



# Rim2alpha determines docking and priming states in insulin granule exocytosis

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**Title: Rim2 $\alpha$  determines docking and priming states in insulin granule exocytosis**

**Running Title:** Role of Rim2 $\alpha$  in insulin granule exocytosis

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## Summary

Insulin secretion is essential for maintenance of glucose homeostasis, but the mechanism of insulin granule exocytosis, the final step of insulin secretion, is largely unknown. Here we investigated the role of Rim2 $\alpha$  in insulin granule exocytosis including the docking, priming, and fusion steps. We found that interaction of Rim2 $\alpha$  and Rab3A is required for docking, which is considered a brake on fusion events, and that docking is necessary for K<sup>+</sup>-induced exocytosis but not for glucose-induced exocytosis. Furthermore, we found that dissociation of the Rim2 $\alpha$ /Munc13-1 complex by glucose stimulation activates Syntaxin1 by Munc13-1, indicating that Rim2 $\alpha$  primes insulin granules for fusion. Thus, Rim2 $\alpha$  determines docking and priming states in insulin granule exocytosis depending on its interacting partner, Rab3A or Munc13-1, respectively. Since Rim2 $\alpha$ <sup>-/-</sup> mice exhibit impaired secretion of various hormones stored as dense-core granules, including glucose-dependent insulinotropic polypeptide, growth hormone, and epinephrine, Rim2 $\alpha$  plays a critical role in exocytosis of dense-core granules.

## Introduction

Stimulus-secretion coupling has been characterized in neurons and neuroendocrine and endocrine cells (Burgoyne and Morgan, 2003; Sudhof, 2004; Seino and Shibasaki, 2005). Pancreatic  $\beta$ -cells, in which insulin is stored as dense-core vesicles, play a central role in glucose homeostasis. Although understanding of the molecular mechanisms of cell signaling in insulin secretion has deepened remarkably in recent years, the mechanisms of insulin granule exocytosis, the final step in the insulin secretory process, are largely unknown.

Recently, various molecules interacting with the insulin granules have been identified (Brunner et al., 2007), including Rab27A (Kasai et al., 2005), granuphilin (Gomi et al., 2005), ZnT8 (Nicolson et al., 2009), Noc2 (Matsumoto et al., 2004), collectrin (Fukui et al., 2005), and Rap1 (Shibasaki et al., 2007). Mutation of the Rab27A gene was discovered from Griscelli syndrome in human characterized by hypopigmentation and loss of cytotoxic killing activity by cytotoxic T lymphocytes

(Menasche et al., 2000). The same mutation found in the mouse coat-color mutant *ashen* causes a defect in insulin granule exocytosis (Kasai et al., 2005). *Granuphilin*<sup>-/-</sup> mice, *ZnT8*<sup>-/-</sup> mice, *Noc2*<sup>-/-</sup> mice, and transgenic mice overexpressing collectrin also exhibit impaired insulin secretion and abnormal glucose homeostasis.

We previously identified Rab3-interacting molecule 2 (Rim2) by yeast two-hybrid screen of a clonal pancreatic  $\beta$ -cell (MIN6) cDNA library (Ozaki et al., 2000). Rim2 is now known as a multidomain protein that occurs as three variants including Rim2 $\alpha$ , Rim2 $\beta$ , and Rim2 $\gamma$  (Wang and Sudhof, 2003). Rim2 $\alpha$ , the full-length form of Rim2, is composed of an N-terminal Zn<sup>2+</sup>-finger domain, a central PDZ and C<sub>2</sub>A domains, and a C-terminal C<sub>2</sub>B domain. Rim2 $\beta$  lacks the N-terminal Zn<sup>2+</sup>-finger domain of Rim2 $\alpha$ , while Rim2 $\gamma$  is composed only of the C-terminal C<sub>2</sub>B domain of Rim2 $\alpha$  with flanking sequences. Rim2 $\alpha$  interacts with Rab3A (Ozaki et al., 2000), Munc13-1 (Dulubova et al., 2005), and Rab8 (Fukuda, 2003) at the N-terminal region. In addition to interaction with these proteins, Rim2 $\alpha$  binds to cAMP-GEFII (also referred to as Epac2) (Ozaki et al., 2000; Shibasaki et al., 2004) and ELKS (Ohara-Imaizumi et al., 2005; Inoue et al., 2006) through the central PDZ domain, to Piccolo through a C<sub>2</sub>A domain (Fujimoto et al., 2002), to RIM-BPs through a PXXP sequence between the two C<sub>2</sub>-domains (Hibino et al., 2002), and to Liprin- $\alpha$ 1 and synaptotagmin 1 through the C-terminal C<sub>2</sub>B domain (Schoch et al., 2002). These findings suggest that Rim2 $\alpha$  functions as a scaffold protein and that it is involved in regulated exocytosis. Indeed, Rim2 $\alpha$  has been found to be involved in cAMP-potentiated insulin secretion through the Epac2 pathway in *in vitro* studies (Ozaki et al., 2000; Kashima et al., 2001). Although Rim2 $\alpha$  is expressed mainly in endocrine and neuroendocrine cells such as pancreatic  $\beta$ -cells, pituitary, and adrenal gland, it is unknown how Rim2 $\alpha$  acts in the steps in the process of exocytosis including recruitment, docking, priming, and fusion.

Rim1 $\alpha$ , an isoform of Rim2 $\alpha$ , is expressed mainly in brain and a putative Rab3 effector involved in the regulation of synaptic vesicle fusion (Wang et al., 1997). Studies of Rim1 $\alpha$ <sup>-/-</sup> mice (Castillo et al., 2002; Lonart et al., 2003) and the null mutation of Rim in *C. elegans* (Koushika et al., 2001)

indicate that Rim1 $\alpha$  is important for long-term potentiation by increasing neurotransmitter release at mossy-fiber synapses in the CA3 region in hippocampus of cerebrum and that it is involved in a post-docking step. To clarify the role of Rim2 $\alpha$  in endocrine function both *in vitro* and *in vivo*, we generated Rim2 $\alpha^{-/-}$  mice. In the course of our study, Schoch et al also generated Rim2 $\alpha^{-/-}$  mice and reported the phenotype of the mice (Schoch et al., 2006). They found no apparent developmental abnormalities of these mice, but there were slight behavioral differences and slightly lower survival rates compared to Rim2 $\alpha^{+/+}$  mice. They also found no changes in body fat content or blood glucose levels in Rim2 $\alpha^{-/-}$  mice. However, no study of endocrine or neuroendocrine functions in Rim2 $\alpha^{-/-}$  mice was reported.

In the present study, we show that Rim2 $\alpha$  is required for the docking and priming states in insulin granule exocytosis through interaction with Rab3A and Munc13-1, respectively, and that although docking is necessary for K<sup>+</sup>-induced exocytosis, it is unnecessary for glucose-induced exocytosis. In addition, we found secretory defects in various hormones including glucose-dependent insulintropic polypeptide (GIP), growth hormone (GH), and epinephrine in Rim2 $\alpha^{-/-}$  mice *in vivo*. Thus, Rim2 $\alpha$  is a key molecule in insulin granule exocytosis and also is required in normal secretion of hormones associated with glucose homeostasis.

## Results

### Insulin and GIP secretion are impaired in Rim2 $\alpha^{-/-}$ mice *in vivo*

The Rim2 gene has three independent promoters that create three variant forms of Rim2, Rim2 $\alpha$ , Rim2 $\beta$ , and Rim2 $\gamma$  (Wang and Sudhof, 2003) (Figure S1A). We found that among the three variants Rim2 $\alpha$  is predominantly expressed in pancreatic islets and insulin secreting MIN6 cells (Figure S1B and S1C). We generated Rim2 $\alpha^{-/-}$  mice in which the fourth exon of the Rim2 gene was replaced by Neo cassette (Figure S1D). The absence of Rim2 $\alpha$  expression in Rim2 $\alpha^{-/-}$  mice was confirmed by northern blot analysis, reverse transcription PCR, and immunoblot analysis (Figure S1E-S1G). The expression levels of Rim2 $\beta$  and Rim2 $\gamma$  were not changed in Rim2 $\alpha^{-/-}$  mice (Figure S1H). Rim2 $\alpha^{-/-}$

mice were viable and fertile.  $Rim2\alpha^{-/-}$  mice exhibited islet hyperplasia ( $Rim2\alpha^{+/+} = 7,887.6 \pm 728.1 \mu m^2$ ;  $Rim2\alpha^{-/-} = 15,620.4 \pm 1486.7 \mu m^2$ ;  $N=4$  and 100 islets;  $p < 0.01$ ) and a relatively increased number of  $\alpha$ -cells ( $Rim2\alpha^{+/+} = 14.7 \pm 1.5\%$  of islets;  $Rim2\alpha^{-/-} = 40.0 \pm 2.5\%$  of islets;  $p < 0.01$ ;  $N=3$ ,  $n=53$  for  $Rim2\alpha^{+/+}$  and  $n=37$  for  $Rim2\alpha^{-/-}$ ) (Figure S1I).

We performed intraperitoneal glucose tolerance test (IPGTT) to examine pancreatic endocrine function in  $Rim2\alpha^{-/-}$  mice. The fasting blood glucose levels of  $Rim2\alpha^{-/-}$  mice were significantly decreased compared those of  $Rim2\alpha^{+/+}$  mice ( $Rim2\alpha^{+/+} = 119 \pm 3.1$  mg/dl;  $Rim2\alpha^{-/-} = 87 \pm 4.7$  mg/dl;  $p < 0.01$ ;  $n=19$  for  $Rim2\alpha^{+/+}$  and  $n=17$  for  $Rim2\alpha^{-/-}$ ). The blood glucose levels of  $Rim2\alpha^{-/-}$  mice 60 min after intraperitoneal glucose administration were significantly higher than those of  $Rim2\alpha^{+/+}$  mice. The insulin response during IPGTT in  $Rim2\alpha^{-/-}$  mice was markedly lower than that in  $Rim2\alpha^{+/+}$  mice (Figure 1A). We also examined changes in blood glucose and insulin levels after oral glucose administration (OGTT). The blood glucose levels of  $Rim2\alpha^{-/-}$  mice during OGTT were significantly higher than those of  $Rim2\alpha^{+/+}$  mice at all time points examined. The insulin levels were markedly reduced in  $Rim2\alpha^{-/-}$  mice compared to those in  $Rim2\alpha^{+/+}$  mice (Figure 1B). Similar results were obtained by oral mixed meal administration (Figure 1C). These results suggest that incretin secretion is decreased in  $Rim2\alpha^{-/-}$  mice. GIP, an incretin secreted by enteroendocrine cells in response to ingestion of nutrients, potentiates glucose-induced insulin secretion (Drucker, 2006). To investigate the possible involvement of incretins in this reduced insulin secretion, we attempted to measure the serum levels of GIP and glucagon-like peptide-1 (GLP-1) in  $Rim2\alpha^{-/-}$  mice. Although GIP-secreting K cells were present in  $Rim2\alpha^{-/-}$  mice (Figure S1J), serum GIP levels in  $Rim2\alpha^{-/-}$  mice were below the sensitivity of assay (Figure 1D). However, the measurement of GLP-1, another incretin, in mouse serum was not possible by using commercially available kits despite several attempts. These results indicate that both insulin and GIP secretion in  $Rim2\alpha^{-/-}$  mice are markedly impaired.

### **Insulin secretion is impaired in pancreatic islets of $Rim2\alpha^{-/-}$ mice**

As shown in Figure 2A, insulin content of the islets of  $\text{Rim2}\alpha^{-/-}$  mice was increased 1.4 fold, compared to that of  $\text{Rim2}\alpha^{+/+}$  mice. Both glucose-induced and  $\text{Ca}^{2+}$ -triggered insulin secretion (assessed by 60 mM  $\text{K}^{+}$  stimulation) from pancreatic islets of  $\text{Rim2}\alpha^{-/-}$  mice were significantly decreased, compared to those from pancreatic islets of  $\text{Rim2}\alpha^{+/+}$  mice (Figure 2B). We then examined insulin secretion potentiated by cAMP in pancreatic islets of  $\text{Rim2}\alpha^{-/-}$  mice. GIP-potentiated insulin secretion from pancreatic islets of  $\text{Rim2}\alpha^{-/-}$  mice was significantly lower than that of  $\text{Rim2}\alpha^{+/+}$  mice (Figure 2C). The cAMP analog 8-Bromo-cAMP-potentiated insulin secretion also was significantly reduced in pancreatic islets of  $\text{Rim2}\alpha^{-/-}$  mice. These results strongly suggest that  $\text{Rim2}\alpha$  plays a major role in insulin secretion.

### **The number of docked insulin granules is decreased in pancreatic $\beta$ -cells of $\text{Rim2}\alpha^{-/-}$ mice**

We investigated the role of  $\text{Rim2}\alpha$  in insulin granule exocytosis. For this purpose, we analyzed the dynamics of insulin granules in living pancreatic  $\beta$ -cells using total internal reflection fluorescence microscopy (TIRFM) (Shibasaki et al., 2007). We confirmed that the modes of insulin granule exocytosis can be classified into three groups depending on the dynamics of the insulin granules: 1) fusion events involving granules that are predocked to the plasma membrane (*old face*); 2) fusion events involving granules that are newly recruited and immediately fused to the plasma membrane without docking (*restless newcomer*); and 3) fusion events involving granules that are newly recruited, docked, and then fused to the plasma membrane (*resting newcomer*).

As shown in Figure 3A, the number of docked insulin granules in pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice was markedly decreased, compared to that in pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{+/+}$  mice. We have previously shown that most  $\text{K}^{+}$ -induced fusion events involve *old face* (Shibasaki et al., 2007). As expected, the number of fusion events caused by  $\text{K}^{+}$  stimulation in pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice was significantly decreased due to the marked reduction in the number of *old face* (Figure 3B). In contrast, glucose-induced fusion events comprised mainly *restless newcomer*. Fusion events, mainly in the first phase, were markedly decreased in pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice, and those

in the second phase were also decreased (Figure 3C). *Resting newcomer*, which docks to the plasma membrane before fusion, were not detected in pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice. These results indicate that  $\text{Rim2}\alpha$  is required in normal regulation of insulin granule exocytosis.

### **The defect in insulin granule exocytosis in $\text{Rim2}\alpha^{-/-}$ mice is not due to changes in expression or distribution of exocytosis-associated proteins**

We intended to examine the expression and subcellular localization of exocytosis-associated proteins as a consequence of  $\text{Rim2}\alpha$  deficiency, using pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice. However, the limited number of isolated pancreatic  $\beta$ -cells of these mice made such an experiment impossible. We therefore established clonal pancreatic  $\beta$ -cells lacking  $\text{Rim2}\alpha$  ( $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells) by crossbreeding  $\text{Rim2}\alpha^{-/-}$  mice and *IT6* mice expressing simian virus 40 large T antigen under human insulin promoter (Miyazaki et al., 1990). The absence of  $\text{Rim2}\alpha$  expression in  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells was confirmed by immunocytochemical and immunoblot analyses using anti-Rim2 antibody (Figure 4A and 4B).

To determine whether deficiency of  $\text{Rim2}\alpha$  affects the expression levels and localization of exocytosis-associated proteins, we compared those of the proteins in MIN6 cells and  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells. We found no significant differences in the expression levels of any of the proteins examined (Figure 4B). The expression levels did not differ in  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells expressing wild-type (WT)  $\text{Rim2}\alpha$  by adenovirus-mediated gene transfer. We examined localization of  $\text{Rim2}\alpha$  and exocytosis-associated proteins in MIN6 cells by immunostaining and subcellular fractionation experiments.  $\text{Rim2}\alpha$  was localized on both insulin granules and plasma membrane (Figure 4A and 4C), whereas Rab3A, Munc13-1, and Syntaxin1 were localized on the plasma membrane (Figure 4C). The localizations of these proteins were the same in  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells.

We then examined insulin secretion in  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells. Both glucose- and  $\text{K}^+$ -induced insulin secretion from  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells were significantly reduced, compared to those from MIN6 cells (Figure 4D). Similarly to the pancreatic  $\beta$ -cells of  $\text{Rim2}\alpha^{-/-}$  mice, the number of docked insulin



granules was significantly decreased in  $\text{Rim2}\alpha^{\text{ko/ko}}$   $\beta$ -cells (Figure 4E). Importantly, the defects in both insulin secretion and the number of docked insulin granules were rescued when WT  $\text{Rim2}\alpha$  was exogenously introduced by adenovirus-based gene transfer (Figure 4D and 4E). These results indicate that  $\text{Rim2}\alpha$  is required for the docking step in insulin granule exocytosis.

### **$\text{Rim2}\alpha$ , Rab3A, and Munc13-1 form a complex regulated by glucose**

Because the expression levels and localization of exocytosis-associated proteins in  $\text{Rim2}\alpha^{\text{ko/ko}}$   $\beta$ -cells did not differ from those in MIN6 cells, we explored the possibility that the interaction of  $\text{Rim2}\alpha$  and  $\text{Rim2}\alpha$ -binding proteins is critical for regulated exocytosis of insulin granules. We performed immunoprecipitation experiments on the interaction of  $\text{Rim2}\alpha$  with Rab3A or Munc13-1 by glucose stimulation. The interaction of  $\text{Rim2}\alpha$  and Munc13-1 was decreased by glucose stimulation (Figure 4F and Figure S2). However, no interaction of  $\text{Rim2}\alpha$  and endogenous Rab3A was detected (Figure 4F). This suggests that most of the endogenous Rab3A is likely to be in the GDP-inactivated state since  $\text{Rim2}\alpha$  binds to the GTP-activated form of Rab3A (Ozaki et al., 2000). In fact, we found that  $\text{Rim2}\alpha$  interacted with the GTP-activated form of Rab3A (Figure 4F). Therefore, we examined the effect of the active form of Rab3A on the interaction of  $\text{Rim2}\alpha$  and Munc13-1. The interaction of  $\text{Rim2}\alpha$  and Munc13-1 in the presence of the constitutively active form of Rab3A (Q81L) (Brondyk et al., 1993) was detected in a glucose concentration-independent manner (Figure 4F and Figure S2). These results indicate that Munc13-1 dissociates from the  $\text{Rim2}\alpha$ /Munc13-1 complex by glucose stimulation.

### **$\text{Rim2}\alpha$ is critical for docking and priming of insulin granules**

The finding of differences in the interactions with these three molecules indicates that  $\text{Rim2}\alpha$  is involved in the recruitment, docking, and/or priming steps. Rab3A and Munc13-1 have been shown to interact with the N-terminal region of  $\text{Rim2}\alpha$  (Dulubova et al., 2005). To determine whether the impaired insulin secretion in  $\text{Rim2}\alpha^{\text{ko/ko}}$   $\beta$ -cells is caused by defective interaction of  $\text{Rim2}\alpha$  with

Rab3A, Munc13-1, or both of them, we prepared a double mutant (E36A/R37S) of Rim2 $\alpha$  that cannot bind to Rab3A but can bind to Munc13-1 (Fukuda, 2004). Using this mutant, we examined insulin secretion and the number of docked insulin granules in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. Although the mutant Rim2 $\alpha$  (E36A/R37S) was able to restore both glucose- and K<sup>+</sup>-induced insulin secretion (Figure 5A upper left panel and Figure 6A), the mutant Rim2 $\alpha$  (E36A/R37S) was unable to restore the number of docked insulin granules (Figure 5A upper right panel), suggesting that the interaction of Rim2 $\alpha$  and Rab3A is required for docking of the insulin granules but not for fusion of the granules to the plasma membrane. Interestingly, because K<sup>+</sup>-induced insulin secretion in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells was significantly higher than that by WT Rim2 $\alpha$  gene transfer, the interaction of Rim2 $\alpha$  and Rab3A may negatively regulate insulin secretion (Figure 5A upper panels, lower left panel and Figure 6A), that is, docking may be a state preventing fusion (i.e., a braking state).

We then prepared a double mutant (K136E/K138E) of Rim2 $\alpha$  that cannot bind to Munc13-1 but can bind to Rab3A (Dulubova et al., 2005). Although the mutant Rim2 $\alpha$  (K136E/K138E) was unable to restore either glucose- or K<sup>+</sup>-induced insulin secretion (Figure 5A, upper left panel and Figure 6A), the mutant was able to restore the number of docked insulin granules (Figure 5A, upper right panel). These results suggest that interaction of Rim2 $\alpha$  and Munc13-1 after the docking step is required for insulin granule exocytosis (Figure 5A, upper panels, lower right panel and Figure 6A).

To examine whether Rim2 $\alpha$  enhances Munc13-1 activity in pancreatic  $\beta$ -cells, we evaluated insulin secretion in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells by phorbol 12-myristate 13-acetate (PMA), which is known to enhance Munc13-1 activity in pancreatic  $\beta$ -cells (Betz et al., 1998; Kang et al., 2006). Glucose-induced insulin secretion was restored in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells in the presence of PMA (Figure 5B, left panel and Figure 6B). However, the number of docked insulin granules was not increased in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells (Figure 5B, middle panel). These results suggest that Rim2 $\alpha$  directly activates Munc13-1 and that it regulates post-docking steps of insulin granule exocytosis (Figure 5B, right panel).

Activation of Munc13-1 is proposed to change Syntaxin1 from the closed state to the open state,

initiating the priming step of granule exocytosis (Madison et al., 2005). Thus, Syntaxin1 might not be activated in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. If that is the case, introduction of exogenous open Syntaxin1 into Rim2 $\alpha^{ko/ko}$   $\beta$ -cells should bypass the interaction of Rim2 $\alpha$  and Munc13-1 to stimulate insulin secretion directly. To investigate this possibility, we examined insulin secretion and the number of docked insulin granules in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells after gene transfer of open Syntaxin1 (Dulubova et al., 1999). Open Syntaxin1 fully restored both glucose- and K<sup>+</sup>-induced insulin secretion, but WT Syntaxin1 did not restore it (Figure 5C left panel and Figure 6C). In contrast, both forms of Syntaxin1 were unable to restore the number of docked insulin granules (Figure 5C, middle panel). These findings indicate that interaction of Rim2 $\alpha$  and Munc13-1 is required in the process of exocytosis between the docking and the fusion steps, suggesting that such interaction underlies priming of insulin granules.

### **Rim2 $\alpha$ is required for Epac2-potentiated insulin secretion**

Rim2 $\alpha$  was originally identified as a molecule that interacts with Epac2 (Ozaki et al., 2000). Because Epac2 is responsible for cAMP-induced, PKA-independent exocytosis, interaction of Rim2 $\alpha$  and Epac2 is thought to be involved in cAMP-potentiated insulin secretion (Ozaki et al., 2000; Kashima et al., 2001). To clarify the role of Rim2 $\alpha$  in cAMP-potentiated insulin secretion, we utilized Rim2 $\alpha^{ko/ko}$   $\beta$ -cells, preparing a triple mutant (R682A, L688A, and G689A) (PDZ-AAA) of Rim2 $\alpha$  that disrupts the interaction of Rim2 $\alpha$  and Epac2 (Shibasaki et al., 2004). This mutant Rim2 $\alpha$  (PDZ-AAA) significantly rescued, although not completely, glucose-induced insulin secretion. Importantly, the glucose-induced insulin secretion potentiated by the Epac specific cAMP analog 8-pCPT-2'-O-Me-cAMP-AM (Vliem et al., 2008) failed to potentiate insulin secretion in these cells (Figure 7). These results make clear that Rim2 $\alpha$  mediates cAMP-induced, Epac2-dependent insulin secretion.

### **Rim2 $\alpha$ deficiency causes multiple defects in hormone secretion**

Systemic analysis of offspring from heterozygous mating revealed that  $\text{Rim2}\alpha^{-/-}$  mice were smaller than littermate  $\text{Rim2}\alpha^{+/+}$  mice (Figure S1K). Both basal and hypoglycemia-induced GH secretion were markedly reduced in  $\text{Rim2}\alpha^{-/-}$  mice (Figure S1L), and insulin-like growth factor-1 (IGF-1) levels were decreased in the mice (Figure S1M). In addition, hypoglycemia-induced epinephrine secretion was significantly decreased in  $\text{Rim2}\alpha^{-/-}$  mice (Figure S1N). In contrast, hypoglycemia-induced glucagon secretion was significantly increased in  $\text{Rim2}\alpha^{-/-}$  mice (Figure S1O), a finding consistent with the increase in the number of  $\alpha$ -cells. Taken together, these results show that  $\text{Rim2}\alpha$  is required for normal regulation of hormone secretion associated with glucose homeostasis.

## Discussion

$\text{Rim1}\alpha$  is a multidomain adaptor protein that was discovered as a putative effector of Rab3 (Wang et al., 1997). The null mutation of Rim in *C. elegans* was shown to decrease the number of fusion-competent vesicles, suggesting a role in the post-docking process (Koushika et al., 2001). We previously identified  $\text{Rim2}\alpha$  as a molecule interacting with Epac2, and found that it was required for cAMP-dependent, PKA-independent insulin secretion *in vitro* (Ozaki et al., 2000; Kashima et al., 2001). However, the role of  $\text{Rim2}\alpha$  in exocytosis has not been clarified. To address this issue, we generated  $\text{Rim2}\alpha^{-/-}$  mice. In the course of our study, Schoch et al. reported the phenotype of  $\text{Rim2}\alpha^{-/-}$  mice, which they generated independently, but no changes in blood glucose levels in  $\text{Rim2}\alpha^{-/-}$  mice were found (Schoch et al., 2006). In the present study, we have analyzed the phenotype of  $\text{Rim2}\alpha^{-/-}$  mice in detail both *in vivo* and *in vitro*.

We recently reported that  $\text{K}^+$ -induced exocytosis comprises mainly granules already docked to the plasma membrane (*old face*), while glucose-induced exocytosis consists almost exclusively of granules that are newly recruited to the plasma membrane (*restless newcomer*) (Shibasaki et al., 2007). In the present study, we show that a mutant  $\text{Rim2}\alpha$  (E36A/R37S) that does not bind to Rab3A did not rescue docked granules in  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells, but was able to rescue glucose-induced insulin

secretion to a degree similar to that rescued by WT Rim2 $\alpha$ . This finding indicates that although docking of insulin granules to the plasma membrane requires interaction of Rim2 $\alpha$  and Rab3A, it is not essential for glucose-induced insulin secretion. Indeed, despite the defect in docking, K<sup>+</sup>-induced insulin secretion is significantly increased in granuphilin<sup>-/-</sup> mice (Gomi et al., 2005).

It has been thought that the priming and fusion steps occur after the docking step in neuronal cells (Verhage et al., 2008). The priming step is proposed to be initiated by unfolding of the SNARE protein Syntaxin1 from the closed to the open state, which can bind SNAP25 and VAMP2, which are other SNARE proteins, to form the SNARE complex. The mutant Syntaxin1 (L166A/E167A), which is a constitutively open form (Dulubova et al., 1999), can rescue the fusion of synaptic vesicles in *C. elegans* Syntaxin null mutants (Richmond et al., 2001). Although Munc13-1 is thought to be a priming factor in large dense-core and synaptic vesicles, and the interaction of Rim1 $\alpha$  and Munc13-1 is thought to regulate priming (Ashery et al., 2000; Betz et al., 2001), the molecular mechanism of the priming step has not been clarified. In Rim1 $\alpha$ <sup>-/-</sup> brain, the expression level of Munc13-1 is decreased (Schoch et al., 2002), and Munc13-1 is not enriched at the active zone of mossy fiber terminals of mouse hippocampus (Andrews-Zwilling et al., 2006). We found that both the expression level and the localization of Munc13-1 are not changed in Rim2 $\alpha$ <sup>ko/ko</sup>  $\beta$ -cells, but that Munc13-1 dissociates from the Rim2 $\alpha$ -Munc13-1 complex by glucose stimulation and activates Syntaxin1 to evoke fusion. Rim2 $\alpha$  is thus a critical molecule in determining both the docking and priming states in accord with its interacting partner Rab3A or Munc13-1, respectively (Figure S3).

We previously showed that Rim2 $\alpha$  is involved in cAMP-induced, PKA-independent insulin secretion through the Epac2 pathway (Ozaki et al., 2000; Kashima et al., 2001). We show here that glucose-induced insulin secretion potentiated by the Epac-specific analog 8-pCPT-2'-O-Me-cAMP-AM is not induced in Rim2 $\alpha$ <sup>ko/ko</sup>  $\beta$ -cells expressing mutant Rim2 $\alpha$  (PDZ-AAA). This further confirms that Rim2 $\alpha$  is required in the Epac2 pathway for potentiation of insulin secretion by cAMP. Because Epac2 also activates Rap1 by cAMP, which may increase the size of the non-docked granule pool and/or facilitate the recruitment of granules to the plasma

membrane (Shibasaki et al., 2007, Seino et al., 2009), both Rim2 $\alpha$  and Rap1 act cooperatively in cAMP-induced, Epac2 dependent insulin secretion.

In this study, we also found that Rim2 $\alpha$  exerts a suppressive effect on the voltage dependence of inactivation of voltage-dependent Ca<sup>2+</sup> channel (VDCC) currents, and controls the intracellular Ca<sup>2+</sup> concentration in pancreatic  $\beta$ -cells (Figure S4), as was found of Rim1 $\alpha$  in neurons (Kiyonaka et al., 2007). Rim2 $\alpha$  has been shown to interact with VDCCs directly or indirectly (Hibino et al., 2002; Shibasaki et al., 2004). It is generally accepted that neuronal secretion represents vesicle fusion in response to Ca<sup>2+</sup> microdomains, which requires close proximity of the vesicles to the sites of focal Ca<sup>2+</sup> entry (Llinas et al., 1992; Barg et al., 2001; Hoppa et al., 2009). These findings together with the role of Rim2 $\alpha$  further suggest the importance of the Ca<sup>2+</sup> microdomain for insulin granule exocytosis in pancreatic  $\beta$ -cells. Rim2 $\alpha$  is expressed predominantly in endocrine and neuroendocrine cells (Ozaki et al., 2000). Since we found defects in the secretion of various hormones including GIP, GH, and epinephrine, all of which are stored as dense-core vesicles, Rim2 $\alpha$  might well play a similar role in exocytosis of these hormones.

## **Experimental procedures**

### **Reagents**

A full list of reagents is provided in Supplemental Experimental Procedures.

### **Generation of Rim2 $\alpha$ <sup>-/-</sup> mice**

Rim2 $\alpha$ <sup>-/-</sup> mice were generated by replacing the amino acid coding sequences in exon 4 of mouse Rim2 with Neo cassette as described (Figure S1). Detailed description and genotyping strategy are provided in Supplemental Experimental Procedures.

### **Generation of Rim2 $\alpha$ -deficient clonal pancreatic $\beta$ -cells**

Clonal pancreatic  $\beta$ -cells lacking Rim2 $\alpha$  (Rim2 $\alpha^{ko/ko}$   $\beta$ -cells) were established by crossbreeding Rim2 $\alpha^{-/-}$  mice and *IT6* mice expressing SV40 large T antigen under human insulin promoter that developed highly differentiated  $\beta$ -cell tumors (Miyazaki et al., 1990). Twenty-seven lines of Rim2 $\alpha^{ko/ko}$   $\beta$ -cells were generated from a 10-week-old mouse lacking Rim2 $\alpha$  and carrying a large T antigen load (Rim2 $\alpha^{-/-}$ ; *IT6* mouse) as previously described (Shibasaki et al., 2007).

### Cell culture

MIN6 cells, Rim2 $\alpha^{ko/ko}$   $\beta$ -cells, and COS-1 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal bovine serum and maintained in a humidified incubator with 95% air and 5% CO<sub>2</sub> at 37°C.

### RNA extraction and quantitative RT-PCR

Total RNA from mouse pancreatic islets and  $\beta$ -cell lines was isolated using the RNeasy Kit. For reverse transcription, ReverTra Ace- $\alpha$ -Kit was used. Quantitative real-time PCR was carried out by SYBR Premix Ex Taq<sup>TM</sup> with the primers for mouse Rim2 $\alpha$  (forward 5'-GGAAAATCATCCTGGCTGTC and reverse 5'-ATGTCACCTGGCAATCTGGTG), mouse Rim2 $\beta$  (forward 5'-ACGAAGTCCATCAGTGTCCA and reverse 5'-GCTCAGACCATTCCAAATCC), and HPRT (forward 5'-TCTTTGCTGACCTGCTGGATT and reverse 5'-GGCTTTGTATTTGGCTTTTCC) using a model 7000 thermal cycler (Applied Biosystems, Foster City, CA). HPRT was used as internal control (Figure S1).

### *In vivo* experiments

Glucose (1.5 g/kg body weight), mixed meal (Twinline: 10 ml/kg body weight), or insulin (0.4 IU/kg body weight) was administered intraperitoneally or orally to overnight (16 h)-fasted male mice at 16-22 weeks of age as described previously (Miki et al., 2005). Blood glucose levels were measured by Antsense III glucose analyzer (Bayer Yakuhi, Osaka, Japan); ELISA system was used for

measurement of serum insulin (Morinaga, Tokyo, Japan), GIP and GH (LINCO Research, St. Charles, MO), and IGF-1 (R&D Systems, Minneapolis, MN). Measurements of epinephrine levels were performed by SRL (Tokyo, Japan).

### **Immunohistological analysis**

The pancreases were removed from  $\text{Rim2}\alpha^{+/+}$  and  $\text{Rim2}\alpha^{-/-}$  mice, and were immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Fixed tissues were dehydrated and embedded in paraffin by conventional procedure. Five-micrometer-thick paraffin sections were stained with guinea pig anti-insulin antibody and mouse anti-glucagon antibody, followed by Alexa Fluor 546-conjugated goat anti-guinea pig IgG antibody and Alexa Fluor 488-conjugated goat anti-mouse IgG antibody, respectively. The immunostained tissues were observed by BZ9000 microscope (Keyence, Osaka, Japan).

### **Immunocytochemical analysis**

MIN6 cells and  $\text{Rim2}\alpha^{ko/ko}$   $\beta$ -cells were fixed with 3.7% formaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min at room temperature and thoroughly rinsed with 0.1 M PBS. After the samples were pretreated with 0.1% Triton X-100 and 10% normal goat serum, they were incubated with guinea pig anti-insulin antibody and rabbit anti-Rim2 antibody, followed by Alexa Fluor 546-conjugated goat anti-guinea pig IgG antibody and Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody, respectively. The immunostained cells were observed by confocal laser scanning microscopy (FV1000; Olympus, Tokyo, Japan).

### **Insulin secretion experiments**

Pancreatic islets were isolated from  $\text{Rim2}\alpha^{+/+}$  and  $\text{Rim2}\alpha^{-/-}$  mice by collagenase digestion and cultured for 2 days as described previously (Kashima et al., 2001). Thirty min after preincubation of isolated islets with HEPES-KRB buffer containing 2.8 mM glucose, five size-matched islets were



collected in each well of a 96-well plate and incubated for 30 min in 100  $\mu$ l of the same buffer containing various stimuli. Insulin released in the incubation buffer and cellular insulin content were measured by ELISA (Medical Biological Laboratories, Nagoya, Japan). The amount of insulin secretion was normalized by cellular insulin content. Insulin secretion experiments in MIN6 cells and Rim2 $\alpha^{ko/ko}$   $\beta$ -cells were performed as described above.

### **TIRFM analysis**

Primary cultured  $\beta$ -cells isolated from mouse pancreatic islets were infected with adenovirus carrying insulin-Venus and subjected to analysis by TIRFM as previously described (Shibasaki et al., 2007). The number of granules docked to the plasma membrane was measured by G-Count software (G-Angstrom K.K, Miyagi, Japan).

### **Subcellular fractionation**

Discontinuous sucrose gradient fractionation of MIN6 cells and Rim2 $\alpha^{ko/ko}$   $\beta$ -cells were carried out as previously described (Sugawara et al., 2009).

### **Co-immunoprecipitation experiments**

Two days before co-immunoprecipitation experiments, COS-1 cells were transiently transfected with plasmids encoding FLAG-tagged Rab3A (Q81L) using Effectene Transfection Reagent (Qiagen) according to the manufacturer's instruction. Co-immunoprecipitation experiments were performed using Pierce Mammalian c-Myc Tag IP/Co-IP Kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacture's instruction. Briefly, Rim2 $\alpha^{ko/ko}$   $\beta$ -cells were infected with Ad- $\beta$ -galactosidase ( $\beta$ -gal) or Ad-Rim2 $\alpha$ . After 2-day culture, the infected cells were preincubated in HEPES-KRB buffer containing 2.8 mM glucose. Thirty min after preincubation, the cells were stimulated with HEPES-KRB buffer containing 2.8 mM glucose or 25 mM glucose for 30 min. The cells were then lysed in buffer containing 10 mM HEPES (pH 7.4), 150 mM NaCl, 0.5% CHAPS, 5

mM MgCl<sub>2</sub>, and protease inhibitor cocktail, and collected in microcentrifuge tubes. The cellular lysates were incubated with 10 µl anti-c-Myc antibody conjugated with agarose in the absence or presence of cellular lysate of COS-1 transfected with FLAG-tagged Rab3A (Q81L). After incubation at 4°C for overnight, the agarose was washed five times with buffer containing 25 mM Tris-HCl, 150 mM NaCl, and 0.05% Tween 20 (pH7.2), and the bound proteins were subjected to SDS-PAGE followed by immunoblot analysis with anti-Rim2, anti-Munc13, and anti-Rab3 antibodies.

### **Construction of adenovirus vectors**

Rat Rab3A was subcloned into pFLAG-CMV-2 vector (Sigma). Site-directed mutagenesis of the N-terminal Zn<sup>2+</sup>-finger domain (E36A/R37S and K136E/K138E mutants) and PDZ domain (PDZ-AAA mutant) in mouse Rim2α and L166A/E167A mutant in mouse Syntaxin1 was performed by the PCR-based method. Recombinant adenovirus carrying c-Myc tagged WT Rim2α (Ad-Rim2α) or c-Myc tagged mutants of Rim2α (Ad-Rim2α mutants) were generated according to the manufacturer's instruction (Stratagene, La Jolla, CA).

### **Perifusion experiments**

Perifusion experiments on insulin secretion of Rim2α<sup>ko/ko</sup> β-cells were performed as described previously (Sugawara et al., 2009). Briefly, the cells were seeded at a density of  $5 \times 10^5$  cells on coverslips. The following day, the cells were infected with Ad-β-gal or Ad-Rim2α mutants or Ad-Syntaxin1 at an MOI of 1 and maintained for 48 h. The cells were then incubated in HEPES-KRB containing 2.8 mM glucose for 50 min and mounted in a perifusion chamber. The cells were perifused in KRBH containing 2.8 mM glucose for 5 min, and the perfusate was then switched to HEPES-KRB containing 25 mM glucose or 60 mM K<sup>+</sup>. Eluted fractions were collected at 1-min intervals, and released insulin in each fraction was measured by insulin assay kit (CIS bio international, Gif sur Yvette, France). The amount of secreted insulin was normalized by cellular insulin contents.

## Statistical Analysis

The data are expressed as means  $\pm$  SEM. Comparisons were made using Student's unpaired *t*-test, Dunnet's method, or Tukey-Kramer's method as indicated in the legends. A probability level of  $p < 0.05$  was considered statistically significant.

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## Figure Legends

### Figure 1. Impaired insulin and GIP secretion in $Rim2\alpha^{-/-}$ mice

(A) Changes in blood glucose levels (left) and serum insulin levels (right) after intraperitoneal glucose load in  $Rim2\alpha^{+/+}$  mice (white) and  $Rim2\alpha^{-/-}$  mice (black).

(B) Changes in blood glucose levels (left) and serum insulin levels (right) after oral glucose load in  $Rim2\alpha^{+/+}$  mice (white) and  $Rim2\alpha^{-/-}$  mice (black).

(C) Changes in blood glucose levels (left) and serum insulin levels (right) after mixed meal load in  $Rim2\alpha^{+/+}$  mice (white) and  $Rim2\alpha^{-/-}$  mice (black).

(D) GIP secretion after mixed meal load in  $Rim2\alpha^{+/+}$  mice (white) and  $Rim2\alpha^{-/-}$  mice (black).

Data were obtained from 3 independent experiments ( $n = 5-19$ ) and expressed as means  $\pm$  SEM. \* $p < 0.01$  (Student's unpaired  $t$ -test).

See also Figure S1.

### Figure 2. Impaired insulin secretion in pancreatic islets of $Rim2\alpha^{-/-}$ mice

(A) Insulin contents in pancreatic islets of  $Rim2\alpha^{+/+}$  mice (white) and  $Rim2\alpha^{-/-}$  mice (black).

(B) Insulin secretion in response to glucose and 60 mM K<sup>+</sup> in pancreatic islets of Rim2 $\alpha$ <sup>+/+</sup> mice (white) and Rim2 $\alpha$ <sup>-/-</sup> mice (black).

(C) Effects of cAMP-increasing agents on insulin secretion in pancreatic islets of Rim2 $\alpha$ <sup>+/+</sup> mice (white) and Rim2 $\alpha$ <sup>-/-</sup> mice (black).

Data were obtained from 2-3 independent experiments (n = 5-9) and expressed as means  $\pm$  SEM. \*p < 0.01 (Student's unpaired *t*-test).

### Figure 3. Rim2 $\alpha$ -mediated insulin granule exocytosis

(A) Comparison of the number of insulin granules docked to the plasma membrane in pancreatic  $\beta$ -cells of Rim2 $\alpha$ <sup>+/+</sup> and Rim2 $\alpha$ <sup>-/-</sup> mice. Primary cultured pancreatic  $\beta$ -cells were preincubated with HEPES-KRB buffer containing 2.8 mM glucose at 37°C for 30 min, fixed, immunostained with anti-insulin antibody and observed by TIRFM. The surrounding lines represent the outline of a cell attached to the cover glass. The number of docked granules was measured in a cell surface area of 200  $\mu\text{m}^2$ . Scale bar, 10  $\mu\text{m}$ . Data were obtained from 3 independent experiments (n = 8) and expressed as means  $\pm$  SEM. \*p < 0.01 (Student's unpaired *t*-test).

(B) Histogram of fusion events at 30-sec intervals in pancreatic  $\beta$ -cells of Rim2 $\alpha$ <sup>+/+</sup> (left) and Rim2 $\alpha$ <sup>-/-</sup> (right) mice stimulated with 60 mM K<sup>+</sup> in a cell surface area of 200  $\mu\text{m}^2$ . Primary cultured pancreatic  $\beta$ -cells were preincubated with HEPES-KRB buffer containing 2.8 mM glucose at 37°C for 30 min. Thirty seconds after acquisition of the image, the primary cultured cells were stimulated with 60 mM K<sup>+</sup>. 2.8 indicates 2.8 mM glucose. *Old face* (Blue): granules that are predocked to the plasma membrane and fused to the membrane by stimulation. *Restless newcomer* (Red): granules that are newly recruited and immediately fused to the plasma membrane by stimulation. *Resting newcomer* (Green): granules that are newly recruited, docked, and fused to the plasma membrane by stimulation. Data were obtained from 5-6 independent experiments (n = 6-7) and expressed as means  $\pm$  SEM.

(C) Histogram of fusion events in pancreatic  $\beta$ -cells of Rim2 $\alpha$ <sup>+/+</sup> (left) and Rim2 $\alpha$ <sup>-/-</sup> (right) mice

stimulated with 25 mM glucose in a cell surface area of 200  $\mu\text{m}^2$ . Fusion events were analyzed as shown in (B). Data were obtained from 5-6 independent experiments ( $n = 6-7$ ) and expressed as means  $\pm$  SEM.

#### **Figure 4. Generation and characterization of Rim2 $\alpha^{ko/ko}$ $\beta$ -cells**

(A) Immunocytochemical analysis of MIN6 cells and Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. Green, Rim2; Red, Insulin. Scale bar, 5  $\mu\text{m}$ .

(B) Comparison of expression levels of exocytosis-related proteins among MIN6 cells, Rim2 $\alpha^{ko/ko}$   $\beta$ -cells, and Rim2 $\alpha^{ko/ko}$   $\beta$ -cells expressing WT Rim2 $\alpha$ .

(C) Subcellular localization of exocytosis-related proteins in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. Rim2 $\alpha^{ko/ko}$   $\beta$ -cells were homogenized, and organelles in the supernatants were separated on discontinuous sucrose gradients. Fractions were subjected to immunoblot analysis with anti-Na<sup>+</sup>-K<sup>+</sup>-ATPase  $\alpha$ -1 (plasma membrane marker), Chromogranin-A (large dense-core granule marker), Rim2, Rab3A, Munc13-1, and Syntaxin1.

(D) Insulin secretion from Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. Data were obtained from 3 independent experiments ( $n = 7-11$ ) and expressed as means  $\pm$  SEM. \* $p < 0.01$  vs. corresponding values in Ad- $\beta$ -gal (Student's unpaired  $t$ -test).

(E) The number of insulin granules docked to the plasma membrane in Rim2 $\alpha^{ko/ko}$   $\beta$ -cells. Data were obtained from 3 independent experiments ( $n = 11-16$ ) and expressed as means  $\pm$  SEM. \* $p < 0.01$  vs. Ad- $\beta$ -gal (Student's unpaired  $t$ -test).

(F) Effect of glucose on the interaction of Rim2 $\alpha$  and Rab3A or Munc13-1. After Rim2 $\alpha^{ko/ko}$   $\beta$ -cells expressing c-Myc-tagged WT Rim2 $\alpha$  were stimulated with 25 mM glucose for 30 min, their lysates were incubated with lysates of COS-1 cells expressing FLAG-tagged Rab3A (Q81L). Their mixture was subjected to immunoprecipitation with anti-c-Myc antibody-conjugated agarose and then to immunoblot analysis with anti-Rim2, Rab3A, and Munc13-1 antibodies.

See also Figure S2.

**Figure 5. Rescue experiments of insulin secretion and the number of docked insulin granules in  $Rim2\alpha^{ko/ko}$   $\beta$ -cells**

(A) Effects of mutant  $Rim2\alpha$  on insulin secretion and the number of insulin granules docked to the plasma membrane. Upper left panel, glucose- and  $K^+$ -induced insulin secretion in  $Rim2\alpha^{ko/ko}$   $\beta$ -cells expressing mutant  $Rim2\alpha$ . Upper right panel, the number of insulin granules docked to the plasma membrane in  $Rim2\alpha^{ko/ko}$   $\beta$ -cells expressing mutant  $Rim2\alpha$ . In the absence of interaction of  $Rim2\alpha$  and Rab3A, insulin granule exocytosis is rather enhanced because the interaction induces docking of insulin granules, which prevents fusion to the plasma membrane (braking) (lower left). In contrast, mutant  $Rim2\alpha$  that cannot bind to Munc13-1 but can bind to Rab3A allows the granules to dock. Since priming is not initiated in this state, fusion events do not occur (lower right). \* $p < 0.01$  vs. corresponding values in Ad- $\beta$ -gal (Dunnett's method). † $p < 0.01$  vs. corresponding values in Ad-WT  $Rim2\alpha$  (Tukey-Kramer method).

(B) Effect of PMA stimulation on insulin secretion (left panel) and the number of insulin granules docked to the plasma membrane (middle panel). Right panel, model for insulin granule exocytosis in  $Rim2\alpha^{ko/ko}$   $\beta$ -cells stimulated with PMA. \* $p < 0.01$  vs. corresponding values in the absence of PMA (Student's unpaired  $t$ -test).

(C) Effect of Syntaxin1 on insulin secretion (left panel) and the number of insulin granules docked to the plasma membrane (middle panel). Right panel, model for insulin granule exocytosis in  $Rim2\alpha^{ko/ko}$   $\beta$ -cells expressing open Syntaxin1. \* $p < 0.01$  vs. corresponding values in Ad- $\beta$ -gal (Dunnett's method).

Data were obtained from 3 independent experiments ( $n = 7-11$ ) and expressed as means  $\pm$  SEM.

Red,  $Rim2\alpha$ ; Blue, Rab3A; Green, Munc13-1.

See also Figure S3.

**Figure 6. Insulin secretion from perifused  $Rim2\alpha^{ko/ko}$   $\beta$ -cells**

(A) Effects of mutant Rim2 $\alpha$  on insulin secretion. In the absence of interaction of Rim2 $\alpha$  and Rab3A, K<sup>+</sup>-induced insulin secretion is enhanced compared to that by WT Rim2 $\alpha$  gene transfer. In contrast, mutant Rim2 $\alpha$  that cannot bind to Munc13-1 does not restore either glucose- or K<sup>+</sup>-induced insulin secretion.

(B) Effect of PMA stimulation on insulin secretion. Insulin secretion is induced by PMA even in the absence of Rim2 $\alpha$ .

(C) Effect of Syntaxin1 on insulin secretion induced by glucose (left) and K<sup>+</sup> (right). Open Syntaxin1 rescues both glucose- and K<sup>+</sup>-induced insulin secretion.

Data were obtained from 2-3 independent experiments (n = 4-5) and expressed as means  $\pm$  SEM.

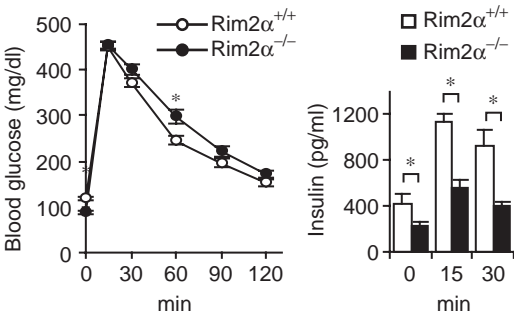
See also Figure S4.

### **Figure 7. Effect of Rim2 $\alpha$ (PDZ-AAA) on Epac2-mediated exocytosis**

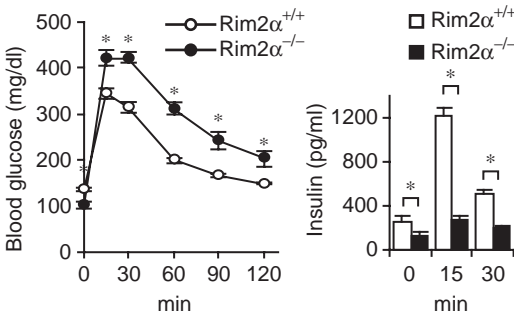
Rim2 $\alpha^{ko/ko}$   $\beta$ -cells expressing Rim2 $\alpha$  (PDZ-AAA) were stimulated with 11.1 mM glucose plus 10  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM. Data were obtained from 3 independent experiments (n = 8-11) and expressed as means  $\pm$  SEM. \*p < 0.01 (Dunnett's method).

Figure 1

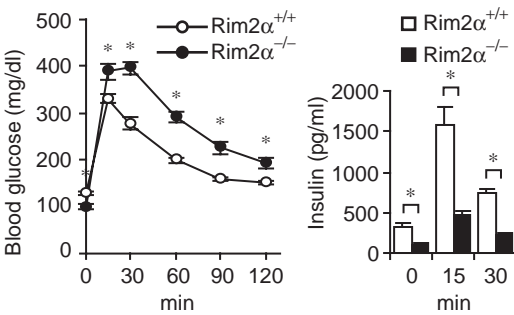
A



B



C



D

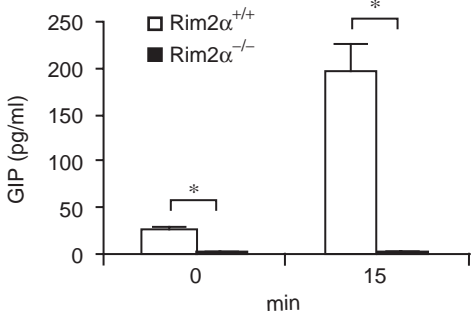


Figure 2

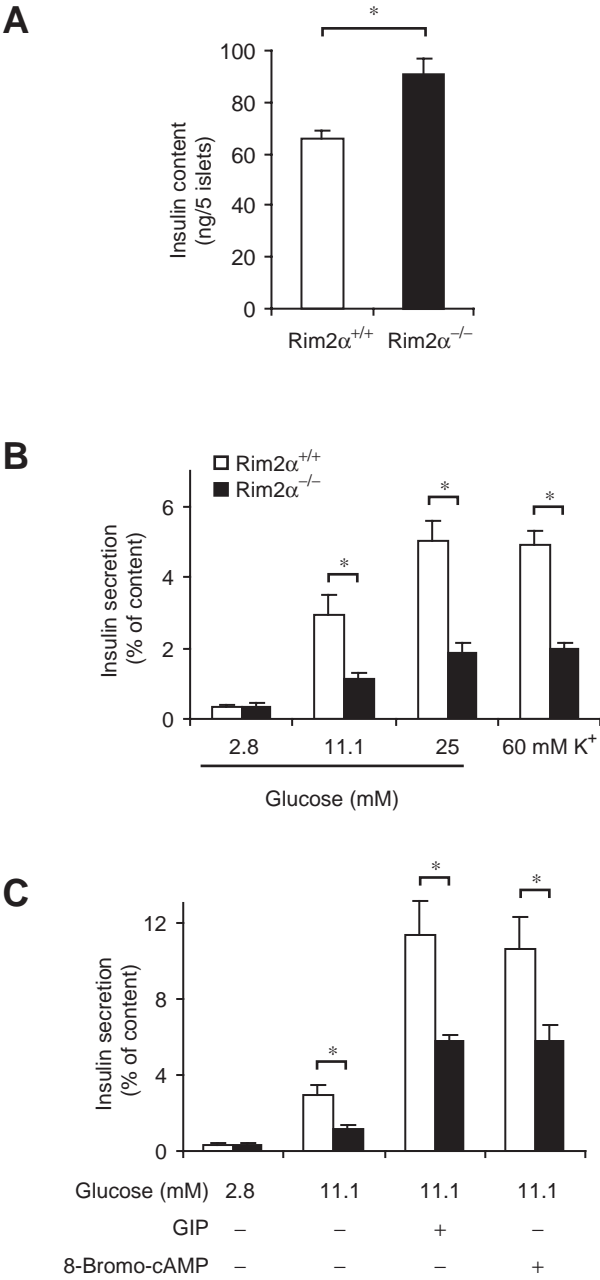




Figure 3

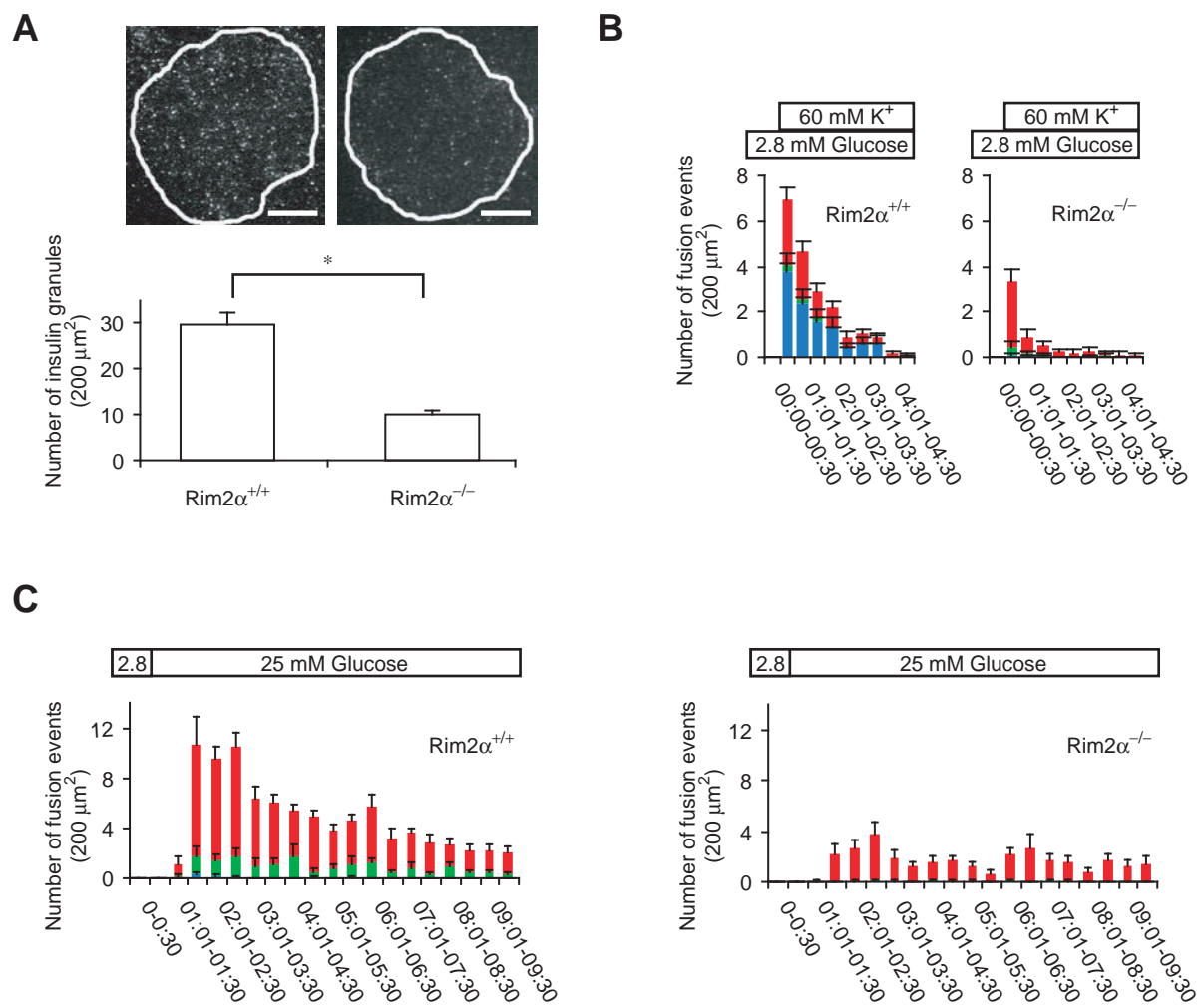
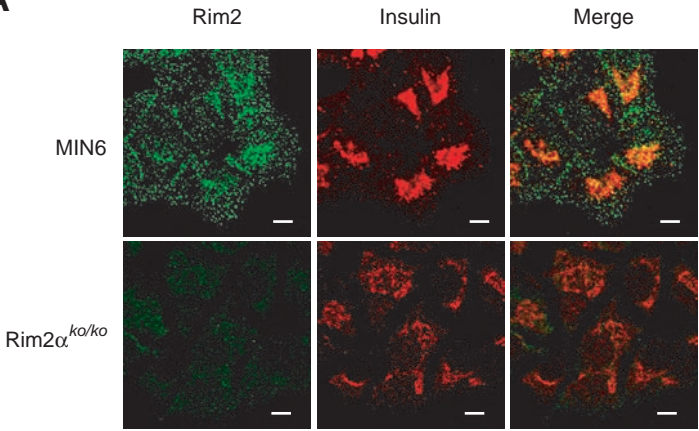
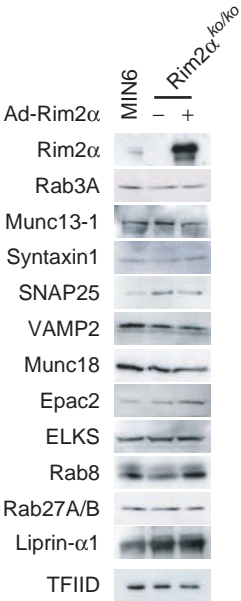


Figure 4

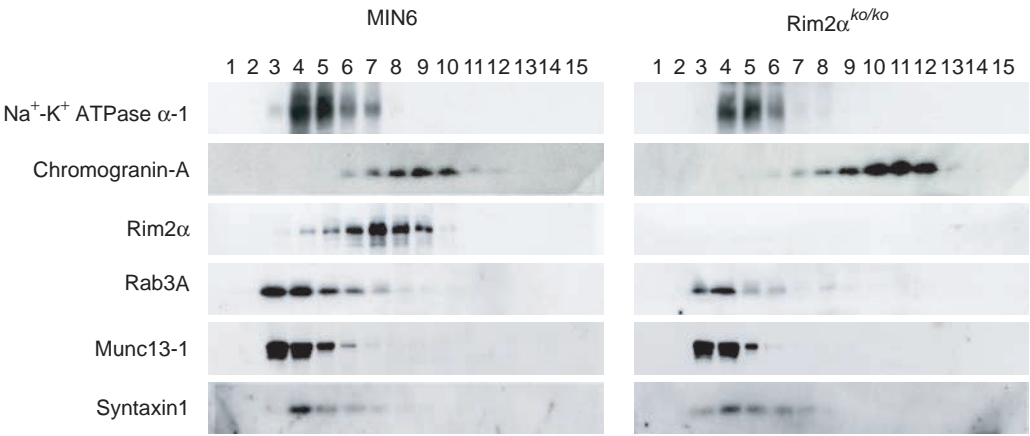
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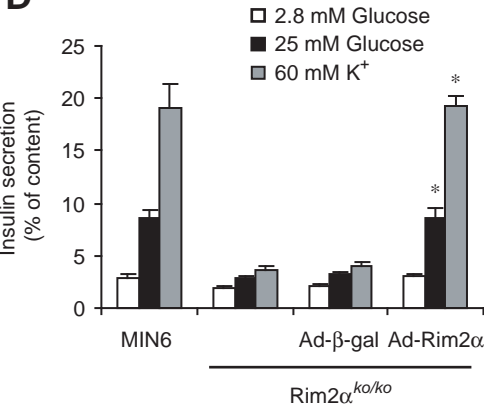
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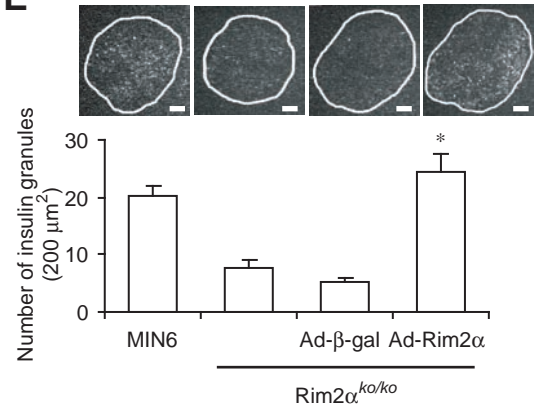
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**D**



**E**



**F**

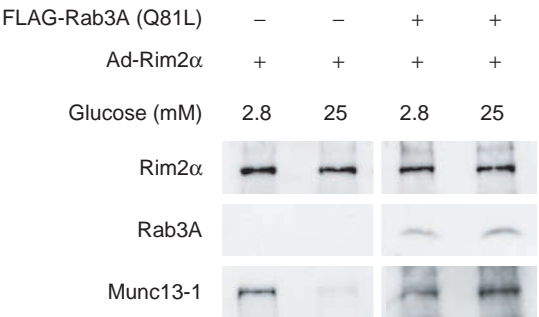
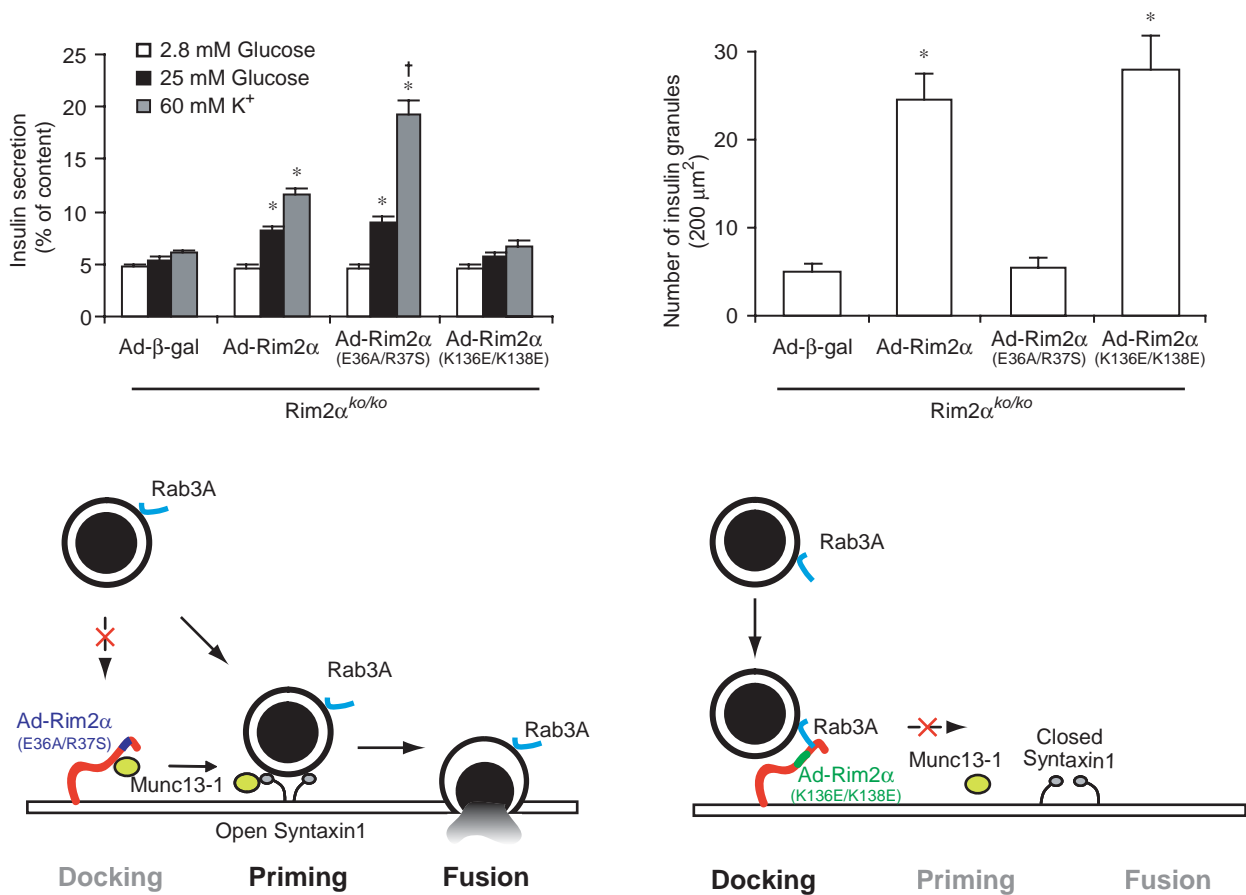
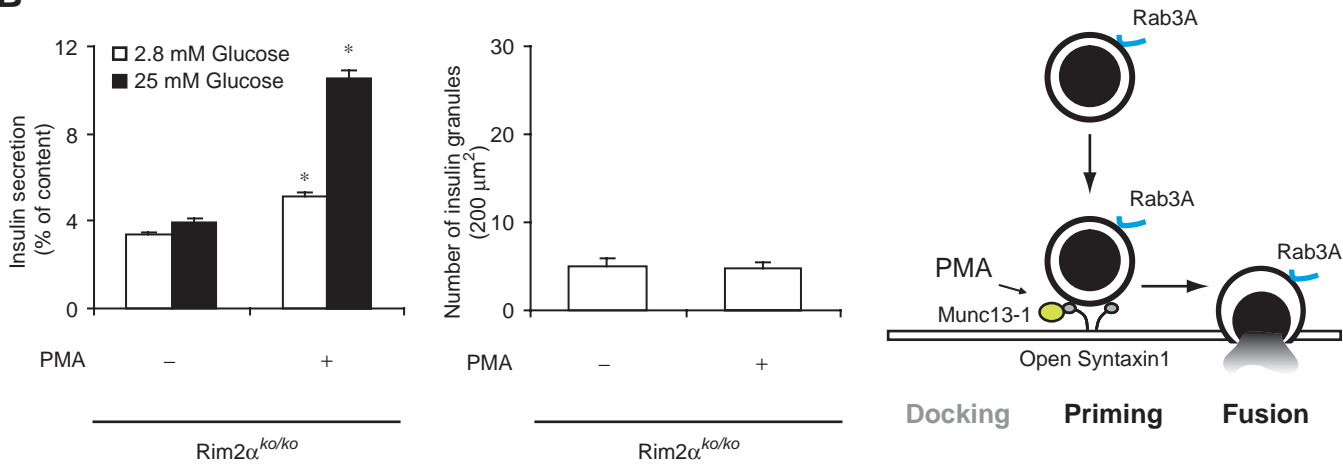


Figure 5

A



B



C

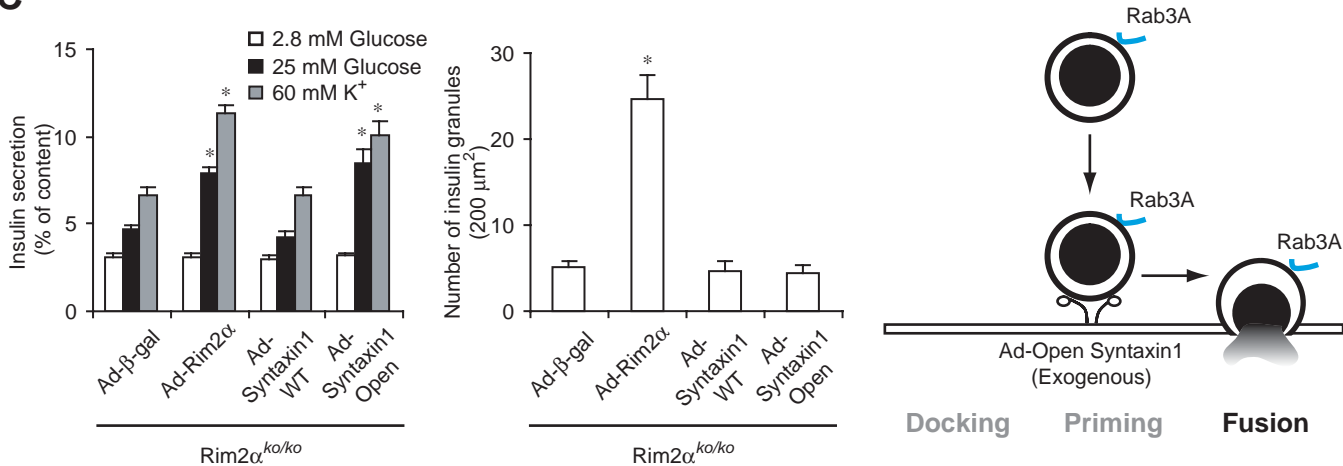
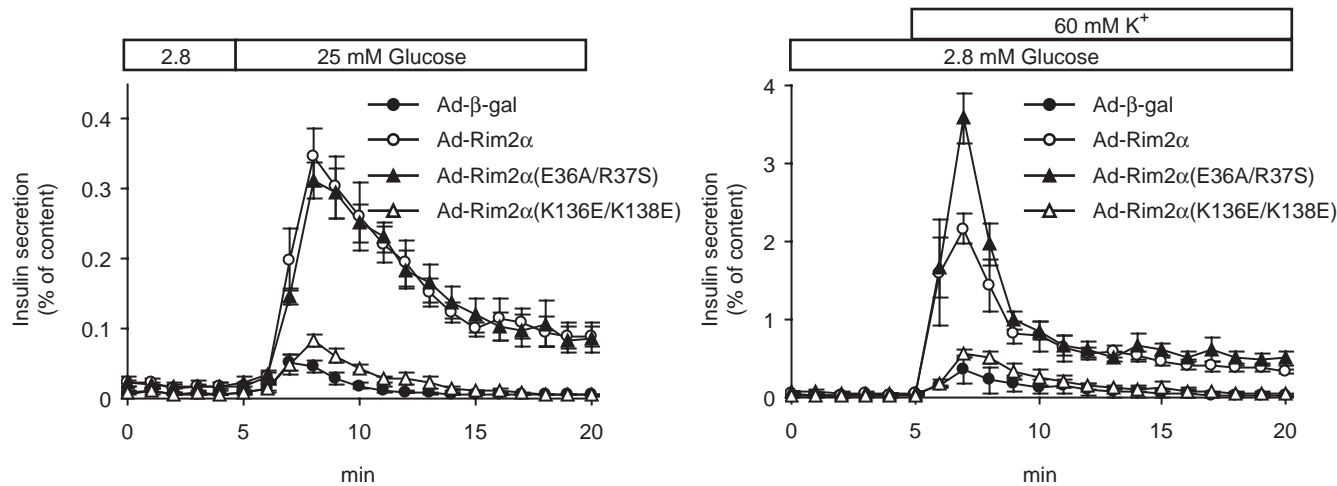
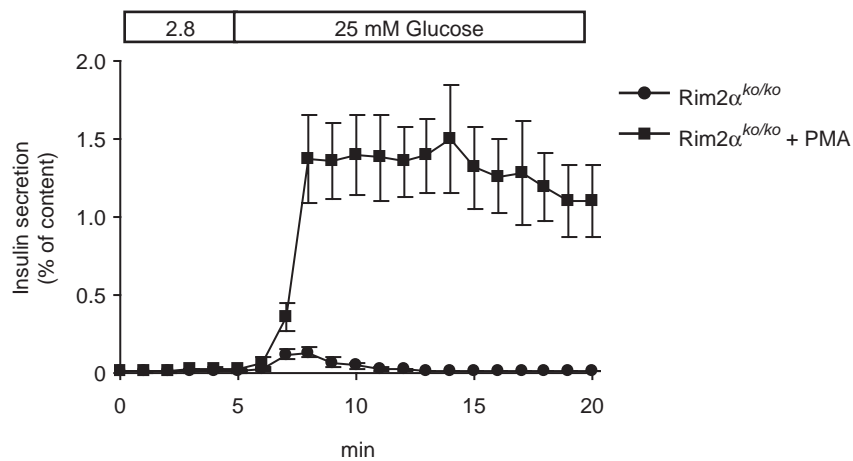


Figure 6

A



B



C

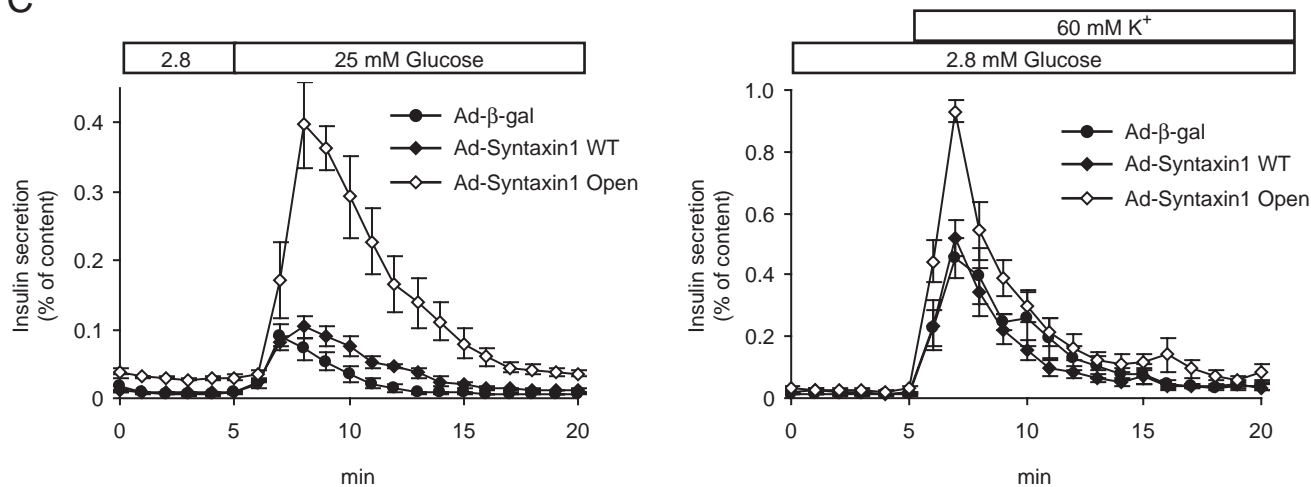


Figure 7

- 2.8 mM Glucose
- 11.1 mM Glucose
- ▒ 11.1 mM Glucose + 10  $\mu$ M 8-pCPT-2'-O-Me-cAMP-AM

