Biochemicals) was applied to the filters, to which autoradiography film (Midwest Scientific, St. Louis, MO) was then exposed.

Macroscopic ^{86}Rb$^+$ efflux assay. COS cells in 12-well plates were incubated for 24 hr in culture medium containing ^{86}RbCl (1 μCi/mL) 2 days post-transfection (transfections were done in parallel). Before measurement of ^{86}Rb$^+$ efflux, cells were washed twice with Ringer's (in mM: 118 NaCl, 2.5 CaCl$_2$, 1.2 KH$_2$PO$_4$, 4.7 KCl, 25 NaHCO$_3$, 1.2 MgSO$_4$, 10 HEPES; pH 7.4) plus metabolic inhibition (MI, 1 mM 2-deoxy-D-glucose and 2.5 μg/mL
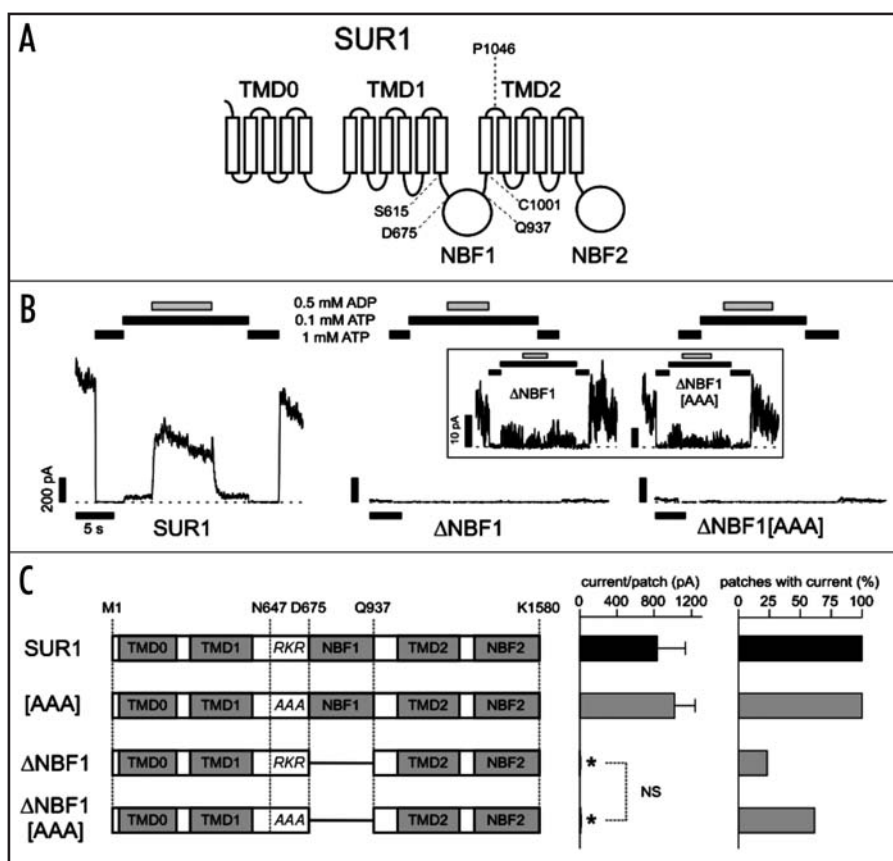
Figure 1. Deletion of NBF1 from SUR1 prevents K_{ATP} channel expression and abolishes MgADP stimulation. (A) Transmembrane topology of SUR1, with relevant residues labeled. TMD, transmembrane domain. NBF, nucleotide binding fold. (B) Representative current traces recorded at -50 mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and either SUR1, SUR1ΔNBF1, or SUR1ΔNBF1[AAA]. All transfections were done in parallel. Patches were exposed to varying concentrations of ATP and ADP, as indicated, in the presence of 0.5 mM free Mg^{2+}. Inset: magnified current traces for SUR1ΔNBF1 and SUR1ΔNBF1[AAA] (note different scale bars for current). (C) Left, schematic representation of SUR1 constructs, with or without NBF1. The length of the TMD1-NBF1 linker (containing the ER retention motif RKR, intact or mutated to AAA) is exaggerated for clarity. Middle, K_{ATP} channel current per patch. Bars indicate mean ± SEM of n = 8–13 patches. *, p < 0.01 as compared to WT by Student's paired t test. Right, percentage of patches with K_{ATP} channels.

oligomycin). At selected time points, the solution was aspirated from the cells and replaced with fresh solution; after completion of the assay, cells were lysed with 1% SDS, aspirated, and radioactivity was assayed in a scintillation solution. Raw data are shown as $^{86}Rb^+$ efflux relative to total counts (including all time points and the lysate for each construct).

The rate constant of K_{ATP}-specific $^{86}Rb^+$ efflux (k_2) was obtained by fitting the data with a single-exponential equation:

$$\text{Relative flux} = 1 - \exp[-(k_1 + k_2) * t]$$

where the apparent rate constant for nonspecific efflux (k_1) was obtained from untransfected cells.

RESULTS

Deletion of NBF1 from SUR1 reduces channel expression and abolishes MgADP stimulation. The demonstration that prokaryotic NBFs form dimers upon nucleotide binding has led to the proposal that formation of a nucleotide-bound SUR NBF1-NBF2 heterodimer underlies Mg-nucleotide stimulation of K_{ATP} channels.[31] Since deletion of NBF2 from SUR1 abolishes channel stimulation by MgADP,[32] we hypothesized that deletion of NBF1 would have similar functional consequences. We therefore deleted the NBF1 domain (D675-D936) from WT SUR1, and coexpressed the resulting SUR1ΔNBF1 construct with Kir6.2 in COS cells (Fig. 1A). Strikingly, deletion of NBF1 inhibited channel expression > 100-fold compared to WT, as assessed by patch-clamping excised membranes in the inside-out configuration (Fig. 1B and C), with K_{ATP} channels being undetectable in most (77%) patches. In the few patches that contained enough channels to assess their functional properties, MgADP stimulation was indeed abolished (Fig. 1B, inset), consistent with NBF1 being an essential domain for SUR1-dependent Mg-nucleotide stimulation.

Interestingly, the ER retention signal RKR is only 25 residues upstream from the site of NBF1 deletion (Fig. 1C). We thus hypothesized that NBF1 deletion alters the folding of the RKR-containing loop such that it is no longer obscured in the fully assembled K_{ATP} channel complex, leading to channel retention in the ER. We therefore mutated the RKR motif to AAA, a manipulation that bypasses the ER retention checkpoint and allows plasma membrane expression of unassociated subunits.[19] Mutation to AAA did increase the percentage of patches containing K_{ATP} channels (62% vs. 23%, Fig. 1C), but the mean channel density was not increased significantly (7 ± 3 vs. 2 ± 1 pA). As expected, MgADP stimulation was also abolished in channels formed by SUR1ΔNBF1[AAA] (Fig. 1B, inset). The lack of a convincing rescue of ΔNBF1 by AAA indicates that the defect of ΔNBF1-containing channels is not merely an inability to exit the ER due to exposure of the RKR domain or due to mismatched channel subunits.

Deletion of NBF1 decreases total SUR1 protein levels. The above results suggest that SUR1 protein levels may be lowered by deletion of NBF1. To test this possibility, COS cell lysates were immunoblotted with anti-FLAG (all constructs discussed are N-terminally tagged with FLAG). SUR1 is detected as a ~150–170 kDa doublet, corresponding to the immature, core-glycosylated ER fraction and the mature, fully-glycosylated plasma membrane fraction (Fig. 2A).[22] SUR1[AAA] exhibits the same pattern, although the ratio of the two bands is shifted such that the mature band is now more prominent than the immature band and hence the fraction of total protein in the plasma membrane is predicted to be increased, consistent with previous studies.[19] Interestingly, SUR1ΔNBF1 is detected as a very faint ~120 kDa single band (Fig. 2A). This single band likely corresponds to the immature (non-plasma membrane) fraction, consistent with the low channel activity detected by patch-clamping.

Multiple PHHI mutations are known to cause reduced channel expression; specifically, some mutations lead to increased degradation
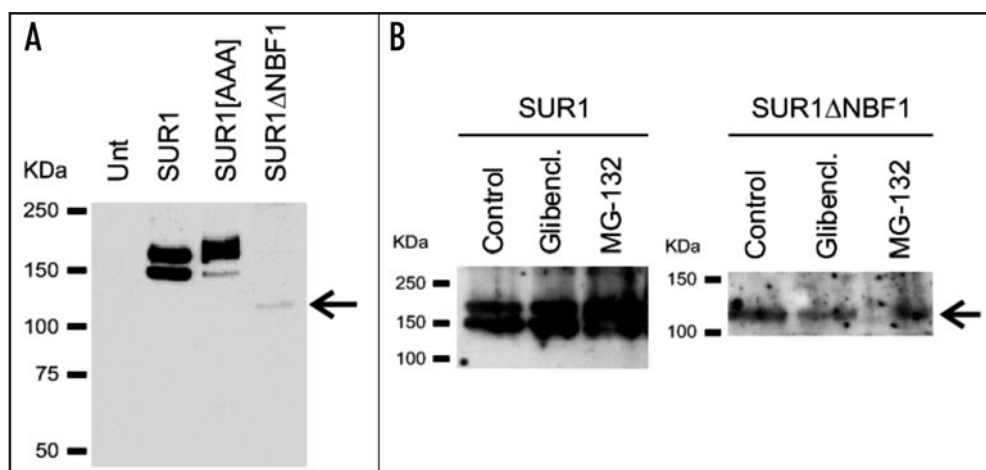
Figure 2. Deletion of NBF1 from SUR1 decreases total SUR1 protein levels. (A) Anti-FLAG Western blot of lysates from COS cells transfected with Kir6.2 and either SUR1, SUR1[AAA], or SUR1ΔNBF1 (all SUR1 constructs are N-terminally FLAG-tagged; all transfections were done in parallel). Untransfected control is also shown. Six micrograms total protein per lane was loaded for each sample. Results are representative of n = 5 experiments. (B) Anti-FLAG Western blot of lysates from COS cells transfected with Kir6.2 and either SUR1 (left) or SUR1ΔNBF1 (right), under either control conditions, incubation with 10 μM glibenclamide for 24 hrs prior to lysis, or incubation with 10 μM MG-132 for 6 hrs prior to lysis. Results are representative of n = 4 experiments.

of SUR1 and thus decreased total SUR1 protein levels.[26] It is therefore possible that the decrease in SUR1 protein levels caused by deletion of NBF1 also results from increased protein degradation. We therefore incubated COS cells with sulfonylureas and the proteasomal inhibitor MG-132, both of which increase SUR1 expression by preventing protein degradation, and are capable of rescuing expression of such PHHI mutants.[27] However, neither sulfonylurea nor MG-132 treatment significantly increased SUR1ΔNBF1 protein levels (Fig. 2B).

Deletion of NBF1 has similar effects on the expression of channels generated by "split" SUR1 constructs. A potential problem resulting from deletion of NBF1 (which does not apply to deletion of NBF2, which is at the C-terminus of SUR1) is that directly linking TMD1 to TMD2 may place structural constraints on the resulting SUR1 protein and lead to improper folding. To circumvent this caveat, we took advantage of the fact that K$_{ATP}$ channels can be reconstituted by coexpression of Kir6.2 with two truncated or "split" SUR1 constructs, one containing the N-terminal "half" of the molecule (residues M1 to S1045, referred to as N-1045), and the other containing the C-terminal "half" (residues P1046 to K1580, referred to as 1046-C).[4]

No channel activity was detected, either by patch-clamp in excised membranes or by Rb-flux assay in metabolically inhibited intact cells, when either N-1045 alone or 1046-C alone was coexpressed with Kir6.2 (Fig. 3A and C). However, coexpression of both N-1045 and 1046-C with Kir6.2 (indicated as N-1045/1046-C) resulted in formation of WT-like K$_{ATP}$ channels that were active in the intact cell, inhibited by ATP, and stimulated by Mg-nucleotides (Fig. 3). The current density per patch was comparable to WT (Fig. 3C), indicating that "splitting" alone does not impair channel formation. Consistent with the results obtained for SUR1ΔNBF1, when N-1045 was C-terminally truncated in order to remove NBF1, no K$_{ATP}$ channel activity was detected in excised membranes or in intact cells (Fig. 3A and C). Thus, even in the "split" SUR1 background, deletion of NBF1 impairs channel expression.

However, because the "splitting" point in the N-1045/1046-C pair is located in the extracellular loop between the first and second

α-helices of TMD2, the "split" pair lacking NBF1 (N-675/1046-C) is also lacking the first α-helix of TMD2 (Fig. 3C), which may in itself be deleterious for channel assembly and expression. We therefore generated another "split" pair, N-936/937-C, which is "split" immediately after NBF1 (Fig. 3C). Neither N-936 alone nor 937-C alone generated K$_{ATP}$ channels when coexpressed with Kir6.2 (Fig. 3A and C), but again, coexpression of both constructs with Kir6.2 (N-936/937-C) resulted in WT-like K$_{ATP}$ channel currents in excised patches. In this background, deletion of NBF1 (N-675/937-C) abolished K$_{ATP}$ channel expression in excised patches, indicating that the expression deficit is due to the absence of NBF1 rather than the first α-helix of TMD2. However, MgADP stimulation was abolished in N-936/937-C channels, resulting in no channel activity in metabolically inhibited intact cells (Fig. 3B and C), as activity in the intact cell is dependent on Mg-nucleotide interactions with the SUR NBFs.[11,17]

Varying the "splitting" point in the vicinity of NBF1 differentially hinders MgADP stimulation. Since N-936/937-C channels exhibited no MgADP stimulation, whereas N-1045/1046-C channels were MgADP-stimulated, we hypothesized that decreased tethering of NBF1 to the membrane (via linkers to TMD1 and TMD2) impairs its ability to dimerize with NBF2 and stimulate the channel. We therefore generated three additional "split" SUR1 pairs with different "splitting" points in the vicinity of SUR1, and assessed the MgADP stimulation of the resulting channels. Splitting immediately after TMD1 (such that the TMD1-NBF1 linker is attached to NBF1) abolished MgADP stimulation, while splitting immediately before NBF1 (such that the TMD1-NBF1 linker is attached to TMD1) resulted in channels that were expressed at similar density to full-length SUR1-containing channels, and that were stimulated by MgADP (Fig. 4A and B). None of these constructs generated functional channels without the relevant partner 'half' (Fig. 3, and data not shown). Interestingly, splitting immediately before TMD2 (such that the NBF1-TMD2 linker is attached to NBF2) also resulted in channels that were stimulated by MgADP. These results indicate that NBF1 need not necessarily be attached to both TMDs in order for MgADP stimulation to be preserved. Rather, they indicate that
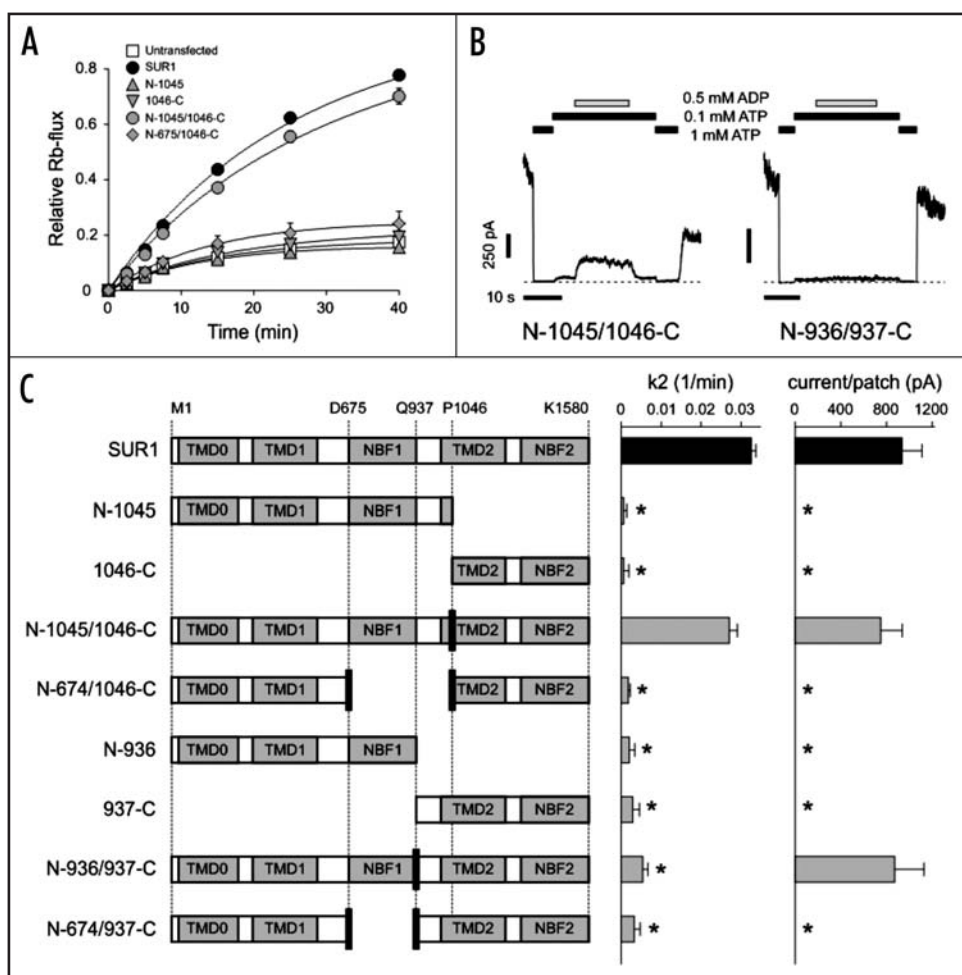
Figure 3. Reconstitution of K$_{ATP}$ channels from "split" SUR1 constructs. (A) Relative ^{86}Rb$^+$ efflux as a function of time, in the presence of metabolic inhibition, from COS cells transfected with Kir6.2 and either WT SUR1, N-1045, 1046-C, N-1045/1046-C, or N-674/1046-C. Untransfected control is also shown. Data points indicate mean ± SEM of n = 4–12 experiments. Data were fitted with a single-exponential function to obtain rate constants for K$_{ATP}$-specific efflux, k_2 (see Methods). (B) Representative current traces recorded at -50 mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and either N-1045/1046-C or N-936/937-C. Patches were exposed to varying concentrations of ATP and ADP, as indicated, in the presence of 0.5 mM free Mg^{2+}. (C) Left, schematic representation of "split" SUR1 constructs, with or without NBF1. Middle, $k2$ values obtained from Rb-fluxes in intact cells. Bars indicate mean ± SEM of n = 4-12 experiments. *:p < 0.01 as compared to WT by Student's paired t-test. Right, K$_{ATP}$ channel current per patch. Bars indicate mean ± SEM of n = 7–23 patches. *, p < 0.01 as compared to WT by Student's paired t test.

N-674/937-C, Fig. 5B), which allowed confirmation that deletion of NBF1 leads to loss of MgADP stimulation in the "split" background as in the full-length background (Fig. 5C). Thus, the effects of deletion of NBF1 in either the "split" or full-length SUR1 background are not due to an inability of the channel to circumvent the ER retention checkpoint.

DISCUSSION

NBF1 is necessary for MgADP stimulation of K$_{ATP}$ channels. Our results indicate that SUR1 NBF1 is necessary for both Mg-nucleotide stimulation of K$_{ATP}$ channels and for channel expression. The former result is not unexpected, as studies on prokaryotic ABC transporters have demonstrated that NBFs form ATP-bound dimers which subsequently hydrolyze ATP and power ABC transporter function.[12-15] In SUR, there are several lines of evidence for formation of a NBF1-NBF2 heterodimer that underlies Mg-nucleotide stimulation of K$_{ATP}$ channels: homology modeling and mutagenesis,[31] cooperativity of azido-nucleotide labeling,[33] single-particle electron microscopy,[34] co-affinity purification of isolated NBFs,[35] and interactions between a soluble NBF1 construct and a TMD2-NBF2 construct in the membrane of insect cells.[36] Since the predicted nucleotide-binding sites are at the dimer interface and formed by sequence elements from both NBFs, deletion of either NBF would be expected to impair nucleotide stimulation of the channel. This has been demonstrated for deletion of NBF2, which abolishes MgADP stimulation of K$_{ATP}$ channels in excised membranes, as well as metabolic inhibition-induced channel activity in the intact cell.[32] We demonstrate here that the same functional consequences result from deletion of NBF1.

NBF1 is also required for K$_{ATP}$ channel expression. Our results also show that deletion of NBF1 severely impairs channel expression levels. This is true in the full-length SUR1 background as well as in the "split" SUR1 background, which addresses the caveat of misfolding due to artificially linking TMD1 and TMD2. The expression deficit is not significantly rescued by ablation of the ER retention motif RKR, although the mean current per patch and the percentage of patches with current are slightly increased, indicative of a minor effect. Since total protein levels are decreased, as detected by immunoblotting, we hypothesize that the SUR1ΔNBF1 construct is less stable than WT and thus increasingly targeted for degradation, such that there is reduced functional channels at the surface membrane, although we cannot formally exclude the possibility that surface protein levels are normal, yet channel

some areas in the vicinity of NBF1 are more sensitive to disruption by "splitting".

Ablation of the RKR motif does not rescue the effects of NBF1 deletion in the "split" SUR1 background. Given the similarity of effects of deletion of NBF1 in the split SUR1 background to those in the full-length SUR1 background, we made further "split" constructs to test whether these effects are also independent of the ER retention motif RKR. In the "split" SUR1 background (N-674/675-C), ablation of the RKR motif by either deletion (N-647/675-C) or mutation to AAA (N-674[AAA]/675-C) enhanced K$_{ATP}$ channel expression without altering MgADP stimulation (Fig. 5A). However, neither manipulation was capable of rescuing expression of the "split" SUR1 lacking NBF1 (N-647/937-C and N-674[AAA]/937-C). A few K$_{ATP}$ channels were, however, detected in some N-674[AAA]/937-C patches (33% of patches vs. 0% patches for either N-647/937-C or
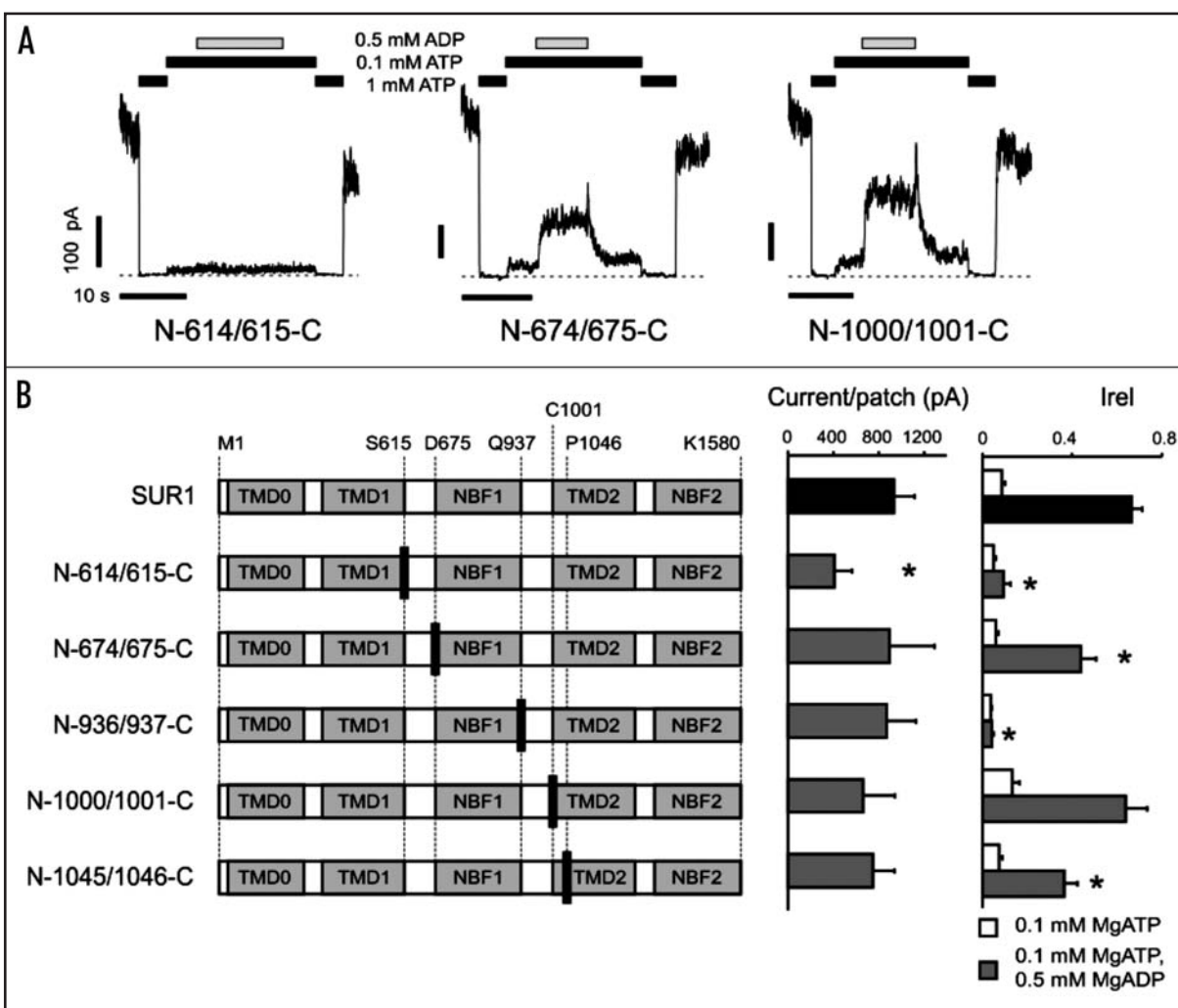
deletion of NBF1.

Figure 4. "Splitting" SUR1 at different locations alters MgADP stimulation of the resulting K$_{ATP}$ channels. (A) Representative current traces recorded at -50 mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and either N-614/615-C, N-674/675-C, or N-1000/1001-C. Patches were exposed to varying concentrations of ATP and ADP, as indicated, in the presence of 0.5 mM free Mg^{2+}. (B) Left, schematic representation of "split" SUR1 constructs. Middle, K$_{ATP}$ channel current per patch. Right, Current in Mg-nucleotides relative to control (no nucleotides (I$_{rel}$)). Bars indicate mean ± SEM of n = 7–20 patches. *, p < 0.05 as compared to WT by Student's paired t test.

activity is absent. The ubiquitination-proteasomal pathway of degradation may be involved: this is the mechanism that underlies the effects of the PHHI mutants A116P and V187D.[26] However, the proteasomal inhibitor MG-132 failed to rescue total ΔNBF1 protein levels, and incubation with sulfonylureas, which facilitates SUR1 folding and prevents its degradation, was also without effect. We therefore suggest that deletion of NBF1 leads to misfolded or unstable protein that is not rescued by sulfonylurea binding and is nonspecifically degraded. Indeed, it has been proposed that rescue of SUR1 trafficking-deficient mutants by SUs or proteasomal inhibitors is only possible in the case of moderate folding defects: severely misfolded constructs, such as the PHHI mutation ΔF1388, may instead accumulate in inclusion bodies or be degraded through other pathways.[27] Such may be the case for the ΔNBF1 constructs as well. An alternative possibility is that there are critical, thus far undefined, anterograde trafficking signals within NBF1.

Deletion of NBF1 from full-length SUR1 by covalently linking TMD1 and TMD2 may potentially lead to misfolding of the resulting protein due to the imposition of artificial structural constraints. This important caveat is circumvented by use of the "split" SUR1 approach. The two "split" SUR1 constructs that constitute the ΔNBF1 pair, N-674 and 937-C, are capable of forming fully functional K$_{ATP}$ channels when coexpressed with the corresponding NBF1-containing "split" construct (N-674 with 675-C, and N-936 with 937-C), demonstrating that neither construct is inherently incapable of forming channels due to improper folding. Rather, it is the absence of NBF1 that is critical. Although it is conceivable that the N-674 and 937-C constructs are unable to interact with each other and thus form functional channels, the fact that all pairs of "split" SUR1 constructs tested were capable of forming channels at levels comparable to WT suggests that this is unlikely. The tight packing between transmembrane domains observed by single-particle electron microscopy in the K$_{ATP}$ channel complex[34] and by X-ray crystallography in full-length prokaryotic ABC transporters[13,37] suggests that TMD1-TMD2 interactions are largely responsible for coassembly of "split" SUR1 pairs into functional channels.

Interestingly, although both NBF1 and NBF2 are required for MgADP stimulation of the channel, deletion of NBF2 does not affect
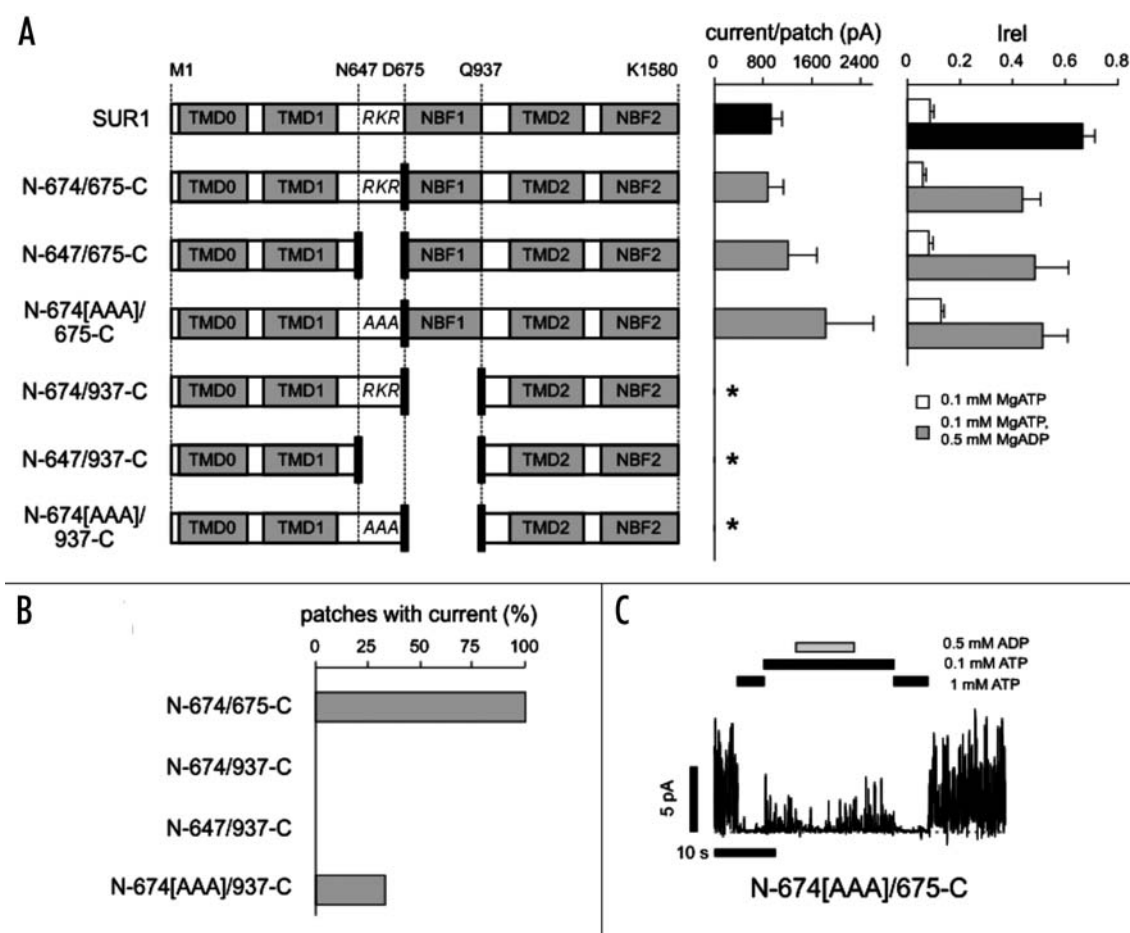
Figure 5. Deletion of NBF1 reduces channel expression and abolishes MgADP stimulation of channels formed by "split" SUR1 constructs. (A) Left, schematic representation of "split" SUR1 constructs, with or without NBF1. The length of the TMD1-NBF1 linker (containing the ER retention motif RKR, intact or mutated to AAA) is exaggerated for clarity. Middle, K_{ATP} channel current per patch. Bars indicate mean ± SEM of n = 6–16 patches. *, p < 0.01 as compared to WT by Student's paired t-test. Right: Current in Mg-nucleotides relative to control (no nucleotides (I_{rel})). Bars indicate mean ± SEM of n = 7–15 patches. (B) Percentage of patches with K_{ATP} channels. (C) Representative current trace recorded at -50 mV from inside-out excised membrane patches from COS cells transfected with Kir6.2 and N-674[AAA]/937-C. Patches were exposed to varying concentrations of ATP and ADP, as indicated, in the presence of 0.5 mM free Mg^{2+}.

channel expression.[32] There are, however, many PHHI mutations located in NBF2 that result in decreased channel expression[23-25,44-46] indicating that preservation of NBF2 structure is also important for expression. In fact, far more PHHI mutations that decrease expression have been identified in NBF2 than in NBF1, suggesting that the role of NBF1 in channel expression is less susceptible to disruption by point mutations, while that of NBF2 is susceptible to point mutations but not domain deletion. To further understand the differences between NBFs in the regulation of channel expression, it would be interesting to assess the effects of mutations in NBF1 that are equivalent to PHHI-causing NBF2 mutations. Despite their overall sequence similarity and proposed dimeric function, important differences between NBF1 and NBF2 have already been documented with respect to azido-nucleotide labeling,[38,39] functional consequences of equivalent mutations,[17] and ability to hydrolyze ATP when expressed as separate constructs.[40] Thus, the involvement of NBF1, but not NBF2, in the regulation of SUR1 expression is an additional element of asymmetry between NBF1 and NBF2.

Interestingly, replacing NBF1 of SUR1 with that of MRP1 only suppresses surface expression of Kir6.2 by ~50% in *Xenopus* oocytes,

and replacing NBF2 actually increases expression.[47] Conversely, replacing TMD0, TMD1, or TMD2 of SUR1 with the corresponding MRP1 domains leads to no expression.[47] These results highlight the relative structural conservation of NBFs, compared to TMDs, across ABC transporters: MRP1 NBFs are able to sufficiently mimic SUR1 NBFs (as determinants of channel trafficking and expression) such that moderate or no impairment of expression results when they are replaced, whereas replacement of either one of the TMDs is prohibitive.

In insect cells, deletion of NBF1 abolishes the ability of SUR1 to bind sulfonylureas.[41] It is unlikely that this results from an inability to reach the plasma membrane, since, unlike in other expression systems, SUR1 alone or Kir6.2 alone can traffic to the membrane of insect cells,[42] and robust sulfonylurea binding can be detected to expressed truncated SUR1 constructs[36,41] that, on their own, should not be capable of channel formation. Thus, the lack of sulfonylurea binding due to deletion of NBF1 may reflect misfolding of SUR1ΔNBF1, which would be consistent with our results.

MgADP stimulation of "split" channels is dependent on the location of the "splitting" point. Reconstitution of K_{ATP} channels at
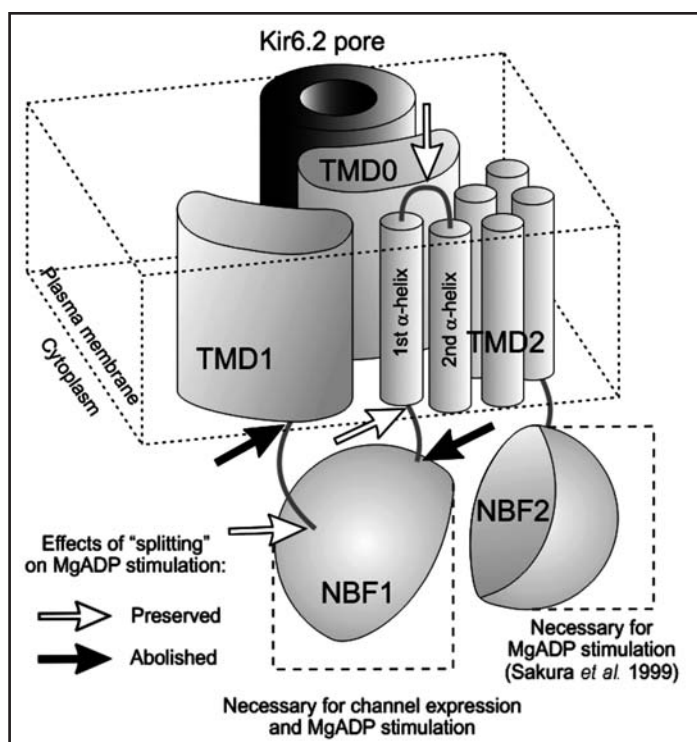
Figure 6. Simplified cartoon representation of NBF1 involvement in K$_{ATP}$ channel expression and MgADP stimulation. Cartoon of SUR1 and Kir6.2 depicting the effects of NBF1 deletion on channel expression and MgADP stimulation, as well as the effects of various "splitting" points on MgADP stimulation (white arrow, preserved stimulation; black arrow, abolished stimulation). For clarity, the size of the NBFs is exaggerated, and only linkers of interest are depicted. TMD2 is shown as a bundle of α-helices in order to explicitly depict the extracellular loop between the first and second helices. Effects of NBF2 deletion[32] are also noted. See text for details.

normal density, from multiple "split" SUR1 pairs demonstrates that the two NBFs do not need to be coexpressed on the same protein. Because the expression levels are similar to wild type, it is reasonable to assume that such split pairs are processed and higher order glycosylated normally. However, these experiments reveal that the location of the "splitting" point is critical in determining the extent to which resulting channels are stimulated by MgADP. The pattern of stimulation of the five "split" SUR1 pairs does not correlate well with the extent of tethering of NBF1 to the membrane (Fig. 6). Rather, it indicates that some regions in the vicinity of NBF1 are more sensitive to "splitting" than others. That stimulation is unchanged when the NBF1-TMD2 linker is severed immediately before TMD2 demonstrates that NBF1 need not be linked to TMD2 in order for MgADP stimulation to be fully preserved, consistent with the modular nature of eukaryotic ABC transporters.

In CFTR, expression of functional channels is unaltered by "splitting" either before or after NBF1,[43] as is the case with our "split" SUR1 constructs. Furthermore, "splitting" CFTR before NBF1 has no significant effects on ligand-dependent channel gating, but "splitting" after NBF1 decreases open probability by increasing the rate of closing.[43] This pattern is similar to that observed in our experiments with SUR1, where "splitting" before NBF1 preserves nucleotide-dependent stimulation while "splitting" after NBF1 abolishes stimulation. Thus, the loop between NBF1 and

TMD2 may be critical for normal NBF1-nucleotide interactions in eukaryotic ABC transporters, or it may be important for positioning NBF1 with respect to NBF2, thus facilitating NBF1-NBF2 heterodimerization. Alternatively, this loop may contain thus far undetected structural elements that, in SUR1, are required for transduction of Mg-nucleotide binding or hydrolysis at the NBFs to the TMDs and ultimately to the Kir6.2 pore. Interestingly, in the crystal structure of a full-length prokaryotic ABC transporter, the cytoplasmic loops connecting α-helices from the TMDs form defined secondary structure elements that may mediate TMD-NBF interactions.[37] Further work is necessary to elucidate the detailed mechanism by which MgADP stimulation is impaired in "split" SUR1 constructs, but our results highlight the critical functional importance of the cytoplasmic linkers connecting NBF1 to TMD1 and TMD2.

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