Supporting Information

Increased dosage of *Ink4/Arf* protects against glucose intolerance and insulin resistance associated with aging

Herminia González-Navarro[1,8,*], Ángela Vinué[2,*], María Jesús Sanz[1,3], Mercedes Delgado[4], Miguel Angel Pozo[4], Manuel Serrano[5], Deborah J. Burks[6], Vicente Andrés [7,8]

[1] Institute of Health Research-INCLIVA, 46010 Valencia, Spain

[2] Vascular Biology Unit, Department of Molecular and Cellular Pathology and Therapy, Instituto de Biomedicina de Valencia (IBV), Spanish Council for Scientific Research (CSIC), 46010 Valencia, Spain

[3] Departamento de Farmacología, Universidad de Valencia-INCLIVA, 46010, Valencia, Spain

[4] CAI Cartografía Cerebral, Instituto Pluridisciplinar, Universidad Complutense de Madrid, 28040, Madrid, Spain

[5] Spanish National Cancer Research Center (CNIO), 28029 Madrid, Spain

[6] CIBER de Diabetes y Enfermedades Metabolicas (CIBERDEM), Centro de Investigación Príncipe Felipe (CIPF), 46012 Valencia, Spain

[7] Laboratory of Molecular and Genetic Cardiovascular Pathophysiology, Department of Epidemiology, Atherothrombosis and Imaging, Centro Nacional de Investigaciones Cardiovasculares (CNIC), 28029 Madrid, Spain

[8]Address for correspondence: e-mail: vandres@cnic.es; e-mail: gonzaleh@uv.es

Experimental Procedures

Immunohistochemical analysis of pancreas. Pancreas were sectioned from mice, washed with PBS, fixed with 4% paraformaldehyde/PBS for 4 hours, transferred to PBS and paraffin-embedded as described (Gonzalez-Navarro et al., 2008). Examination of pancreas, performed by a researcher blinded to genotype, consisted of β-cell mass determination, measured as the area occupied by pancreatic islets relative to pancreatic area (%), and the number of pancreatic islets relative to total pancreatic area. Pancreatic islets were identified by insulin immunostaining (described below) in 10-12 slides per mouse, separated 125 μm. Pancreatic cell proliferation was evaluated in two slides/mouse (5 mice per experimental group) using Ki67 staining as proliferation marker. The immunohistochemistry protocol was: antigen retrieval with Sodium Citrate buffer 10mM, pH 6.5, peroxidase inactivation (H_2O_2 0.3%, methanol), incubation with primary antibodies (anti-insulin: 1/200 dilution, sc9168, SANTACRUZ, SantaCruz, CA, USA; anti-Ki67: a prediluted rabbit monoclonal antibody Clone SP6; MAD-000310QD, VITRO, Granada, Spain;) followed by incubation with a biotinylated anti-rabbit secondary antibody (1/500 dilution, sc-2040, SANTACRUZ, SantaCruz, CA, USA), and streptavidin-HRP (Ref. TS-060-HR, LABVISION Corporation, Thermofisher Fremont, CA USA) and DAB substrate (BUF021A, AbD SEROTEC, Düsseldorf, Germany). Slides were counterstained with hematoxylin and mounted with EUKITT (A10500, DELTALAB, Barcelona, Spain). Images were captured with an OPTIKAM PRO 5 digital camera mounted on a stereo microscope (OPTIKA, Barcelona, Spain) and analyzed by computer-assisted morphometry (SigmaScan, Pro5).

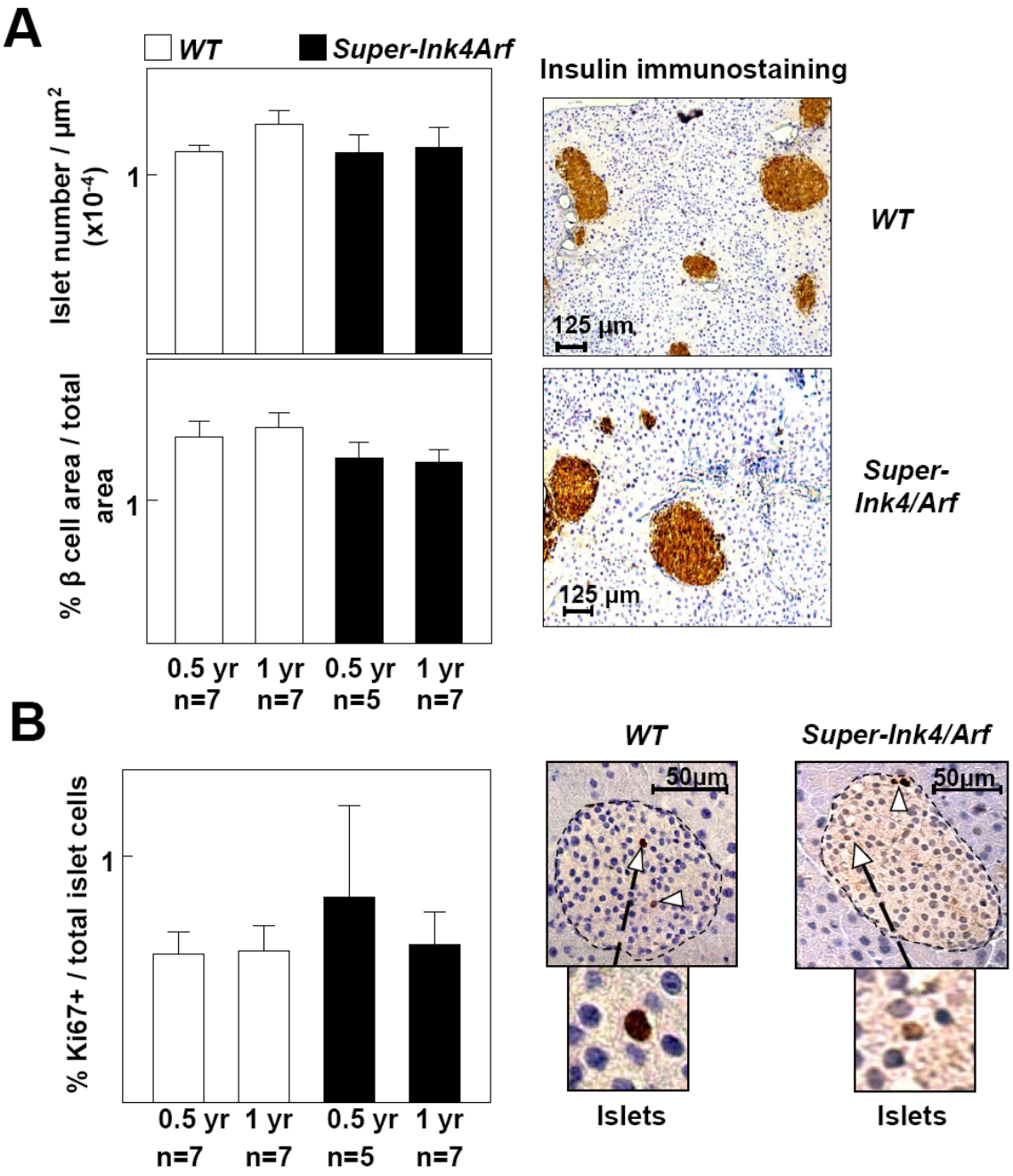

Figure S1. Increased gene dosage of *Ink4/Arf* does not affect pancreatic islet number and area or β-cell proliferation in 1-year-old mice. (A) β-cell islets in paraffin-embedded pancreas were identified by immunohistochemistry using anti-insulin antibody. Islet number was relativized to total pancreatic area and average β-cell area was expressed as percentage of insulin-immunoreactive area relative to total pancreatic area. The number of islets was not affected by extra *Ink4/Arf* gene dosage at any age. Likewise, average β-cell area was similar in *WT* and *Super-Ink4/Arf* mice. **(B)** β-cell proliferation was analyzed in pancreatic islets using anti-Ki67 antibody. Results are expressed as percentage of Ki-67-positive cells relative to total islet cell number. No differences between genotypes were observed in the expression of Ki67, thus excluding potential effects of the *Ink4/Arf* locus on β-cell proliferative potential as a mechanism for the observed phenotype in 1-year-old *Super-Ink4/Arf* mice. Representative micrographs are shown for anti-insulin (A) and anti-Ki-67 (B) immunostaining. The dotted lines in the micrographs limit pancreatic islets and the arrows point to Ki67-immunoreactive cells. Statistical analysis was done using one-way ANOVA.