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MafA controls Genes implicated in Insulin Biosynthesis and Secretion

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Abstract

Aims/hypothesis—Effects of the transcription factor MafA on the regulation of β-cell gene expression and function were investigated.

Methods—INS-1 stable cell lines permitting inducible up- or down-regulation of this transcription factor were established.

Results—MafA overexpression enhanced and its dominant-negative mutant (DN-MafA) diminished binding of the factor to the insulin promoter, correlating with insulin mRNA levels and cellular protein content. Glucose-stimulated insulin secretion was facilitated by MafA and blunted by DN-MafA. This is partly due to alterations in glucokinase expression, the glucose sensor of β-cells. In addition, the expression of important β-cell genes, such as Glut2, Pdx-1, Nkx6.1, GLP-1 receptor, prohormone convertase-1/3 and pyruvate carboxylase was regulated positively by MafA and negatively by DN-MafA.

Conclusions—The data suggest that MafA is not only a key activator for insulin transcription but also a master regulator of genes, implicated in maintaining β-cell function, in particular metabolism-secretion coupling, proinsulin processing and GLP-1 signaling. Our in vitro study provides molecular targets explaining the phenotype of recently reported MafA-null mice. We also demonstrate that MafA is expressed specifically in β-cells of human islets. Glucose influenced DNA-binding activity of MafA in rat islets in a bell-shaped manner. MafA thus qualifies as a master regulator of β-cell-specific gene expression and function.

Keywords

MafA; beta-cells; human islets; insulin secretion; metabolism

Introduction

Type 1 diabetes is characterized by absolute insulin deficiency due to autoimmune destruction of the insulin-producing pancreatic β-cells. These patients require daily injection of insulin or islet transplantation. Because of scarcity of islet donors, generation of insulin producing cells

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from human progenitor/stem cells becomes a challenge for diabetes treatment. The reduction in insulin secretory capacity and β-cell mass are the precipitating factors for development of type 2 diabetes in addition to insulin resistance in the liver, muscle and adipose tissues. Therefore, identification and characterization of key transcription factors regulating β-cell gene expression and insulin secretion are essential not only for treatment of Type 1 diabetes but also for understanding the pathogenesis of β-cell dysfunction in Type 2 diabetes.

The β-cell specificity and glucose-responsiveness of insulin expression are conferred by three conserved enhancer elements in the insulin promoter region. These are E1, A3 and RIPE3b/C1, which respectively bind three transcription factors Beta2/NeuroD, Pdx1/IPF1, and MafA (1-10). Beta2 is expressed in all pancreatic endocrine cell types, whereas Pdx1 and MafA display β-cell restricted expression (3;4;6;7;11). Mouse models with targeted gene disruption have revealed the essential roles of both Beta2 and Pdx1 in pancreatic development and β-cell differentiation (12-14). In addition, mutations in the human *NEUROD1* and *IPF1* genes are associated with, respectively, maturity onset diabetes of the young (MODY) subtype 4 and 6 (15;16). Moreover, adenovirus vector mediated gene therapy using either Beta2 in combination with betacellulin (17) or Pdx1 alone (18) has been shown to induce insulin production in mouse liver and cure streptozotocin-induced diabetes. Similar gene-therapy was recently shown to yield encouraging results taking advantage of synergistic actions of MafA, Pdx1 and Beta2 (19). Furthermore, β-cell replacement therapy in similar animal models has been achieved using *in vitro* differentiated insulin-producing cells, which are generated through expression of Pdx1 in human fetal liver progenitor cells (20).

Large Maf proteins represent a subgroup of the Maf family of basic-leucine zipper transcription factors and include MafA, c-Maf, MafB and Nrl (6;7). MafA is a key regulator of insulin gene transcription, whereas c-Maf and MafB are expressed specifically in adult α-cells and control the glucagon promoter activity (7;21). Recent studies, however, suggest that MafB is not only important to islet α-cell function but may also be involved in regulating genes required for both α- and β-cell differentiation during development (22-24). It is noteworthy that reduced MafA function has been implicated in the development of β-cell dysfunction (25-30). Hyperglycemia suppresses MafA expression in vivo, which is prevented by constitutive overexpression of Foxo1 in β-cells (25). In addition, glucotoxicity and lipotoxicity inhibit insulin expression through regulation of the DNA binding activity or/and expression of MafA and Pdx-1 (29). Moreover, reduced expression of MafA has been reported in β-cells deficient in both insulin and IGF-1 receptors, associated with reduced β-cell mass and diabetes (30).

The role of MafA in regulation of pancreatic β-cell gene expression and insulin secretion has not been fully characterised. Using inducible INS-1 β-cell models, we have defined the function of Pdx1 in the regulation of target gene expression and insulin secretion (31;32), consistent with the results obtained in mice with β-cell-specific deletion of Pdx1 (12;33). Similar approaches were employed in the present study to establish the regulatory role of MafA in β-cell function.

Materials and Methods

Establishment of Inducible INS-1 Stable Cell Lines

The first step stable and INS-1-derived INS-rβ (also refer to as r9) cell line, which carries the reverse tetracycline/doxycycline-dependent transactivator (34) was described previously (31; 35). The plasmids used in the secondary stable transfection were constructed by subcloning the cDNA encoding either wild type MafA (6) or its dominant-negative mutant DN-MafA (3) into the expression vector PUHD10-3 (34) (a generous gift from Dr. H. Bujard). Both MafA and DN-MafA were epitope-tagged with HA (hemagglutinin). The procedures for stable transfection, clone selection, and screening were described previously (35).

Cell Culture

The standard rat insulinoma INS-1-derived stable cell lines were cultured in RPMI1640 containing 11.2 mmol/l glucose (36), unless otherwise indicated.

Immunofluorescence in INS-1 cells

Cells grown on polyornithine treated glass coverslips were treated for 24 h with or without 500 ng/ml doxycycline. Cells were then washed, fixed in 4% paraformaldehyde, permeabilized with 0.1% Triton X-100 in phosphate-buffered saline containing 1% BSA (PBS-BSA). The preparation was then blocked with PBS-BSA before incubating with the first antibody, anti-HA-tag (a kind gift from MBL/LabForce, Nunningen, Switzerland) (1:100 dilution), followed by the second antibody labeling.

Nuclear Protein Extraction and Electrophoretic Mobility-Shift Assay (EMSA)

Nuclear extracts from INS-1 cells were prepared according to Schreiber et al. (37). Rat islets were isolated by collagenase digestion as described (38) and their nuclear proteins were extracted as previously reported (37). The double-stranded oligonucleotides corresponding to the RIPE3b/C1 element of the rat insulin II promoter, 5′tggaaactgcagcttcagcccctctg3′, was used as a probe (7). EMSA procedures including conditions for probe labeling and binding reactions were performed as previously described (39). Anti-MafA antibody (BETHYL, Montgomery, TX, USA) was used for supershift experiments.

Western blotting

For Western blotting cells were cultured with 0, 75, 150, and 500 ng/ml doxycycline for 24 h. Nuclear extracts and total cellular proteins were fractionated by 11% SDS-PAGE. The dilution for MafA antibody is 1:4,000. Immunoblotting procedures were performed as described previously (40) using enhanced chemiluminescence (Pierce, Rockford, Illinois) for detection.

Measurements of Insulin Secretion and Cellular Insulin Content

Insulin secretion in INS-1-derived cells was measured in 24-well plates over a period of 30 min, in Krebs-Ringer-Bicarbonate-HEPES buffer (KRBH, mmol/l: 140 NaCl, 3.6 KCl, 0.5 NaH_2PO_4, 0.5 $MgSO_4$, 1.5 $CaCl_2$, 2 $NaHCO_3$, 10 HEPES, 0.1% BSA) containing indicated concentrations of glucose. Cellular insulin content was determined after extraction with acid ethanol following the procedures of Wang et al. (39). Insulin was detected by radioimmunoassay using rat insulin as a standard (39).

Total RNA Isolation and Northern Blotting

Total RNA was extracted and blotted to nylon membranes as described previously (35). The membrane was prehybridized and then hybridized to ^{32}P-labeled random primer cDNA probes according to Wang and Iynedijian (35). To ensure equal RNA loading and even transfer, all membranes were stripped and re-hybridized with a ″house-keeping gene″ probe cyclophilin. cDNA fragments used as probes for MafA, glucokinase, Glut-2, insulin, Hnf1a, Hnf4a, NeuroD/Beta2, and Pdx1 mRNA detection were digested from the corresponding plasmids. cDNA probes for rat GLP-1 receptor (GLP-1R), Nkx6.1, glucagon, prohormone convertase-1/3 (PC-1/3), pyruvate carboxylase, mitochondrial uncoupling protein 2 (UCP2), Tfam, short-chain 3-hydroxyacyl-CoA dehydrogenase (Schad), acyl-CoA oxidase (ACO), and cyclophilin were prepared by RT-PCR and confirmed by sequencing.

Human islet isolation and culture

Freshly isolated human islets from three different donors, obtained from D. Bosco (The Cell Isolation and Transplantation Center, Department of Surgery, Geneva; Switzerland), were

maintained in CMRL-1066 (Invitrogen) at 5.6 mM glucose supplemented with 10% FCS, 100 Units/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml gentamycin (Sigma) for 2-4 days before experiments. The present investigation has been approved by the institutional ethics committee. Handpicked islets were then exposed to 5.6 or 11 mmol/l glucose with or without the presence of the GLP-1R agonist exendin-4 (Ex-4, 10 nM) for 24 h.

Immunohistochemistry

After stimulation, human islets were partially dispersed by trypsin treatment and cells were subjected to cytospin on SuperFrost®Plus slides (Menzel GmbH & Co KG, Braunschweig, Germany) and fixed in 4% paraformaldehyde pH 7.0 for 30 minutes at room temperature. After three PBS washes, two incubations with boiling 10 mmol/l citrate pH 6.0 for three minutes, cells were permeabilized with 0.2% Triton PBS for 20 minutes. Primary rabbit antibodies for MafA, Isl-1 and IPF1/Pdx-1 (dilutions 1:250) and mouse anti-insulin (1:1000, Sigma) were diluted in 0.05% triton PBS and used for an overnight incubation at 4°C. After three PBS washes, signals for the transcription factors were amplified with biotin-conjugated goat anti-rabbit-IgG antibody (1:250, Jackson ImmunoResearch Laboratories) and cells were incubated with streptavidin-Alexa 488 and Alexa 568 labeled anti-mouse antibody ((both 1:300; Molecular Probes)). After washings, cell were incubated three minutes in 10 μg/ml 4',6-diamidino-2-phenylindole (DAPI), washed three times and mounted in Dakocytomation fluorescent mounting medium (DakoCytomation AG, Untermüli, Switzerland). Images were acquired with a Zeiss Axiocam Imaging System (Bioimaging Core Facility, Medical Faculty, Geneva University).

Statistics

Results are expressed as Mean ± s.e.m and statistical analyses were performed by unpaired Student's t-test.

Results

Establishment of stable INS-1 cell lines permitting inducible expression of MafA and DN-MafA

We have established over 10 clones positively expressing MafA and DN-MafA, respectively, using parental INS-rβ cells (31). The clones designated as MafA*22 and DN-MafA*39 were selected for the present study, because these clones displayed robust levels of MafA or DN-MafA mRNAs after doxycycline induction and showed undetectable background expression under non-induced conditions.

Characterization of INS-1 clones expressing MafA of DN-MafA

Immunofluorescence with an antibody against the N-terminal epitope HA-tag illustrated that nuclear localized MafA (Fig. 1a) and DN-MafA (Fig. 1b) proteins were induced in an all-or-none manner. Both MafA*22 (Fig. 1a) and DN-MafA*39 cells (Fig. 1b) were cultured with 500 ng/ml doxycycline for 24 h.

Western blotting (Fig. 2a) with an antibody against the C-terminus of MafA demonstrated that MafA*22 cells expressed the transgene-encoded protein in a doxycycline dose-dependent manner. The protein levels of MafA in cells cultured for 24 h with 75, 150, and 500 ng/ml doxycycline, were approximately 3, 6, and 15-fold, respectively, of endogenous level of MafA protein (Fig. 2a). As expected, the same MafA antibody also recognized DN-MafA, which was induced about 5-fold comparing with endogenous wild type MafA after 500 ng/ml doxycycline treatment for 24 h (Fig 2a). Most importantly, DN-MafA*39 cells did not show detectable expression of DN-MafA protein under non-induced conditions (Fig. 2a).

EMSA with the RIPE3b/C1 element of the insulin promoter demonstrated that doxycycline induced MafA expression increased its DNA-binding activity dose-dependently (left panel of Fig. 2b). The intensity of retarded MafA binding complexes, performed with nuclear extracts from cells cultured for 24 h with 75, 150, and 500 ng/ml doxycycline, was estimated at 5, 15, and 15-fold, respectively, comparing with that of endogenous MafA (left panel of Fig. 2b). DN-MafA lacks the N-terminal transactivation domain but contains the intact DNA-binding activity (3). It therefore exerts its dominant-negative function through competing with endogenous MafA for the cognate DNA-binding. Indeed, Induction of DN-MafA almost completely abolished the MafA DNA-binding activity (right panel of Fig. 2b). Again, no leakage binding activity of DN-MafA was observed under none-induced conditions. The specificity of retarded binding complexes of MafA and DN-MafA were confirmed by the supershift experiments with MafA antibody.

MafA controls the expression of β-cell-specific genes

To explore the gene expression patterns regulated by MafA, we performed quantitative Northern blotting using total RNAs extracted from MafA*22 and DN-MafA*39 cells under induced and non-induced conditions. As shown in Fig. 3a, graded overexpression of MafA resulted in stepwise increase in the mRNA levels of Pdx-1 and Nkx6.1. Overexpression of MafA approximately 3-fold above the endogenous level already induced maximum expression of insulin and GLP-1R (Fig. 3a). To avoid artifact caused by super-physiological overexpression of MafA, we also investigated the gene expression profile in MafA*22 cells cultured with increasing concentrations of glucose in the presence of 75 ng/ml doxycycline (Fig 3b). Induction of MafA by 3- to 4-fold drastically increased the mRNA levels of five β-cell-specific genes including insulin, GLP-1R, Pdx-1, Nkx6.1, and PC-1/3 (12;41-46). GLP-1R mediates the positive effects of GLP-1 on β-cell growth and insulin secretion (42), whereas PC-1/3 plays a dominant role in processing of proinsulin (47). In contrast, the expression of Hnf1α, Hnf4α, and Beta2/NeuroD, which are ubiquitously distributed in all islet endocrine cells (13;48), was not induced by MafA (Fig 3b). It is noteworthy that MafA represents so far the most potent transcription factor in the regulation of the expression of insulin and possibly other β-cell-specific genes in our systematic screening of INS-1 cell models. The increase of endogenous insulin expression has never been observed in similar INS-1 cell lines expressing Pdx-1 (31), Hnf1α (40), and Hnf4α (49), as well as Beta2/NeuroD or Pax4 (our unpublished data).

Accordingly, induction of DN-MafA led to marked reduction in the transcript levels of insulin, GLP-1R, Pdx-1, Nkx6.1, and PC-1/3, whereas the expression of Hnf1α and Hnf4α was unaltered (Fig 3c). However, the Beta2 expression was suppressed by DN-MafA (Fig. 3c).

We and others have previously shown that Pdx-1 and Nkx6.1 suppresses the glucagon expression and causes α- and β-phenotype shift (31;45). We therefore examined whether MafA has similar function. Unexpectedly, induction of MafA did not inhibit the expression of glucagon, as demonstrated by Northern blotting after prolonged film exposure (Fig. S1). More intriguingly, induction of DN-MafA diminished the mRNA levels of glucagon (Fig. S1), which is opposite to suppression of Pdx-1 or Nkx6.1 activity in INS-1 cells. Therefore, the effects of MafA can not be simply explained as functioning through Pdx-1 or Nkx6.1.

MafA regulates the expression of genes implicated in glucose metabolism

We investigated further the effects of MafA on other β-cell-specific genes involved in glucose metabolism. Using quantitative Northern blotting analysis we found that overexpression of MafA robustly up-regulated the expression of two β-cell-restricted genes, Glut2 and pyruvate carboxylase (Fig. 4a and b). In addition, dominant-negative suppression of MafA drastically down-regulated transcript levels of these two genes (Fig. 4c). Glut2 is the major glucose

transporter expressed in rodent β-cells (50), while pyruvate carboxylase participates in the regulation of metabolism-secretion coupling in β-cells (51; 52).

Although glucokinase is expressed in both islet α- and β-cells, it has been shown to confer the β-cell glucose-sensing (35;53). The mRNA levels of glucokinase were elevated by MafA and reduced by DN-MafA (Fig. 4). Such effects seem to be unique to MafA, since glucokinase expression remained constant in our INS-1 cells permitting either overexpression or dominant-negative suppression of other pancreatic transcription factors, such as Pdx-1 (31), Hnf1α (40), and Hnf4α (49), as well as Beta2/NeuroD or Pax4 (our unpublished data).

The expression of other genes implicated in metabolism-secretion coupling, such as UCP2, Tfam, Schad, and ACO (54-56), was not regulated by manipulation of MafA function in INS-1 cells (Fig. 4). UCP2 and Tfam modulate, respectively, the membrane potential and biogenesis of mitochondria, whereas Schad and ACO are involved in fatty-acid metabolism.

MafA regulates glucose-stimulated insulin secretion

The effects of MafA on the expression of glucokinase and pyruvate carboxylase as well as Glut2 suggest that MafA could be implicated in the regulation of metabolism-secretion coupling in β-cells. We therefore examined the impact of overexpression or dominant-negative suppression of MafA on insulin secretion in INS-1 cells. As shown in Fig. 5a, overexpression of MafA caused a left-ward shift of insulin secretion in response to glucose. The MafA induced increase in glucokinase expression (Fig 4a and b) should explain, at least in part, for the left-shift of glucose responsiveness, because similar results have been obtained in INS-1 cells overexpressing glucokinse (35), the rate-limiting enzyme for β-cell glucose metabolism. In contrast, dominant-negative suppression of MafA led to blunted glucose-induced insulin secretion, whereas KCl-depolarization-elicited insulin release remained constant (Fig. 5b). These results suggest that MafA regulates metabolism-secretion coupling in steps upstream of exocytosis. INS-1 cells represent a unique model to assess the implications of pyruvate carboxylase in metabolism-secretion coupling, because these insulinoma cells express low levels of moncarboxylate transporter but sufficient to use pyruvate as secretogue (57;58), in contrast to native islet β-cells. We therefore evaluated the consequences of DN-MafA on pyruvate-stimulated insulin secretion in INS-1 cells. As demonstrated in Fig 5b, induction of DN-MafA also impaired insulin secretion in response to pyruvate, which could be explained by markedly reduced pyruvate carboxylase expression (Fig. 4c). The insulin secretion data were expressed as percentage of cellular insulin, because induction of MafA and DN-MafA resulted in, respectively, 23% increase and 27% decrease in the cellular insulin content.

MafA is expressed specifically in β-cells of human islets

To characterize the MafA expression in adult human islets, we have performed immunofluorescence staining with antibodies against Isl1, MafA, Pdx1/IPF1 and insulin. As shown in Fig 6a, MafA is specifically localized in nuclei of insulin positive cells, whereas Isl1 is expressed in both β- and none-β-cells. Pdx1 also almost exclusively localized in nuclei of β-cells (Fig. 6a). In addition, subcellular distribution of MafA was not strikingly different in human islets cultured or 24 h, respectively, in 5 and 11.2 mmol/l glucose (Fig. 6b). The negative effects of GLP-1R agonist, exendin-4, were pronounced (Fig. 6b). Since immunostaining is semi-quantitative, small changes in protein levels may not be obvious. We therefore performed EMSA to evaluate the glucose-responsiveness of MafA DNA binding.

Glucose regulates MafA DNA-binding activity in rat islets

It has been very confusing in terms of glucose-regulation of MafA (6;25;26;59). Zhao and colleagues (59) have reported that both MafA protein levels and its binding to the RIPE3b/C1 element are markedly increased in rat islets cultured for 24 h with 16.7 mmol/l glucose, in

comparison with control islets treated with 2.8 mmol/l glucose. Similar glucose-induced MafA DNA-binding is demonstrated in insulinoma MIN6 and β-TC6 cells (6). In contrast, reduced MafA DNA-binding activity and its protein levels have been shown, respectively, in INS-1 cells cultured with 16.7 mmol/l glucose for 48 h (28) and in HIT-T15 cells treated chronically with 11.1 mmol/l glucose (27). To clarify this controversy, we have performed EMSA with nuclear extracts from rat islets cultured with, respectively, 2.5, 5, 15, and 30 mmol/l glucose for 48 h. As shown in Fig 7, MafA DNA-binding is regulated by glucose in a bell-shaped manner. The DNA-binding activity was incrementally induced in the range of 2.5 to 15 mmol/l glucose, but reduced at 30 mmol/l glucose. These results not only support the notion that glucose enhances MafA DNA-binding (6;59), but are also in agreement with the previous finding that glucotoxicity diminishes the DNA binding activity (25;27;28). The discrepancy between the studies in rat islets and insulinoma cells could be related to decreased sensitivity of the former to high glucose.

Discussion

Using two INS-1 stable cell lines permitting inducible overexpression or dominant-negative suppression of MafA, we have assessed the effects of MafA on insulin secretion. Overexpression of MafA caused left-shift of insulin secretion in response to glucose, whereas induction of DN-MafA blunted nutrient-stimulated insulin release. This was not due to alterations in insulin content, since the data were expressed as percentage of cellular insulin. Recently, Zhang and colleagues (60) have reported that mice-deficient in MafA developed diabetes due to impaired insulin secretion. However, the authors did not define the molecular targets responsible for the phenotype. We have defined the target genes of MafA through quantitative gene expression profiling of our novel INS-1 cell lines under induced and non-induced conditions. The results demonstrate that MafA is not only a key activator for insulin transcription but also a master regulator of many β-cell genes, implicated in maintaining β-cell phenotype, metabolism-secretion coupling, proinsulin processing and GLP-1 signaling.

Overexpression of MafA alone is sufficient to raise the endogenous insulin mRNA levels. This is unique to MafA, because induction of Pdx-1 (31) or Beta2 either separately or combined in the similar INS-1 cell system (our unpublished data) did not reproduce the effects of MafA. Similarly, other key β-cell transcription factors such as Hnf1α and Hnf4α as well as Pax4 do not show such potency in our systematic series of INS-1 cell models (40;49). Transduction of MafA has been shown previously to increase the endogenous insulin transcript expression in rat islets (59). We therefore propose that MafA could be a potent and target transcription factor employed for β-cell generation through either *in vivo* gene therapy or *in vitro* differentiation of stem/progenitor cells.

Accordingly, dominant-negative suppression of MafA decreased insulin expression as revealed by mRNA and protein contents. We have also identified the β-cell-enriched PC-1/3 and GLP-1R as two novel targets of MafA. DN-MafA drastically suppressed the expression of PC-1/3 and GLP-1R, whereas induction of MafA enhanced their transcript levels. PC-1/3 is required for insulin maturation (47), whereas GLP-1R is necessary for GLP-1-mediated β-cell growth and insulin secretion (42). Similar reduction of PC-1/3 and GLP-1R expression has also been observed in our INS-1 cells expressing DN-Pdx-1, which causes defective proinsulin processing and GLP-1 signaling (32). MafA has been previously reported to bind the Pdx-1 promoter (61). Indeed, we found that Pdx-1 is robustly up- or down-regulated following respectively induction and suppression of MafA function. Decreased expression of Pdx-1 has also been demonstrated in MafA-deficient islets (60). We therefore postulate that MafA regulates expression of PC-1/3 and GLP-1R, at least in part by acting through Pdx-1.

In addition, we have also identified two other common target genes of MafA and Pdx-1: Glut2 and Nkx6.1. Like Pdx-1, MafA also controls the expression of these two β-cell-restricted genes. Decreased expression of Glut2 has also been reported in the islets of MafA-null mice (60). However, this could not explain the impaired insulin secretion (60). As the capacity of this hexose transporter is in large excess of the activity of glucokinase and other glycolytic enzymes, this decrease would not affect glucose metabolism (50). To delineate the molecular targets responsible for the regulatory effects of MafA on insulin secretion, we have evaluated the expression patterns of genes involved in metabolism-secretion coupling with or without induction of MafA or DN-MafA. The β-cell-enriched pyruvate carboxylase has been identified as another novel target of MafA in the present study. Pyruvate carboxylase plays an essential role in metabolism-secretion coupling in both native β-cells and insulinoma cells (51;52). Approximately 50% of glucose-derived pyruvate enters mitochondrial metabolism via carboxylation catalyzed by pyruvate carboxylase, while the remainder is converted into acetylcoA by the pyruvate dehydrogenase complex (51). This provides anaplerotic input to the tricarboxylic acid cycle (62). INS-1 cells, which express low levels of monocarboxylate transporter, nonetheless sufficient to take up pyruvate (57;58), provide a suitable model to assess the impact of decreased pyruvate carboxylase expression on insulin secretion. Induction of DN-MafA not only impaired glucose-stimulated but also pyruvate-induced insulin release. As pyruvate is metabolised exclusively by the mitochondria, the down-regulation of pyruvate carboxylase certainly contributes together with glucokinase to the insulin secretory defect. On the other hand, the MafA-evoked overexpression of pyruvate carboxylase may partially contribute to the left shift of insulin secretion in response to glucose. However, the β-cell glucose-sensor, glucokinase, should be the major player, since similar left-shift of insulin secretory response has been documented in INS-1 cells overexpressing glucokinase (35). At variance with MafA-deficient mice, we found that mRNA levels of glucokinase were regulated positively by MafA and negatively by DN-MafA. This is also unique to MafA because glucokinase expression remains constant in our INS-1 stable cell lines allowing overexpression or suppression of other pancreatic transcription factors, including Pdx-1 (31), Hnf1α (40), Hnf4α (49), Beta2/NeuroD or Pax4 (Wang and Wollheim, unpublished data). The reason for the apparent difference in glucokinase regulation in the MafA mutant mice and INS-1 cells is not clear. Moreover, K^+-induced insulin secretion was inhibited in islets from MafA-/- mice but not affected in INS-1 cells with suppressed MafA function. This could be due to effects on L-type voltage-gated Ca^{2+}-channels in native β-cells. INS-1 cells express a broader spectrum of such Ca^{2+}-channels than native β-cells (63), which may explain the preserved insulin secretion following membrane-depolarization.

Another intriguing result with MafA is that it does not promote the α- and β-phenotype shift observed with Pdx1 and Nkx6.1 (31;45), although our data suggest that MafA regulates their expression. We have previously shown that Pdx1 suppresses glucagon expression and regulates the α-/β-phenotype shift in INS-1 cells (31). A recent elegant study in INS-1-derived cells suggest that Pdx1 acts through Nkx6.1 (45). Therefore, the effects of MafA on β-cell-specific gene expression can not be explained simply by an action on Pdx1. Another argument supporting this notion is that overexpression of Pdx1 does not potentate glucose-stimulated insulin secretion (32). Furthermore, in agreement with the MafA-null mice (59), induction of DN-MafA diminished the expression of Beta2 in INS-1 cells, although MafA overexpression did not affect its transcript levels.

We also demonstrate that MafA is specifically expressed in the β-cells of human islets and that its subcellular distribution is not clearly regulated by physiological concentrations of glucose. However, the DNA-binding activity of MafA is indeed regulated in a glucose-concentration-dependent and a bell-shape manner. We therefore clarified the apparent controversies in the literature regarding the glucose-responsiveness of MafA DNA-binding (6;27;28;59). To summarize data from the present study and previous reports, we suggest that glucose induces

MafA DNA-binding activity at physiological concentrations, but suppresses the binding at both sub- and supra-physiological levels.

In conclusion, we have employed an inducible INS-1 cell system, which allows direct assessment of MafA target genes. We demonstrate that MafA controls many β-cell-specific genes including, insulin, Glut2, Pdx-1, Nkx6.1, GLP-1R, PC-1/3, and pyruvate carboxylase. Pyruvate carboxylase in combination with glucokinase may represent the molecular targets responsible for the regulatory effects of MafA on metabolism-secretion coupling. Furthermore, MafA regulates expression of genes essential for insulin transcription and processing, as well as for GLP-1 signaling. Therefore, we propose that MafA is a candidate gene for diabetes and a target gene for restoring β-cell function in type 2 diabetes. In addition, it is also a potential target for treatment of type 1 diabetes through MafA-mediated *in vitro* β-cell generation from progenitor/stem cells or *in vivo* gene therapy.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

PC-1/3, prohormone convertase1/3; DN-MafA, dominant-negative mutant of MafA; MODY, maturity onset diabetes of the young; EMSA, electrophoretic mobility-shift assay; KRBH, Krebs-Ringer-Bicarbonate-HEPES buffer; UCP2, mitochondrial uncoupling protein-2; Schad, 3-hydroxyacyl-CoA dehydrogenase; ACO, acyl-CoA oxidase.

References

1. Sharma A, Stein R. Glucose-induced transcription of the insulin gene is mediated by factors required for beta-cell-type-specific expression. Mol Cell Biol 1994;14:871–879. [PubMed: 8289826]
2. Onheda K, Mirmira RG, Wang J, Johnson JD, German MS. The homeodomain of PDX-1 mediates multiple protein-protein interactions in the formation of a transcriptional activation complex on the insulin promoter. Mol.Cell.Biol 2000;20:900–911. [PubMed: 10629047]
3. Olbrot M, Rud J, Moss LG, Sharma A. Identification of beta-cell-specific insulin gene transcription factor RIPE3b1 as mammalian MafA. Proc Natl Acad Sci U S A 2002;99:6737–6742. [PubMed: 12011435]
4. Naya FJ, Stellrecht CM, Tsai MJ. Tissue-specific regulation of the insulin gene by a novel basic helix-loop-helix transcription factor. Genes Dev 1995;9:1009–1019. [PubMed: 7774807]
5. Marshak S, totary H, Cerasi E, Melloul D. Putification of the beta-cell glucose-sensitive factor that transactivates the insulin gene differentially in normal and transformed islet cells. Proc.Natl.Acad.Sci.USA 1996;93:15057–15062. [PubMed: 8986763]
6. Kataoka K, Han SI, Shioda S, Hirai M, Nishizawa M, Handa H. MafA is a glucose-regulated and pancreatic beta-cell-specific transcriptional activator for the insulin gene. J Biol Chem 2002;277:49903–49910. [PubMed: 12368292]
7. Matsuoka TA, Zhao L, Artner I, Jarrett HW, Friedman D, Means A, Stein R. Members of the large Maf transcription family regulate insulin gene transcription in islet beta cells. Mol Cell Biol 2003;23:6049–6062. [PubMed: 12917329]

8. Le Lay J, Stein R. Involvement of PDX-1 in activation of human insulin gene transcription. J Endocrinol 2006;188:287–294. [PubMed: 16461554]
9. Aramata S, Han SI, Yasuda K, Kataoka K. Synergistic activation of the insulin gene promoter by the beta-cell enriched transcription factors MafA, Beta2, and Pdx1. Biochim Biophys Acta 2005;1730:41–46. [PubMed: 15993959]
10. Nishimura W, Salameh T, Kondo T, Sharma A. Regulation of insulin gene expression by overlapping DNA-binding elements. Biochem J 2005;392:181–189. [PubMed: 16050808]
11. Ohlsson H, Karlsson K, Edlund T. IPF1, a homeodomain-containing transactivator of the insulin gene. EMBO.J 1993;12:4251–4259. [PubMed: 7901001]
12. Ahlgren U, Johnson J, Johnson L, Simu K, Edlund H. Beta-cell-specific inactivation of the mouse Ipf1 / Pdx1 gene results in loss of the beta-cell phenotype and maturity onset diabetes. Gene & Development 1998;12:1763–1768.
13. Naya FJ, Huang H-P, Qiu Y, Mutoh H, DeMayo FJ, Leiter AB, Tsai M-J. Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2 / NeuroD-deficient mice. Genes & Development 1997;11:2323–2334. [PubMed: 9308961]
14. Johnson J, Carisson L, Edlund T, Edlund H. Insulin-promoter-factor 1 is required for pancreas development in mice. Nature 1994;371:606–609. [PubMed: 7935793]
15. Stoffers DA, Ferrer J, Clarke WL, Habener JF. Early-onset type-II diabetes mellitus (MODY4) linked to IPF1. Nat Genet 1997;17:138–139. [PubMed: 9326926]
16. Malecki MT, Jhala US, Antonellis A, Fields L, Doria A, Orban T, Saad M, Warram JH, Montminy M, Krolewski A. Mutations in NEUROD1 are associated with the development of type 2 diabetes mellitus. Nature Genet 1999;23:323–328. [PubMed: 10545951]
17. Kojima H, Fujimiya M, Matsumura K, Younan P, Imaeda H, Maeda M, Chan L. NeuroD-betacellulin gene therapy induces islet neogenesis in the liver and reverses diabetes in mice. Nat Med 2003;9:596–603. [PubMed: 12704384]
18. Ferber S, Halkin A, Cohen H, Ber I, Einav Y, Goldberg I, Barshack I, Seijffers R, Kopolovic J, Kaiser N, Karasik A. Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia. Nature Medicine 2000;6:568–572.
19. Kaneto H, Matsuoka TA, Nakatani Y, Miyatsuka T, Matsuhisa M, Hori M, Yamasaki Y. A crucial role of MafA as a novel therapeutic target for diabetes. J Biol Chem 2005;280:15047–15052. [PubMed: 15664997]
20. Zalzman M, Gupta S, Giri RK, Berkovich I, Sappal BS, Karnieli O, Zern MA, Fleischer N, Efrat S. Reversal of hyperglycemia in mice by using human expandable insulin-producing cells differentiated from fetal liver progenitor cells. Proc Natl Acad Sci U S A 2003;100:7253–7258. [PubMed: 12756298]
21. Kataoka K, Shioda S, Ando K, Sakagami K, Handa H, Yasuda K. Differentially expressed Maf family transcription factors, c-Maf and MafA, activate glucagon and insulin gene expression in pancreatic islet alpha- and beta-cells. J Mol Endocrinol 2004;32:9–20. [PubMed: 14765989]
22. Artner I, Le Lay J, Hang Y, Elghazi L, Schisler JC, Henderson E, Sosa-Pineda B, Stein R. MafB: an activator of the glucagon gene expressed in developing islet alpha- and beta-cells. Diabetes 2006;55:297–304. [PubMed: 16443760]
23. Nishimura W, Kondo T, Salameh T, El Khattabi I, Dodge R, Bonner-Weir S, Sharma A. A switch from MafB to MafA expression accompanies differentiation to pancreatic beta-cells. Dev Biol 2006;293:526–539. [PubMed: 16580660]
24. Tsuchiya M, Taniguchi S, Yasuda K, Nitta K, Maeda A, Shigemoto M, Tsuchiya K. Potential roles of large mafs in cell lineages and developing pancreas. Pancreas 2006;32:408–416. [PubMed: 16670624]
25. Kitamura YI, Kitamura T, Kruse JP, Raum JC, Stein R, Gu W, Accili D. FoxO1 protects against pancreatic beta cell failure through NeuroD and MafA induction. Cell Metab 2005;2:153–163. [PubMed: 16154098]
26. Hagman DK, Hays LB, Parazzoli SD, Poitout V. Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of Langerhans. J Biol Chem 2005;280:32413–32418. [PubMed: 15944145]

27. Harmon JS, Stein R, Robertson RP. Oxidative stress-mediated, post-translational loss of MafA protein as a contributing mechanism to loss of insulin gene expression in glucotoxic beta cells. J Biol Chem 2005;280:11107–11113. [PubMed: 15664999]

28. Pino MF, Ye DZ, Linning KD, Green CD, Wicksteed B, Poitout V, Olson LK. Elevated glucose attenuates human insulin gene promoter activity in INS-1 pancreatic beta-cells via reduced nuclear factor binding to the A5/core and Z element. Mol Endocrinol 2005;19:1343–1360. [PubMed: 15650027]

29. Poitout V, Hagman D, Stein R, Artner I, Robertson RP, Harmon JS. Regulation of the insulin gene by glucose and fatty acids. J Nutr 2006;136:873–876. [PubMed: 16549443]

30. Ueki K, Okada T, Hu J, Liew CW, Assmann A, Dahlgren GM, Peters JL, Shackman JG, Zhang M, Artner I, Satin LS, Stein R, Holzenberger M, Kennedy RT, Kahn CR, Kulkarni RN. Total insulin and IGF-I resistance in pancreatic beta cells causes overt diabetes. Nat Genet 2006;38:583–588. [PubMed: 16642022]

31. Wang H, Maechler P, Ritz-Laser B, Hagenfeldt KA, Ishihara H, Philippe J, Wollheim CB. Pdx1 level defines pancreatic gene expression pattern and cell lineage differentiation. J Biol Chem 2001;276:25279–25286. [PubMed: 11309388]

32. Wang H, Iezzi M, Theander S, Antinozzi PA, Gauthier BR, Halban PA, Wollheim CB. Suppression of Pdx-1 perturbs proinsulin processing, insulin secretion and GLP-1 signalling in INS-1 cells. Diabetologia 2005;48:720–731. [PubMed: 15756539]

33. Li Y, Cao X, Li LX, Brubaker PL, Edlund H, Drucker DJ. beta-Cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. Diabetes 2005;54:482–491. [PubMed: 15677506]

34. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, Bujard H. Transcriptional activation by tetracyclines in mammalian cells. Science 1995;268:1766–1769. [PubMed: 7792603]

35. Wang H, Iynedjian PB. Modulation of glucose responsiveness of insulinoma beta-cells by graded overexpression of glucokinase. Proc Natl Acad Sci U S A 1997;94:4372–4377. [PubMed: 9113996]

36. Asfari M, Janjic D, Meda P, Li G, Halban PA, Wollheim CB. Establishment of 2-mercaptoethanol-dependent differentiated insulin- secreting cell lines. Endocrinology 1992;130:167–178. [PubMed: 1370150]

37. Schreiber E, Matthias P, Muller MM, Schaffner W. Identification of a novel lymphoid specific octamer binding protein (OTF-2B) by proteolytic clipping bandshift assay (PCBA). Embo J 1988;7:4221–4229. [PubMed: 3072196]

38. Rubi B, Ishihara H, Hegardt FG, Wollheim CB, Maechler P. GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. J Biol Chem 2001;276:36391–36396. [PubMed: 11473118]

39. Wang H, Maechler P, Hagenfeldt KA, Wollheim CB. Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. Embo J 1998;17:6701–6713. [PubMed: 9822613]

40. Wang H, Maechler P, Hagenfeldt KA, Wollheim CB. Dominant-negative suppression of HNF-1alpha function results in defective insulin gene transcription and impaired metabolism-secretion coupling in a pancreatic beta-cell line. Embo J 1998;17:6701–6713. [PubMed: 9822613]

41. Zhu X, Zhou A, Dey A, Norrbom C, Carroll R, Zhang C, Laurent V, Lindberg I, Ugleholdt R, Holst JJ, Steiner DF. Disruption of PC1/3 expression in mice causes dwarfism and multiple neuroendocrine peptide processing defects. Proc Natl Acad Sci U S A 2002;99:10293–10298. [PubMed: 12145326]

42. Preitner F, Ibberson M, Franklin I, Binnert C, Pende M, Gjinovci A, Hansotia T, Drucker DJ, Wollheim C, Burcelin R, Thorens B. Gluco-incretins control insulin secretion at multiple levels as revealed in mice lacking GLP-1 and GIP receptors. J Clin Invest 2004;113:635–645. [PubMed: 14966573]

43. Mirmira RG, Watada H, German MS. Beta-cell differentiation factor Nkx6.1 contains distinct DNA binding interference and transcriptional repression domains. J.Biol.Chem 2000;275:14743–14751. [PubMed: 10799563]

44. Watada H, Mirmira RG, Leung J, German MS. Transcriptional and transcriptional regulation of beta-cell differentiation factor Nkx6.1. J.Biol.Chem 2000;275:34224–34230. [PubMed: 10938085]

45. Schisler JC, Jensen PB, Taylor DG, Becker TC, Knop FK, Takekawa S, German M, Weir GC, Lu D, Mirmira RG, Newgard CB. The Nkx6.1 homeodomain transcription factor suppresses glucagon expression and regulates glucose-stimulated insulin secretion in islet beta cells. Proc Natl Acad Sci U S A 2005;102:7297–7302. [PubMed: 15883383]
46. Iype T, Francis J, Garmey JC, Schisler JC, Nesher R, Weir GC, Becker TC, Newgard CB, Griffen SC, Mirmira RG. Mechanism of insulin gene regulation by the pancreatic transcription factor Pdx-1: application of pre-mrna analysis and chromatin immunoprecipitation to assess formation of functional transcriptional complexes. J Biol Chem 2005;280:16798–16807. [PubMed: 15743769]
47. Zhu X, Orci L, Carroll R, Norrbom C, Ravazzola M, Steiner DF. Severe block in processing of proinsulin to insulin accompanied by elevation of des-64,65 proinsulin intermediates in islets of mice lacking prohormone convertase 1/3. Proc Natl Acad Sci U S A 2002;99:10299–10304. [PubMed: 12136131]
48. Wang H, Hagenfeldt-Johansson K, Otten LA, Gauthier BR, Herrera PL, Wollheim CB. Experimental models of transcription factor-associated maturity-onset diabetes of the young. Diabetes 2002;51 (Suppl 3):S333–342. [PubMed: 12475772]
49. Wang H, Maechler P, Antinozzi PA, Hagenfeldt KA, Wollheim CB. Hepatocyte nuclear factor 4alpha regulates the expression of pancreatic beta -cell genes implicated in glucose metabolism and nutrient-induced insulin secretion. J Biol Chem 2000;275:35953–35959. [PubMed: 10967120]
50. Thorens B. GLUT2 in pancreatic and extra-pancreatic gluco-detection (review). Mol Membr Biol 2001;18:265–273. [PubMed: 11780755]
51. Schuit F, De Vos A, Farfari S, Moens K, Pipeleers D, Brun T, Prentki M. Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells. J Biol Chem 1997;272:18572–18579. [PubMed: 9228023]
52. Lu D, Mulder H, Zhao P, Burgess SC, Jensen MV, Kamzolova S, Newgard CB, Sherry AD. 13C NMR isotopomer analysis reveals a connection between pyruvate cycling and glucose-stimulated insulin secretion (GSIS). Proc Natl Acad Sci U S A 2002;99:2708–2713. [PubMed: 11880625]
53. Matschinsky FM. Banting Lecture 1995. A lesson in metabolic regulation inspired by the glucokinase glucose sensor paradigm. Diabetes 1996;45:223–241. [PubMed: 8549869]
54. Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB, Zheng XX, Wheeler MB, Shulman GI, Chan CB, Lowell BB. Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. Cell 2001;105:745–755. [PubMed: 11440717]
55. Wang J, Silva JP, Gustafsson CM, Rustin P, Larsson NG. Increased in vivo apoptosis in cells lacking mitochondrial DNA gene expression. Proc Natl Acad Sci U S A 2001;98:4038–4043. [PubMed: 11259653]
56. Clayton PT, Eaton S, Aynsley-Green A, Edginton M, Hussain K, Krywawych S, Datta V, Malingre HE, Berger R, van den Berg IE. Hyperinsulinism in short-chain L-3-hydroxyacyl-CoA dehydrogenase deficiency reveals the importance of beta-oxidation in insulin secretion. J Clin Invest 2001;108:457–465. [PubMed: 11489939]
57. Ishihara H, Wang H, Drewes LR, Wollheim CB. Overexpression of monocarboxylate transporter and lactate dehydrogenase alters insulin secretory responses to pyruvate and lactate in beta cells. J Clin Invest 1999;104:1621–1629. [PubMed: 10587526]
58. Sekine N, Cirulli V, Regazzi R, Brown LJ, Gine E, Tamarit-Rodriguez J, Girotti M, Marie S, MacDonald MJ, Wollheim CB, et al. Low lactate dehydrogenase and high mitochondrial glycerol phosphate dehydrogenase in pancreatic beta-cells. Potential role in nutrient sensing. J Biol Chem 1994;269:4895–4902. [PubMed: 8106462]
59. Zhao L, Guo M, Matsuoka TA, Hagman DK, Parazzoli SD, Poitout V, Stein R. The islet beta cell-enriched MafA activator is a key regulator of insulin gene transcription. J Biol Chem 2005;280:11887–11894. [PubMed: 15665000]
60. Zhang C, Moriguchi T, Kajihara M, Esaki R, Harada A, Shimohata H, Oishi H, Hamada M, Morito N, Hasegawa K, Kudo T, Engel JD, Yamamoto M, Takahashi S. MafA Is a Key Regulator of Glucose-Stimulated Insulin Secretion. Mol Cell Biol 2005;25:4969–4976. [PubMed: 15923615]

61. Samaras SE, Zhao L, Means A, Henderson E, Matsuoka TA, Stein R. The islet beta cell-enriched RIPE3b1/Maf transcription factor regulates pdx-1 expression. J Biol Chem 2003;278:12263–12270. [PubMed: 12551916]

62. Newgard CB. While tinkering with the beta-cell...metabolic regulatory mechanisms and new therapeutic strategies: American Diabetes Association Lilly Lecture, 2001. Diabetes 2002;51:3141–3150. [PubMed: 12401704]

63. Horvath A, Szabadkai G, Varnai P, Aranyi T, Wollheim CB, Spat A, Enyedi P. Voltage dependent calcium channels in adrenal glomerulosa cells and in insulin producing cells. Cell Calcium 1998;23:33–42. [PubMed: 9570008]

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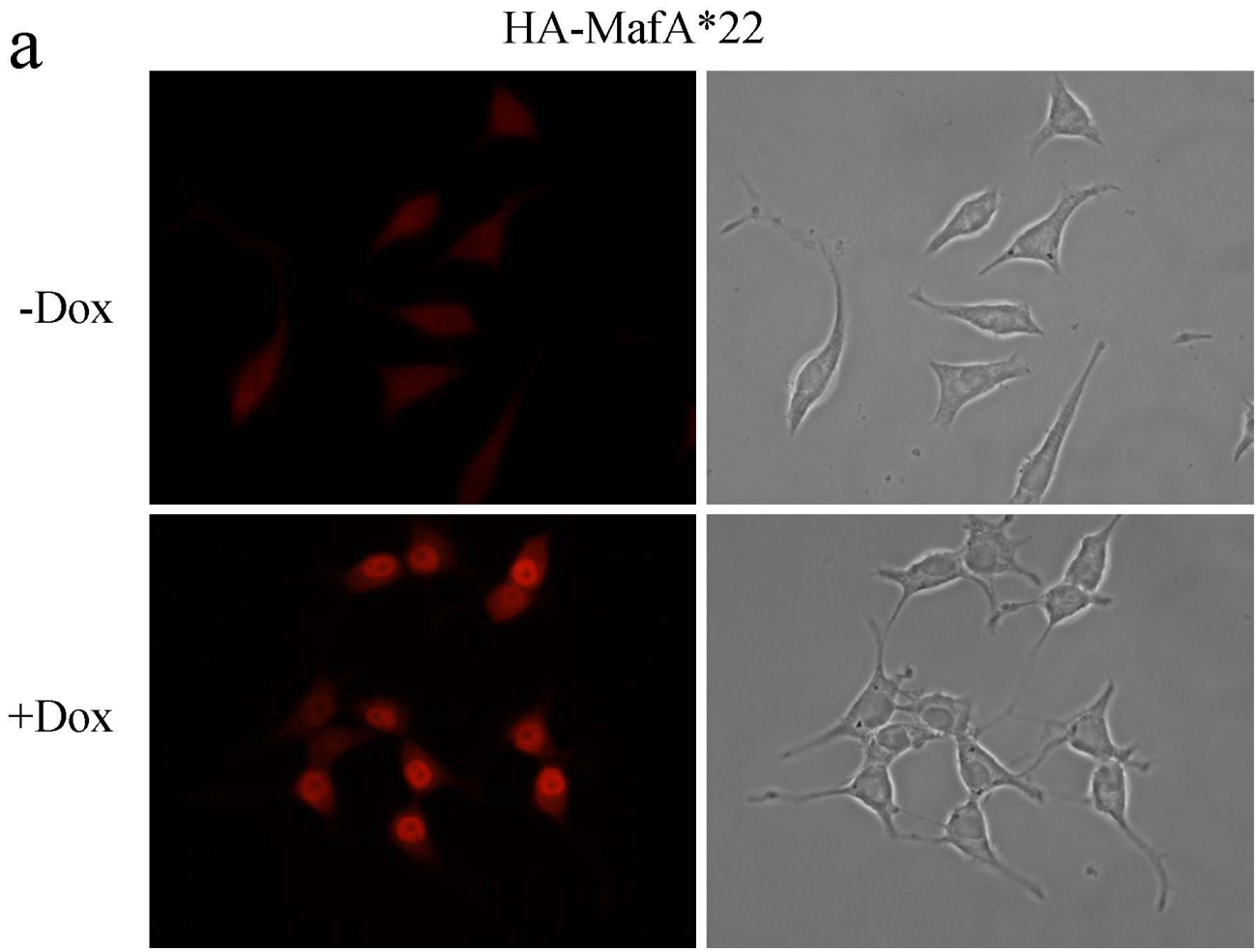


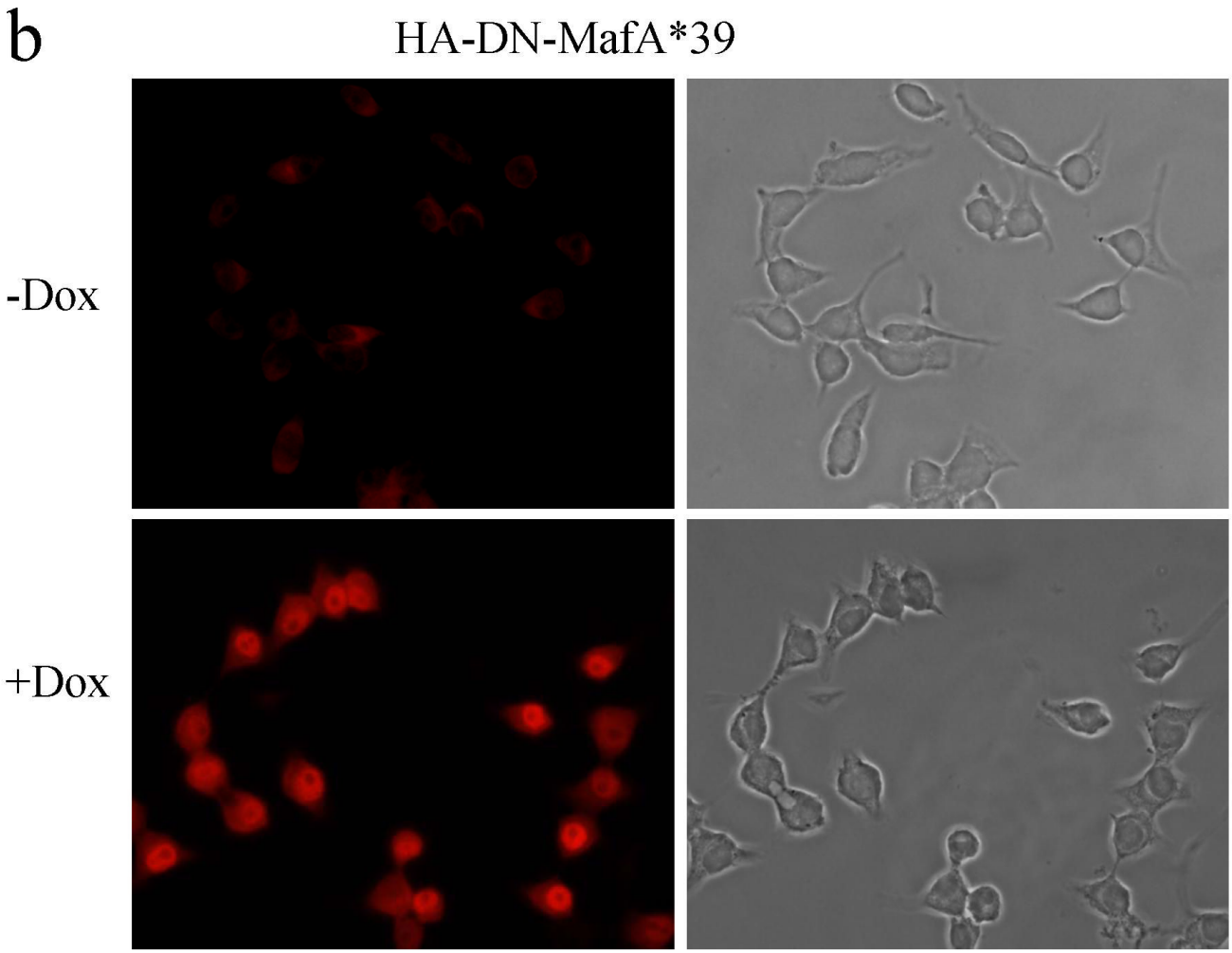
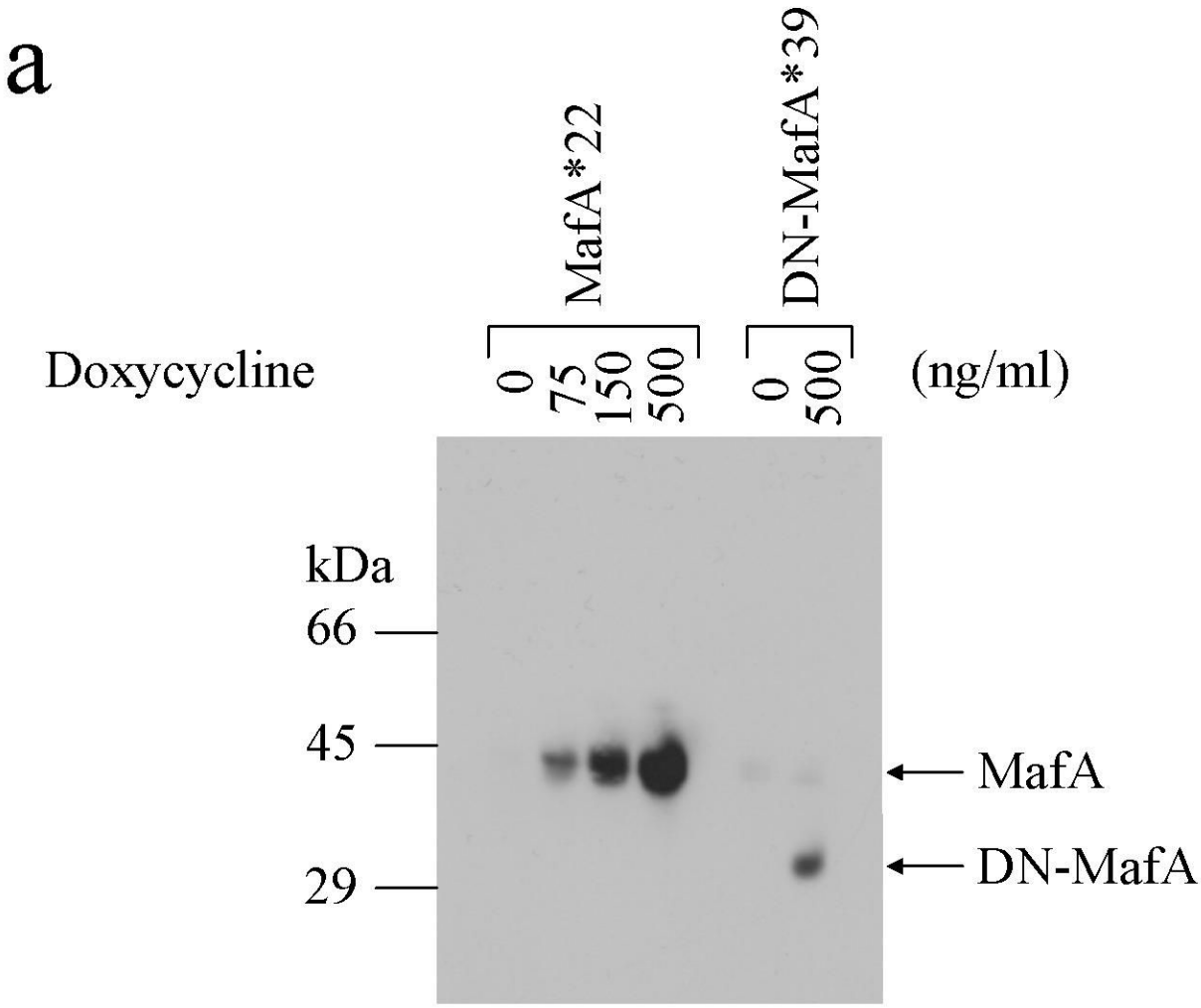


Fig 1.

Both MafA and DN-MafA were induced in an all-or-none manner. Immunofluorescence staining with antibody against HA-tag showed that both MafA (**a**) and DN-MafA (**b**) proteins were induced by doxycycline in an all-or-none manner. The phase contrast images were shown in the right panel. MafA*22 (**a**) and DN-MafA*39 (**b**) cells were cultured with (+Dox) or without (-Dox) 500 ng/ml doxycycline for 24 h.



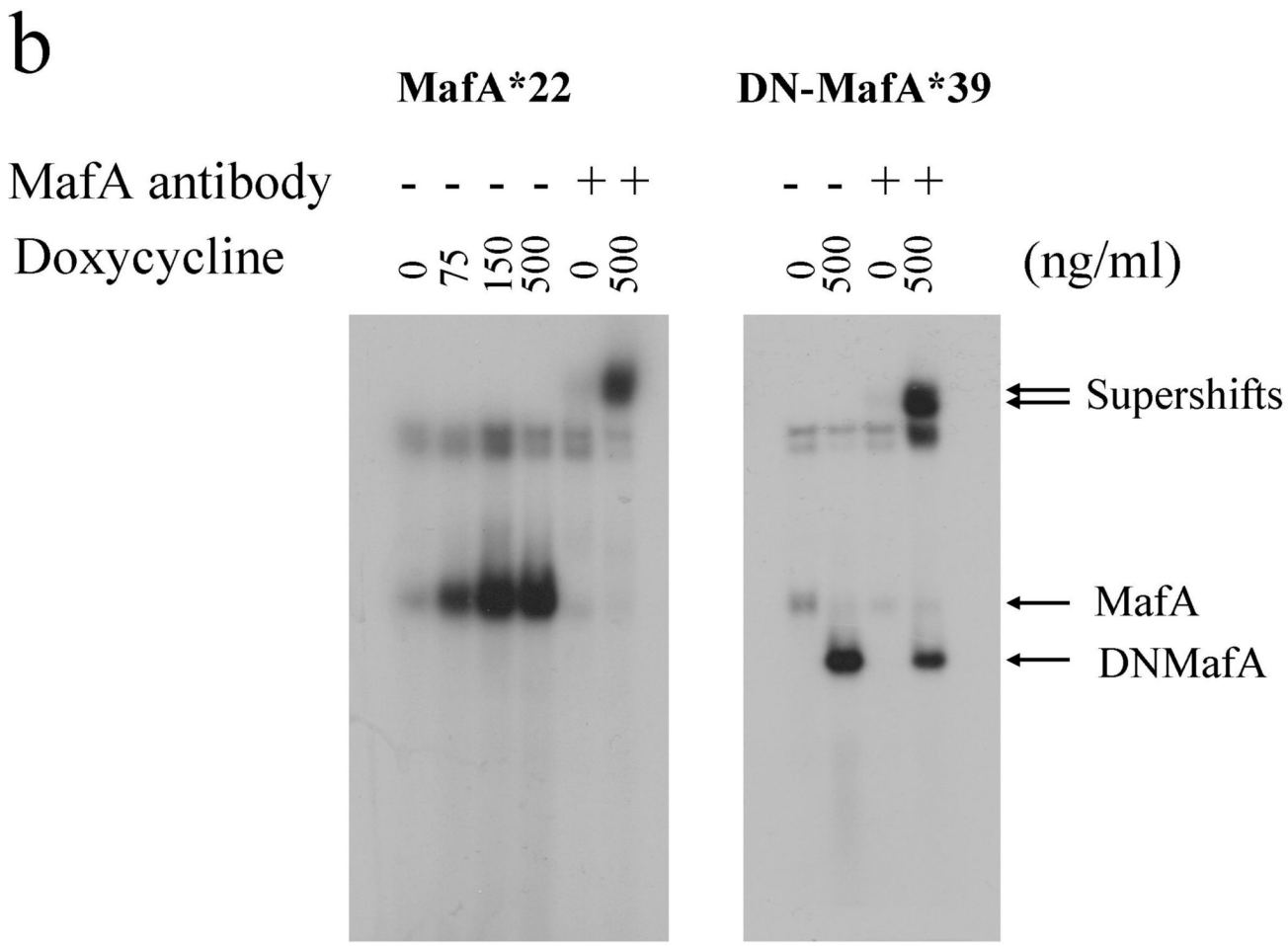


Fig 2.

MafA binding to the insulin promoter was enhanced by overexpression of MafA and diminished by induction of DN-MafA. **a** Immunoblotting with antibody against MafA demonstrated the graded overexpression of MafA protein after induction with incremental doses of doxycycline (*left*). DN-MafA protein was induced by doxycycline in an all-or-none manner (*right*). Nuclear proteins were extracted, respectively, from MafA*22 and DN-MafA*39 cells induced for 24 h with indicated concentrations of doxycycline. **b** EMSA with the RIPE3b/C1 element of the insulin promoter demonstrated that the MafA DNA binding activity was incrementally increased by graded overexpression of MafA (**left**) and nearly abolished by induction of DN-MafA (**right**). The antibody against the C-terminus of MafA supershifted both MafA and DN-MafA as expected. Nuclear proteins were extracted, respectively, from MafA*22 and DN-MafA*39 cells induced for 24 h with indicated concentrations of doxycycline. Two to four independent experiments were performed with similar results.

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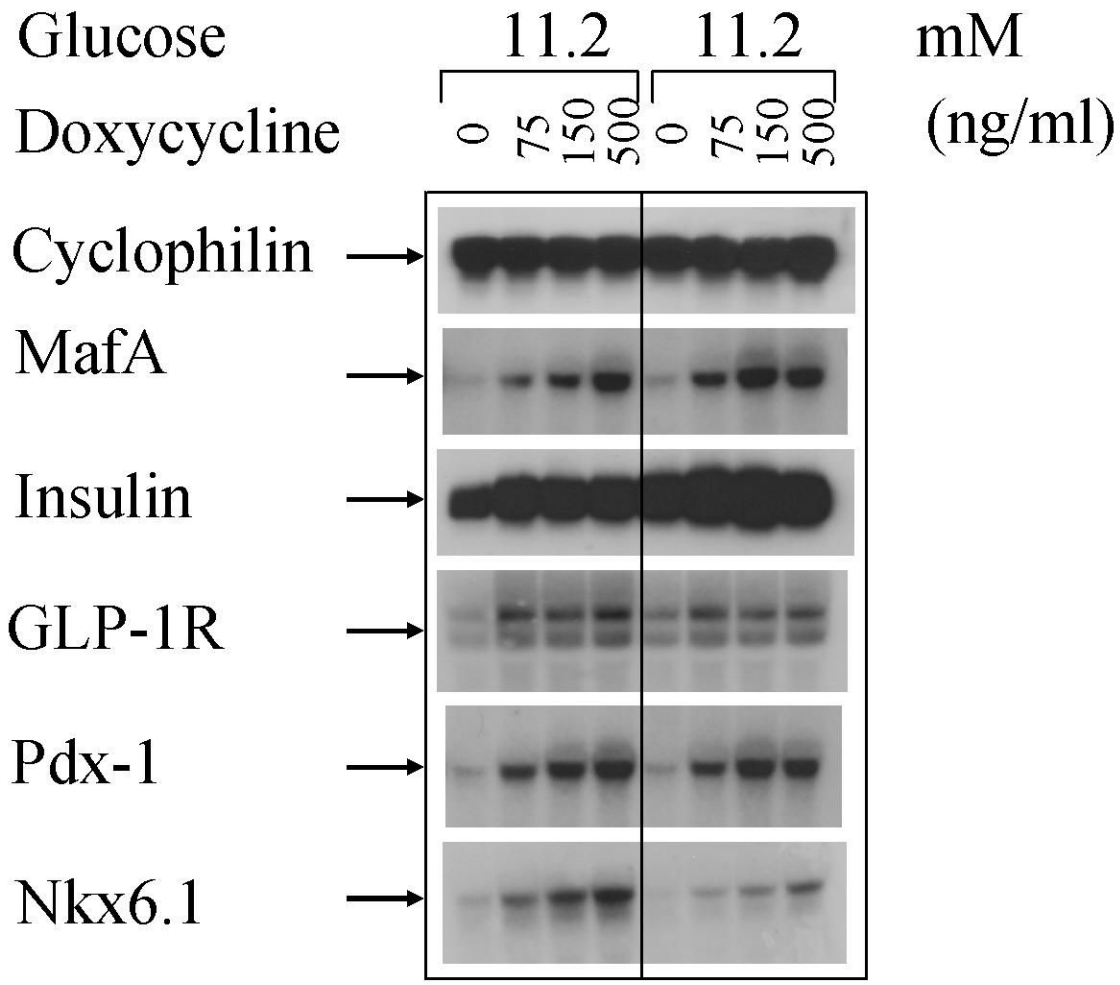

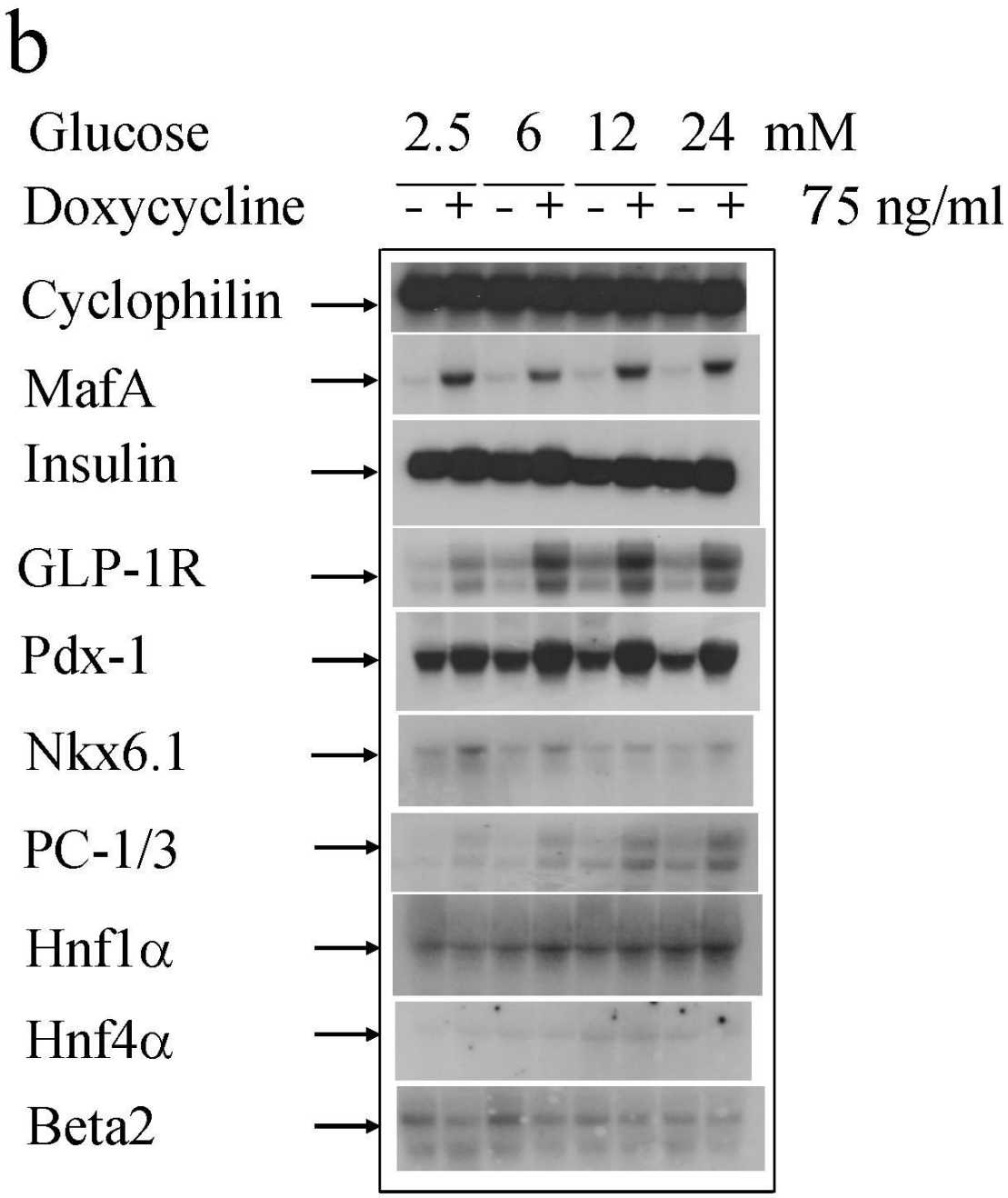

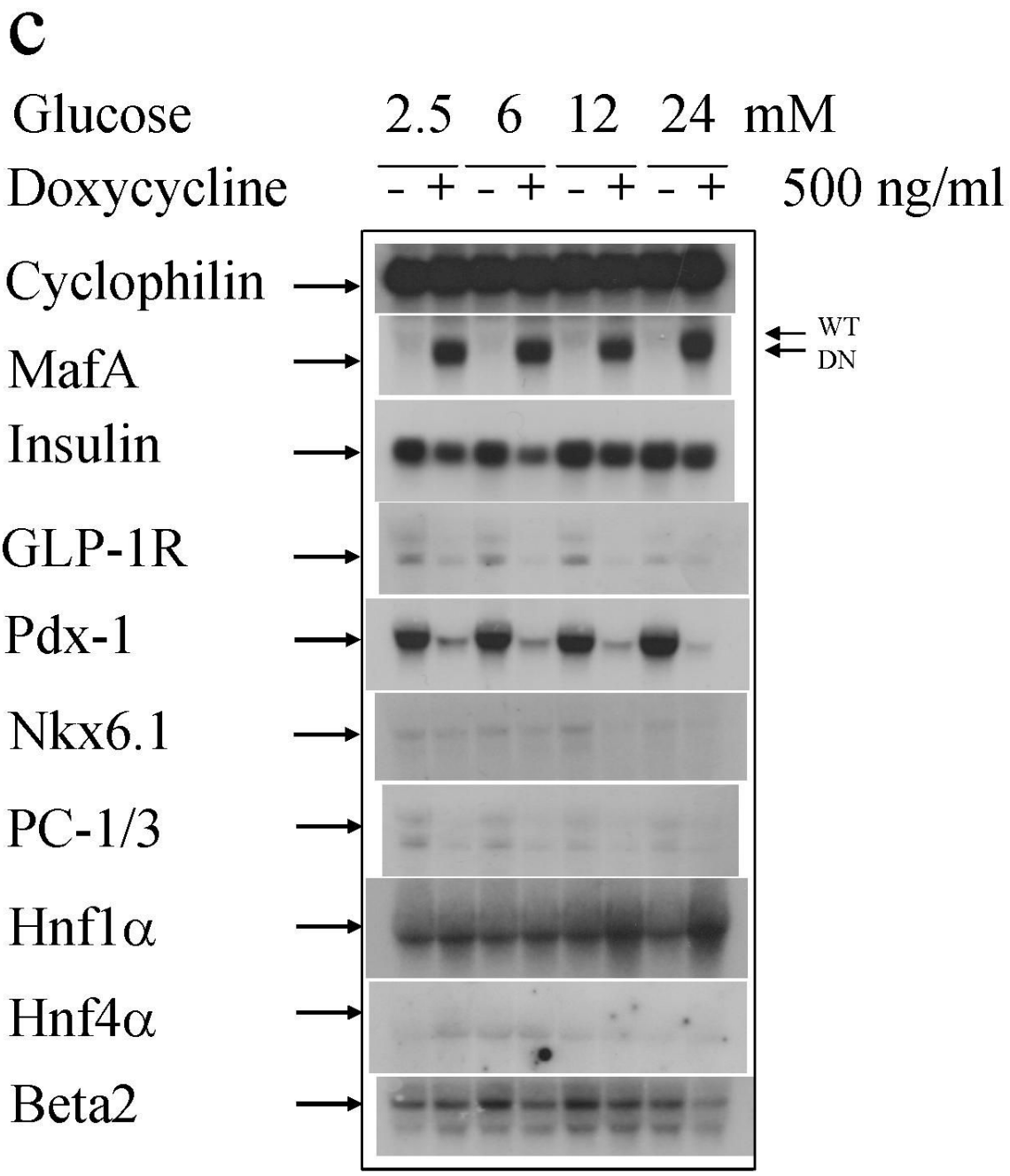

Fig 3.

MafA targets β-cell-specific genes. The gene expression pattern in these cells was quantitatively evaluated by Northern blotting. 20 μg total RNA samples were analysed by hybridising with indicated cDNA probes. The experiments were repeated twice with similar results. **a** MafA*22 cells were cultured in standard medium (11.2 mM glucose) with 0, 75, 150 or 500 ng/ml doxycycline for 24 hours. Two independent experiments were blotted side by side to demonstrate the reproducibility. At 75, 150, and 500 ng/ml doxycycline concentrations, MafA was induced, respectively, by 3.2-, 8.7-, and 11.6-fold. This caused correspondingly increased expression of insulin (by 1.8-, 1.8-, and 1.5-fold), GLP-1R (by 2.4-, 2.2-, and 2.3-fold), Pdx-1 (3.1-, 4.7-, and 4.8-fold), and Nkx6.1 (2.7-, 3.1- and 4.2-fold). Data represent

average of two experiments. **b** MafA*22 cells were cultured with or without 75 ng/ml doxycycline in 2.5 mM glucose medium for 16 h, and then incubated for further 8 h at indicated glucose concentrations. The 3.4-fold induction of MafA resulted in increased expression of insulin (by 1.9-fold), GLP-1R (by 3.2-fold), Pdx-1 (by 4.1-fold), and Nkx6.1 (by 2.3-fold). Data represent the average of experiments at four glucose concentrations. **c** DN-MafA*39 cells were cultured with or without 500 ng/ml doxycycline in 2.5 mM glucose medium for 16 h, and then incubated for further 8 h at indicated glucose concentrations. Induction of DN-MafA led to decreased mRNA levels of insulin (by 62%), GLP-1R (by 74%), Pxd-1 (by 91%), Nkx6.1 (by 31%), and PC1/3 (by 52%). Data represent the average at the four glucose concentrations.

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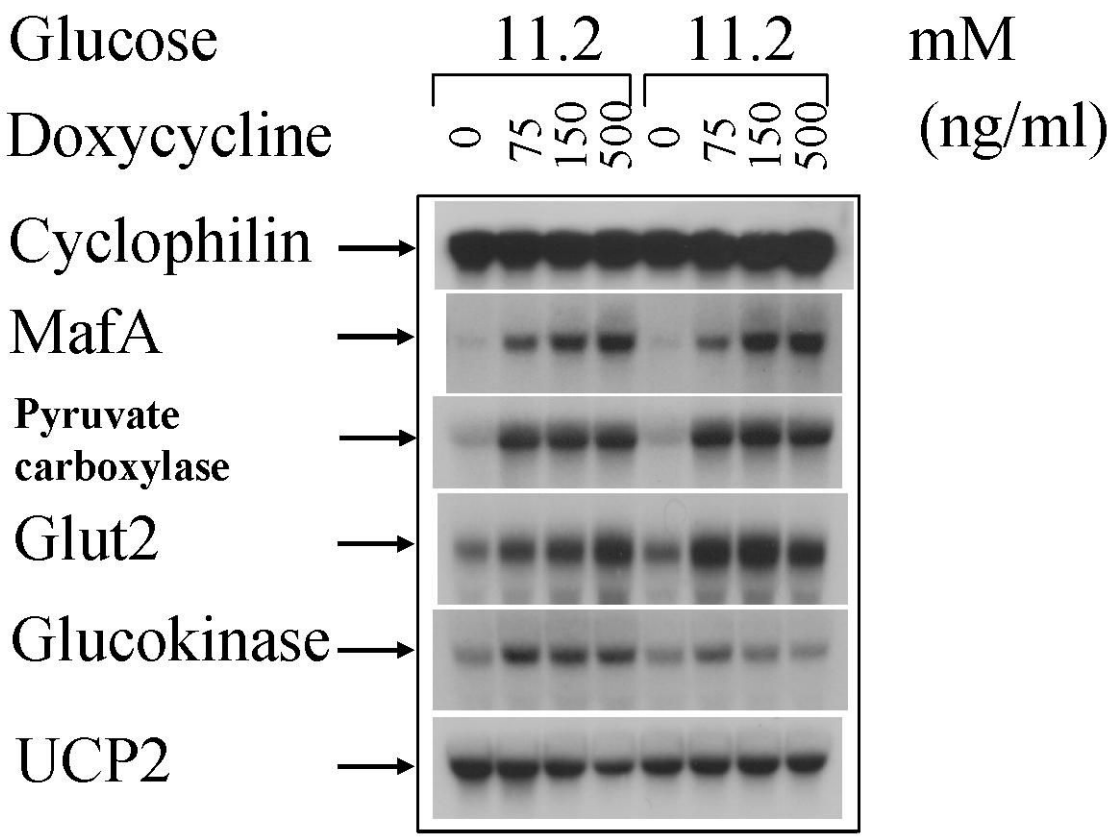

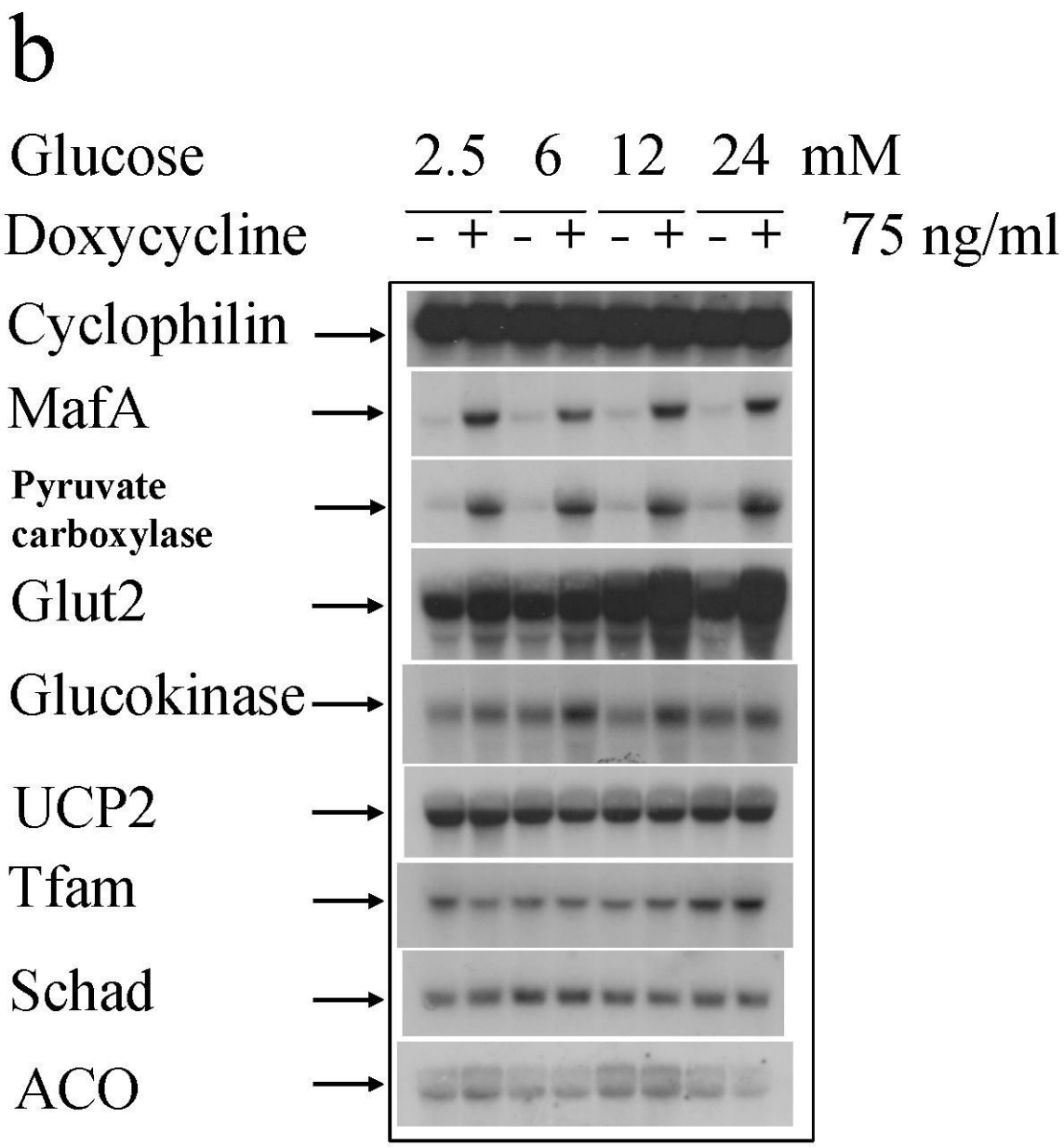

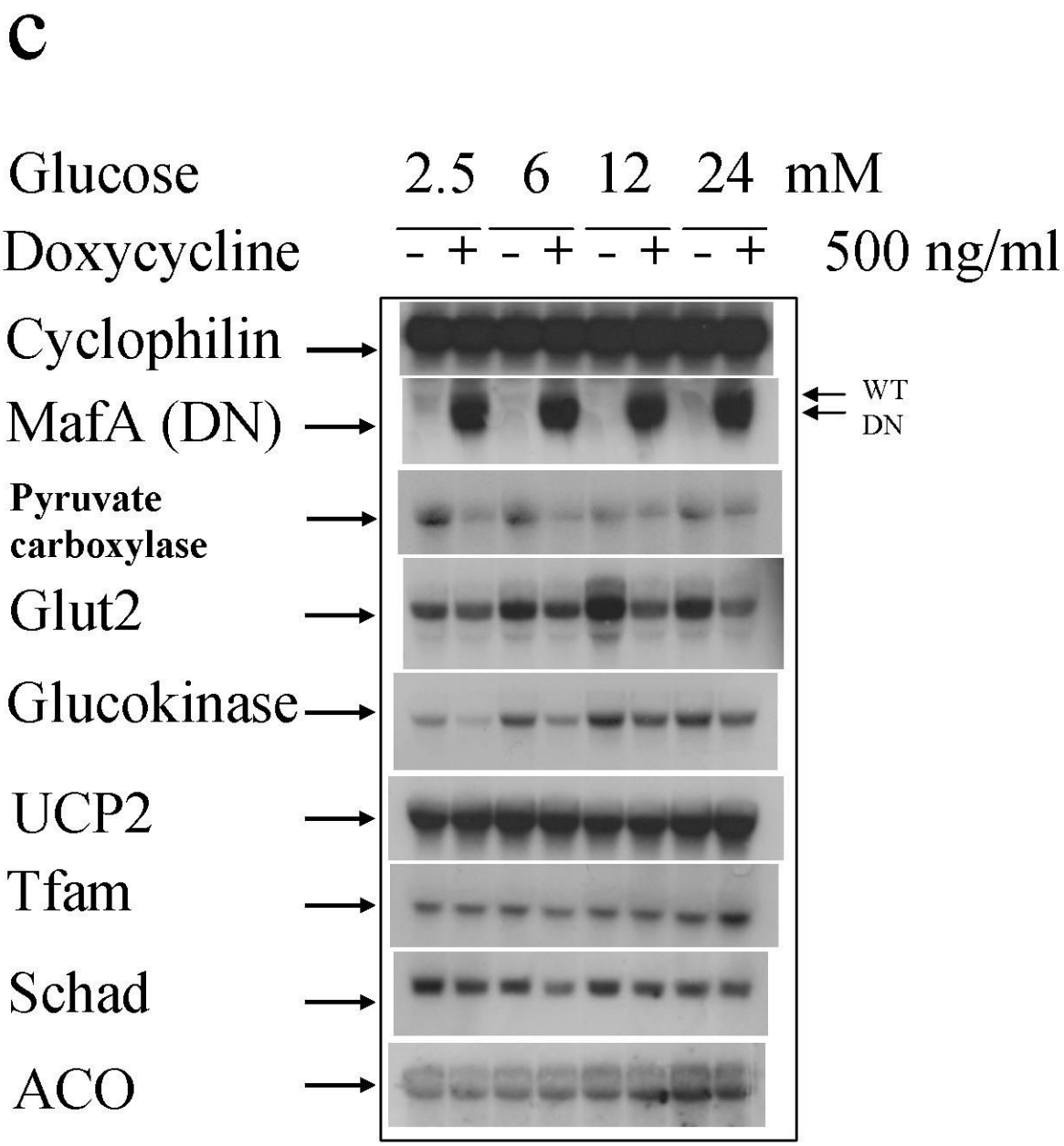

Fig 4.

MafA targets genes essential for metabolism-secretion coupling. Total RNA samples (20 μg) were analysed by hybridising with indicated cDNA probes. The experiments were repeated twice with similar results. **a** MafA*22 cells were cultured as described in Fig. 3a. Two seperate experiments were blotted side by side to demonstrate the consistency. MafA was induced, respectively, by 3.4-, 8.3-, and 10.2-fold by 75, 150, and 500 ng/ml doxycycline. This resulted in corresponding increases in the mRNA levels of pyruvate carboxylase (by 4.4-, 4.8-, and 4.5-fold), Glut2 (by 3.1-, 3.7-, and 3.3-fold), and glucokinase (by 2.2-, 2.1-, and 1.8-fold). Data represent the average of two experiments. **b** MafA*22 cells were cultured as described in Fig. 3b. 3.6-fold induction of MafA caused increased expression of pyruvate carboxylase, Glut2, and glucokinase, respectively, by 4.3-, 2.4-, and 1.9-fold. Data represent the average at four

glucose concentrations. **c** DN-MafA*39 cells were cultured as described in Fig. 3c. The gene expression pattern in these cells was quantitatively evaluated by Northcern blotting. The equilibration with 2.5 mM glucose is necessary to observe the glucose-responsive gene expression, such as Glut2. Induction of DN-MafA led to decreased expression of pyruvate carboxylase, Glut2, and glucokinase, respectively, by 62%, 46%, and 37%. Data represent the average at four glucose concentrations.

Fig 5.

MafA regulates nutrient-stimulated insulin secretion. **a** MafA*22 cells were cultured in standard medium (11.2 mM glucose) with (+Dox) or without 75 ng/ml doxycycline (-Dox) for 19 h, and then equilibrated in 2.5 mmol/l glucose medium for a further 5 h. Insulin-release from MafA*22 cells in KRBH buffer containing indicated concentrations of glucose was determined by radio-immunoassay and expressed as percentage of cellular insulin content. Cellular insulin content was increased by 23% (-Dox: 2.16 ± 0.23 μg/mg protein; +Dox: 2.65 ± 0.25 μg/mg protein, n=6, $p<0.01$) after MafA induction. **b** DN-MafA*39 cells were cultured in standard medium (11.2 mM glucose) with (+Dox) or without 500 ng/ml doxycycline (-Dox) for 19 h, and then equilibrated in 2.5 mmol/l glucose medium for a further 5 h. Induction of DN-MafA resulted in defective insulin-release induced by (mM:) 24 glucose, 5 pyruvate, and 20 KCl. Insulin secretion from DN-MafA*39 cells stimulated by (mM:) 24 glucose, 5 pyruvate, and 20 KCl was measured in KRBH containing 2.5 mmol/l glucose (Basal) was expressed as percentage of cellular insulin content. Cellular insulin content was reduced by 27% (-Dox: 2.245 ± 0.22; +Dox: 1.64 ± 0.20 μg/mg protein, n=6, $p<0.005$). Data represent mean ± SEM of six independent experiments. *$p<0.05$, **$p<0.001$.

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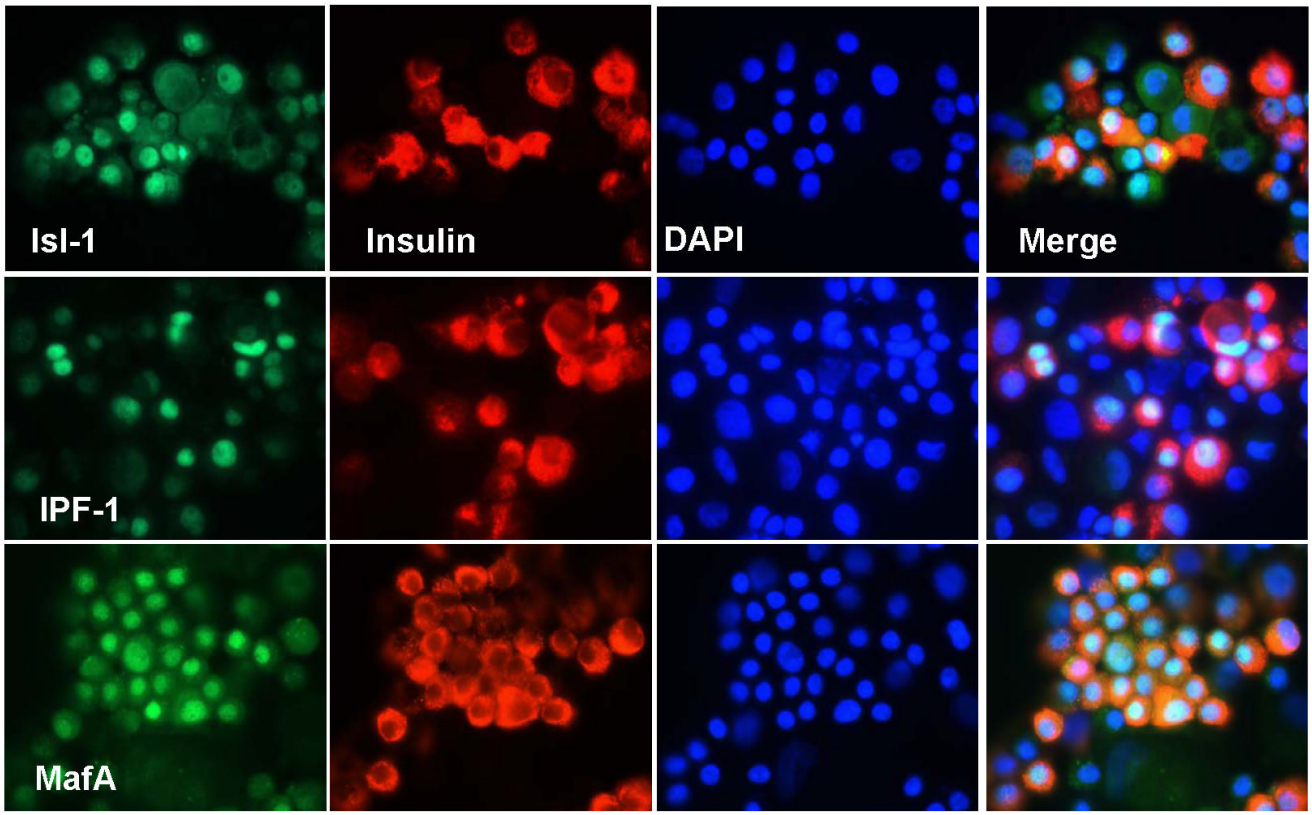

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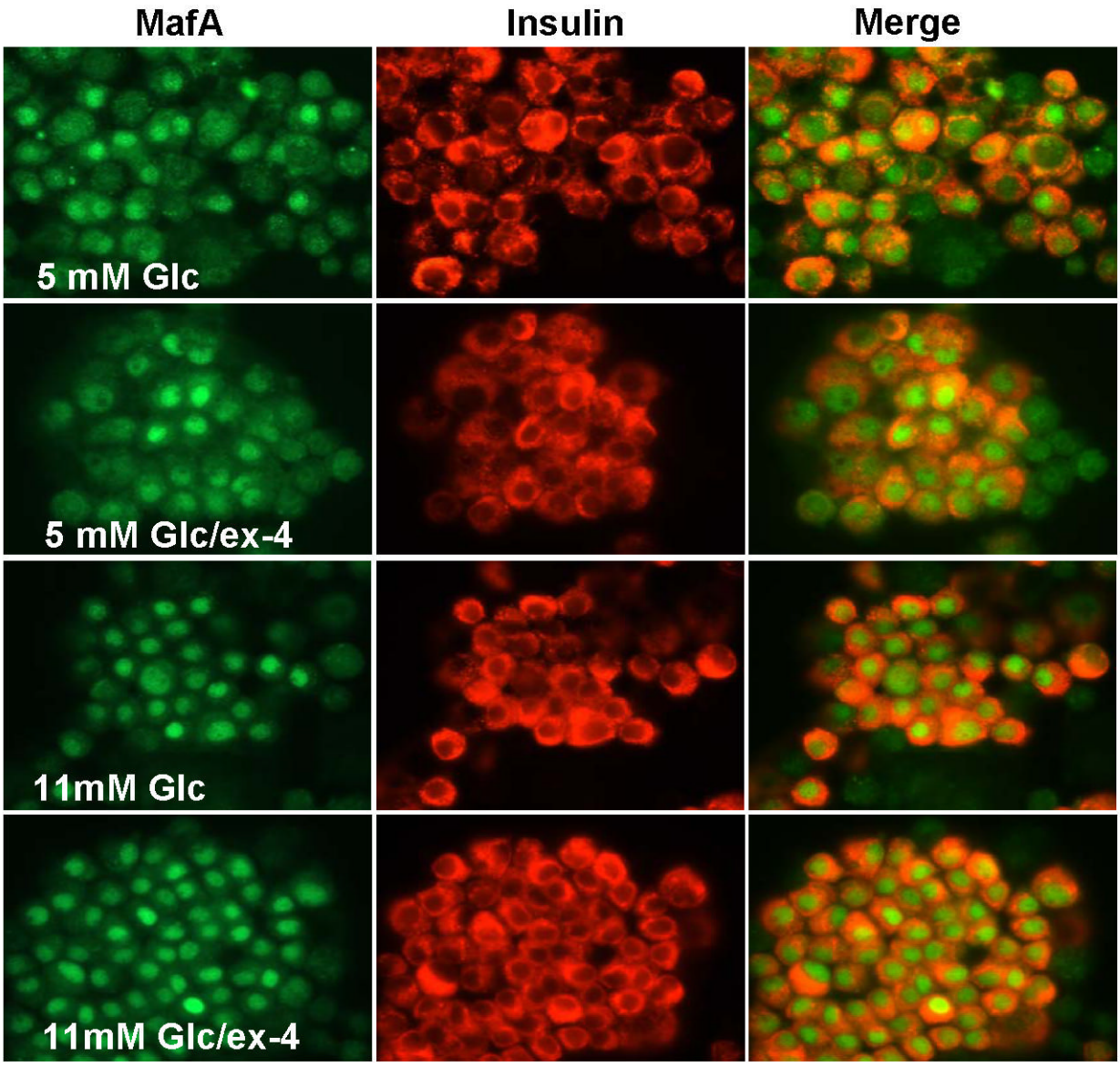

Fig 6.

MafA is expressed specifically in β-cells of human islets. **a** Immunofluorescent detection of the islet transcription factors Isl-1, IPF-1 or mafA (green), insulin (red) as well as DAPI nuclei staining (blue) in dispersed human islet cells cultured at 11.2 mM glucose for 24 h. Similar to IPF-1, MafA is exclusively detected in the nuclei of insulin positive cells. **b** Immunocytochemical detection of mafA (green) and β-cells (insulin in red) was performed 24 hr after incubation with the indicated concentrations of glucose with or without the presence of the GLP-1R agonist exendin-4 (Ex-4, 10 nM). The merge image of the MafA and insulin expressing β-cells is shown.

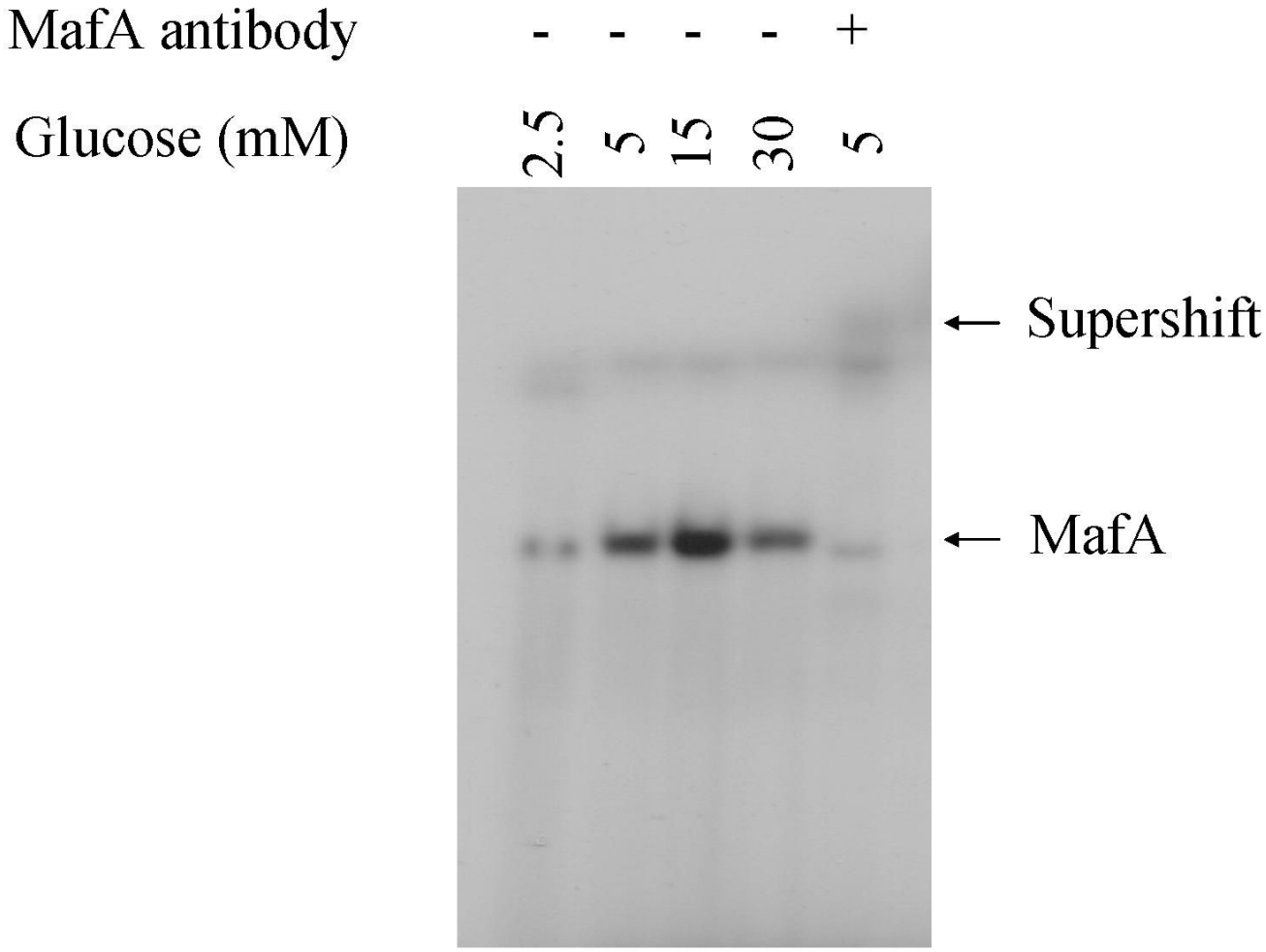

Fig 7.

Bell-shaped DNA-binding of MafA in response to glucose in demonstrated in rat islets. EMSA was performed using with the RIPE3b/C1 element of the insulin promoter. Nuclear proteins were extracted from rat islets cultured with 2.5, 5, 15, and 30 mM glucose for 48 h. DNA-binding activity was increased, respectively, by 3.3 ± 0.7, 12.1 ± 2.5, and 2.2 ± 0.4 fold (n=3), at 5, 15, and 30 mM glucose concentrations. Data were calculated by scanning three independent EMSA experiments using rat islets from two separate isolations.