Methods to Study Roles of β-Arrestins in the Regulation of Pancreatic β-Cell Function

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Abstract

Novel findings reveal important functional roles for β-arrestin 1 and β-arrestin 2 in the regulation of insulin secretion, β-cell survival, and β-cell mass plasticity not only by glucose, but also by G-protein coupled receptors, such as the glucagon-like peptide-1 (GLP-1) and the pituitary adenylate cyclase-activating polypeptide (PACAP) receptors or GPR40, or tyrosine kinase receptors, such as the insulin receptor. Here, we describe experimental protocols to knockdown β-arrestins by small interference RNA, to follow subcellular localization of β-arrestins in the cytosol and nucleus of the insulinoma INS-1E rat pancreatic β-cell line, to analyse β-arrestin protein expression by western blot using INS-1E cells and isolated mouse or human pancreatic islets. We also provide details on how to genotype β-arrestin 2 knockout (*Arrb2*$^{-/-}$) mice, and to evaluate β-arrestin-mediated roles in β-cell mass plasticity and β-cell signalling using immunocytochemistry on pancreatic sections or on primary dispersed β-cells from wild type mice and *Arrb2*$^{-/-}$ mice.

Key Words

Pancreatic β-cell, Small interference RNA, Subcellular fractionation, Western blot, Genotyping, Immunocytochemistry, Immunofluorescence, β-arrestins

1 Introduction

Type 2 diabetes is a major public health issue with worldwide increasing incidences. It is the most prevalent form of diabetes that accounts for ~90% of all diabetic subjects. Typically recognized as a metabolic disease with disrupted glucose metabolism, type 2 diabetes is characterized by abnormally prolonged high blood glucose levels [1]. This chronic hyperglycemia is considered as a consequence of the complex heterogeneous nature, of polygenic origin, of the illness, which combines insulin-resistance in peripheral tissues (failure of insulin action) and impaired insulin-secretion from pancreatic β-cells. Although the relative importance of each of these disorders in the etiology of type 2 diabetes has been disputed, both are necessary for the development of the disease, which appears, even in the case of severe insulin-resistance, only when an impaired insulin secretion exists [2,3]. Moreover, a progressive decrease in β-cell mass has been reported in patients with type 2 diabetes [4,2]. Together, these evidences support the notion that β-cells play a central role in the etiology of type 2 diabetes. Therapeutic strategies designed to protect and to preserve a functional pancreatic β-cell mass are therefore essential to fight diabetes.

Pancreatic β-cell proliferation, survival and secretion are well described to be regulated by signalling pathways linked to glucose, G-protein coupled receptors (GPCRs), such as the glucagon-like peptide-1 (GLP-1), the pituitary adenylate cyclase-activating polypeptide (PACAP) receptors, or GPR40, and tyrosine kinase receptors (TKRs), such as the insulin receptor. Novel findings, including those from our group, reveal important functional roles for β-arrestin 1 (ARRB1) and β-arrestin 2 (ARRB2) in the regulation of insulin secretion, β-cell survival, β-cell proliferation, and β-cell mass plasticity [5-15]. This chapter details various step by step methods that we routinely use in the laboratory to investigate the roles of β-arrestins (ARRBs), in particular, we detail how 1) to evaluate levels of ARRB expression, 2) to analyze subcellular localization of ARRBs, 3) to knockdown *Arrbs* by small interference

RNA approaches in β-cells, 4) to genotype *Arrb2* knockout (*Arrb2*$^{-/-}$) mice, and 5) how to evaluate ARRBs roles in β-cell signalling and β-cell mass plasticity using immunocytochemistry on pancreatic sections or on primary dispersed β-cells from wild type mice and *Arrb2*$^{-/-}$ mice.

2 Materials

Prepare all the solutions with ultrapure water. Keep the reagents at room temperature (unless indicated otherwise). Follow the regulation for waste disposal.

2.1 Arrb knockdown by small interference RNA in INS-1E cells

a. Silencers used and preparation (Life Technologies)

Arrb1 Silencer® Select siRNA (5 nmol, catalog reference s129662), *Arrb2* Silencer® Select siRNA (5 nmol, catalog reference s129664) and Silencer® Select Negative Control No. 1 siRNA (5 nmol, catalog reference 4390843). Briefly, centrifuge the tube to ensure that the dried siRNA is at the bottom of the tube. Resuspend the 5 nmol siRNA using 100 µl of nuclease-free water provided for a final concentration of 50 µM. Store at - 20°C until use.

b. Reagents

Lipofectamine® 3000, Opti-MEM® Medium and RPMI medium (all Life Technologies)

2.2 Protein extraction and immunoblots from INS-1E cells and islets

Keep the solutions and materials at 4°C.

2.2.1 Protein extraction

1. 50 mM Tris-HCl buffer (pH 7.5): In a glass beaker, mix 250 ml of water and 3.03 g of Tris-base. Once the mixture is homogenized, adjust the pH to 7.5 by adding hydrochloric acid (HCl). Make up the volume to 500 ml and store at 4°C.

2. 1 M NaCl buffer: In a glass beaker, mix 90 ml of water and 5.84 g of NaCl. Make up the volume to 100 ml and store at 4°C.

3. 100 mM dithiothreitol (DTT): In 10 ml of water, mix 154 mg of DTT. Make aliquots of 500 µl and store at - 20°C.

4. 1 M sodium fluoride (NaF): In 10 ml of water, mix 419.9 mg of NaF. Make aliquots of 500 µl and store at - 20°C.

5. 100 mM sodium orthovanadate (Na_3VO_4): In 10 ml of water, mix 184 mg of Na_3VO_4. Make aliquots of 500 µl and store at - 20°C.

6. Protease inhibitor cocktail powder (Catalog reference P2714-1BTL, Sigma): Resuspend the powder in 10 ml of water (10 X solution). Make aliquots of 100 µl and store at - 20°C.

7. Nonidet P-40 (NP-40) or IGEPAL® (Sigma).

8. Pre-Buffer: In a glass beaker, mix 191 ml of water with 240 ml of 50 mM Tris-HCl, pH 7.5, 90 ml of 1 M NaCl and 1.04 ml of 1 M $MgCl_2$. Keep at 4°C.

9. Cell lysis buffer: 0.5% NP-40, 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM $MgCl_2$, 1 mM DTT, 5 mM NaF, 1 mM Na_3VO_4. In 43.5 ml of pre-buffer, mix 250 µl of NP-40, 500 µl of 100 mM DTT, 250 µl of 1 M NaF and 500 µl of 100 mM Na_3VO_4. Make aliquots of 900 µl and store at - 20°C. When needed, extemporaneously add 100 µl of protease inhibitors (10X solution) to this 900 µl aliquot.

10. Mini-centrifuge

11. Phosphate buffered saline (PBS).

12. Plastic scraper.

13. Ice.

2.2.2 Protein assay

1. Bicinchoninic Acid solution (Catalog reference B9643, Sigma).

2. Copper (II) sulfate pentahydrate 4% solution (Catalog reference C2284, Sigma).

3. Protein standard solution, 2 mg/ml bovine serum albumin (BSA).

4. 96-well plates.

5. Spectrophotometer.

6. Water bath.

2.2.3 Immunoblots

1. NuPAGE® LDS sample buffer (4X) (Novex, catalog reference NP007, Life Technologies).

2. NuPAGE® sample reducing agent (10X) (Catalog reference NP004, Life Technologies).

3. Boiling recipients.

4. Running buffer: Mix 950 ml of water with 50 ml of NuPAGE® MES SDS Running Buffer (20 X) (Catalog reference NP0002, Life Technologies).

5. NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm (Life Technologies).

6. NuPAGE® Antioxidant (Catalog reference NP005, Life Technologies).

7. SeeBlue® Plus2 pre-stained protein standard (Catalog reference LC5925, Life Technologies).

8. Methanol.

9. Transfer buffer: Mix 375 ml of water with 25 ml of NuPAGE® Transfer Buffer (20 X) (Catalog reference NP0006-1, Life Technologies) and 100 ml of methanol.

10. FluoroTrans® polyvinylidene difluoride (PVDF) membrane 0.2 μm.

11. Blotting tweezer to hold the PVDF membrane.

12. Blotting roller.

13. Orbital Shaker.

14. Small containers where the membrane can fit with 10 ml liquid.

15. Restore™ Western blot stripping buffer (21059, Life Technologies).

16. 10 X TBS: 200 mM Tris-HCl, pH 7.6, 1.37 M NaCl buffer. In 800 ml of water, dissolve 48.2 g of Tris-base and 160 g of NaCl. Once the mixture is homogenized, adjust the pH to 7.6 by adding hydrochloric acid (HCl). Make up the volume to 2 L and store at room temperature.

17. Tween® 20.

18. Washing solution (TBS-T): 1 X TBS, 0.1 % Tween® 20. Dilute 100 ml of 10X TBS in 800 ml of water. Add 1 ml of Tween® 20 and make up the volume up to 1 L. Store at room temperature.

19. Blotting-grade blocker, nonfat dry milk (Bio-Rad).

20. Blocking solution: TBS-T, 5% milk: Weigh 2.5 g of milk and mix gently with 40 ml of TBS-T in a beaker with the help of a stirrer. Make up the volume to 50 ml with TBS-T and store at 4°C for up to two days.

21. Primary antibodies: anti-ARRB2 mouse monoclonal antibody (Catalog reference ab54790, Abcam), anti-ARRB1 mouse monoclonal antibody (Catalog reference 610551, Becton Dickinson).

22. Secondary antibody: Goat Anti-Mouse IgG (H + L)-horseradish peroxidase enzyme (HRP) conjugate.

23. Immobilon Western Chemiluminescent HRP substrate (Millipore).

24. Transparent plastic sheet protector.

25. Plastic bag sealer.

26. Chemiluminescence Imaging System.

27. Image J software for blot analysis.

2.3 Cytoplasmic and nuclear protein extraction

1. NER-PER Nuclear and Cytoplasmic Extraction Reagents (Catalog reference 78833, Life Technologies).

2. Halt Protease Inhibitor Cocktail 100X (Catalog reference 78425, Life Technologies).

3. Cytoplasmic Extraction Reagent I solution: 500 µl of CERI + 5 µl Halt Protease Inhibitor Cocktail (100 X).

4. Nuclear Extraction Reagent (NER) solution: 100 µl NER + 1 µl Halt Protease Inhibitor Cocktail (100 X).

5. Phosphate buffered saline (PBS).

6. Mini-centrifuge.

7. Vortex.

2.4 Genotyping of Arrb2 knockout mice

1. Kapa mouse genotyping kit (KK7301 or KK7302)

2. PCR-grade water.

3. Primers for our mice (see subheading 3.4) are: Arrb2WT-forward GATCAAAGCCCTCGATGATC (intron 2), Arrb2KO-forward GCTAAAGCGCATGCTCCAGA (Neomycin), Arrb2-reverse ACAGGGTCCACTTTGTCCA (exon 3). Arrb2WT-forward and Arrb2KO-forward are specific for the wild type and knockout allele respectively, while the primer Arrb2-reverse ACAGGGTCCACTTTGTCCA is common for both wild type and knockout. Amplicon lengths are 605 bp and 300 bp for Arrb2WT and Arrb2KO alleles, respectively.

4. 25 mM $MgCl_2$.

5. 50x TAE. In 300 ml of water, dissolve 96.8 g of Tris-base and 1.48 g of EDTA. Add 22.84 ml of glacial acetic acid. Adjust pH to 8.2 by adding glacial acetic acid. Make up the volume to 400 ml and store at room temperature.

6. 1% (w/v) agarose gel in TAE containing 0.2-0.5 µg/ml ethidium bromide.

7. 0.5 ml tubes.

8. PCR-tubes.

9. Mini-centrifuge.

10. Cycler for PCR (Mastercycler, thermocycler…).

11. Ice.

12. Heating block or water bath.

2.5. Immunocytochemistry on pancreatic sections from Arrb2$^{-/-}$ mice

1. Phosphate buffered saline containing Ca^{2+}/Mg^{2+} (PBS).

2. Tween® 20. Prepare 0.05% Tween in PBS.

3. 37% formaldehyde solution (Sigma). Prepare 4% formaldehyde solution in PBS.

4. Absolute ethanol.

5. 70% ethanol (500 ml) for step 3.5.1.

6. 90% and 70% ethanol (200 ml each) for step 3.5.3.

7. Xylene.

8. Citrate buffer pH 6. Prepare 200 ml of 1 X citrate buffer.

9. MilliQ water.

10. 1% BSA in PBS.

11. Primary antibodies of interest. Here are the antibodies used in figure 4: Guinea pig anti-insulin antibody (Abcam, 1/200 in 1% BSA in PBS), Mouse anti-glucagon antibody (Sigma-Aldrich, 1/400 in 1% BSA in PBS).

12. 4',6-diamidino-2-phenylindole (DAPI) to stain nuclei

13. Secondary antibodies of interest. Here are the antibodies used in figure 4: Cy3 AffiniPure donkey anti guinea pig antibody (Jackson immunoresearch, 1/500 in 1% BSA in PBS), Alexa Fluor®488 AffiniPure donkey anti mouse antibody (Jackson immunoresearch, 1/500 in 1% BSA in PBS).

14. Mowiol® 4-88: Weigh 6 g of glycerol and add 2.4 g of Mowiol. Mix with a magnetic stirrer for at least 3 h. Add 6 ml of milliQ water and mix with a magnetic stirrer overnight. Add 12 ml of Tris-HCl 0.2M, pH 8.5. Mix with a magnetic stirrer 1 h at room temperature.

Incubate 20 min at 50°C. Mix gently during the incubation period. Transfer into a 50 ml tube. Centrifuge at 20°C at 5,000 g during 15 min. Make 500 µl aliquots in 1.5 ml tube and store at - 20°C.

15. Flat embedding cassettes (Catalog reference 410-02S Klinipath).

16. Sponge for embedding cassettes (Catalog reference F/PP15-8010 Microm Microtech).

17. Microscope slides.

18. Microscope coverslips (24mm x 60mm).

19. Dako Pen or any water-repelling pen.

20. Immunocytochemistry racks.

21. Opaque plastic or metallic box with a sealed lid.

22. Fluorescence microscope. The model used to generate the images was an AxioImager microscope (Zeiss).

2.6. Immunocytochemistry on dispersed pancreatic islet cells from Arrb2$^{-/-}$ mice

1. Phosphate buffered saline containing Ca^{2+}/Mg^{2+} (PBS).

2. 37% formaldehyde solution. Prepare 4% formaldehyde solution by diluting in PBS.

3. 100 mM glycine in PBS from 1 M stock. 1 M stock: 0.75 g in 10 ml, pH 8.5 with NaOH.

4. 10% FBS in PBS.

5. 0.2% Triton in PBS.

6. 3% BSA in PBS.

7. Parafilm-M®.

8. Microscope coverslips (15 or 18 mm diameter)

9. Microscope slides

10. Opaque plastic or metallic box with a sealed lid.

11. Primary antibodies of interest. Here are the antibodies used in figure 5: Guinea pig anti-insulin antibody (Abcam, 1/200 in 1% BSA in PBS), Rabbit antibody raised against our protein of interest (1/150 in 1% BSA in PBS).

12. Secondary antibodies of interest. Here are the antibodies used in figure 5: Alexa Fluor®488 AffiniPure donkey anti guinea pig antibody (Jackson immunoresearch, 1/5000 in 1% BSA in PBS), Cy3 AffiniPure donkey anti rabbit antibody (Jackson immunoresearch, 1/6000 in 1% BSA in PBS).

13. Mowiol® 4-88: Prepare as in section 2.5.14

3. Methods

3.1 Arrb knockdown by small interference RNA in INS-1E cells

1. Seed the INS-1E cells to be 60-80% confluent at transfection (i.e. 800,000 INS-1E cells per well in 6-well plates).
2. The next day, dilute Lipofectamine 3000 in Opti-MEM Medium: 3.3 µl Lipofectamine in 110 µl Opti-MEM.
3. Dilute siRNA in Opti-MEM Medium. For a 50 nM siRNA final concentration in the well: 1.2 µl siRNA in 118.8 µl Opti-MEM.
4. Add 110 µl of diluted siRNA to 110 µl of diluted lipofectamine.
5. Incubate for 5 min.
6. During this time, rinse wells with Opti-MEM Medium. Add 800 µl of Opti-MEM medium per well.
7. Add 200 µl siRNA-lipid complex to INS-1E cells (total volume per well is 1 ml). Incubate cells at 37°C.
8. 24 h after transfection, replace Opti-MEM medium with 2 ml of complete RPMI culture medium.
9. Lyse for protein extraction (see step 3.2) or perform immunocytochemistry (see step 3.6) of INS-1E cells 48 h or 72 h after transfection.

3.2 Protein extraction and immunoblots from INS-1E cells and islets

Detailed protocols from our lab to isolate the islets of Langerhans [15,16] have already been published. In this section, we have described procedures that we have already used for ARRBs and other proteins of interest [17,15,8,9].

3.2.1. Protein extraction

Pre-chill down the material to be used and keep everything on ice.

1. Aspirate culture medium (from culture dishes for INS-1E cells, or from 1.5 ml tubes for islets).
2. Rinse twice with cold PBS.
3. Add 110 µl lysis buffer per well (for INS-1E cells in 6-well plates) or 30 µl lysis buffer per 100 islets. Lyse for 10 min on ice.
4. Scrape cells with a plastic scraper and collect the lysate into 1.5 ml tube. For the islets only: sonicate the sample at low power, 3 times 5 s on ice.
5. Centrifuge for 10 min at 10,000 rpm at 4°C in mini-centrifuge to remove cellular debris and transfer the supernatant into 1.5 ml tubes.
6. Keep the samples at - 80°C until subsequent protein assay determination using bicinchoninic acid solution (BCA) and immunoblotting.

3.2.2. Protein assay

Protein determination by the Bicinchoninic Acid (BCA) method was performed according to the manufacturer's instructions.

3.2.3. Immunoblots

Run INS-1E cell or islet lysates (35-40 µg of protein) in a NuPAGE® Novex 4-12% Bis-Tris gel. Transfer the proteins from the gel to a PVDF membrane (*see* notes 1 and 2). All procedures can be done at room temperature unless specified otherwise.

1. Place the PVDF membrane in a dish and add the blocking solution until it covers the membrane. Shake gently for 1 h at room temperature on an orbital shaker.
2. Prepare the solution containing primary antibody by adding anti-ARRB1 or anti-ARRB2 antibodies in new blocking solution. Use 8 µl of antibody to 2 ml of blocking solution (1/250).

3. Place the membrane in a small plastic bag. Add the antibody solution and seal the plastic (see note 3). Incubate overnight shaking gently on the orbital shaker in cold room.
4. Discard the antibody solution.
5. Wash the membrane 3 times for 5 min in washing solution under gentle agitation.
6. Discard the washing solution.
7. Prepare the solution containing the secondary antibody in new blocking solution. Use 1 µl of antibody to 2 ml of blocking solution (1/2000).
8. Place the membrane in a small plastic bag. Add the secondary antibody solution and seal the plastic. Incubate 1 h shaking gently on the orbital shaker at room temperature.
9. Wash the membrane 3 times for 5 min in washing solution under gentle agitation.
10. Apply the Immobilon Western Chemiluminescent HRP substrate as indicated by the manufacturer and incubate for 1 min.
11. Place the membrane between a transparent plastic sheet protector.
12. Proceed to signal detection using a chemiluminescence imaging system (Figure 1).

3.3 Cytoplasmic and nuclear protein extraction

Pre-chill down the material to be used and keep everything on ice.

1. Aspirate culture medium from INS-1E cells plated in 6-well plates.
2. Rinse once with cold PBS.
3. Add 1 ml of cold PBS to each well.
4. Scrap the cells with a plastic scraper and collect the cells into 1.5 ml tube.
5. Centrifuge for 1 min at 2655 x g, in a mini-centrifuge, at 4 °C to pellet the cells.
6. Carefully remove and discard the supernatant, leaving cell pellet as dry as possible.
7. Add 100 µl of ice-cold CERI solution to the pellet. Resuspend the cells.
8. Vigorously vortex the tube on the highest setting for 15 s.

9. Incubate the tube on ice for 10 min.

10. Add 5.5 μl of ice-cold Cytoplasmic Extraction Reagent II (CER II) solution to the tube.

11. Vortex the tube on the highest setting for 5 s.

12. Incubate the tube on ice for 1 min.

13. Vortex the tube on the highest setting for 5 s.

14. Centrifuge for 5 min, in a mini-centrifuge, at 20,817 x g at 4°C.

15. Transfer the supernatant (cytoplasmic extract) to a clean tube and keep the sample at -80°C until immunoblot analysis.

16. Rinse the insoluble (pellet) fraction, which contains nuclei, with 100 μl of CERI solution.

17. Vortex the tube on the highest setting for 8 s.

18. Centrifuge for 5 min at 20,817 x g at 4°C.

19. Discard the supernatant.

20. Suspend the pellet fraction in 28 μl of ice-cold NER solution.

21. Vortex the tube on the highest setting for 15 s.

22. Place the sample on ice and continue vortexing for 15 s every 10 min, for a total of 40 min.

23. Centrifuge the tube for 10 min, in a mini-centrifuge, at 20,817 x g at 4 °C.

24. Transfer the supernatant (nuclear extract) to a clean tube and keep the sample at - 80°C until immunoblot analysis (*see section 3.2.3*). Figure 2 illustrates a typical ARRB1 immunoblot image obtained after cytoplasmic and nuclear protein extraction.

3.4. Genotyping of Arrb2$^{-/-}$ mice

Our original *Arrb2* heterozygous mice were obtained from Dr. Robert J. Lefkowitz (Duke University Medical Center, Durham, NC, USA). *Arrb2*$^{-/-}$ mice under different strain are available from the Jackson Laboratory (www.jax.org/mouse-search?searchTerm=arrb2). As

already reported [9] our mice were subsequently backcrossed onto C57BL/6J background (Charles River, Lyon, France). *Arrb2*$^{-/-}$ mice and their wild-type littermate (*Arrb2*$^{+/+}$) are generated by breeding heterozygous animals in our EOPS animal facility at the Institut de Génomique Fonctionnelle, Montpellier, France. Animals are housed in standard conditions with a 12 h light/dark cycle, stable temperature (22 ± 1°C), controlled humidity (55 ± 10%) and water and food *ad libitum*.

DNA extraction and PCR can be performed using classical techniques (*see* note 4). In the laboratory we use a slightly adapted protocol from KAPA mouse genotyping kit (www.kapabiosystems.com).

3.4.1. DNA extraction

1. Warm up the heating block (or water bath) to 75°C.

2. Prepare a mix for the extraction. For each extraction tube: 44 µl PCR-grade water, 5 µl express extract buffer (vortex before adding), 1 µl express extract enzyme (never vortex, mix with pipette).

3. Add 50 µl of the mix on mouse tail or ear tissue.

4. Incubate for 10 min at 75°C (during this step cells are lysed, nucleases and proteins degraded and DNA released).

5. Incubate for 5 min at 95°C (to inactivate the thermostable KAPA express extract protease).

6. Incubate the samples on ice (2-3 min to cool down the samples).

7. Vortex for 2-3 sec, centrifuge at high speed, with a mini-centrifuge, for 1 min, and transfer 40 µl of supernatant to fresh 0.5 ml tubes or alternatively use immediately the sample for PCR.

3.4.2. PCR reaction

1. Prepare a mix containing for each sample reaction (protocol slightly adapted from KAPA mouse genotyping kit): 3.625 µl PCR-grade water, 0.25 µl $MgCl_2$ (from 25 mM stock), 0.625 µl of 10 µM primer *Arrb2*WT-forward, 0.625 µl of 10 µM primer *Arrb2*KO-forward, 0.625 µl of 10 µM primer *Arrb2*-reverse, 6.25 µl KAPA2G fast genotyping mix (do not vortex, mix by hand). Prepare four extra tubes for the mix to have control lanes (1 WT, 1 KO, 1 H_2O only, and 1 extra).

2. Add 0.5 µl of extracted DNA in PCR tubes.

3. Add 12 µl of the PCR mix to samples.

4. Make a short spin of samples in a mini-centrifuge.

5. Run the PCR, repeating steps b-d below for 35 cycles

a. 95°C, 3min

b. 95°C, 15sec

c. 58°C, 15sec

d. 72°C, 30sec

e. 72°C, 7min

f. 4°C

6. Load samples on agarose gel (1% in TAE) containing ethidium bromide, and run for 45 min at 100 V (Figure 3).

3.5. Immunocytochemistry on pancreatic sections from Arrb2$^{-/-}$ mice

In this section we have described the procedure that we have used in *Ravier et al. 2014 Diabetologia* [9] (see note 5).

3.5.1. Pancreas setting into cassettes

1. Sacrifice the animal by cervical dislocation according to your animal-care institutionally approved procedures.

2. Locate the pancreas and immediately harvest.

3. Spread into flat embedding cassette with fixed lid containing a PBS humidified sponge.

4. At this stage you can also weigh the pancreas. This step is required if you want to assess the pancreatic islet or β-cell mass.

5. Place a second PBS humidified sponge, and close the cassette.

6. Place the cassette in 4% formaldehyde under a fume hood and leave 20-24 h under stirring at room temperature.

7. Wash two times with PBS, each time for 5 min.

8. Wash with 70% ethanol to dehydrate the pancreas and store at 4°C.

3.5.2. Dehydration and paraffinising of the pancreas

For this step, our samples are processed by the RHEM facility in Montpellier (https://www.rhem.cnrs.fr/) for final steps of dehydration and paraffin embedding. The pancreas is longitudinally sectioned through pancreatic head-to-tail axis (4 μm thick). We use three pancreatic sections per mouse, separated by at least 100 μm. Detailed protocols can be found elsewhere (www.protocolsonline.com/histology/sample-preparation/paraffin-processing-of-tissue).

3.5.3. Deparaffinising and rehydrating pancreatic sections

1. Place slides (pancreatic sections) in an immunocytochemistry rack.

2. Wash with xylene for 10 min under a fume hood.

3. During this washing step prepare 200 ml of 1 X citrate buffer, pH 6. It has to be freshly prepared. Warm-up the solution directly in a proper immunocytochemistry rack (98°C, water bath).

4. At the end of step 2, wash with a second bath of xylene for 5 min.

5. Wash with absolute ethanol (99.9%) for 5 min.

6. Wash with 90% ethanol for 5 min.

7. Wash with 70% ethanol for 5 min.

8. Rehydrate with Milli-Q water for 5 min.

3.5.4. Antigen retrieval and immunohistochemical staining

Day 1

1. Place the slides in the citrate buffer rack, pH 6 that has been prepared in section 3.5.3(3), and incubate for 30 min at 98°C.

2. Leave the citrate buffer rack to cool down for 30 min at room temperature.

3. Remove the slides from the rack, drain for few seconds and wipe around the section with tissue paper to remove the citrate buffer.

4. Use a water-repelling pen such as Dako pen to draw a circle around the pancreatic section.

5. Place the slides horizontally in a humidified chamber (*see* note 6).

6. Cover pancreatic slices with the blocking buffer (PBS with 1% BSA) and leave for 30 min at room temperature.

7. Drain slides and wipe around the sections with tissue paper.

8. Add primary antibodies diluted in PBS with 1% BSA to cover the entire pancreatic section.

9. Shut the lid of the box and keep at 4°C overnight.

Day 2

10. Drain slides and wash three times in Tween 0.05% in PBS, each time for 10 min at room temperature.

11. From now on, the following steps should be performed with as little exposure to light as possible.

12. Add secondary antibodies and DAPI (to stain nuclei) diluted in PBS with 1% BSA (1/1000) to cover the entire pancreatic section.

13. Shut the lid of the box and keep at room temperature for 45 min.

14. Drain slides and wash two times in Tween 0.05% in PBS, each time for 10 min at room temperature.

15. Wash with PBS for 10 min at room temperature.

16. Wash with Milli-Q water for 10 min at room temperature.

17. Mount slides with Mowiol or any other mounting agent recommended for fluorescence (Dako Glycergel, DABCO, Vectashield, etc.) (*see* note 7). Place a drop (~ 40 µl) of mounting medium on the slide (*see* note 8) and put a glass coverslip on the top.

18. Let slides dry at 4°C overnight in the dark before examining the cells under a fluorescence microscope. Figure 4 illustrates a typical immunocytochemistry experiment observed in sections from *Arrb2*$^{+/+}$and *Arrb2*$^{-/-}$ mouse pancreas.

19. After imaging, slides mounted in Mowiol can be stored at - 20°C, and can be re-imaged several months after, depending on the stability of antibodies.

3.6. Immunocytochemistry on dispersed pancreatic islet cells from Arrb2$^{-/-}$ *mice*

A detailed protocol to prepare dispersed islet cells has already been published [16]. Compared to immunocytochemistry on pancreatic sections, this protocol allows to stimulate the cells with drugs or to re-express our protein of interest (ARRB2) by adenoviral infection of

pancreatic β-cells (Figure 5). In this section, we have described the procedure that we currently use in our lab [18] (*see* note 9).

Day 1

1. If cells are expressing a fluorescent protein, such as GFP, from now, the following steps should be performed with as little exposure to light as possible.

2. Fix cells with 3.7% formaldehyde in PBS containing Ca^{2+}/Mg^{2+} at room temperature for 20 min. If you plan doing an immunofluorescence experiment against a phosphorylated protein, you should add 80 mM NaF (to protect from phosphatases).

3. Wash cells with PBS for 5 min.

4. Wash cells with 100 mM glycine in PBS for 5 min (*see* note 10).

5. Wash cells with blocking buffer 10% FBS in PBS for 5 min.

6. Wash cells twice with PBS.

7. Permeabilization of cells with 0.2% Triton in PBS for 20 min.

8. Wash cells 3 times with PBS.

9. Wash cells with blocking buffer with 3% BSA in PBS at room temperature for 15 min.

10. Drain the coverslip and wipe around the sections with tissue paper.

11. On Parafilm-M® placed in a humidified chamber (*see* note 6), for a 15 mm diameter coverslip, add 60 µl of primary antibody in 3% BSA in PBS (*see* notes 8 and 11).

12. Place the coverslip inverted on the Parafilm-M®, cells facing the primary antibody/Parafilm-M® (*see* note 12).

13. Shut the lid of the box and incubate overnight at 4°C in the dark.

Day 2

14. Wash cells 3 times with PBS, each time for 10 min.

From now, the following steps should be performed with as little exposure to light as possible if it was not already required in step 1 from day 1.

15. On Parafilm-M® placed in a humidified chamber (*see* note 6), for a 15 mm diameter coverslip, add 60 µl of secondary antibody and DAPI (to stain nuclei) diluted in PBS with 3% BSA (1/1000) (*see* notes 8 and 11).

16. Place the coverslip inverted on the Parafilm-M®, cells facing the secondary antibody /Parafilm-M® (*see* note 12).

17. Shut-off the lid of the box and incubate 1 h at room temperature in the dark.

18. Wash cells 3 times with PBS, each time for 10 min.

19. Drain the coverslip and wipe around the sections with tissue paper.

20. Mount slides with Mowiol or any other mounting agent recommended for fluorescence (Dako Glycergel, DABCO, Vectashield, etc.) (*see* note 7). Place a drop (~ 20 µl) of mounting medium on the slide (*see* note 8) and put a glass coverslip on the top.

21. Let slides dry at 4°C overnight in the dark before examining the cells under a fluorescence microscope. Figure 5 illustrates a typical immunocytochemistry observed in dispersed islet cells from *Arrb2*$^{-/-}$ mice.

22. After imaging, slides mounted in Mowiol can be stored at - 20°C, and can be reimaged several months after, depending on the stability of antibodies.

4 Notes

1. PVDF membranes need to be activated in methanol for 30 s before use.

2. It is important to handle the membrane with blotting tweezers and to keep it wet at all time.

3. Verify that you don't have bubbles at the interface between your membrane and the antibody solution.

4. Commercially available kits from several companies can be found elsewhere: www.quantabio.com/accustart-ii-pcr-genotyping-kit; www.systembio.com/genome-engineering-ez-genotyping-kit;www.neuvitro.com/rapid-genotyping-system.htm. Mice are genotyped by PCR using genomic DNA isolated from tail, but DNA isolated from ear can also be used.

5. Other detailed protocols can be found elsewhere: www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol; www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if.

6. Usually we place humidified tissue paper at the bottom of an opaque plastic or metallic box with a sealed lid.

7. The mounting medium prevents dehydration, optimizes the refractive index for microscopy, prevents photobleaching and preserves samples for long-term storage.

8. Avoid making bubbles. Burst bubbles with a thin needle or remove with the pipette.

9. Other similar detailed protocols can be found elsewhere: www.abcam.com/protocols/immunocytochemistry-immunofluorescence-protocol; www.cellsignal.com/contents/resources-protocols/immunofluorescence-general-protocol/if.

10. Glycine will bind free aldehyde groups that occur with fixative such as formaldehyde. This step will reduce the background due to non-specific binding of primary and secondary antibodies.

11. This procedure reduces the volume of the antibody that has to be used.

12. Check that you don’t have bubbles at the interface between your cells and the antibodies.

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Figure legends

Figure 1. ARRB2 protein expression in *Arrb2*$^{+/+}$, *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice. Representative immunoblots and quantitative analysis by densitometry using Image J of Arrb2 protein from *Arrb2*$^{+/+}$, *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice. Reproduced from [9] with permission from Springer.

Figure 2. Detection of β-arrestin 1 in cytoplasmic and nuclear fractions from INS-1E cells. INS-1E cells were subjected to subcellular fractionation. Cytoplasmic and nuclear fractions resolved by SDS-PAGE and immunoblotted with anti-β-arrestin 1, anti-GAPDH (loading control for cytoplasmic fraction), anti-PARP antibody (loading control for nuclear fraction).

Figure 3. Genotyping of *Arrb2*$^{+/+}$, *Arrb2*$^{+/-}$ and *Arrb2*$^{-/-}$ mice. Mouse genotyping was performed by DNA extraction and PCR as described in section 3.4. Samples were loaded on an agarose gel (1% in TAE) containing Ethidium bromide, and run for 45 min at 100V. MW: Molecular weight. Amplicon lengths are 605 bp and 300 bp for *Arrb2*$^{+/+}$ and *Arrb2*$^{-/-}$ alleles, respectively. Reproduced from [9] with permission from Springer.

Figure 4. Immunocytochemistry on islets. (**a**) Representative immunofluorescence staining of insulin and glucagon from a pancreatic section. Nuclei have been labelled with DAPI. Quantification of beta and alpha cell number per islet (**b**) and beta cell size (**c**) within the islets from *Arrb2*$^{+/+}$ vs *Arrb2*$^{-/-}$ mice, using the calibrated Image J analysis program. **$p < 0.01$ compared with *Arrb2*$^{+/+}$ mice. White, *Arrb2*$^{+/+}$, black, *Arrb2*$^{-/-}$. Reproduced from [9] with permission from Springer.

Figure 5. Immunocytochemistry on dispersed islet cells from *Arrb2*$^{-/-}$ mice that re-express ARRB2. *Arrb2*$^{+/+}$ mouse β-cells were immunostained for insulin and our protein of interest was immunostained in red. When ARRB2 expression was restored (ARRB2-GFP) in *Arrb2*$^{-/-}$ β-cells, it reduced the fluorescence intensity of our protein of interest in the nucleus (compare cell 1 with cell 2 or *Arrb2*$^{+/+}$ mouse β-cells). Scale bar, 5 μm.

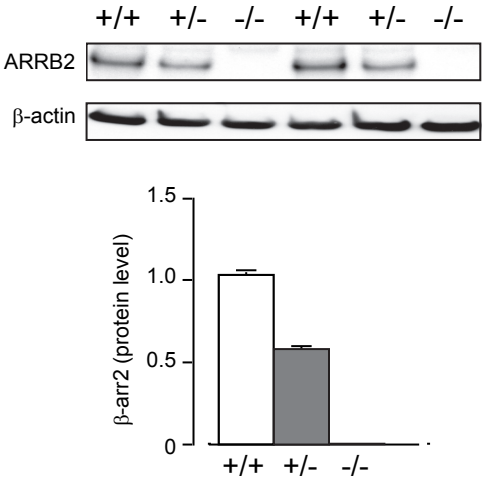

Figure1

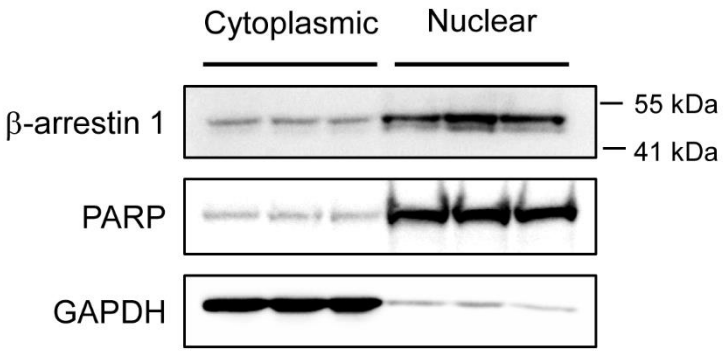

Figure 2

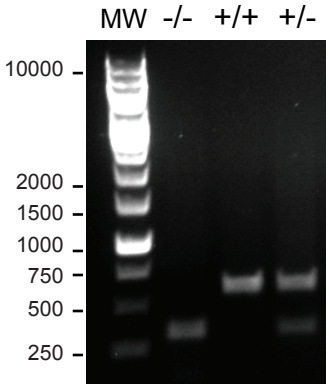

Figure 3

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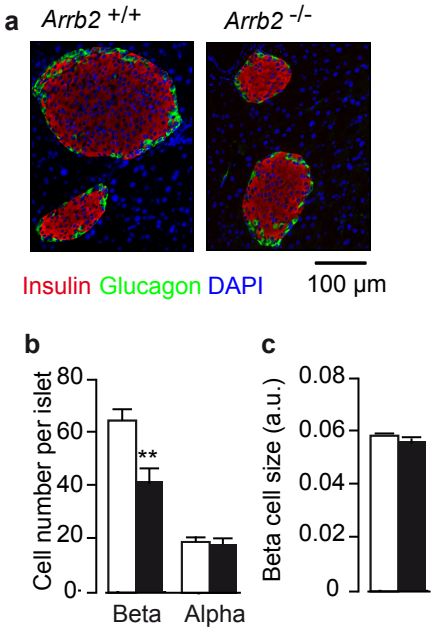
a
Arrb2 +/+
Arrb2 -/-
Insulin Glucagon DAPI
100 µm
b
Cell number per islet
80
60
40
20
0
**
Beta
Alpha
c
Beta cell size (a.u.)
0.08
0.06
0.04
0.02
0

Figure 4

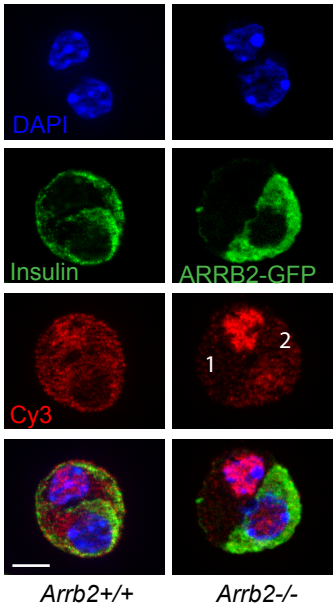

Figure 5