# Deficiency in RNA editing enzyme ADAR2 impairs regulated exocytosis

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ABSTRACT **Mammalian RNA editing catalyzed by adenosine deaminases acting on RNA (ADARs) ADAR1 and ADAR2 plays pivotal roles in the brain through functional modifications of neurotransmitter receptors and ion channels. We have demonstrated previously that RNA editing by ADAR2 is regulated metabolically in pancreatic β cells. To investigate the cellular functions of ADAR2 in professional secretory cells, we studied the effects of ADAR2 knockdown on regulated exocytosis. Selective knockdown of ADAR2 expression markedly impaired glucose-stimulated insulin secretion in the rat insulinoma INS-1 cells and primary pancreatic islets and significantly diminished KCl-stimulated secretion of exogenous human growth hormone or endogenous chromogranin B protein in the rat adrenal pheochromocytoma PC12 cells. Notably, restored overexpression of catalytically active but not editing-deficient mutant ADAR2 could rescue the impairment in stimulated secretion from ADAR2 knockdown cells. Moreover, ADAR2 suppression significantly attenuated $Ca^{2+}$-evoked membrane capacitance increases and appreciably reduced the number of membrane-docked insulin granules in INS-1 cells. Interestingly, the secretory defects resulting from ADAR2 deficiency were coupled to decreased expression of Munc18-1 and synaptotagmin-7, two key molecules in the regulation of vesicle exocytosis. Thus, these findings reveal an important aspect of ADAR2 actions in regulated exocytosis, implicating RNA editing in the control of cellular secretory machinery.—Yang, L., Zhao, L., Gan, Z., He, Z., Xu, J., Gao, X., Wang, X., Han, W., Chen, L., Xu, T., Li, W., Liu, Y. Deficiency in RNA editing enzyme ADAR2 impairs regulated exocytosis. *FASEB J.* 24, 3720–3732 (2010). www.fasebj.org**

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MAMMALIAN RNA EDITING through the deamination conversion of adenosine (A) to inosine (I) represents a genetic recoding mechanism (1) that modulates a variety of cellular processes ranging from pre-mRNA splicing, coding capacity changes to microRNA biogenesis/maturation (2, 3). This process is catalyzed by two main members of the ADAR (adenosine deaminase acting on RNA) family, ADAR1 and ADAR2, which have a similar catalytic domain near their C termini but possess distinct A to I editing site selectivities (4–7). ADAR2 is a nuclear protein with two RNA binding motifs, whereas ADAR1 is under the transcriptional control of different promoters and exists in two isoforms with three repeated RNA binding motifs: a constitutively expressed 110-kDa protein (ADAR1-p110) localized exclusively in the nucleus (8) and an interferon-inducible 150-kDa protein (ADAR1-p150) with a long N-terminal extension containing two Z-DNA binding domains (9), localized both in the cytoplasm and nucleus.

Extensive studies have shown that ADAR-catalyzed RNA editing plays critical roles in the function and development of the central nervous system (1, 2). As inosine is recognized as guanosine by the cellular protein translational machinery, site-selective RNA editing at crucial positions within pre-mRNAs encoding neurotransmitter receptors and ion channels, such as the ionotropic glutamate receptor (GluR), G-protein-coupled serotonin-2C subtype receptor, and Kv1.1 potassium channel, leads to codon changes and consequent alterations in their functional activities (10–13). Despite recent progress with regard to the search for cellular RNA substrates targeted by ADARs (14), the function of A to I RNA editing in tissues other than the brain remains unclear, largely due to the severe lethal

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phenotypes exhibited by mice with genetic ablation of ADAR1 or ADAR2 (10, 15).

Previously, we have examined the possible physiological actions of ADARs in the peripheral tissues and found that RNA editing by ADAR2 is up-regulated selectively in pancreatic islets from overnutrition-induced obese mice with insulin resistance. Moreover, in the rat insulinoma INS-1 cells, the expression of ADAR2 and ADAR2-mediated RNA editing is augmented prominently in response to glucose stimulation (16). These findings raised the possibility that ADAR2 enzyme may play a hitherto unrecognized regulatory role in β cells in particular, and in professional secretory cells in general. Pancreatic β cells are central to the maintenance of glucose homeostasis in mammals, sensing and responding to glucose changes in the blood by regulated insulin biosynthesis and secretion (17). Insulin secretion involves a sequential cascade of cellular events. Elevation in the ATP/ADP ratio as a result of glucose metabolism causes closure (inactivation) of ATP-sensitive $K^+$ ($K_{ATP}$) channels and leads to membrane depolarization and activation of voltage-dependent $Ca^{2+}$ channels. Subsequently, a rise in the cytosolic calcium flux (18) triggers the tightly controlled exocytosis of insulin granules (19), which has been shown to be partly mediated by the $Ca^{2+}$-sensing synaptotagmin-7 (20). Regulated exocytosis is a highly organized sequential process that involves the physical movement of granules to the subplasma membrane region, their tethering and docking at release sites on the plasma membrane, switching to a fully releasable state, releasing granule contents through triggered membrane fusion, and retrieval of the granule membrane (21). A large family of evolutionarily conserved proteins known as the SNAREs (soluble-N-ethylmaleimide-sensitive factor attachment protein receptor), which includes synaptobrevin 2/VAMP2, SNAP25, and syntaxin-1 as well as their regulators Munc18-1 and $Ca^{2+}$-binding synaptotagmins, play a key role in intracellular vesicular transport and exocytosis (22). While major progress has been made over the past decade (23–28) about the detailed cellular mechanisms by which the assembly of SNARE complex is regulated, it remains totally unexplored whether ADAR-mediated RNA editing is linked functionally to the cellular exocytotic machinery. Here we sought to investigate the functional importance of ADAR2 in hormone and neuropeptide secretion using two professional secretory cells: pancreatic β cells and the rat adrenal pheochromocytoma PC12 cells.

## MATERIALS AND METHODS

### Cell culture and pancreatic islet isolation

Rat INS-1 832/13 or 832/3 β cells (a generous gift from Dr. Christopher B. Newgard, Duke University Medical Center, Durham, NC, USA) that possess robust glucose-stimulated insulin secretion properties were maintained under conditions as described (29). Rat pheochromocytoma PC-12 cells were maintained in RPMI 1640 (Gibco, North Andover, MA, USA) supplemented with 15% horse serum and 3% fetal bovine serum (Invitrogen, Carlsbad, CA, USA). Human embryonic kidney (HEK) 293, HEK293T, and HeLa cells were cultured in DMEM (Gibco) containing 10% FBS (Gibco). C57BL/6N male mice (Shanghai Laboratory Animal Co. Ltd., Shanghai, China) were housed in laboratory cages at a temperature of 23 ± 3°C and a humidity of 35 ± 5% under a 12-h light-dark cycle (lights on at 6 AM). Pancreatic islets from C57BL/6N male mice were isolated using the Liberase (Roche, Rotkreuz, Switzerland) digestion method. Pancreas was perfused with the digestion buffer and islets were isolated as described previously (16). All protocols for animal experiments were approved by the Institutional Animal Care and Use Committees at the Institute for Nutritional Sciences, SIBS, CAS.

### RNAi knockdown by transient transfection

Duplex siRNA oligonucleotides were from Genepharma. Ltd. (Shanghai, China) for the following target genes: siADAR2, 5′-GCAGCUGAACGAGAUCAAAdTdT-3′, directed against the coding region from nt 371 downstream of the start codon of rat *Adar2* (GenBank accession number NM_012894); siADAR1–160, 5′-GCAGAUAGAGUUUCUCAAAdTdT-3′, and siADAR1–3079, 5′-GCAUCUGACCCGUGCUAUUdTdT-3′, against the coding regions from 160 and from 3079 downstream of the start codon of rat *Adar1* (GenBank NM_031006), respectively; siGck-294, 5′-GCUCAGAAGUCGGAGACUUdTdT-3′, and siGck-994, 5′-GGUACGACUUGUGCUGCUUdTdT-3′, targeting the coding regions from 294 and from 994 downstream of the start codon of rat *Glucokinase* (GenBank NM_012565), respectively; and siCON, 5′-GCAGUAAGCGAUACGCAAAdTdT-3′, a nonspecific scrambled control. Mixtures of two siRNA duplexes were used to knock down expression of ADAR1 and GCK. INS-1 cells were transfected with siRNA duplexes at 100 nM using the Amaxa Nucleofection™ system (Amaxa, Cologne, Germany) and cultured 48 h post-transfection before GSIS assays.

### Knockdown by virus-delivered short-hairpin RNAs

For lentivirus-mediated ADAR2 knockdown, lentiviral vector FG12 and packaging plasmids pRSV/REV, pMDLG/pRRE and pHCMVG (generous gifts from Dr. Yingying Le, Institute for Nutritional Sciences) were used. To construct the hairpin siRNA expression cassette, two synthesized complementary DNA oligonucleotides (see below) were annealed and inserted immediately downstream of the U6 promoter of pBS-SKII plasmid, and the derived cassette was subcloned into FG12 vector as described previously (30). Recombinant lentiviruses LV-shADAR2 #1 (5′-GCAGCTGAACGAGATCAAACC-3′) and LV-shADAR2 #2 (5′-GCATCAACGGCGAATACATGA-3′) were generated, expressing shRNA directed against two coding regions from nt 371 and from 1249 downstream of the start codon of rat ADAR2. LV-shCON (5′-GATGTTGTCAACGACTAGTTT-3′) virus that expresses a scrambled shRNA sequence was prepared as a control. Lentiviral particles were produced in HEK-293T cells as described (31) and subsequently concentrated 100-fold by sucrose gradient ultracentrifugation. The titers were measured in HeLa cells using fluorescence-activated cell sorter (FACS) analysis based on GFP expression. INS-1 cells were infected with concentrated lentivirus stocks at a multiplicity of infection (MOI) of 3 viral particles/cell and cultured for 60 h prior to subsequent experimental assays.

For ADAR2 knockdown by adenovirus-delivered shRNA,

BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen) was used according to manufacturer's instructions. Recombinant adenoviruses Ad-shADAR1 and Ad-shADAR2 #1 were generated by introducing DNA fragments encoding the same targeting sequences as in siADAR1 and siADAR2 into the pENTR™/U6 entry vector (Invitrogen). Ad-shLacZ, Ad-shADAR1, and Ad-shADAR2 #1 were produced and amplified in 293A cells before purification by 1-step ultracentrifugation in cesium chloride gradient. After subsequent dialysis, viral titers were determined by the tissue culture infectious dose 50 (TCID50) methods. INS-1 cells or islets were infected with viruses at MOI of 10 or 20 or $3 \times 10^5$ pfu/islet (~100 pfu/cell).

### Adenoviral ADAR2 overexpression

Recombinant adenoviruses for rat ADAR2 overexpression were generated using AdEasy™ Adenoviral Vector System (Stratagene, La Jolla, CA, USA) according to the manufacturer's instructions. Briefly, the coding sequence of EGFP (pEGFP-N1; Clontech, Mountain View, CA, USA) was subcloned into pShuttle-CMV vector digested with *BglII* and *SalI*, and the RT-PCR-amplified cDNA fragment of wild-type rADAR2 was subcloned into pShuttle-CMV-EGFP after digestion with *SalI* and *NotI*. To generate the ADAR2-H394A: E396A mutant, substitution mutations were introduced using the Muta-direct Site-directed Mutagenesis Kit (SBS, Shanghai, China). The shuttle plasmids and pAdEasy-1 were subsequently cotransformed into the BJ5183 bacteria to produce recombinant adenoviral DNA, which was then used for transfection of HEK293 cells. Recombinant viral particles were obtained from the cell lysates and purified by CsCl gradient ultracentrifugation. INS-1 cells at ~60% confluence were infected at MOI of 2 or 20 as indicated.

### Antibodies and Western immunoblot

ADAR2 (C-15), ADAR1 (L-15) and chromogranin B antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Munc18-1, Syntaxin-1, and Synaptotagmin-7 antibodies were purchased from Sigma (St. Louis, MO, USA), Medical & Biological Laboratories (Nagoya, Japan) and Synaptic Systems(Göttingen, Germany), respectively. Protein extracts were prepared from INS-1 and PC12 cells or isolated primary islets, and Western blotting was performed as described previously (16).

### Insulin/amylin secretion assay and insulin content analysis

Insulin or amylin secretion from INS-1 cells was assayed as described previously (29). Briefly, confluent cells in 24-well plates were washed with HEPES-balanced salt solution (HBSS) followed by a 2 h preincubation in the same buffer. Secreted insulin or amylin was then measured after static incubation for a 2 h period in HBSS containing the secretagogues as indicated. Cell lysates were collected for subsequent analysis of target protein or mRNA levels. For GSIS analysis with mouse pancreatic islets, adenovirus-infected islets were maintained in HBSS with 2.8 mM glucose for 1 h and aliquoted into 20 islets per well, followed by incubation in HBSS with 2.8 mM glucose for 1 h at 37°C for basal secretion assessment. Islets were subsequently transferred and incubated in HBSS with 16.7 mM glucose for stimulated secretion analysis at 15 or 50 min incubation. To determine the intracellular insulin content, INS-1 cells were lysed in 0.1 M acetic acid containing 0.1% BSA by mild sonication on ice. After centrifugation for 5 min at 12,000 *g* at 4°C, the extracts were neutralized by dilution in PBS. Insulin levels were measured using an RIA Kit (Linco Research, St. Charles, MO, USA), and amylin was analyzed with an ELISA Kit (Bachem, Bubendorf, Switzerland).

### Measurement of protein secretion from PC12 cells

PC12 cells were cotransfected transiently with the plasmid pcDNA3 expressing hGH (kindly provided by Philippe A. Halban, University of Geneva, Geneva, Switzerland, and Romano Regazzi, University of Lausanne, Lausanne, Switzerland) along with FG12-shADAR2 #1 or FG12-shCON at a ratio of 1:3. Cells were washed twice with HBSS at 72 h post-transfection and precultured in the same buffer for 2 h at 37°C. Subsequently, cells were incubated for 2 h at 37°C in HBSS containing 2.8 mM glucose, followed by stimulation for 2 h in HBSS containing 55 mM KCl. Basal and stimulated incubation buffers were recovered, and cells were extracted with acid ethanol. The amount of hGH release in the medium was measured by ELISA (Roche). Stimulated secretion of the endogenous protein CgB was assayed as described previously with some modifications (32). Briefly, adenovirus-infected PC12 cells in 6-well plates were maintained in 1.5 ml RPMI 1640 for 2 h and then cultured as basal conditions in 1.5 ml RPMI 1640 for 2 h. Cells were then subjected to stimulation by incubation in RPMI 1640 with 55 mM KCl for 2 h. The medium was collected and precipitated with trichloroacetic acid for 30 min at 4°C, and the precipitated samples were subjected to Western immunoblot analysis. The cells were collected and the total cell number was counted after the experiments.

### Cellular ATP measurement

INS-1 cells were incubated in HBSS buffer with 2.8 or 16.7 mM glucose for 30 min and suspended in Somatic Cell Releasing Agent (Sigma). The amount of ATP was measured using the Bioluminescent Somatic Cell Assay Kit (Sigma) according to the manufacturer's instructions.

### Intracellular $Ca^{2+}$ measurement

Intracellular calcium fluxes were measured by the FlexStation (Molecular Devices, Sunnyvale, CA, USA) (33). Lentivirus-infected INS-1 cells were loaded with 1 μM of Fura-2 AM (Molecular Probes, Eugene, OR, USA) for 30 min in the presence of 0.01% pluronic F-127 (Molecular Probes) at 37°C. Washed cells were resuspended in the emission buffer (137 mM NaCl; 2.7 mM KCl; 10 mM $Na_2HPO_4$; 2 mM $KH_2PO_4$; 1 mM $CaCl_2$; 0.76 mM $MgCl_2$; 2.8 mM glucose, pH 7.4) and were dispensed at $3 \times 10^5$ in 70 μl/well into black-wall clear-bottom 96-well plates (Corning, Big Flats, NY, USA). Kinetic calcium fluxes were measured at 37°C following the addition of glucose to 16.7 mM or KCl to 30 mM. Fluorescence emission intensity at 510 nm was monitored at intervals of 4 or 5 s with excitation wavelengths at 340 and 380 nm. Data were analyzed as the ratios of the two intensity values using SoftmaxPro Software (Molecular Devices).

### Quantitative RT–PCR and RNA editing analysis

Total cellular RNA from INS-1 or PC12 cells was isolated using TRIzol reagent (Invitrogen). SYBR green-based real-

time quantitative PCR was performed with an ABI 7500 Fast Real-Time PCR System (ABI, Foster City, CA, USA). Oligonucleotide primers for the target genes were listed in Supplemental Table 1. Quantitative analysis of editing at the Q/R site of GluR-B pre-mRNA was performed by *Tau*I (Fermentas, Burlington, ON, Canada) digestion as described previously (16).

### Electron microscopy

INS-1 cells infected by lentiviruses were preincubated in HBSS buffer containing 2.8 mM glucose at 37°C for 2 h, followed by 15 min in HBSS containing 16.7 mM glucose. Cells were fixed and embedded as described (34), and ultrathin sections (60–80 nm) were contrasted with uranyl acetate and lead citrate before examination by transmission electron microscope (H-7650; Hitachi, Tokyo, Japan) equipped with a charge-coupled device camera (ER-B; AMT, Moorebank, NSW, Australia). Micrographs were randomly taken at ×1000 and ×8000. The areas of cytoplasm were assessed from images at ×1000 by ImageJ (http://rsb.info.nih.gov/ij), and granules were counted from those at ×8000.

### Electrophysiology and membrane capacitance analysis

Electrophysiological experiments were conducted with standard whole-cell recordings using an EPC-10 patch-clamp amplifier (Heka, Lambrecht, Germany) (35). INS-1 cells were transfected with FG12-shADAR2 #1 or FG12-shCON 72 h prior to patch-clamp experiment performed at 30°C, using the extracellular solution (118 mM NaCl; 20 mM TEA-Cl; 5.6 mM KCl; 1.2 mM $MgCl_2$; 2.6 mM $CaCl_2$; 10 mM HEPES; and 5 mM glucose, pH 7.4). $Ca^{2+}$ infusion was performed with the pipette solution (125 mM K-glutamate; 10 mM KCl; 10 mM NaCl; 1 mM $MgCl_2$; 5 mM HEPES; 3 mM Mg-ATP; 10 mM EGTA; and 9 mM $CaCl_2$, pH 7.2). The membrane capacitance increases ($\Delta C$) were measured during the first minute after establishment of the whole-cell configuration, and analyzed using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA).

### Statistical analysis

Data are shown as means ± SE. Student's *t* test was performed for 2 groups and 1-way ANOVA for >2 groups, with values of $P < 0.05$ considered statistically significant.

## RESULTS

### ADAR2 knockdown impairs glucose-stimulated insulin secretion in β cells

To test the idea that glucose-regulated ADAR2 deaminase is implicated in modulation of the endocrine function of β cells, we specifically suppressed the expression of ADAR2 by RNA interference in β cells and assessed its effect on glucose-stimulated insulin secretion (GSIS). In comparison with a scrambled small interfering (si) RNA control (siCON), transient transfection of INS-1 β cells with siRNAs directed to ADAR1 (siADAR1) or ADAR2 (siADAR2) selectively and efficiently reduced (by ~70–80%) the expression of the targeted ADAR deaminases (Supplemental Fig. 1*A*). Indeed, knockdown of ADAR2 but not ADAR1 significantly decreased GSIS (by ~40%, from 8.0±1.0- to 4.7±0.6-fold), similar to the extent of secretion impairment (by ~55%, from 8.0±1.0- to 3.6±1.4-fold) resulting from siRNA knockdown of glucokinase (*Gck*), an essential enzyme in glucose metabolism and GSIS (Supplemental Fig. 1*B*). To avoid possible off-target effects, we generated recombinant lentiviruses (LV-shADAR2 #1 and #2) expressing two small hairpin RNAs (shRNAs) that targeted two different coding regions of ADAR2. In contrast to the control virus expressing the scrambled shRNA (LV-shCON), infection of INS-1 cells by LV-shADAR2 #1 and #2 resulted in ~70 and 80% decreases, respectively, in ADAR2 expression levels, while showing no effect on ADAR1 (p110) expression (**Fig. 1*A***). To monitor the intracellular ADAR2 deaminase activities, editing efficiency was assessed for the Q/R site (16), whereby ADAR2 selectively converts a glutamine (Q) codon (C*A*G) to an arginine (R) codon (C*I*G) within the pre-mRNA encoding the glutamate receptor subunit B (GluR-B). In parallel with the diminished ADAR2 expression, editing at the Q/R site in cells infected by LV-shADAR2 #1 and #2 was reduced markedly (from 78.5±5.9 to 35.8±11.6 and 41.6±11.6%, respectively) in comparison to that in LV-shCON-infected control cells (Fig. 1*B*). Similar to the results from the transient siRNA transfection experiments, lentivirus-mediated ADAR2 knockdown markedly reduced the fold stimulation of insulin secretion in response to 16.7 mM glucose, with ~60% decreases observed for LV-shADAR2 #1- or #2-infected INS-1 cells (Fig. 1*C*; from 5.2±0.3- to 2.2±0.3- and to 2.1±0.5-fold, respectively).

To confirm ADAR2 knockdown's specific effect on insulin secretion, we examined GSIS from INS-1 cells infected with adenoviruses encoding shRNAs against ADAR1 (Ad-shADAR1, presumably targeting the ADAR1 mRNAs encoding both the p150 and p110 isoforms) or ADAR2 (Ad-shADAR2 #1, expressing the same shRNA as its lentiviral counterpart LV-shADAR2 #1). In comparison with the control virus Ad-shLacZ, Ad-shADAR1 infection dramatically decreased the abundance of both the p150 and p110 ADAR1 proteins but did not significantly affect GSIS (**Fig. 2*A***); by contrast, knockdown of ADAR2 by Ad-shADAR2 #1 led to an ~55% reduction in GSIS, indicating the specific coupling of ADAR2 but not ADAR1 to the process of insulin secretion on glucose stimulation. Next, to determine whether ADAR2 knockdown causes defects in the secretory process of insulin-containing vesicles rather than specifically influencing insulin itself, we analyzed the secretion of amylin, a 37-aa peptide hormone that is known to be costored and cosecreted with insulin in pancreatic β cells (36). Indeed, glucose-induced amylin secretion was also reduced by ~70% in ADAR2-deficient INS-1 cells infected with Ad-shADAR2 #1 as compared with Ad-LacZ-infected cells (Fig. 2*B*; 2.22±0.38- *vs.* 7.36±0.86-fold).

To verify the effects *ex vivo* of ADAR2 deficiency on GSIS further, we infected isolated mouse pancreatic islets with Ad-shADAR2 #1. In contrast to the Ad-shLacZ control,

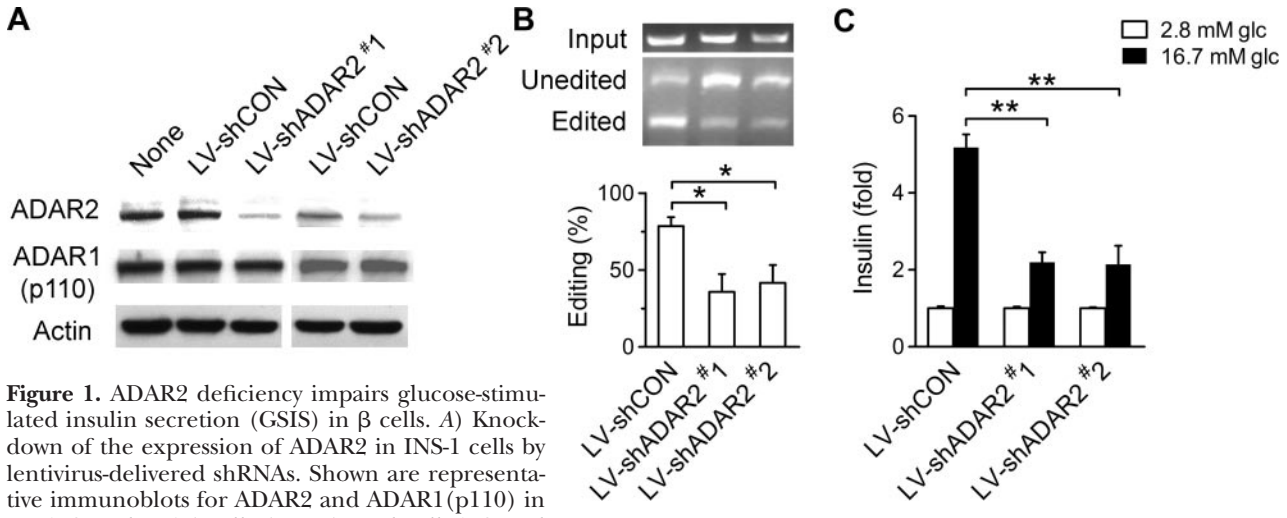


**Figure 1.** ADAR2 deficiency impairs glucose-stimulated insulin secretion (GSIS) in β cells. *A*) Knockdown of the expression of ADAR2 in INS-1 cells by lentivirus-delivered shRNAs. Shown are representative immunoblots for ADAR2 and ADAR1(p110) in noninfected INS-1 cells (None), and cells infected for ~60 h by control lentivirus LV-shCON *vs.* ADAR2-targeting viruses LV-shADAR2 #1 and #2. β-Actin was used as a loading control. *B*) ADAR2 knockdown reduces cellular RNA editing levels. Editing efficiency at the Q/R site of GluR-B in lentivirus-infected INS-1 cells was measured by editing-dependent *Tau*I digestion of the RT-PCR products derived from GluR-B pre-mRNA. *C*) ADAR2 knockdown decreases GSIS in INS-1 cells. Secreted insulin was measured in response to stimulation by 16.7 mM glucose (glc) from cells ~60 h postinfection by LV-shCON *vs.* LV-shADAR2 #1 and #2. Shown are fold increases in stimulated insulin secretion relative to their corresponding basal levels at 2.8 mM glucose. Data represent mean ± SE values from ≥3 independent experiments. $*P < 0.05$, $**P < 0.01$ *vs.* LV-shCON.

infection by Ad-shADAR2 #1 efficiently reduced (by ~57%) the expression of ADAR2 protein in the islets and significantly impaired insulin secretion when stimulated by 16.7 mM glucose for 15 and 50 min, leading to decreases in fold stimulation by ~37 and 27% (Fig. 2*C*; from 3.2±0.3- to 2.0±0.3-fold and from 10.1±0.8- to 7.3±0.5-fold), respectively. Together, these results demonstrate that ADAR2 enzyme is specifically and functionally connected to the cellular capacity for insulin secretion in response to glucose stimulation, suggesting an important role of ADAR2 in the regulated operation of the secretory machinery in pancreatic β cells.

### The ADAR2 editing activity is required for mediating its effect on stimulated secretion in β cells

To determine whether the deaminase activity of ADAR2 is involved directly in mediating its effects on glucose-stimulated secretion in β cells, we performed rescue experiments in the ADAR2 knockdown INS-1 cells by overexpressing either the wild-type (rADAR2) or a catalytically inactive mutant (rADAR2-Mut) of rat ADAR2, generated with substitution replacement with alanine of $His^{394}$ and $Glu^{396}$, the two highly conserved and enzymatically critical residues (H394A and E396A) within its catalytic domain (37, 38) (**Fig. 3*A*** and Supplemental Fig. 2*A*). Adenoviral overexpression as the GFP-fusion protein of rADAR2-Mut significantly decreased the Q/R site editing of the endogenous GluR-B pre-mRNA (from 84.1±11.2 to 57.2±16.9%) in INS-1 cells (Fig. 3*A* and Supplemental Fig. 2*B*), whereas rADAR2 overexpression led to nearly complete editing at this site. Under moderate ADAR2-knockdown conditions (Fig. 3*B*) using a lower dose of Ad-shADAR2 #1 where an ~25% reduction in GSIS was observed from infected INS-1 cells (Fig. 3*C*), coinfection with Ad-GFP-rADAR2 efficiently restored the GSIS capacity, with up to ~94% fold stimulation observed relative to that from control cells coinfected with Ad-shLacZ and Ad-GFP (Fig. 3*C*); in sharp contrast, Ad-GFP-rADAR2-Mut was totally unable to reverse the impairing effect of ADAR2 deficiency on GSIS.

To circumvent moderate ADAR2 knockdown efficiency as a result of coinfection with multiple adenoviruses for GSIS analysis, we then developed an alternative secretion assay by cotransfecting INS-1 cells with shADAR2 #1 and pCMV plasmids expressing Flag-tagged rADAR2 or rADAR2-Mut along with that expressing human growth hormone (hGH), which is known to be targeted to secretory granules and cosecreted with hormone peptides in response to secretagogue (39). Similar to our observations from GSIS analysis, a marked decrease in glucose-stimulated hGH secretion (by ~50%) was detected in ADAR2 knockdown INS-1 cells (Fig. 3*D*); moreover, simultaneous overexpression of rADAR2 but not rADAR2-Mut resulted in prominent rescue of the deficit in glucose-stimulated hGH secretion, with ~90% of the fold stimulation observed relative to that from cells cotransfected with control plasmids (Fig. 3*D*). These data further suggest that the RNA editing activity of ADAR2 enzyme may work as a key component of ADAR2-mediated modulation of cellular secretion in β cells.

### Cellular mechanisms that mediate ADAR2 deficiency-dependent GSIS attenuation

Two signaling mechanisms in β cells are known to mediate GSIS, the $K_{ATP}$ channel-dependent triggering (40) and -independent amplifying (41) pathways. The

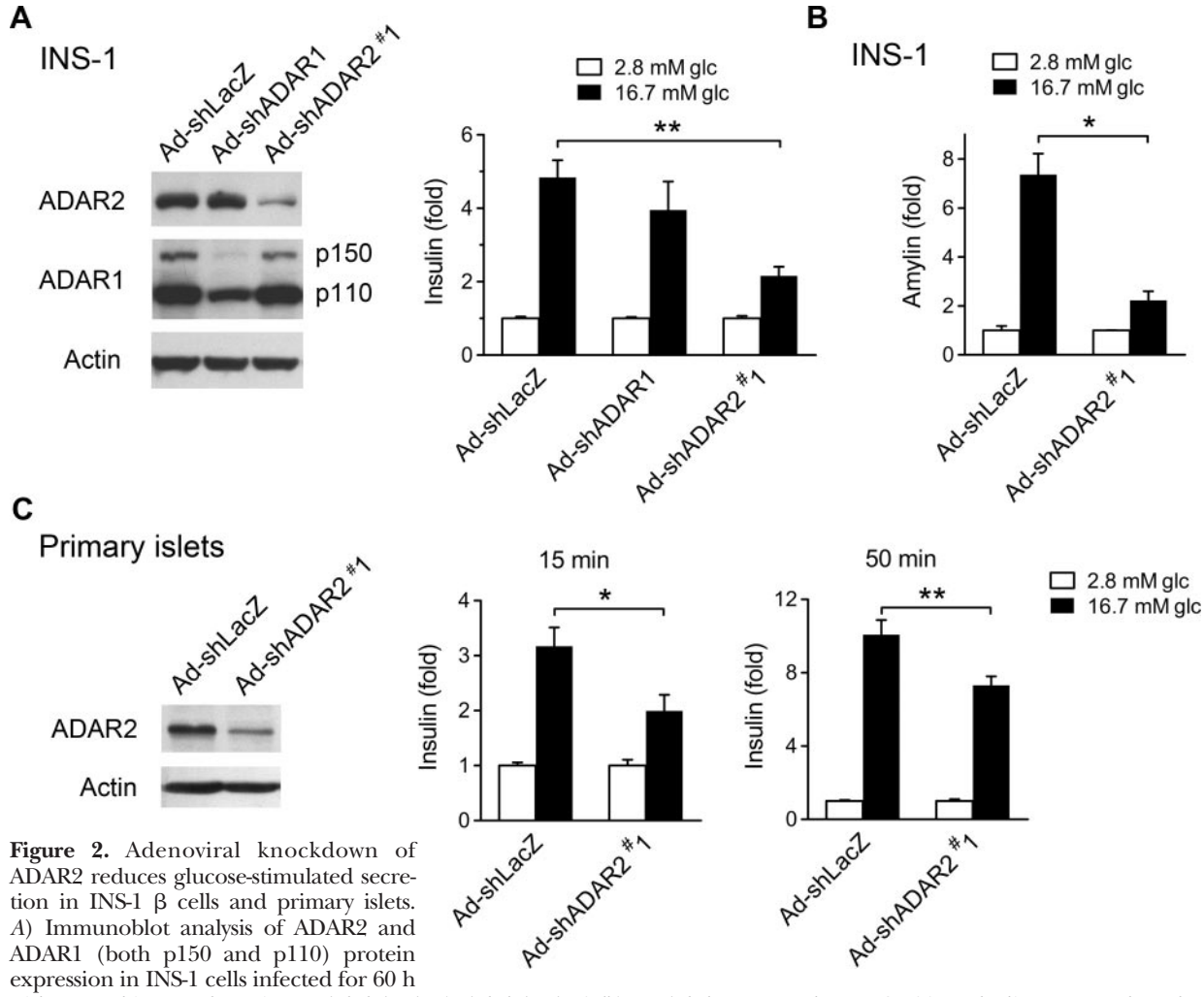


**Figure 2.** Adenoviral knockdown of ADAR2 reduces glucose-stimulated secretion in INS-1 β cells and primary islets. *A*) Immunoblot analysis of ADAR2 and ADAR1 (both p150 and p110) protein expression in INS-1 cells infected for 60 h with recombinant adenoviruses Ad-shADAR1, Ad-shADAR2 #1, or Ad-shLacZ, each at MOI 20. Tubulin was used as the loading control. Secretion of insulin was determined from the infected INS-1 cells in response to stimulation for 2 h by 16.7 mM glucose. Shown are fold increases in stimulated secretion relative to the basal levels in cells cultured at 2.8 mM glucose. Data represent means ± SE from 3 independent experiments. $^{**}P < 0.01$ *vs.* control cells infected by Ad-shLacZ. *B*) Amylin secretion was likewise measured as for GSIS and shown as fold increases in stimulated secretion relative to the basal levels. Data represent the means ± SE from 3 independent experiments. $^{*}P < 0.05$ *vs.* control cells infected by Ad-shLacZ. *C*) ADAR2 knockdown diminishes GSIS in primary islets. Isolated mouse islets were infected with Ad-shLacZ or Ad-shADAR2 #1 for 60–72 h. Representative immunoblot shows ADAR2 knockdown efficiency in the islets. Static insulin secretion was determined at 15 and 50 min from islets stimulated by 16.7 mM glucose relative to that maintained at 2.8 mM glucose. Data are shown as average ± SE fold increases in secretion from 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs.* Ad-shLacZ.

former involves ion channels for the depolarization of the cell membrane, whereas the latter acts *via* mechanisms that have yet to be defined (18). To determine the mechanisms by which ADAR2 enzyme exerts its actions on GSIS, we examined the effect of ADAR2 knockdown on insulin secretion in INS-1 cells in response to various stimulatory secretogogues. Similar to the results from the GSIS experiments, ADAR2 deficiency blunted by 2- to 3-fold the induction of insulin secretion by 30 mM KCl (**Fig. 4*A***; from 6.2±1.0 to 2.8±0.4- and to 1.9±0.5-fold in cells infected with LV-shADAR2 #1 and LV-shADAR2 #2, respectively), which can depolarize the cell membrane in the absence of high glucose. This finding indicates that ADAR2 deficiency impairs the $K_{ATP}$ channel-dependent pathway that mediates the stimulation of insulin secretion, downstream of the membrane depolarization elicited by glucose or KCl. To examine whether ADAR2 deficiency also affects the $K_{ATP}$ channel-independent mechanisms, we analyzed GSIS in the presence of 35 mM KCl as well as 250 μM diazoxide, an agent that opens the $K_{ATP}$ channel. Under these conditions whereby the $K_{ATP}$ channel-dependent pathway was bypassed, deficient ADAR2 expression in cells infected by LV-shADAR2 #1 substantially disabled glucose to stimulate insulin secretion, resulting in an ~80% decrease as compared to control cells infected with LV-shCON (Fig. 4*B*; from 1.54±0.05- to 1.1±0.02-fold). Thus, ADAR2 deficiency brings about secretory defects at key steps that are common to the $K_{ATP}$ channel-dependent and -independent pathways in GSIS.

To dissect further the cellular secretory cascades

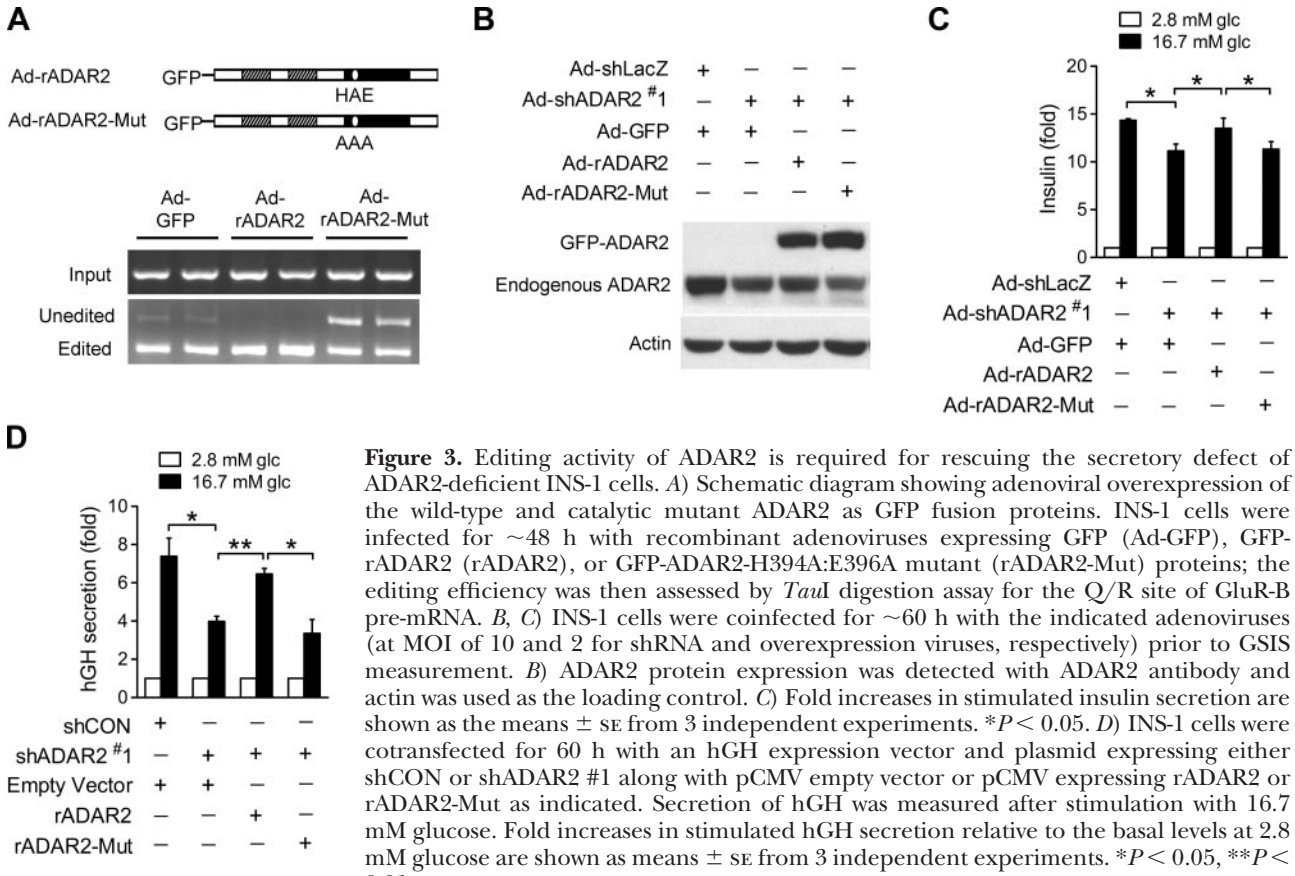


**Figure 3.** Editing activity of ADAR2 is required for rescuing the secretory defect of ADAR2-deficient INS-1 cells. *A*) Schematic diagram showing adenoviral overexpression of the wild-type and catalytic mutant ADAR2 as GFP fusion proteins. INS-1 cells were infected for ~48 h with recombinant adenoviruses expressing GFP (Ad-GFP), GFP-rADAR2 (rADAR2), or GFP-ADAR2-H394A:E396A mutant (rADAR2-Mut) proteins; the editing efficiency was then assessed by *Tau*I digestion assay for the Q/R site of GluR-B pre-mRNA. *B, C*) INS-1 cells were coinfected for ~60 h with the indicated adenoviruses (at MOI of 10 and 2 for shRNA and overexpression viruses, respectively) prior to GSIS measurement. *B*) ADAR2 protein expression was detected with ADAR2 antibody and actin was used as the loading control. *C*) Fold increases in stimulated insulin secretion are shown as the means ± SE from 3 independent experiments. $*P < 0.05$. *D*) INS-1 cells were cotransfected for 60 h with an hGH expression vector and plasmid expressing either shCON or shADAR2 #1 along with pCMV empty vector or pCMV expressing rADAR2 or rADAR2-Mut as indicated. Secretion of hGH was measured after stimulation with 16.7 mM glucose. Fold increases in stimulated hGH secretion relative to the basal levels at 2.8 mM glucose are shown as means ± SE from 3 independent experiments. $*P < 0.05$, $**P < 0.01$.

influenced by ADAR2 enzyme, we examined whether ADAR2 deficiency causes defects in glucose metabolism, insulin biosynthesis, or cellular calcium mobilization. In INS-1 cells, infection by LV-shADAR2 #1 led to, as analyzed by quantitative RT-PCR, a reduction by ~84% in the mRNA level of *ADAR2* but no apparent changes in that of *ADAR1* or the crucial glycolytic enzyme *Gck* (Supplemental Fig. 3*A*). ADAR2 knockdown did not influence the total cellular ATP production on glucose stimulation as compared to control cells infected by LV-shCON (Fig. 4*C*; 1.7±0.2- *vs.* 1.6±0.1-fold). Moreover, ADAR2 deficiency displayed little effect on insulin biosynthesis, as neither the mRNA levels of *Insulin 1* or *2* (Supplemental Fig. 3*B*) nor the total cellular insulin contents (Supplemental Fig. 3*C*; 170.9±10.6 *vs.* 162.6±2.4 ng/$10^6$ cells) were significantly altered in LV-shADAR2 #1-infected cells relative to the control cells. These results argue against the possibility that ADAR2 deficiency results in impairment in glucose metabolism or insulin biosynthesis, consistent with the earlier observation that the defects in insulin secretion occurred downstream of membrane depolarization on glucose stimulation.

We next tested whether ADAR2 deficiency leads to cellular defects in the molecular components responsible for triggering intracellular $Ca^{2+}$ fluxes *via* monitoring by FlexStation the intracellular $Ca^{2+}$ concentration $[Ca^{2+}]_i$ in INS-1 cells labeled with Fura-2 (Fig. 4*D, E*). Similar $[Ca^{2+}]_i$ oscillations or elevations were recorded in ADAR2-deficient INS-1 cells infected by LV-shADAR2 #1 as in LV-shCON-infected control cells, either on stimulation by 16.7 mM glucose (Fig. 4*D*) or depolarization by 30 mM KCl (Fig. 4*E*). Thus, these data suggest that ADAR2 deficiency affects vesicle exocytosis through mechanisms downstream of the intracellular calcium mobilization.

### ADAR2 deficiency leads to exocytotic impairment in β cells

Regulated exocytosis occurs through the fusion of the membrane of secretory vesicles with the plasma membrane, usually evoked by calcium signaling (18). To investigate whether ADAR2 is coupled directly to insulin exocytosis in β cells, we examined the effect of ADAR2 knockdown on calcium-evoked increases in membrane capacitance ($C_m$) arising from granule fusion with cell membrane during stimulated vesicle release (42). As shown by measurement of increases in the whole-cell $C_m$ triggered by intracellular infusion of $Ca^{2+}$ at ~1.5 μM (**Fig. 5*A***), knockdown of ADAR2 expression in shADAR2 #1-transfected INS-1 cells markedly attenuated $Ca^{2+}$-elicited capacitance increase ($\Delta C/\Delta t$) as compared to shCON-transfected control cells, exhibiting an averaged decrease of ~56% in $\Delta C/\Delta t$ (Fig. 5*B*; 1.9±0.7 *vs.* 4.3±0.9 fF/s). To confirm further that ADAR2 deficiency parallels defective insulin exocytosis, we examined the insulin granules by electron microscopy (Fig. 5*C*). Quantitation analysis of the electron micrographs revealed a

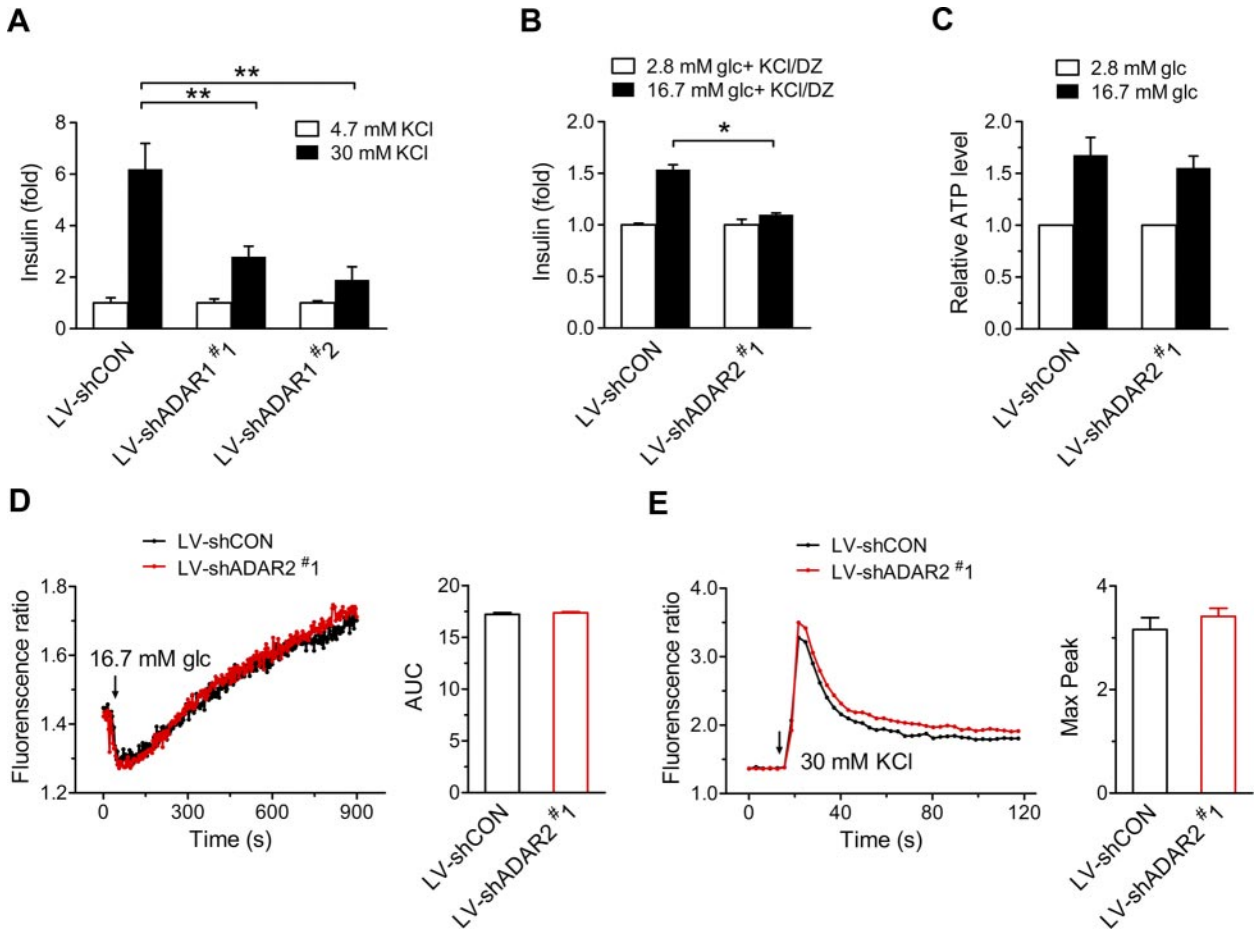


**Figure 4.** Cellular mechanisms by which ADAR2 deficiency reduces stimulated insulin secretion in β cells. INS-1 cells maintained at 11.1 mM glucose were infected by LV-shCON or LV-shADAR2 #1 viruses as indicated, and insulin secretion was measured ~60 h postinfection in response to the indicated secretagogues for 2 h. *A*) KCl-stimulated insulin secretion. Shown are fold increases in secretion stimulated by 30 mM KCl relative to that at 4.7 mM KCl. *B*) Glucose-induced insulin secretion through $K^+_{ATP}$-independent pathways. Insulin secretion was measured from infected cells maintained at low (2.8 mM) or high (16.7 mM) concentrations of glucose (glc) in the presence of 35 mM KCl and 250 μM diazoxide (DZ). *C*) ADAR2 knockdown does not affect ATP production. Infected INS-1 cells were stimulated by 16.7 mM glucose for 30 min, and intracellular ATP levels were determined and shown as relative to those in cells incubated at 2.8 mM glucose. *D, E*) ADAR2 deficiency has no effects on intracellular $Ca^{2+}$ fluxes. INS-1 cells infected for ~48 h were precultured at 2.8 mM glucose for 18 h and labeled by fura-2AM. Intracellular $[Ca^{2+}]_i$ was monitored following stimulation with 16.7 mM glucose (*D*) or 30 mM KCl (*E*). Shown are representative glucose- or KCl-induced intracellular $Ca^{2+}$ fluxes, recorded as the ratio of fluorescence at 340 nm/380 nm. Bar graphs indicate the quantification of $[Ca^{2+}]_i$, presented as area under the curve (AUC; *D*) or maximal signal (*E*). Data are means ± SE from 3 independent experiments. $*P < 0.05$, $**P < 0.01$ *vs.* LV-shCON.

significant reduction (by ~25%) in the number of secretory granules morphologically docked at the plasma membrane in ADAR2-deficient INS-1 cells maintained at 2.8 mM glucose as compared to control cells (Fig. 5*D*; 0.55±0.06 *vs.* 0.43±0.06 docked granules/$\mu m^2$ cytoplasm area). However, no noticeable alterations were found in the number of total insulin granules (Fig. 5*D*; 1.46±0.16 *vs.* 1.42±0.18 granules/$\mu m^2$ cytoplasm area). Thus, these observations suggest that ADAR2 deficiency leads to dysregulated insulin vesicle docking and/or fusion, the critical exocytotic steps that are exquisitely controlled in response to glucose stimulation.

### ADAR2 knockdown disrupts cellular protein secretion in PC12 cells

Neuroendocrine cells bear a number of resemblances to pancreatic β cells with respect to cellular secretory functions, particularly the regulatory machinery involved in vesicle exocytosis (19). To investigate whether ADAR2 deficiency-dependent exocytotic impairment is unique to pancreatic β cells, or applies to regulated exocytosis in general, we analyzed the effect of ADAR2 knockdown on KCl-stimulated peptide secretion in PC12 cells. First, we cotransfected PC12 cells with plasmids expressing shADAR2 #1 and the human growth hormone (hGH). Indeed, the release of hGH in response to 55 mM KCl from shADAR2 #1-transfected cells was significantly decreased as compared with shCON-transfected control cells (**Fig. 6*A***; from 2.87±0.02- to 1.92±0.17-fold). Next, we examined further the effect of ADAR2 knockdown on the secretion of an endogenous protein, chromogranin B (CgB), which is an acidic soluble proprotein known to be ubiqui-

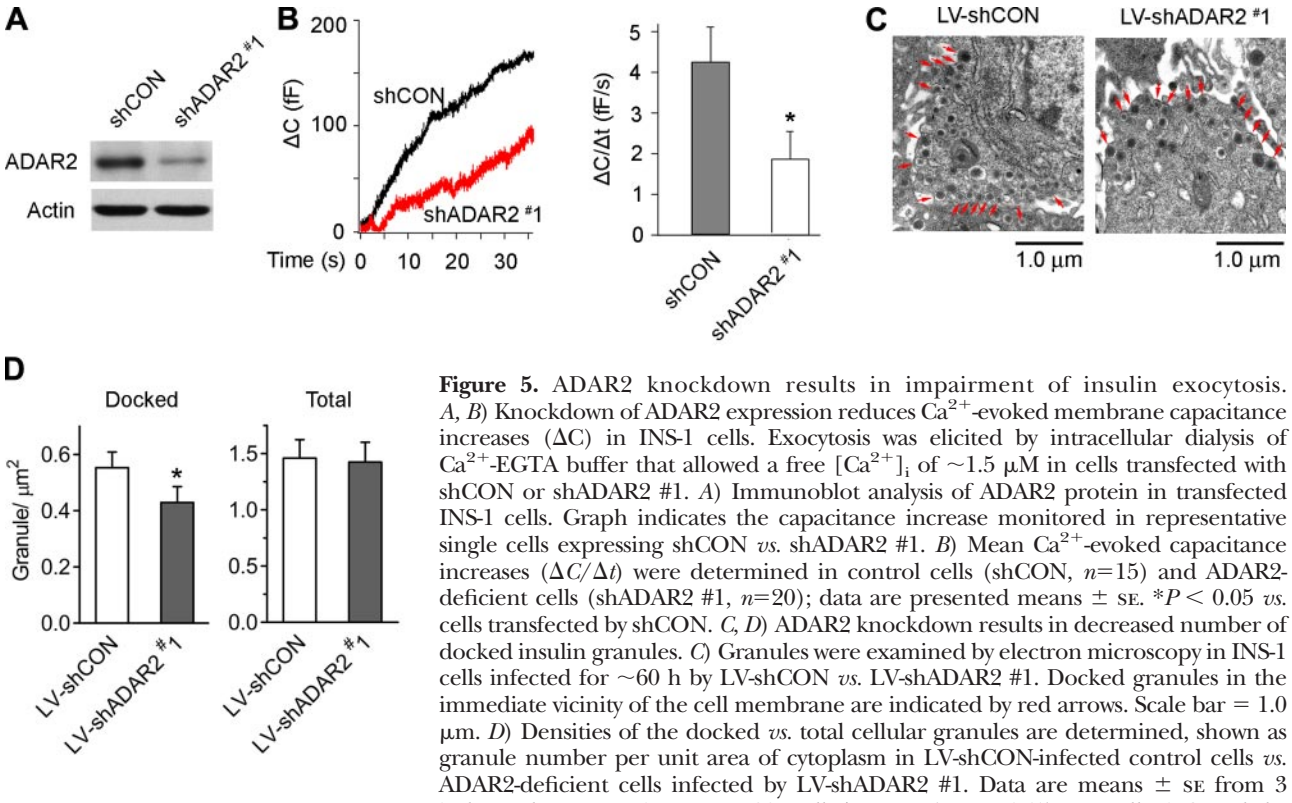


**Figure 5.** ADAR2 knockdown results in impairment of insulin exocytosis. *A, B*) Knockdown of ADAR2 expression reduces $Ca^{2+}$-evoked membrane capacitance increases (ΔC) in INS-1 cells. Exocytosis was elicited by intracellular dialysis of $Ca^{2+}$-EGTA buffer that allowed a free $[Ca^{2+}]_i$ of ~1.5 μM in cells transfected with shCON or shADAR2 #1. *A*) Immunoblot analysis of ADAR2 protein in transfected INS-1 cells. Graph indicates the capacitance increase monitored in representative single cells expressing shCON *vs.* shADAR2 #1. *B*) Mean $Ca^{2+}$-evoked capacitance increases ($\Delta C/\Delta t$) were determined in control cells (shCON, $n$=15) and ADAR2-deficient cells (shADAR2 #1, $n$=20); data are presented means ± SE. *$P < 0.05$ *vs.* cells transfected by shCON. *C, D*) ADAR2 knockdown results in decreased number of docked insulin granules. *C*) Granules were examined by electron microscopy in INS-1 cells infected for ~60 h by LV-shCON *vs.* LV-shADAR2 #1. Docked granules in the immediate vicinity of the cell membrane are indicated by red arrows. Scale bar = 1.0 μm. *D*) Densities of the docked *vs.* total cellular granules are determined, shown as granule number per unit area of cytoplasm in LV-shCON-infected control cells *vs.* ADAR2-deficient cells infected by LV-shADAR2 #1. Data are means ± SE from 3 independent experiments; ~30 cells/group. *$P < 0.05$ *vs.* cells infected by LV-shCON.

tously expressed and secreted in endocrine, neuroendocrine, and neuronal cells (43). Consistently, secreted CgB in the culture medium on stimulation of 55 mM KCl, as assessed by Western immunoblot, was reduced by ~30% from PC12 cells infected with Ad-shADAR2 #1 in comparison with control cells infected with Ad-shCON (Fig. 6*B*; from 4.14±0.02-fold to 2.83±0.17-fold). Thus, these data indicate that ADAR2 exerts important and general functional actions on protein exocytosis, not only in β cells but also in neuroendocrine cells.

### ADAR2 deficiency is associated with altered expression of key exocytotic molecules

Pancreatic β cells possess the full repertoire of key molecules that are orchestrated to mediate the controlled release of large-density core vesicles for classical neurotransmitters in neuron cells (19), including SNARE proteins, SNARE-regulators (*e.g.*, Munc18) and calcium-sensing molecules (*e.g.*, synaptotagmins). Munc18-1 interacts with the target membrane SNARE protein syntaxin-1 and is thought to be the chief regulator of the exocytotic SNARE complexes (24, 44). Of intriguing note, synaptotagmin-1, an essential $Ca^{2+}$-sensing component of the cellular apparatus in synaptic release, has recently been identified in *Drosophila* as a dADAR-targeted substrate with four A to I editing sites within the highly conserved C2B domain (45). To investigate further the molecular nature of ADAR2 deficiency-induced exocytotic defects, we first examined whether ADAR2 affected insulin exocytosis in β cells through editing modification of the mammalian homologues of synaptotagmin molecules. However, direct sequencing of the RT-PCR DNA fragments encoding synaptotagmin-1, -3 to -5, -7, and -9 to -13, the synaptotagmins found to be expressed in INS-1 cells (46), revealed no detectible conserved editing events within the homologous regions harboring the edited sites occurring in the fly (unpublished observations). Surprisingly, in comparison with Ad-shLacZ-infected control cells, ADAR2 knockdown in Ad-shADAR2 #1-infected cells substantially reduced the protein expression levels of not only Munc18-1 (by ~50%) but also synaptotagmin-7 (by ~40%), another key molecule in mediating exocytotic secretion in pancreatic α and β cells (20, 47, 48); however, ADAR2 knockdown exhibited little effect on the expression of Syntaxin-1, the interacting SNARE partner of Munc18-1 (**Fig. 7*A***). In accordance with the specificity of the impairing effect of ADAR2 deficiency on GSIS, knockdown of ADAR1 expression resulted in no significant alterations in the expression of these exocytotic regulatory proteins (Fig. 7*A*). Furthermore, moderate decreases were detected by quantitative RT-PCR analysis in the mRNA levels of *Munc18-1* and *Syt 7* (by ~40 and 35%, respectively), while little changes were detected in that of *Munc18-2* (Supplemental Fig. 4). Similarly, ADAR2 deficiency led to significantly down-regulated expression of Munc18-1 and Syt 7 proteins (by ~55 and 50%, respectively) in Ad-shADAR2 #1-infected PC12 cells (Fig. 7*B*). These results thus suggest that the expression of Munc18-1 and synaptotagmin-7 may be selectively targeted for modu-

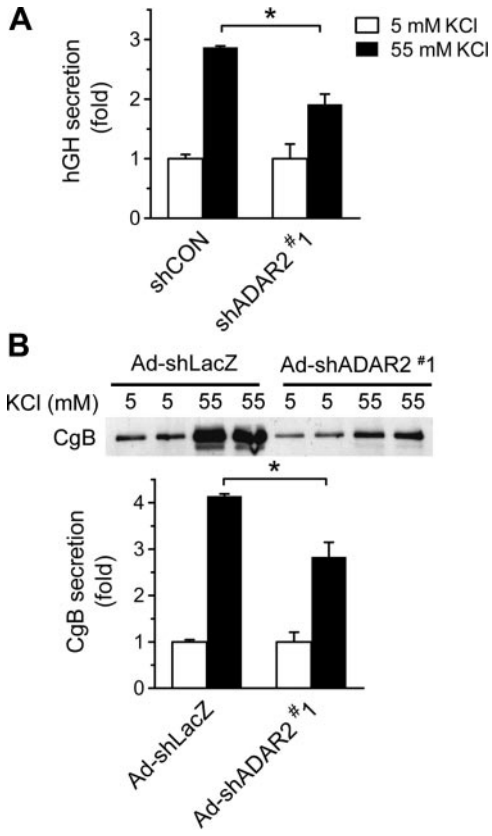


**Figure 6.** ADAR2 deficiency leads to impairment in stimulated exocytosis in PC12 cells. *A*) ADAR2 knockdown reduces KCl-stimulated secretion of human growth hormone. PC12 cells were cotransfected for ~72 h with the plasmid encoding human growth hormone (hGH) and plasmids expressing shADAR2 #1 *vs.* shCON. Secretion of hGH was measured by ELISA from cells stimulated by 55 *vs.* 5 mM KCl. Fold increases in hGH secretion are shown as means ± SE from 3 independent experiments. $*P < 0.05$ *vs.* shCON. *B*) ADAR2 knockdown attenuates KCl-stimulated chromogranin B secretion. At ~72 h postinfection by Ad-shADAR2 #1 *vs.* Ad-shLacZ, secreted chromogranin B (CgB) levels were analyzed by Western immunoblot from PC12 cells in response to stimulation by KCl at 55 mM relative to 5 mM. Fold increases in CgB secretion were quantified by densitometry from the immunoblots after normalization to cell numbers. Data are means ± SE from 4 independent experiments. $*P < 0.05$ *vs.* Ad-shLacZ.

lation by ADAR2, explaining at least in part the influence of ADAR2 deficiency on the stimulated protein secretion both in β cells and PC12 cells.

## DISCUSSION

In our exploratory attempt to gain further insights into the cellular function of ADAR-mediated RNA editing, several lines of evidence from the present study revealed a functional connection between ADAR2 editing enzyme and regulated exocytosis in professional secretory cells. First, we found that selective knockdown by RNAi of the expression of ADAR2 but not ADAR1 resulted in impairment in the stimulated secretion of large dense-core secretory granules in INS-1 β cells as well as PC12 cells. Second, the deaminase activity of ADAR2 appears to be an essential component in mediating its effect on cellular secretion. Third, suppression of ADAR2 in β cells also led to significant attenuation of $Ca^{2+}$-evoked increases in membrane capacitance. Fourth, ADAR2 deficiency-elicited secretory defects were associated with down-regulated expression of Munc18-1 and synaptotagmin-7, two key regulatory molecules involved in vesicle exocytosis. These findings suggest that ADAR2-dependent action may serve as a hitherto unappreciated novel mechanism in the control of cellular secretory machinery.

In pancreatic β cells, action of ADAR2 editing enzyme may exert an additional mode of control in fuel-regulated insulin secretion by affecting the formation of SNARE complex. While we were unable to identify editing modifications for the mammalian synaptotagmin proteins in β cells as those reported for its *Drosophila* homologue (45), we did observe significant suppression of the expression of Munc18-1 and synaptotagmin-7 as a result of ADAR2 deficiency in INS-1 β cells as well as PC12 cells. These results are in accordance with the importance of Munc18-1 expression in cellular exocytosis, which has been suggested as a powerful mechanism in controlling the size of readily releasable vesicle pool (49). In addition to its crucial role in synaptic release (50), Munc18-1 has also been demonstrated to exert a positive action in regulated insulin secretion from β cells (51). However, the way in which Munc18-1 expression is physiologically regulated remains largely unclear, and the precise mechanism whereby ADAR2 deficiency leads to its expression down-regulation is yet to be deciphered. Given A to I RNA editing as a newly recognized regulatory mechanism in microRNA maturation or its targeting redirection (3, 52, 53), it is tempting to speculate that ADAR2 may operate through a microRNA-mediated mechanism to exert its effect on the expression of key exocytotic molecules such as Munc18-1.

The physiological importance of ADAR2-mediated action in cellular exocytosis remains to be defined. Previously we observed that ADAR2 in the pancreatic islets of mice was selectively up-regulated on the increased demand for insulin secretion in the state of diet-induced obesity (16). Here our results showed that ADAR2 knockdown in INS-1 cells and primary islets led to dampened insulin secretion on glucose stimulation, indicating a role of ADAR2 enzyme in glucose metabolism. However, whether ADAR2 exerts critical metabolic actions *in vivo* on systemic glucose homeostasis through its effect on the secretory properties of β cells is currently an open question. In the brain, ADAR-mediated RNA editing of three AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) receptor subunits (GluR-B, -C, and -D) at a position termed the R/G site has been implicated in controlling the kinetic properties (*i.e.*, the recovery rates from desensitization) of these channels (54), whereas editing at the Q/R site of GluR-B subunit by ADAR2 leads to reduced calcium permeability of the gated channels (55). Interestingly,

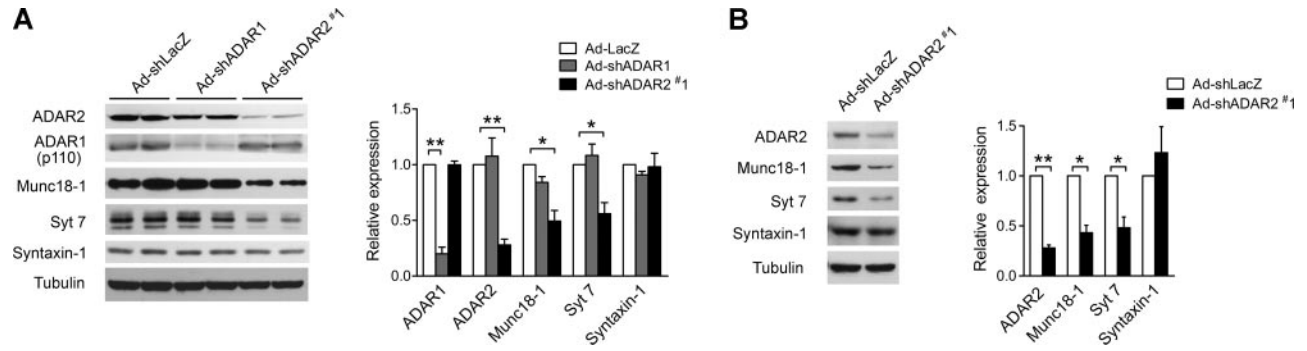


**Figure 7.** ADAR2 deficiency is associated with decreased expression of key exocytotic molecules. *A*) Cell lysates of INS-1 cells infected for 60 h with Ad-shADAR1, Ad-shADAR2 #1, or Ad-shLacZ were subjected to Western immunoblot analysis, using the indicated antibodies for the detection of ADAR1, ADAR2, Munc18-1, Syt 7, and Syntaxin-1, respectively. Relative expression levels of the indicated proteins were quantified by densitometry from the immunoblots after normalization to tubulin, which was used as the loading control. *B*) Cell lysates of PC12 cells infected for 72 h with Ad-shADAR2 #1 *vs.* Ad-shLacZ were analyzed by Western immunoblotting for the detection of the indicated proteins. Shown are representative immunoblots as well as quantification of the relative expression levels of each indicated protein after normalization to tubulin. Data are means ± SE from ≥3 independent experiments. $*P < 0.05$, $**P < 0.01$ *vs.* Ad-shLacZ.

glutamate is known to be cosecreted with glucagon from α cells (56), and intracellular glutamate has been shown to exert crucial actions on insulin secretion in β cells (57). Given that GluR receptor channels are expressed abundantly in β cells (16, 58), it is currently unclear whether ADAR2-dependent editing of glutamate receptors is involved in coordinating cell-cell communications (*e.g.*, between α and β cells) within pancreatic islets in response to various physiological stimulatory conditions.

A wide range of cellular RNA species are thought to undergo targeted editing modifications by ADAR enzymes (14, 59), including coding as well as noncoding RNA molecules such as miRNAs. It is most likely that RNA editing by ADAR2 acts on multiple RNA targets related to cellular secretory pathways, providing a fine-tuning mechanism in the control of the orchestrated coupling process of stimulated vesicle exocytosis. In this scenario, ADAR2-mediated actions might play a profound part in the CNS through modulating the dynamics of synaptic vesicle docking and fusion (18, 34). While our findings suggest a functional role for ADAR2 in the regulation of glucose metabolism, ADAR2 has been also implicated in the central control of energy balance (38). Singh *et al.* (38) previously reported that mice expressing either the wild-type or deaminase-deficient ADAR2 transgenes exhibited hyperphagia and developed adult-onset obesity. Whereas the exact mechanism by which ADAR2 affects the neuronal circuitry involved in body weight regulation remains poorly understood, our results provide additional clues with respect to the potential action of ADAR2 in metabolic control. In the CNS, glucose-responsive (GR) neurons may possess similar glucose-sensing mechanisms as in pancreatic β cells; *e.g.*, through ATP-mediated closure of $K_{ATP}$ channels (60), thereby altering their firing rate when the brain glucose levels rise (61). Given the various neurotransmitters/neuropeptides found to be released from GR neurons (62), further elucidation of the detailed molecular events mediated by ADAR2 editing enzyme in regulated exocytosis (*e.g.*, in response to glucose stimulation) will allow us to better understand the physiological function of A to I RNA editing in the control of neuropeptide secretion and neurotransmitter release, in addition to the endocrine function of β cells. In this regard, tissue- or neuron-specific gene-targeting strategies would be required to further dissect the central or peripheral actions of ADAR2 in metabolic homeostasis. FJ

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