Rab35 GTPase positively regulates endocytic recycling of cardiac $K_{\text{ATP}}$ channels

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Abstract

ATP-sensitive $K^+$ ($K_{\text{ATP}}$) channel couples membrane excitability to intracellular energy metabolism. Maintaining $K_{\text{ATP}}$ channel surface expression is key to normal insulin secretion, blood pressure and cardioprotection. However, the molecular mechanisms regulating $K_{\text{ATP}}$ channel internalization and endocytic recycling, which directly affect the surface expression of $K_{\text{ATP}}$ channels, are poorly understood. Here we used the cardiac $K_{\text{ATP}}$ channel subtype, Kir6.2/SUR2A, and characterized Rab35 GTPase as a key regulator of $K_{\text{ATP}}$ channel endocytic recycling. Electrophysiological recordings and surface biotinylation assays showed decreased $K_{\text{ATP}}$ channel surface density with co-expression of a dominant negative Rab35 mutant (Rab35-DN), but not other recycling-related Rab GTPases, including Rab4, Rab11a and Rab11b. Immunofluorescence images revealed strong colocalization of Rab35-DN with recycling Kir6.2. Rab35-DN minimized the recycling rate of $K_{\text{ATP}}$ channels. Rab35 also regulated $K_{\text{ATP}}$ channel current amplitude in isolated adult cardiomyocytes by affecting its surface expression but not channel properties, which validated its physiologic relevance and the potential of pharmacologic target for treating the diseases with $K_{\text{ATP}}$ channel trafficking defects.

Keywords

$K_{\text{ATP}}$ channel; Rab35; endocytic recycling; surface density; cardiomyocytes

Introduction

Sarcolemmal ATP-sensitive $K^+$ ($K_{\text{ATP}}$) channels uniquely couple intracellular energy metabolism to cell membrane excitability, regulating diverse physiologic processes including insulin secreting, blood flow, neurotransmitter release and action potential duration adaptation [1,2]. Decreased $K_{\text{ATP}}$ channel surface expression caused by genetic variations or pathophysiologic conditions are associated with hypoglycemia, diabetes mellitus, atrial fibrillation, dilated cardiomyopathy and cardiac ischemia [1]. Therefore, strategies to maintain $K_{\text{ATP}}$ channel surface density have a high therapeutic potential.

$K_{\text{ATP}}$ channel is composed of four pore-forming inward rectifier potassium channel (Kir6) subunits and four regulatory sulfonylurea receptor (SUR) subunits. Both Kir6 and SUR subunits have the ER
retention motif (RKR), and the formation of $K_{\text{ATP}}$ channel octamer has been proposed to shield this motif to facilitate ER exit [3]. Further, SUR subunits are glycosylated in the Golgi apparatus before reaching the cell surface [4,5]. Several scaffolding proteins, including ankymins, have been shown to anchor the $K_{\text{ATP}}$ channels on cell membrane [6–8]. Internalization of $K_{\text{ATP}}$ channels occurs mainly through clathrin-coated vesicles [9]. Experiments with antibody capture assays have demonstrated that $K_{\text{ATP}}$ channels can be recycled, which occurs at a time scale of 10–15 min [10]. However, the molecular mechanism of $K_{\text{ATP}}$ channel recycling is poorly understood.

Rab family members serve as multifaceted organizers of almost all membrane trafficking processes by consuming GTP in eukaryotic cells. More than 60 Rab protein family members are distributed in different intracellular membranes, controlling multiple processes such as the generation, movement, fusion, and recruitment of intracellular vesicles that transport membrane proteins. Within the Rab family, Rab4 mediates fast endocytic recycling directly from early endosomes, whereas Rab11a, Rab11b and Rab35 mediate slow endocytic recycling through recycling endosomes [11,12].

The aim of this study is to investigate the involvement of Rab GTPases in $K_{\text{ATP}}$ channel endocytic recycling, and our data demonstrate that, of the four recycling-related Rab GTPases, only Rab35 promotes $K_{\text{ATP}}$ channel endocytic recycling and increases its surface expression in both heterologous cell system and cardiomyocytes.

### Materials and methods

#### cDNA constructs

Plasmids of Kir6.2 and SUR2A without tags are kindly supplied by Dr. Lei Chen (Peking University, Beijing, China) [13]. Dominant negative Rab constructs (GFP-Rab4-S22N, GFP-Rab11a-S25N, GFP-Rab11b-S25N, GFP-Rab35-S22N) and Avi-Kir6.2–4HA plasmids were synthesized by Genscript. Constitutively active Rab35 (GFP-Rab35-Q67L) plasmid were synthesized by HanBio. HA-Kir6.2 was previously used [14].

#### Inside-out $K_{\text{ATP}}$ current recording

Inside-out patch clamping was performed by using an Axopatch 200B amplifier, and data were recorded with a Digidata 1550B and Clampex 11 software, digitized at 5 kHz. The cytosolic ATP concentration was changed by using MappingLab PVG-08. The resistance of the pipette was 4 ~ 5 MΩ when filled with pipette solution (in mM: 30 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, 110 potassium gluconate, and pH 7.4). ATP was dissolved in base solution (in mM: 30 KCl, 1 EGTA, 1 MgCl₂, 10 HEPES, 110 potassium gluconate, and pH 7.2). After patch excision, pipette potential was held at +80 mV and currents were recorded immediately to minimize current rundown. Current traces were analyzed by Clampfit 11 for open probability and single channel current. Recordings in the absence of ATP for 10 seconds were filtered at 1 kHz and subjected to all-point histograms, which were further fitted with Gaussian distributions. The parameters (A, μ, σ) generated from Gaussian fitting were used to calculate open probability and single channel current.

#### Biotinylation assay

HEK293 cells transfected with Avi-Kir6.2–4HA/SUR2A (PolyJet™ In Vitro DNA Transfection Reagent, SL100688, SignaGen Laboratories) were incubated at 4°C for 1 h with 0.33 mg/ml biotin in PBS buffer, and reaction was terminated by quenching solution. After washing twice with TBS buffer, cell lysates were prepared in RIPA buffer containing 1% protease inhibitor cocktail, 0.2% PMSF and 5 mM EDTA. Lysates were mixed with Neutravidin Agarose Beads (89881, Thermo Fisher Scientific), and rotated by the shaker at 4°C overnight. Centrifugated at 1000 g for 1 min, supernatants were discarded, and agarose beads were washed three times with PBS, and then mixed with 4x Laemmli Protein Sample Buffer (1610747, Biorad) containing 50 mM DTT for 1 h at room temperature to elute biotinylated proteins.

#### Western blotting

Total cell lysates and biotinylated proteins were subjected to SDS-PAGE electrophoresis. The
primary antibodies used include mouse anti-HA (901501, 1:5000, BioLegend), mouse anti-GAPDH (FD0063, 1:10,000, FuDeBio), mouse anti-Akt (sc-5298, 1:2000, Santa Cruz Biotechnology), rabbit anti-p-Akt (4060, 1:2000, Cell Signaling Technology). The secondary antibodies used were IRDye® 800CW Goat anti-Mouse IgG Secondary Antibody (926–32210, LI-COR Biosciences), IRDye® 680RD Goat anti-Rabbit IgG Secondary Antibody (926–68071, LI-COR Biosciences). LY294002 (L832989, 10 μM, Macklin) was applied for 24 h to inhibit PI3K signaling.

**Immunofluorescence**

Hela cells transfected with HA-Kir6.2/SUR2A and GFP-tagged dominant negative Rab GTPases were incubated overnight with mouse anti-HA antibody (901501, 1:200 dilution in DMEM containing 10% donkey serum, BioLegend). Cells were fixed with 4% paraformaldehyde in PBS for 15 min, permeabilized by 1% Triton X-100 for 15 min, and blocked with 5% donkey serum in PBS for 30 min. Cy3 AffiniPure Donkey anti-mouse IgG (715–165-151, 1: 500, Jackson ImmunoResearch Laboratories) was diluted in blocking solution, and incubated at room temperature in dark room for 45 min. After washing with PBS three times, cells were mounted with DAPI Fluoromount-G (0100–20, SouthernBiotech). Images were collected by Olympus FV3000 confocal Microscope.

For recycling double staining assay, after overnight HA antibody incubation, the cells were washed with DMEM and surface-bound antibodies were labeled at room temperature for 0.5 h by Alexa Fluor647 AffiniPure Donkey anti-mouse IgG (715–605-151, 1: 500, Jackson ImmunoResearch Laboratories). The cells were then incubated at 37°C for 2 h to allow recycling of internalized channels back to cell surface. The cells were then fixed with 4% paraformaldehyde and HA-antibody marked channels recycled back to the cell surface were labeled with Cy3 AffiniPure Donkey anti-mouse IgG (715–165-151, 1: 500, Jackson ImmunoResearch Laboratories) and non-recycled channels were labeled with Alexa Fluor647 AffiniPure Donkey Anti-mouse IgG (715–605-151, 1: 500, Jackson ImmunoResearch Laboratories) after permeabilization.

**Isolation and infection of adult rat ventricular cardiomyocytes**

Ventricular cardiomyocytes were isolated from male Sprague-Dawley rats (9–11 weeks old, purchased from Vital River). Hearts were rapidly excised after anesthesia and rinsed with ice-cold Tyrode’s solution (in mM: 137 NaCl, 5.4 KCl, 10 HEPES, 1 MgCl2, 0.33 NaH2PO4, 1.8 CaCl2, 10 glucose, and pH 7.4). The hearts were cannulated and perfused in Langendorf mode with Ca2+-free Tyrode’s solution for 2 min. The perfusate was then switched to Ca2+-free Tyrode’s solution containing 1.56 mg/ml collagenase type II (LS004176, Worthington) for 20 min. The enzyme was washed out by 2 min perfusion with KB solution (in mM: 20 taunine, 50 L-glutamic acid, 10 HEPES, 10 Glucose, 1 EGTA, 3 MgCl2, 20 KH2PO4, 40 KCl, and pH 7.2). Hearts were removed from the cannula and cut into pieces and the supernatant was kept after resuspending by KB solution followed by MEM medium (PM150410, Procell) containing 1% penicillin-streptomycin. Cells were plated on laminin (L2020, 20 μg/ml, Sigma)-coated coverslips and cultured in MEM medium. Adenoviruses that carry Rab35-S22N or Rab35-Q67L (purchased from HanBio) were added at a multiplicity of infection of 1000 for a 24 h incubation. Infected cardiomyocytes were labeled by mCherry expression. Cultured cardiomyocytes were used for patch at 48 h post-infection. The animal study protocol was approved by the Ethics Committee of Soochow University (SUDA20210930A03).

**Reverse transcription – polymerase chain reaction**

Rab35 expression in HEK293 and rat heart were analyzed by reverse transcription – polymerase chain reaction. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was analyzed as control. Total RNAs were isolated from HEK293 cells and rat heart using Trizol reagents (Invitrogen, 15596018) to make first-strand cDNAs with the HiScript III 1st Strand cDNA Synthesis kit (Vazyme, R312-01). PCR amplification of Rab35 transcripts was done using forward (5’-AAGCTGCAGATCTGGGACAC –3’) and reverse
(5’- CGTCGTAACCAATGACCC – 3’) primers in 30 cycles of 15s denaturation at 95°C, 20s annealing at 60°C, and 30s elongation at 72°C. As control, primers for GAPDH (forward primer 5’- CCTGCACCACAACTGCTTA – 3’, reverse primer 5’- AGTGATGGCATGGACTGTGG – 3’) were included in parallel reactions. Both Rab35 and GAPDH primers were designed to fully match human and rat sequences. Amplified PCR products were examined by agarose gel electrophoresis.

**Statistical analysis**

Western blotting gray value and Manders coefficient were obtained with Image J and python scripts. Data processing of patch traces were analyzed by Clampfit 11. When comparing two groups, the Student’s t-test is applied. One-way or two-way ANOVA was used for comparison of multiple groups, followed by the Holm-Sidak’s analysis. Values of $P < 0.05$ were considered significant.

**Results**

**Dominant negative Rab35 downregulates $K_{\text{ATP}}$ channel current amplitude**

To examine the involvement of Rab GTPases in $K_{\text{ATP}}$ channel endocytic recycling, we individually co-expressed GDP-locked dominant-negative (DN) mutants of Rab4, Rab11a, Rab11b and Rab35 [15], with $K_{\text{ATP}}$ channel subunits in HEK293 cells. Each of the Rab GTPase mutants were concatenated with EGFP at the N-terminus. We measured $K_{\text{ATP}}$ channel currents with inside-out membrane patches in ATP-free solutions. $K_{\text{ATP}}$ channel mean patch current was significantly decreased in cells expressing dominant-negative Rab35 mutant (Rab35-DN), whereas Rab4-DN, Rab11a-DN and Rab11b-DN had no effect on $K_{\text{ATP}}$ channel current amplitude (Figure 1).

**Rab35 does not affect $K_{\text{ATP}}$ channel intrinsic properties**

We next determined whether Rab35-DN has a direct effect on $K_{\text{ATP}}$ channel opening. Using the inside-out patch clamp configuration, we determined the unitary current amplitude of $K_{\text{ATP}}$ channels using all-point histograms and curve fitting to Gaussian distributions (Figure 2 (a)). The unitary $K_{\text{ATP}}$ channel current and open probability were 5.82 ± 0.08 pA and 0.41 ± 0.10 for control, and were unaffected by co-expression with Rab35-DN, with 5.74 ± 0.19 pA and 0.35 ± 0.05 respectively (Figure 2(b,c)). The $K_{\text{ATP}}$ channel unitary current and open probability co-expressed with Rab4-DN, Rab11a-DN or Rab11b-DN were also unchanged (Supplementary Figure S1).

**Dominant-negative Rab35 decreases $K_{\text{ATP}}$ channel surface density**

Electrophysiological data are consistent with the concept that Rab35-DN impairs $K_{\text{ATP}}$ channel surface expression. We tested this possibility biochemically using an engineered Kir6.2 expressing an extracellular Avi tag and C-terminal HA tags (Avi-Kir6.2–4HA). Avi-Kir6.2–4HA/SUR2A

![Figure 1](image-url). Of the four Rab GTPases related to endocytic recycling, only Rab35-DN (dominant negative mutant) reduces $K_{\text{ATP}}$ current amplitude. (a) Representative inside-out current traces in control and Rab35-DN groups recorded from HEK293 cells transfected with Kir6.2/SUR2A. The presence of 1 mM ATP is indicated. (b) Summary of mean $K_{\text{ATP}}$ currents of all control and four dominant negative Rab GTPases (Rab4-DN, Rab11a-DN, Rab11b-DN, Rab35-DN). n ≥ 18 current traces in each group. *$P < 0.05$ vs. the control group determined by one-way ANOVA followed by the Holm-Sidak’s analysis.
channels were expressed in HEK293 cells. Surface proteins were purified using a surface biotinylation assay, and Avi-Kir6.2–4HA was detected with immunoblotting. We found that co-expression of Rab35-DN with Avi-Kir6.2–4HA/SUR2A did not affect the total amount of Kir6.2, but significantly decreased its surface expression (Figure 3). The total and surface expression of $K_{\text{ATP}}$ channels co-expressed with Rab4-DN, Rab11a-DN and Rab11b-DN were also investigated and found to be unchanged (Supplementary Figure S2). Both the electrophysiological and biochemical data demonstrate that dominant-negative Rab35, but not Rab4, Rab11a or Rab11b, downregulates $K_{\text{ATP}}$ channel surface density.

**Dominant-negative Rab35 retains recycled $K_{\text{ATP}}$ channels in intracellular vesicles**

To investigate the location and trafficking of recycled $K_{\text{ATP}}$ channels, we used a Kir6.2 subunit with an HA tag in its extracellular loop (HA-Kir6.2) to label only recycled $K_{\text{ATP}}$ channels but not newly synthesized channels. After an incubation for 2 h with HA antibody, surface and recycling $K_{\text{ATP}}$ channels were labeled. We then performed an immunofluorescence experiment to test the colocalization of recycling $K_{\text{ATP}}$ channels with dominant negative Rab GTPases (Figure 4(a)).

Statistically, recycling $K_{\text{ATP}}$ channels had a significantly higher colocalization coefficient with Rab35-DN compared to Rab4-DN, Rab11a-DN and Rab11b-DN (Figure 4(b)), indicating that $K_{\text{ATP}}$ channels recycled through Rab35-driven compartments, and Rab35-DN induced accumulation of $K_{\text{ATP}}$ channels in these intracellular vesicles, shown by enlarged vesicle size (Figure 4(c) and Supplementary Figure S3).

**$K_{\text{ATP}}$ channel recycling is minimized by dominant negative Rab35**

To directly investigate the recycling process of $K_{\text{ATP}}$ channels, we introduced a double staining assay [16] using Kir6.2 with an extracellular HA tag (HA-Kir6.2). HA antibody incubation labeled the $K_{\text{ATP}}$ channels in the process of endocytosis and recycling. $K_{\text{ATP}}$ channels recycled back to the cell membrane were labeled with Cy3-conjugated secondary antibody, while the $K_{\text{ATP}}$ channel pool for recycling were labeled with Alexa Fluor 647-conjugated secondary antibody. The ratio of Cy3 to Alexa Fluor647 fluorescence intensity indicated the $K_{\text{ATP}}$ channel recycling rate. As
Figure 3. Rab35-DN reduces $K_{ATP}$ channel surface density. (a) HEK293 cells transfected with Avi-Kir6.2–4HA/SUR2A were surface biotinylated, and western blotting was performed with anti-HA and anti-GAPDH Abs. Representative blots of total (t) and biotinylated surface (s) Kir6.2 and GAPDH are shown. (b) Ratios of total Kir6.2 to total GAPDH and surface Kir6.2 to total Kir6.2 are shown for control and Rab35-DN groups. n ≥ 10 blots/group. *P < 0.05 vs. the control group with student’s t-test.

Figure 4. Rab35-DN induces intracellular accumulation of $K_{ATP}$ channels. (a) Representative immunostaining images obtained from HeLa cells transfected with HA-Kir6.2/SUR2A (red) and GFP-tagged Rab4-DN, Rab11a-DN, Rab11b-DN or Rab35-DN (green). (b) Manders M2 colocalization coefficient was calculated as the presence of HA-Kir6.2 in the GFP-labeled vesicles. (c) Average diameters of the vesicles containing $K_{ATP}$ channels compared in Rab4-DN, Rab11a-DN, Rab11b-DN and Rab35-DN groups. n ≥ 11 images in each group. Scale bar, 5 µm. **P < 0.01 determined by one-way ANOVA followed by Holm-Sidak test.
shown in Figure 5(a), at t = 0 min, no recycled $K_{\text{ATP}}$ channels were detected, while at t = 120 min, a significant amount of Cy™3 labeled channels were observed. When co-expressed with Rab35-
DN, the amount of $K_{\text{ATP}}$ channels recycled back to the cell membrane was dramatically reduced (Figure 5(b)), indicating a direct involvement of Rab35 in $K_{\text{ATP}}$ channel recycling.

**Rab35 regulates $K_{\text{ATP}}$ channel recycling independent of PI3K signaling**

Rab35 directly binds to and controls the activity of p85α, the regulatory subunit of PI3K [17]. Several studies showed that Rab35 may act as an upstream activator of PI3K/Akt signaling [17,18] and PI3K activation has been shown to increase $K_{\text{ATP}}$ channel surface density in neurons and pancreatic β-cells [19,20]. To test the possibility of the involvement of PI3K in Rab35-mediated $K_{\text{ATP}}$ channel recycling, we used the PI3K inhibitor LY294002 to impair PI3K activation, which decreased Akt phosphorylation (Figure 6(a,b)). Without changing the total $K_{\text{ATP}}$ channel expression (Figure 6(c,d)), a constitutively active Rab35 (Rab35-CA) significantly increased $K_{\text{ATP}}$ channel mean patch current (Figure 6(e)) and the current amplitudes in both control and Rab35-CA groups were unaffected by PI3K inhibitor LY294002 (Figure 6(e)), demonstrating that Rab35 regulates $K_{\text{ATP}}$ channel recycling independent of PI3K signaling.

**Active Rab35 enhances $K_{\text{ATP}}$ channel surface density in cardiomyocytes**

To elucidate the physiological relevance of the regulation of $K_{\text{ATP}}$ channel recycling by Rab35, we studied the functional role of Rab35 in isolated rat adult cardiomyocytes by adenoviral delivery of Rab35-DN, Rab35-CA, or mCherry as a control. Consistent with the observations in transfected HEK293 cells, $K_{\text{ATP}}$ channel mean patch current was significantly increased by Rab35-CA (Figure 7(a)) without changing intrinsic $K_{\text{ATP}}$ channel properties (Figure 7(b,c)). However, Rab35-DN was without effect on $K_{\text{ATP}}$ channel current amplitude in rat cardiomyocytes (Figure 7(a)). Several databases, including The Human Protein Atlas and Proteomics DB, show a low expression of Rab35 in the heart among different tissues, and we confirmed that the Rab35 mRNA expression level was significantly lower in rat heart compared to HEK293 cells (Supplementary Figure S4), indicating that Rab35-DN could not decrease $K_{\text{ATP}}$ channel current in rat cardiomyocytes due to the low expression of endogenous Rab35.

**Discussion**

Several Rab GTPases have been reported to be directly involved in the trafficking of ion channels in cardiomyocytes, including Rab4, Rab5, Rab7, Rab9, and Rab11 [21]. A novel finding of this study is the identification of $K_{\text{ATP}}$ channel as the first ion channel in cardiomyocytes recycled in a Rab35-dependent manner.

Rab35 contains an evolutionarily conserved polybasic C-terminal tail and localizes both at the intracellular vesicles and plasma membrane [22,23]. Rab35 plays an important role in promoting endocytic recycling of various cargoes, including Ca$^{2+}$-activated K$^+$ channel KCa2.3 in terms of ion channels. Rab35-DN, used in this work as well, resulted in accumulation of KCa2.3 in an intracellular compartment and a decrease in steady-state plasma membrane expression in endothelial cells.

![Figure 7](image-url) **Figure 7.** Rab35 regulates native $K_{\text{ATP}}$ channels in ventricular cardiomyocytes. (a) Summary of mean $K_{\text{ATP}}$ current amplitudes, (b) $K_{\text{ATP}}$ channel unitary current and (c) open probability of control, Rab35-DN and Rab35-CA groups. $n \geq 5$ in each group. *$P < 0.05$ vs. the control group determined by one-way ANOVA followed by the Holm-Sidak's analysis.
However, KCa3.1 recycled in a Rab35-independent manner [24]. Rab4 or Rab11 does not affect K ATP channel trafficking in our study but regulates Kv1.5 recycling in atrial myocytes [16], indicating the cargo specificity of Rab GTPases in mediating intracellular trafficking of ion channels.

The number of surface K ATP channels varies dynamically, and the increase of surface K ATP channels can occur within minutes [25], indicating that this regulation is not at the transcription or translation level, but through recruitment of stored K ATP channels in intracellular vesicles to traffic to cell surface. Rab4 mediates fast endocytic recycling directly from early endosomes, whereas Rab11 and Rab35 mediate slow endocytic recycling through recycling endosomes. K ATP channel recycling occurs at a time scale of 10–15 min [10], indicating the involvement of slow endocytic recycling. Indeed, we observed colocalization of endocytosed K ATP channels with Rab35-DN positive vesicles (Figure 4) and Rab35-DN decreased the recycling rate of K ATP channels (Figure 5), demonstrating that Rab35 is a key mediator of K ATP channel recycling. We previously reported that Rab11a was also colocalized with K ATP channels, and regulated K ATP channel surface expression [14]. However, the colocalization experiment performed in the previous study was between Rab11a and total K ATP channels while in the present study between Rab35 and recycling K ATP channels. Moreover, based on the calculation of current amplitude difference, the amount of expressed K ATP channels in the previous experiment with Rab11a was ~100 fold higher than that with Rab35 in the present study, which indicates that Rab35 may be the primary regulator of K ATP channel recycling, while overexpressed K ATP channel may utilize Rab11a-containing vesicles for trafficking as well.

In the regulation of K ATP channel trafficking, another Rab GTPase, Rab8a, has also been shown to affect K ATP channel surface density [26]. Rab8a colocalized and co-immunoprecipitated with Kir6.2 subunit and knockdown of Rab8a reduced K ATP channel surface expression [26]. Rab8a is not a marker protein for ERC, but can be recruited to ERC by Rab35 through the interaction with molecule interacting with CasL-like protein 1 (MICAL)-L1, which is a Rab35 effector [27,28], indicating that Rab8a may be a cooperator of Rab35 in regulating K ATP channel recycling. The Rab35/MICAL-L1 complex can recruit not only Rab8a, but also Rab13, Rab36 and Eps15 homology domain protein family 1 (EHD1) [27,29,30]. However, a dominant negative EHD1 construct was reported to have no effect on K ATP channel recycling and surface density [14,31], suggesting that not all Rab35 effectors contribute to regulate K ATP channel recycling and whether Rab13 or Rab36 affect K ATP channel recycling needs further investigation. It is well known that leptin promotes K ATP channel recycling via AMPK and PI3K pathways [20,25], and ischemia preconditioning maintains K ATP channel surface density to protect cardiomyocytes against ischemic injury in an AMPK-dependent and PKC-dependent manner [32,33]. AMPK, PI3K and PKC are all phosphorylation kinases and Rab35 has been shown to be phosphorylated at Thr72 by LRRK2 in the process of Parkinson’s disease [34]. Whether Rab35 phosphorylation is involved in regulating K ATP channel recycling and cardioprotection needs further investigation.

We and others have shown that K ATP channels at the surface membrane mediate the protective effect of ischemic preconditioning [2,32,35]. Maintaining and increasing K ATP channel surface density protect cardiomyocytes against ischemic injury [14]. Constitutively active Rab35 mutant increased endogenous K ATP channel current, indicating that stimulating Rab35 may represent as a future therapy for treating ischemic cardiac injury. Indeed, it is well known that exercise is protective to the heart, partially mediated by surface K ATP channels, and in an exercised mouse model, swimming increased the expression of Rab35 at both the mRNA and protein levels [36]. The role of the link between Rab35 and K ATP channel in cardioprotection needs to be further investigated.

**Conclusion**

This study demonstrated that Rab35 is the key regulator of K ATP channel endocytic recycling. Inactivation of Rab35 downregulates K ATP channel current amplitude by reducing K ATP channel.
surface expression without affecting channel properties. Activation of Rab35 in cardiomyocytes upregulates $K_{\text{ATP}}$ channel current, indicating Rab35 as the potential pharmacologic target for treating the diseases due to $K_{\text{ATP}}$ channel trafficking defects.

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**Disclosure statement**

No potential conflict of interest was reported by the author(s).

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**Data availability statement**

The data that support the findings of this study are available from the corresponding author upon reasonable request. https://doi.org/10.5061/dryad.w6m905qs0

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