

# PI3K/Akt signalling-mediated protein surface expression sensed by 14-3-3 interacting motif

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## Keywords

14-3-3; Akt; GPR15; PI3K; regulation of surface expression

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The regulation of protein expression on the cell surface membrane is an important component of the cellular response to extracellular signalling. The translation of extracellular signalling into specific protein localization often involves the post-translational modification of cargo proteins. Using a genetic screen of random peptides, we have previously identified a group of C-terminal sequences, represented by RGRSWTY-COOH (termed 'SWTY'), which are capable of overriding an endoplasmic reticulum localization signal and directing membrane proteins to the cell surface via specific binding to 14-3-3 proteins. The identity of the kinase signalling pathways that drive phosphorylation and 14-3-3 binding of the SWTY sequence is not known. In this study, we report that the activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway by the over-expression of active kinases, stimulation with fetal bovine serum or growth factors can: (a) phosphorylate the SWTY sequence; (b) recruit 14-3-3 proteins to SWTY; and (c) promote surface expression of the chimeric potassium channel fused with the SWTY sequence. The expression of the dominant negative Akt inhibited the enhancement of surface expression by fetal bovine serum. In addition, the activation of PI3K significantly enhanced the 14-3-3 association and cell surface expression of GPR15, a G protein-coupled receptor which carries an endogenous SWTY-like, C-terminal, 14-3-3 binding sequence and is known to serve as a HIV co-receptor. Given the wealth and specificity of both kinase activity and 14-3-3 binding sequences, our results suggest that the C-terminal SWTY-like motif may serve as a sensor that can selectively induce the cell surface expression of membrane proteins in response to different extracellular signals.

## Structured digital abstract

- MINT-7233053: *PKA* (uniprotkb:P17612) *phosphorylates* (MI:0217) *Kir2.1* (uniprotkb:P35561) by *protein kinase assay* (MI:0424)
- MINT-7233066: *GPR15* (uniprotkb:Q9BG77) *physically interacts* (MI:0915) with *14-3-3 protein beta* (uniprotkb:P31946) by *anti tag coimmunoprecipitation* (MI:0007)

## Abbreviations

Akt, protein kinase B; CaMKII, calcium and calmodulin-dependent kinase II; ER, endoplasmic reticulum; HA, hemagglutinin; GPR15, G protein-coupled receptor 15; GST, glutathione S-transferase; PDGF, platelet-derived growth factor; PI3K, phosphoinositide 3-kinase; PKA/C, protein kinase A/C; SIV, simian immunodeficiency virus.

## Introduction

Membrane proteins comprise approximately 30% of the proteome [1]. For plasma membrane proteins, their density on the cell surface is often a key determinant of their overall function in a cell. In addition to transcriptional regulation, the surface expression of membrane proteins is tightly regulated at various post-transcriptional and post-translational levels in response to extracellular signals.

Increasing evidence suggests that the phosphorylation signal plays a critical role in protein targeting to the plasma membrane. For example, extracellular signalling that activates phosphoinositide 3-kinase (PI3K) causes a significant increase in the surface expression of voltage-gated calcium channels and transient receptor potential channels [2,3]. In the KCNK3 potassium channel, the deletion of a 14-3-3 binding motif results in a substantial loss of surface expression, implicating the influence of the phosphorylation signal on the cargo protein [4,5]. In this channel, the recognition of phosphorylated peptide signals by 14-3-3 proteins and the concurrent steric masking of the adjacent endoplasmic reticulum (ER) localization signal allow efficient forward transport of the channel [6,7]. However, no experimental evidence is yet available regarding the signalling pathways responsible for the phosphorylation of this 14-3-3 binding signal.

Using a novel random peptide display system, we have previously isolated a series of peptide motifs that override the ER localization signal and confer surface expression of reporter membrane protein. Among them, the C-terminal 'SWTY' peptides (represented by RGRSWTY-COOH) were found to mediate surface expression by interacting with 14-3-3 [8]. Bioinformatics analysis identified native membrane proteins that carry SWTY-like sequences, including an HIV co-receptor GPR15 [8].

14-3-3 proteins have been reported to interact with more than 300 proteins and regulate a wide variety of biological pathways [9,10]. Most of the 14-3-3 interactions are mediated by two canonical internal binding motifs, mode I (RSXpS/pTXP) and mode II (RX $\phi$ XpS/pTXP) ( $\phi$  is an aromatic or aliphatic amino acid, X is any amino acid, pS/pT represents phosphorylated Ser or Thr) [11,12]. However, a number of earlier reports recognized the interaction between 14-3-3 and the C-termini of membrane proteins. These include plant plasma membrane H<sup>+</sup>-ATPase and the Ib $\alpha$  subunit of the glycoprotein complex Ib-X-V, to which 14-3-3 binding is now known to cause significant changes in structure and function (see review [13]). With increasing evidence of specific and functional

interactions of 14-3-3 with protein C-termini, including SWTY, this characteristic binding is proposed as mode III [13–17].

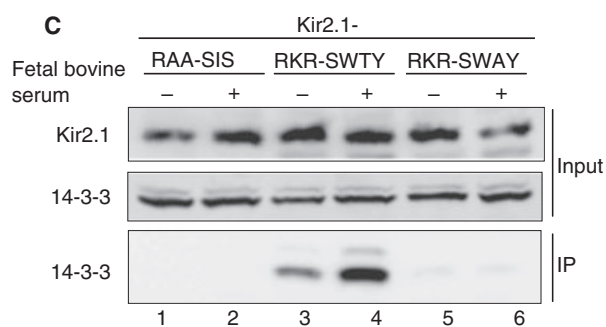
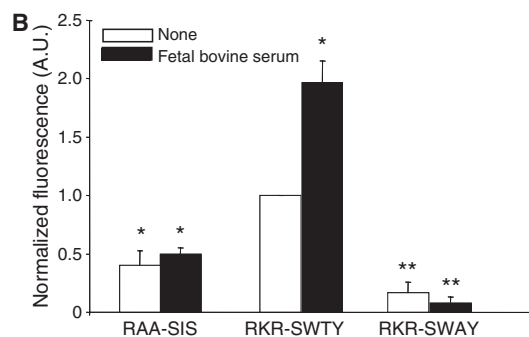
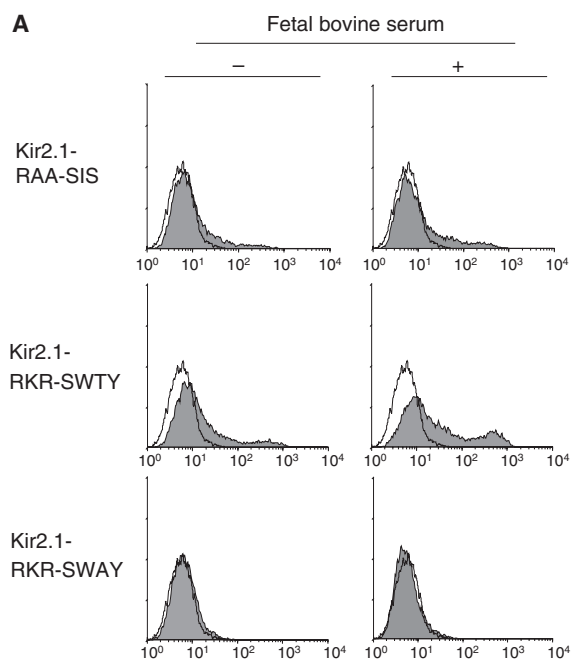
14-3-3 binding to the SWTY motif is dependent on phosphorylation and thus relies on the activity of kinases that phosphorylate the motif [8]. To understand the physiological role of 14-3-3-mediated cell surface transport in membrane proteins, it is essential to identify the signalling pathways that drive the phosphorylation of target proteins and the recruitment of 14-3-3. In this study, we demonstrate that SWTY motifs serve as sensors that translate extracellular signals into the cell surface localization of membrane proteins through protein kinase B (Akt) activity. Akt, a downstream kinase of PI3K, directly phosphorylates the SWTY sequence on fetal bovine serum or growth factor stimulation, and recruits 14-3-3, which subsequently confers the cell surface expression of the SWTY-carrying reporter potassium channel. In addition, we show that the surface expression of GPR15, a G protein-coupled receptor that bears a C-terminal SWTY-like 14-3-3 binding motif [8], is promoted by the activation of PI3K.

## Results

### Serum-induced surface expression of an SWTY reporter protein

In our earlier study, we observed that the fusion of the ER localization signal 'RKR' [18] to the surface membrane potassium channel Kir2.1 efficiently retained this channel intracellularly [8,19]. The SWTY motif, when placed at the extreme C-terminus of this chimeric channel, overrode the RKR signal and potentiated surface expression; Kir2.1-RKR-SWTY showed a four- to six-fold higher surface expression than that of wild-type Kir2.1. Specific interaction of 14-3-3 proteins with the SWTY motif was found to be necessary and sufficient to confer surface expression in HEK293 cells [8]. With the strict phosphorylation dependence of the SWTY–14-3-3 interaction in an *in vitro* binding assay [8], HEK293 cells should possess active protein kinase(s) phosphorylating the SWTY sequence.

We first tested whether serum components can regulate SWTY-mediated surface expression. HEK293 cells transfected with chimeric Kir2.1 constructs were cultured for 24 h in serum-deprived medium, and then stimulated with 10% fetal bovine serum. Figure 1A shows that fetal bovine serum treatment caused a two-fold increase in the surface expression of Kir2.1-RKR-



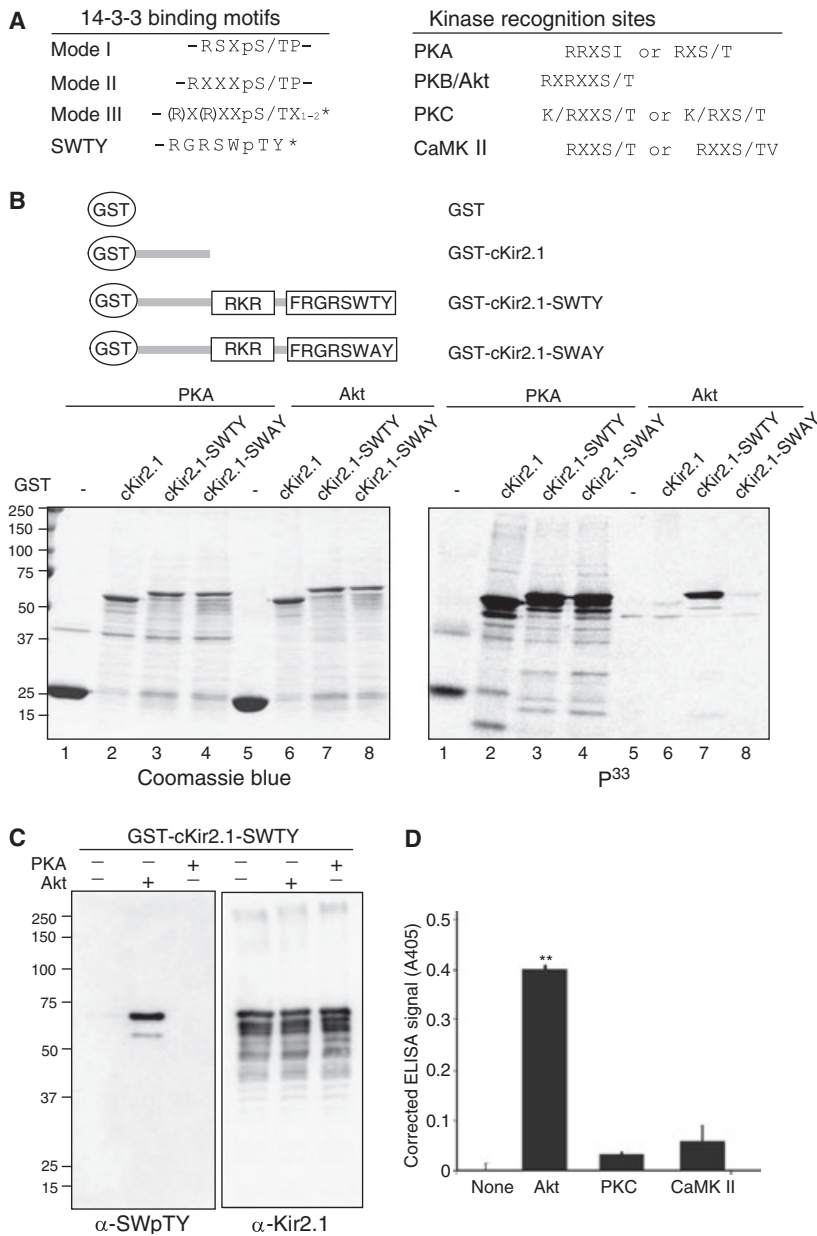
SWTY, but no changes for the Kir2.1-RAA-SIS control, which carries neither the RKR nor SWTY signal. Furthermore, mutation of Thr at the -2 position (RKR-SWAY), which abrogates 14-3-3 binding [8], abolished both the surface expression and sensitivity to fetal bovine serum treatment. Quantification of the surface expression indicated that, under fetal bovine

**Fig. 1.** Serum-induced surface expression of SWTY reporter protein. (A) Flow cytometric analyses of hemagglutinin (HA)-tagged Kir2.1 channel in HEK293 cells. The shaded areas are staining signals using anti-HA IgG. Histograms display the cell number (vertical axis) versus the logarithmic fluorescence intensity (horizontal axis). Mock-transfected cells stained with primary and secondary antibodies served as background (unshaded areas). The transfected constructs are as indicated. After starvation, cells were incubated for 6 h with (+) or without (-) fetal bovine serum. (B) Normalized cell surface fluorescence intensity. Geometric means of surface HA staining signals from cells transfected with three different constructs are normalized against the signal of Kir2.1-RKR-SWTY with no treatment with fetal bovine serum. The results shown are the means  $\pm$  standard errors (SE) (bars) of three independent experiments. Student's *t*-tests were used to analyse differences. \**P* < 0.05. \*\**P* < 0.01. (C) SWTY-specific increase in 14-3-3 association by fetal bovine serum induction. Kir2.1-RAA-SIS, Kir2.1-RKR-SWTY and Kir2.1-RKR-SWAY were expressed in HEK293 cells in medium without fetal bovine serum for 24 h. The cells were then further incubated for 6 h in the absence (lanes 1, 3 and 5) or presence (lanes 2, 4 and 6) of fetal bovine serum. Top and middle panels are immunoblots of total protein lysates (input) probed by antibodies for either Kir2.1 or 14-3-3 as indicated. The bottom panel exhibits an immunoblot by an anti-14-3-3 IgG after immunoprecipitation of Kir2.1 with anti-HA (IP).

serum treatment, SWTY conferred a four-fold higher expression than that of the Kir2.1-RAA-SIS control (Fig. 1B). Immunoblot analyses of protein expression indicated little change in total Kir2.1 or 14-3-3 proteins among different constructs with or without fetal bovine serum treatment (Fig. 1C). Examination of 14-3-3 binding revealed both specificity for Kir2.1-RKR-SWTY and an approximate two-fold increase in binding on fetal bovine serum treatment (Fig. 1C, lanes 3 and 4). These results demonstrate that an SWTY motif confers sensitivity to fetal bovine serum induction and the fetal bovine serum-induced increase in surface expression correlates with 14-3-3 binding.

### Akt phosphorylation of SWTY signals

We have previously shown that phosphorylation at the -2 position of an SWTY motif is necessary for 14-3-3 interaction [8]. Various kinases, including protein kinase A (PKA), Akt, protein kinase C (PKC), and calcium and calmodulin-dependent kinase II (CaMK II), have been implicated in the generation of 14-3-3 binding sites [20–25], which comprise the internal peptides defined as canonical mode I and II 14-3-3 binding motifs (Fig. 2A). However, little is known about the kinases responsible for the generation of C-terminal 14-3-3 binding sites, recently proposed as new mode III 14-3-3 binding (see review [13]). Figure 2A compares the SWTY motif with mode I, II and III 14-3-3 binding



**Fig. 2.** *In vitro* phosphorylation of an SWTY motif by Akt. (A) Sequence comparison of an SWTY sequence with 14-3-3 binding motifs and kinase recognition sites. (R) indicates either one or both arginines are allowed at this position. \*C-terminus of the protein (stop codon). (B) Phosphorylation of an SWTY motif *in vitro*. GST fusion proteins of the C-terminal domains of Kir2.1 (GST-cKir2.1), GST-cKir2.1 with RKR-SWTY and RKR-SWAY sequences (GST-cKir2.1-SWTY and GST-cKir2.1-SWAY, respectively) were generated as shown and affinity purified. The GST fusion proteins were *in vitro* phosphorylated with recombinant PKA or Akt. The reaction products were resolved by SDS-PAGE and subjected to Coomassie blue staining (left) and autoradiography (right). (C) Akt recognition of -2 Thr of the SWTY sequence. Purified GST-cKir2.1-RKR-SWTY proteins were *in vitro* phosphorylated with either Akt or PKA and analysed by an anti-phospho-SWTY IgG (left) and an anti-Kir2.1 IgG (right). (D) Akt-specific phosphorylation of an SWTY motif. After *in vitro* phosphorylation with Akt, PKC and CaMK II, biotinylated SWTY peptide was bound to a streptavidin-coated 96-well plate and subjected to ELISA with anti-SWpTY. The absorbance at 405 nm (A405) was corrected against signal with no kinase control. Values are the means  $\pm$  standard errors (SE) (bar) of three independent experiments. Student's *t*-tests were used to analyse differences; \*\**P* < 0.01.

consensus sequences. We tested whether these kinases can phosphorylate the SWTY motif *in vitro* using purified glutathione *S*-transferase (GST) fusions of the C-terminal cytoplasmic domains of Kir2.1, Kir2.1-RKR-SWTY and Kir2.1-RKR-SWAY. Figure 2 shows the results for Akt and PKA by autoradiography. On incubation with recombinant PKA, robust phosphorylation was observed in all three GST fusion proteins (Fig. 2B, lanes 2–4), consistent with the known PKA site present in the C-terminal domains of Kir2.1 [26]. However, Akt treatment resulted in significant <sup>33</sup>P incorporation for GST-cKir2.1-SWTY, whereas GST alone, GST-cKir2.1 and GST-cKir2.1-SWAY

proteins showed only background incorporation (Fig. 2B, lanes 5–8). To demonstrate that the incorporation of phosphate by Akt really occurred at the -2 Thr position, we generated a phosphor-specific antibody for RGRSWpTY-COOH (Fig. S1). Consistent with the results from radioisotope incorporation, Akt but not PKA was capable of phosphorylating the -2 Thr of the SWTY motif, hence conferring specific immunoreactivity to the anti-SWpTY IgG.

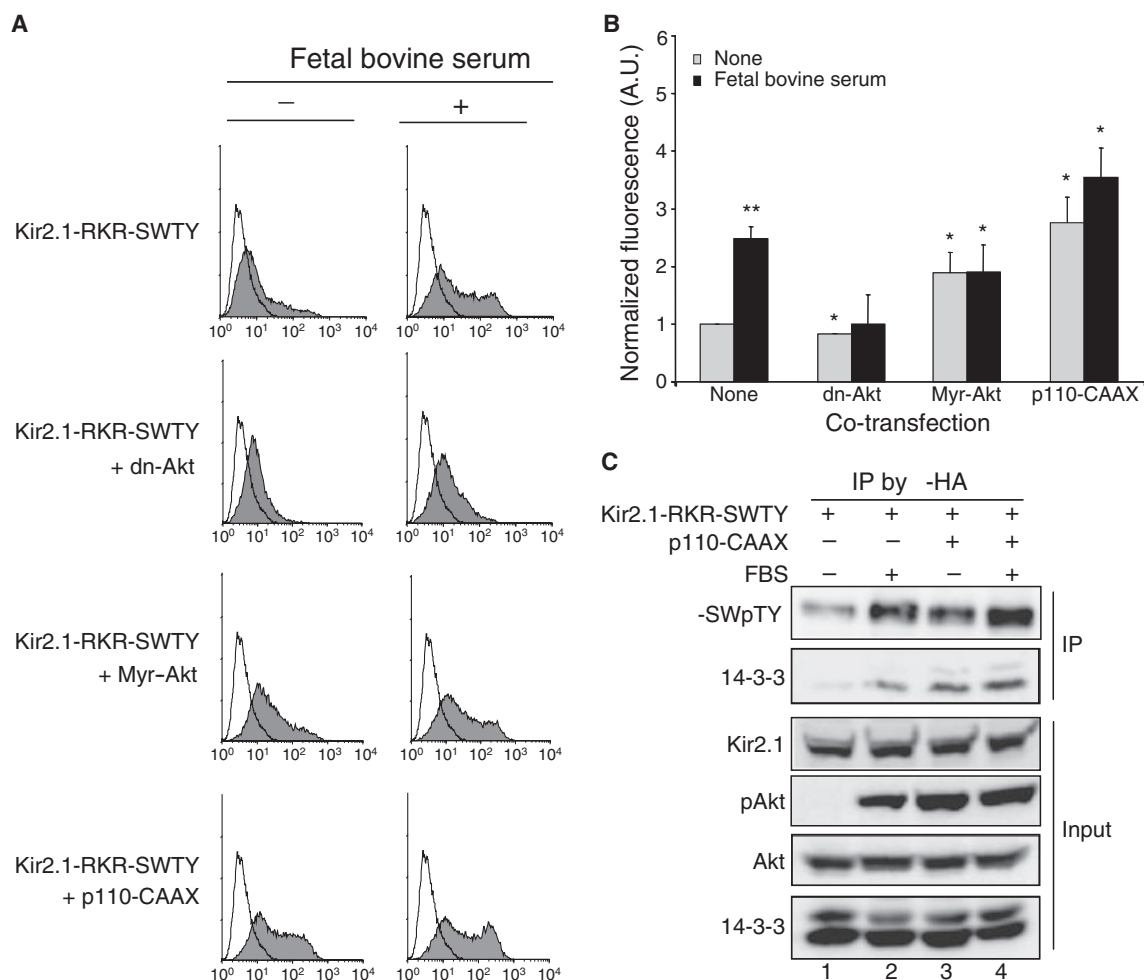
To determine whether SWTY can be phosphorylated *in vitro* by PKC and CaMK II, ELISA experiments were performed. Incubation of SWTY peptide with PKC and CaMK II resulted in basal levels of signal in

ELISA using the anti-SWpTY IgG, further supporting preferential phosphorylation by Akt (Fig. 2D).

### PI3K/Akt-dependent regulation of SWTY-mediated surface expression *in vivo*

To demonstrate the causal link between the activation of Akt and SWTY-mediated surface expression, the Akt activity in the cells was manipulated by the co-expression of constitutively active or dominant neg-

ative Akt mutants. Figure 3A, B shows that the inducible effects of fetal bovine serum were blocked by the co-expression of a dominant negative K179M Akt (dn-Akt). Furthermore, under serum starvation conditions, the constitutively active Myr-Akt conferred surface expression at a similar level to that of fetal bovine serum-treated control cells (Fig. 3B, None). Both Akt constructs masked the effect of fetal bovine serum stimulation. These data provide evidence that Akt activation is both necessary and sufficient for the



**Fig. 3.** Akt phosphorylation of SWTY and regulation of SWTY-mediated surface expression *in vivo*. (A) Flow cytometric analyses of an HA-tagged Kir2.1-RKR-SWTY channel in HEK293 cells. Surface expression of either Kir2.1-RKR-SWTY alone or co-expressed with the dominant negative form of Akt (dn-Akt), constitutively active form of Akt (Myr-Akt) or PI3K (p110-CAAX) was monitored under starvation and stimulation by fetal bovine serum. (B) Normalized fluorescence of surface expression. Histogram displays the geometric means of the surface staining signals of Kir2.1-RKR-SWTY normalized against the signal of no stimulation after 24 h of starvation. The results shown are the means  $\pm$  standard errors (SE) (bars) of three independent experiments. Student's *t*-tests were used to analyse differences; \**P* < 0.05; \*\**P* < 0.01. (C) Protein expression and binding induced by p110-CAAX. HA-tagged Kir2.1-RKR-SWTY was expressed alone or together with p110-CAAX in HEK293 cells without fetal bovine serum for 24 h. The cells were then further incubated for 6 h in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of serum. The top two panels show immunoblots by anti-SWpTY or anti-14-3-3 IgG after immunoprecipitation with anti-HA IgG (IP). The bottom four panels show immunoblots of total protein lysates probed by anti-Kir2.1, anti-pAkt, anti-Akt and anti-14-3-3 IgGs, respectively.

fetal bovine serum-induced surface expression of the SWTY-carrying Kir2.1 channel.

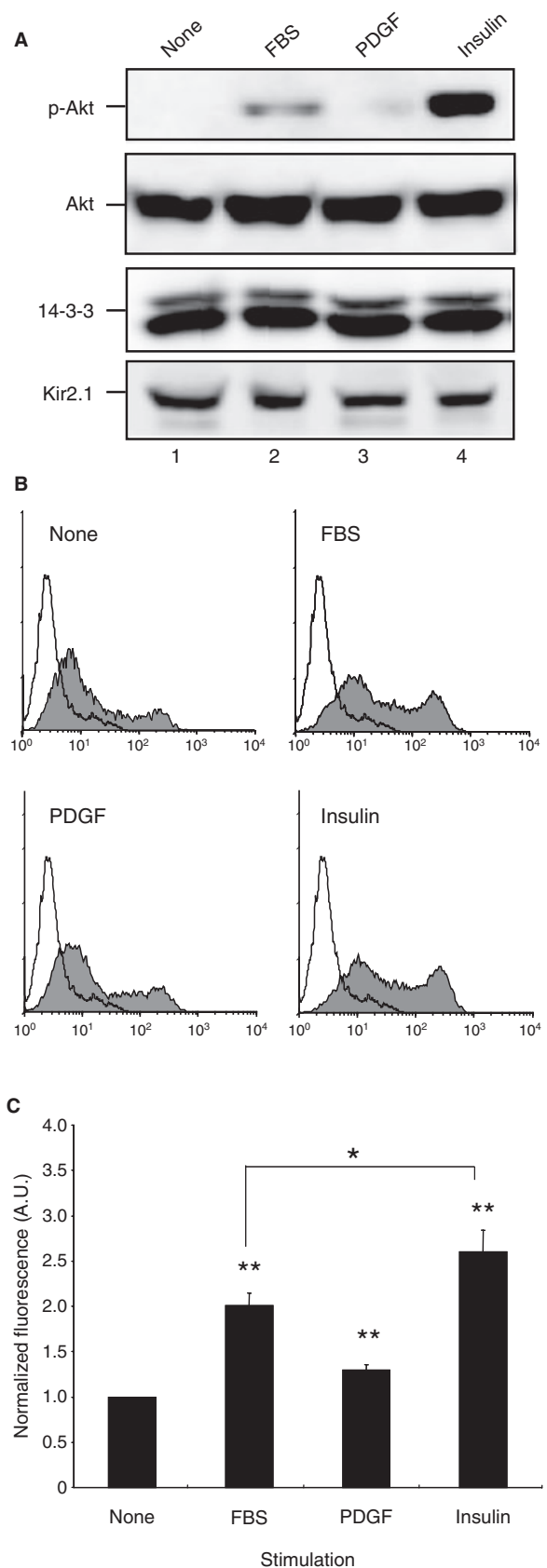
Because Akt is commonly activated by PI3K, a membrane-targeted constitutively active catalytic subunit of PI3K (p110-CAAX) was tested for its effects on SWTY-mediated surface expression. Indeed, under serum starvation conditions, the co-expression of p110-CAAX conferred a comparable surface expression to that by fetal bovine serum treatment (Fig. 3A, B). Consistent with its action on the Akt pathway, the co-expression of p110-CAAX induced phosphorylation of Akt, as probed by an anti-phospho-Akt IgG. Furthermore, both fetal bovine serum treatment and p110-CAAX expression induced an increase in anti-SWpTY signal, accompanied by an enhanced interaction between Kir2.1-RKR-SWTY and 14-3-3 (Fig. 3C, lanes 2 and 3). The inhibitors for either PI3K (wortmannin) or Akt (API-2), but not MEK inhibitor (PD98059), reduced the induction of the surface expression by fetal bovine serum (Fig. S2).

Akt is a downstream Ser/Thr kinase of PI3K activated by insulin and other growth factors, such as platelet-derived growth factor (PDGF), epidermal growth factor and insulin-like growth factor, in different cells [27]. To investigate the specific signalling pathway(s) activating Akt to direct SWTY-mediated surface expression, HEK293 cells transfected with Kir2.1-RKR-SWTY were starved and stimulated with different growth factors, including PDGF and insulin. Insulin gave rise to the highest phospho-Akt signal and surface expression, whereas there were no changes in the total protein expression of Akt and 14-3-3 on stimulation (Fig. 4). Therefore, insulin signalling may be one pathway that critically regulates SWTY-mediated surface expression through the activation of the PI3K/Akt pathway.

### Regulation of surface expression of GPR15 by PI3K signalling pathway

Our discovery of the SWTY motif by random peptide screening has led to the identification of proteins that

**Fig. 4.** Induction of SWTY-mediated surface expression by different growth factors. (A) Phosphorylation of Akt by different growth factors. HEK293 cells transfected with HA-tagged Kir2.1-RKR-SWTY were cultured for 24 h in the absence of fetal bovine serum (FBS) and stimulated either with fetal bovine serum (10%), PDGF (60 ng·mL<sup>-1</sup>) or insulin (100 ng·mL<sup>-1</sup>) for 3 h. Lysates were separated by SDS-PAGE and immunoblotted with antibodies for p-Akt, Akt, 14-3-3 and Kir2.1. (B) Flow cytometric analyses of HA-tagged Kir2.1-RKR-SWTY in HEK293 cells. (C) Normalized surface expression levels determined by fluorescence from flow cytometry in (B). Values are means ± standard errors (SE) (bar) of four independent experiments. Student's *t*-tests were used to analyze differences; \**P* < 0.05; \*\**P* < 0.01.



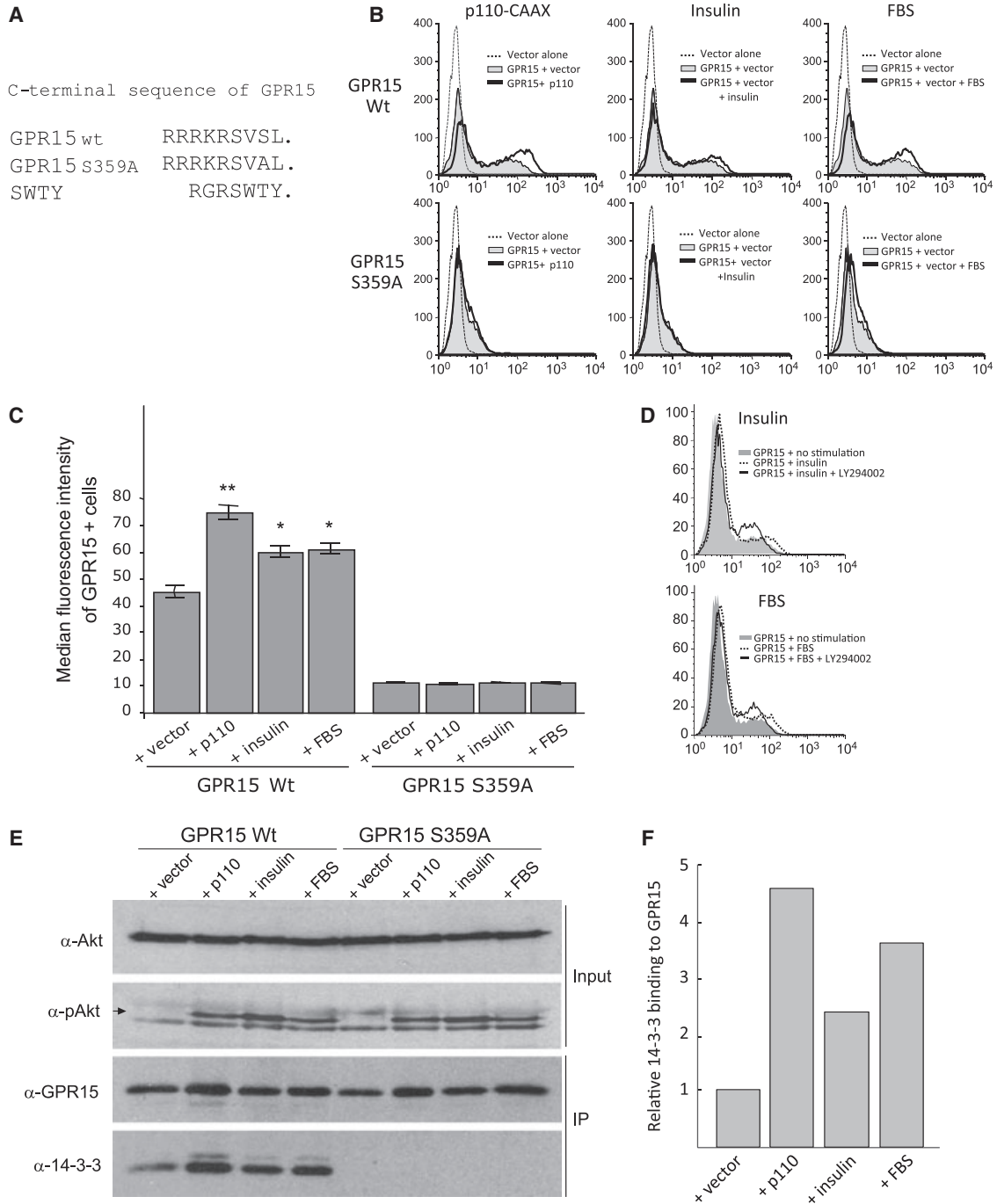
require a C-terminal, SWTY-like signal for efficient surface expression [8]. These include GPR15 (see Fig. 5A for C-terminal sequence), a G protein-coupled receptor that serves as a co-receptor for HIV and simian immunodeficiency virus (SIV) entry, and has also been suggested to mediate regenerative enteropathy on viral infection [28]. Mutation of -2 Ser to Ala (S359A) abolishes the binding of 14-3-3 and substantially reduces the surface expression of GPR15 ([8] and Fig. 5B). To investigate whether the GPR15 C-terminal sequence plays a similar role to SWTY by sensing PI3K signalling, we tested the effects of the co-expression of active PI3K or stimulation with insulin or serum on the surface expression of GPR15. As the S359A mutant lacks the ability to bind to 14-3-3, the change in surface expression level of the S359A mutant caused by cell treatment would represent 14-3-3-independent effects. Under the condition in which PI3K expression and cell treatments all activated Akt (see Fig. 5E, pAkt panel), the surface expression of wild-type GPR15, but not S359A, was significantly elevated by the over-expression of active PI3K (Fig. 5B, C). Stimulation of cells with serum or insulin also caused a significant increase in surface expression only for wild-type GPR15. The enhancement by these treatments was efficiently blocked by the PI3K inhibitor LY294002, which supports the concept that PI3K activation is necessary for the growth factor-induced surface expression of GPR15 (Fig. 5D). Importantly, the quantification of 14-3-3 proteins co-immunoprecipitated with GPR15 demonstrated that PI3K co-expression and growth factor treatments all enhanced 14-3-3 binding to GPR15 by two- to four-fold (Fig. 5E, F). Together, these results suggest that GPR15 surface expression may be induced by the extracellular signals that activate the PI3K pathway through the enhancement of 14-3-3 binding to the GPR15 C-terminal sequence.

## Discussion

The recent identification of the SWTY (RGRSWTY-COOH) sequence by genetic screening has reinforced the concept of a new mode III 14-3-3 binding, which is characterized by a restricted C-terminal location and phosphorylation-dependent interaction with 14-3-3 [8,15,29]. Although various kinases have been implied in internal mode I and II 14-3-3 binding, a kinase(s) recognizing a specific C-terminal sequence and conferring 14-3-3 interaction has not been studied. In this study, we provide evidence by both *in vitro* and *in vivo* assays that Akt is responsible for the phosphorylation of the SWTY sequence that leads to 14-3-3 binding.

Akt is a Ser/Thr kinase that is commonly activated by PI3K. When extracellular signals, such as insulin and other growth factors, activate PI3K through receptor tyrosine kinases, the resultant phosphoinositide products recruit Akt to the cell membrane, where Akt is phosphorylated and activated by phosphoinositide-dependent kinase 1. On activation, Akt phosphorylates a variety of proteins at the Ser/Thr residues of a consensus sequence, RxRxxS/T, thereby inducing diverse biological responses, such as the inhibition of programmed cell death, promotion of cell proliferation and regulation of protein trafficking [27,30,31]. Although Akt has been known to control the activity of a number of cytosolic proteins by enabling 14-3-3 binding on phosphorylation [11,29], our study demonstrates, for the first time, that C-terminal recognition by Akt could lead to 14-3-3 binding and the promotion of the cell surface expression of the membrane protein. This provides a basis for the potential development of assay technology and therapeutic intervention by engineering membrane proteins of which surface expression can be regulated by specific extracellular signals. Functional positioning at the protein C-terminus makes the SWTY motif suitable for such purposes.

We have shown that PI3K/Akt activity or a signalling pathway activating PI3K/Akt induces phosphorylation of the SWTY sequence, recruits 14-3-3 and confers elevated surface expression of the chimeric Kir2.1 channel. Because a variety of kinases can recognize the 14-3-3 binding site, receptors with different 14-3-3 binding motifs may sense different signalling pathways. It is of particular interest that many of the proteins reported to carry C-terminal 14-3-3 binding motifs are membrane proteins [4,8,15,32,33]. Among them is GPR15, an orphan G protein-coupled receptor that functions as a co-receptor for HIV and SIV entry [28,34,35]. We have demonstrated previously that the integrity of the -2 positioned Ser of GPR15 is important for binding to 14-3-3, and hence surface expression [8]. In this study, we show that the surface expression of GPR15 can be promoted by PI3K activation that enhances 14-3-3 binding to this receptor (Fig. 5). It is known that HIV infection activates the pro-survival PI3K/Akt pathway and thereby extends the lifespan of infected macrophages [36]. Our results suggest that the PI3K signalling pathway may play further roles in HIV infection by regulating viral selectivity and infection efficiency [34] through alteration of the cell surface expression level of the HIV co-receptor GPR15. GPR15 is also involved in the regenerative enteropathy in HIV/SIV infection, where cell surface GPR15 seems to transmit the apoptotic signal in the



intestinal cell on contact with the virus [28,35]. Further studies on endogenous GPR15 protein are necessary to better understand the role of PI3K signalling in the 14-3-3-mediated surface expression of GPR15 and HIV pathology.

The molecular mechanisms underlying 14-3-3-mediated surface expression, for example how 14-3-3

protein binding can override the ER localization signal and cell surface transport, are yet to be elucidated [14]. Nevertheless, considering the wealth of 14-3-3 binding sequences [9,37], it is conceivable that there are more membrane proteins than currently known that are regulated by 14-3-3 binding for their specific cellular localization. As multiple Ser/Thr kinases are

**Fig. 5.** Regulation of surface expression of GPR15 by PI3K signalling. (A) C-terminal sequence of GPR15. C-terminal nine amino acid residues of human GPR15 wild-type and S359A mutant were aligned with the SWTY sequence. (B) Flow cytometric analyses of the surface expression of GPR15. The GPR15 wild-type or S359A mutant was co-transfected with pCDNA3.1 (+) vector, full line with shaded area) or p110-CAAX (+p110, full line with unshaded area) and cultured for 24 h in the absence of fetal bovine serum (FBS). For growth factor treatment, cells were co-transfected with GPR15 and pCDNA3.1 plasmids, cultured for 24 h in the absence of fetal bovine serum, and treated with insulin (100 ng·mL<sup>-1</sup>) or fetal bovine serum (10%) for 6 h. Cells were stained with PE-labelled anti-GPR15 monoclonal IgG. The broken line represents the background signal of antibody stain from the cells transfected with pCDNA3.1 vector alone. (C) Statistical analysis of the effects of PI3K, insulin and fetal bovine serum on GPR15 surface expression. The cell populations that showed a positive signal for GPR15 were selected and analysed for the median fluorescence intensity by FLOJO software. Values are means  $\pm$  standard errors (SE) (bar) of triplicate samples and were analysed for differences by Student's *t*-test. \**P* < 0.05; \*\**P* < 0.005. (D) Effects of PI3K inhibitor on insulin- and fetal bovine serum-induced surface expression of GPR15. LY294002 (10  $\mu$ M) was added to the cell culture 30 min prior to the cell treatment with insulin or fetal bovine serum. Overlaid histograms are from GPR15-transfected cells with no stimulation (shaded area), with insulin (broken line) and with LY294002 and insulin (full line with unshaded area). (E) Effects of PI3K, insulin and fetal bovine serum on 14-3-3 binding to GPR15. Cells prepared as described in (B) were lysed and subjected to the co-immunoprecipitation of 14-3-3 proteins. Total cell lysate (Input) and the eluates from the HA antibody immunoprecipitant (IP) were resolved by SDS-PAGE and immunoblotted with the corresponding antibodies. In the pAkt panel, the arrow indicates the bands of the phosphorylated Akt signal. (F) Quantitative analysis of co-immunoprecipitated 14-3-3 proteins. The immunoblots developed by chemiluminescence using the imager were quantified for band intensity. The bar graph is a representative of one of three experiments.

known to phosphorylate 14-3-3 binding sites [9,29], different extracellular signals may confer selective induction of 14-3-3-mediated surface expression of membrane proteins. For example, the KCNK3 potassium channel has a C-terminal sequence of RRSSV-COOH, which binds 14-3-3 in a phosphorylation-dependent manner and critically regulates surface expression [5–8]. This sequence matches the consensus site of PKA rather than Akt, but the identity of the responsible kinases is not known. Identification and investigation of more 14-3-3 binding membrane proteins will facilitate our understanding of the molecular mechanisms underlying the signal-induced 14-3-3-mediated protein transport.

## Materials and methods

### Plasmids

Extracellularly HA-tagged mouse Kir2.1 was fused with the C-terminal cytoplasmic tail of the Kir6.2 channel with modifications, as described previously [8]. These were termed Kir2.1-RAA-SIS (Kir2.1-LLDALTLASSRG-PLRAASVAVAKAKPKFSISPDSL), Kir2.1-RKR-SWTY (Kir2.1-LLDALTLASSRGPLRKRSVAVAKAKPKFRGR-SWTY), Kir2.1-RKR-SWAY (Kir2.1-LLDALTLASSRG-PLRKRSVAVAKAKPKFRGRSWAY). Human GPR15 plasmids with (S359A) or without (wild-type) Ala mutation were cloned in pCDNA3.1(+) vector (Invitrogen, Carlsbad, CA, USA), as described previously [8]. Plasmids expressing a constitutively active PI3K (p110-CAAX, p110 subunit fused with a C-terminal isoprenylation motif), constitutively active Akt (Myr-Akt, Akt fused with an N-terminal myristoylation motif) and kinase-dead dominant negative Akt (K179M, mutation in lysine at amino acid 179) were identi-

cal to those described previously [38]. GST fusion constructs were prepared in pGEX-4T2 vector (GE Healthcare, Piscataway, NJ, USA).

### Antibodies

The rabbit anti-pan-Akt and anti-phospho-Akt IgGs were purchased from Cell Signaling Technologies (Danvers, MA, USA). Mouse monoclonal anti-HA and rabbit polyclonal anti-14-3-3 $\beta$  (this antibody reacts with all seven 14-3-3 isoforms) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Mouse monoclonal anti-human GPR15 IgG was purchased from R&D Systems (Minneapolis, MN, USA) and labelled with *R*-phycoerythrin (Invitrogen) before use. The rabbit polyclonal anti-Kir2.1 serum was raised against the C-terminal cytoplasmic region corresponding to amino acids 188–428. The rabbit polyclonal serum specifically recognizing phosphorylated SWTY at the Thr residue was generated by Sigma-Genosys (The Woodlands, TX, USA). Specificity to the phosphorylated SWTY sequence was confirmed by ELISA using N-terminally biotinylated FRGRSWpTY-COOH (pT, phosphorylated Thr) and FRGRSWTY-COOH peptides and alkaline phosphatase-conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA, USA) (see Fig. S1).

### In vitro phosphorylation

The GST-fused C-terminal cytoplasmic tails of Kir2.1, Kir2.1-RKR-SWTY and Kir2.1-RAA-SWTY were expressed in BL21 *Escherichia coli* (Invitrogen) and purified by glutathione-conjugated Sepharose beads (GE Healthcare). *In vitro* phosphorylation was performed by incubating 2  $\mu$ g of each GST fusion protein with 50 ng of recombinant Akt1 or PKA (Upstate Biotechnology, Lake Placid, NY,

USA) at 30 °C for 60 min in dilution buffer containing 50 mM Tris/HCl (pH 7.5), 0.1 mM EGTA, 15 mM dithiothreitol, 10 mM MgCl<sub>2</sub>, 1 mM ATP and 0.125 Ci·mL<sup>-1</sup> [<sup>33</sup>P]ATP[γP]. Reaction products were resolved by SDS-PAGE and subjected to Coomassie blue staining and autoradiography. For immunoblotting with anti-SWpTY IgG, the phosphorylation reaction was performed at 30 °C for 30 min in the absence of [<sup>33</sup>P]ATP[γP].

### Cell culture and transfection

HEK293 cells were maintained in 50% DMEM/50% F12 medium containing 10% fetal bovine serum. For the stimulation of cells, the transfected cells were serum starved for 24 h and then left untreated (control) or stimulated with fetal bovine serum (10%), insulin (100 ng·mL<sup>-1</sup>; Sigma, St. Louis, MO, USA) or PDGF (60 ng·mL<sup>-1</sup>; Sigma) for the indicated times. For inhibitor studies, cells were pre-treated with various concentrations of LY294002 (Sigma), wortmannin (Sigma), API-2 (Calbiochem, La Jolla, CA, USA) or PD098059 (Cell Signaling Technologies) for 30 min. The carrier concentration (0.1% dimethylsulfoxide) was maintained constant for all cell treatments. Transient transfections were performed using FuGENE6 (Roche Applied Sciences, Indianapolis, IN, USA), 30 min after initiation of starvation.

### Flow cytometry

Transfected HEK293 cells were harvested and washed with Hanks' balanced salt solution supplemented with 5 mM Hepes (pH 7.3) and 2% fetal bovine serum (staining medium). All antibody incubations and washes were performed in staining medium at 4 °C. For the HA-tagged Kir2.1 channel, the cells were stained with anti-HA IgG for 30 min on ice, followed by 10 min staining with Alexa Fluor 488-conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR, USA). For GPR15, the cells were stained with R-phycoerythrin-labelled anti-GPR15 IgG for 30 min on ice. The stained cells were examined for cell surface fluorescence with FACSCalibur (BD Biosciences, San Jose, CA, USA) and analysed with FloJo software (Tree Star Inc., Ashland, OR, USA).

### Immunoprecipitation and immunoblot

For immunoprecipitation, transfected cells were washed with NaCl/P<sub>i</sub> once and lysed with lysis buffer (1% NP40, 25 mM Tris, 150 mM NaCl, pH 7.5) with protease inhibitor cocktails (Sigma) for 20 min at 4 °C. After centrifugation for 20 min at 11 000 g, the supernatant was mixed with protein A-conjugated agarose beads (Sigma) pre-incubated with 1 µg of anti-HA IgG. After overnight incubation, the beads were washed three times with lysis buffer, and

then the precipitated proteins were eluted with 2× sample buffer for SDS-PAGE analysis and immunoblot, as described previously [39]. The samples resolved in SDS-PAGE gels were transferred to nitrocellulose and blotted with the corresponding primary antibodies, followed by horseradish peroxidase-conjugated secondary antibodies. The immunoblots were developed with the ECL system (GE Healthcare). In some experiments, the blots were developed using Molecular Imager (Bio-Rad, Hercules, CA, USA) for the quantification of the band intensity.

### Statistical analysis

Values are expressed as the means ± standard error. The significance of the difference between the means was calculated by Student's *t*-test.

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## Supporting information

The following supplementary material is available:

**Fig. S1.** Specificity of anti-phospho SWTY IgG.

**Fig. S2.** Effects of kinase inhibitors on fetal bovine serum-induced surface expression of Kir2.1-RKR-SWTY.

This supplementary material can be found in the online version of this article.

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