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## Altered MENIN expression disrupts the MAFA differentiation pathway in insulinoma

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### Abstract

The protein MENIN is the product of the *MEN1* gene. Altered MENIN expression is one of the few events that are clearly associated with foregut neuroendocrine tumours (NETs), classical oncogenes or tumor suppressors being not involved. One of the current challenges is to understand how alteration of MENIN expression contributes to the development of these tumours. We hypothesised that MENIN might regulate factors maintaining endocrine-differentiated functions. We chose the insulinoma model, a paradigmatic example of well-differentiated pancreatic NETs, to study whether MENIN interferes with expression of MAFA, a master glucose-dependent transcription factor in differentiated  $\beta$  cells. Immunohistochemical analysis of a series of human insulinomas revealed a correlated decrease of both MENIN and MAFA. Decreased MAFA expression resulting from targeted *Men1* ablation was also consistently observed in mouse insulinomas. *In vitro* analyses using insulinoma cell lines showed that MENIN regulated MAFA protein and mRNA levels, and bound to *MafA* promoter sequences. MENIN knockdown concomitantly decreased mRNA expression of both *MafA* and  $\beta$ -cell differentiation markers

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### Declaration of interest

The authors have no potential conflicts of interest to disclose.

### Author contributions

MC-B is the guarantor of this work and as such, had full access to all of the data in this study and assumes responsibility for the integrity of the data and accuracy of the data analysis. ZH, CV AB-L, WB, JL and RB performed the experiments. CXZ, CP, PB, CR, JYS and AC contributed to valuable discussions. MC-B, CV and ZH wrote the manuscript. CP and RS reviewed and edited the manuscript RS provided MAFA promoter and luciferase plasmids.

(Ins1/2, Gck, Slc2a2 and Pdx-1), and in parallel, increased the proliferation rate of tumours as measured by bromodeoxyuridine incorporation. Interestingly, MAFA knockdown alone also increased proliferation rate but did not affect the expression of candidate proliferation genes regulated by MENIN. Finally, MENIN variants with missense mutations detected in patients with MEN1 lost the wild-type MENIN properties to regulate MAFA. Together, our findings unveil a previously unsuspected MENIN/MAFA connection regarding control of the  $\beta$ -cell differentiation/proliferation balance, which could contribute to tumorigenesis.

## Keywords

MENIN; MEN1; insulinoma; MAFA; endocrine tumour; differentiation

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## Introduction

A rise in the incidence and prevalence of neuroendocrine tumours (NETs) has been observed in recent decades, but there has been no improvement in patient survival, mainly owing to a lack of clarity regarding the underlying molecular mechanisms (Modlin *et al.* 2008; Rindi and Wiedenmann 2012). The *MEN1* tumour suppressor gene is one of the few genes for which there is clear evidence of involvement in the pathogenesis of foregut NETs.

Alterations of *MEN1* are present in patients with multiple endocrine neoplasia type I (MEN1) syndrome (Agarwal *et al.* 1997; Wautot *et al.* 2002) characterised by an increased incidence of endocrine tumours in several target tissues (mainly the foregut, the pituitary, parathyroid and thymus glands), as well as in a significant proportion of sporadic pancreatic neuroendocrine tumours (PETs) including insulinomas (Corbo *et al.* 2010; Jiao *et al.* 2011).

Insulinomas are tumours characterised by the maintenance of a high level of differentiation (including the capacity to secrete amounts of insulin sufficient to induce functional symptoms), a low proliferation rate and a usually benign behaviour; nevertheless, some cases behave as malignant tumours with metastatic dissemination (de Herder *et al.* 2006; Metz and Jensen 2008; Shin *et al.* 2010). In mouse models, targeted *Men1* ablation in pancreatic  $\beta$  cells is consistently associated with insulinoma development (Bertolino *et al.* 2003; Crabtree *et al.* 2003). A current major challenge in insulinoma and NETs research is to understand how the loss of appropriate expression of the *Men1* gene product, MENIN, contributes to the development of these endocrine tumours (Gracanin *et al.* 2009). MENIN is a protein that is ubiquitously expressed even in non-endocrine tissues. It is found mainly in the nucleus and can function either as a tumour suppressor in several types of endocrine cells or as a tumour promoter in other tissue types (Gracanin *et al.* 2009). MENIN is translated from several transcripts of about 2.8 kb in size and is initiated from a large number of transcription start sites (Fromaget *et al.* 2003). Multiple MENIN-interacting proteins have been described, suggesting that the protein has a wide spectrum of activities (Yang and Hua 2007). Regulation of endocrine-specific determinants remains to be clarified.

Accumulating evidence suggests that MENIN mediates chromatin interactions, especially between transcription factors (TFs) and their targets and is involved, either directly or indirectly, in the fine tuning of the balance between proliferation/apoptosis and

differentiation (Yang and Hua 2007). It has been shown in several cellular contexts that decreased MENIN expression is associated with increased proliferation and modified apoptotic sensitivity (Bazzi *et al.* 2008; Hussein *et al.* 2007; Schnepf *et al.* 2004; Wu and Hua 2011); however, the consequences of MENIN alterations on endocrine cell differentiation, the mechanisms of such alterations and their contributions to NET tumorigenesis have not yet been explored. Insulinoma offers an attractive model to study these interactions. In contrast to other NETs there are several animal and cellular models of insulinoma currently available that provide opportunities for exploring the role of MENIN in the control of differentiation processes. In addition, the mechanisms of  $\beta$ -cell differentiation and of the TFs involved are intensively studied. MENIN has been implicated in the control of adaptive  $\beta$ -cell proliferation, both in normal physiological contexts such as during pregnancy, and in pathological conditions such as type 1 diabetes, but the underlying mechanisms remain unknown (Karnik *et al.* 2007; Yang *et al.* 2010). The molecular mechanisms responsible for MENIN loss of function and the resulting irreversible proliferation/survival leading to tumour development also remain to be elucidated.

MAFA (v-MAF musculoaponeurotic fibrosarcoma oncogene homolog A), is a protein belonging to the large-MAF TF family, whose members have been shown to function as oncogenes or tumour suppressors in different tissues but not in  $\beta$ -cells (Pouponnot *et al.* 2006). In addition, MAFA is a master glucose-regulated TF that contributes to the maintenance of  $\beta$ -cell differentiation and controls, either directly or indirectly, the expression of target genes implicated in  $\beta$ -cell functions, including *Ins-1/2* (encoding insulin), *Slc2a2* (encoding the glucose transporter GLUT2), *Gck* (encoding glucokinase) and *Pdx-1* (Aramata *et al.* 2007; Yang and Cvekl 2007). The level of MAFA in  $\beta$  cells is tightly controlled mainly by glucose, the major growth and differentiation regulator of  $\beta$  cells. Short-term glucose stimulation (up to 24 h) induces a transient increase in MAFA expression and exerts coordinate stimulatory effects both on hormone synthesis and cell proliferation (Liu *et al.* 2009), whereas prolonged glucose exposure (>24 h) decreases MAFA expression and is deleterious to  $\beta$ -cell function and survival (Martens and Pipeleers 2009). MAFA is thus considered a barometer of  $\beta$ -cell health (Hang and Stein 2011).

In this study, we investigated whether MENIN can regulate the expression of MAFA, a major TF that maintains  $\beta$ -cell differentiation (Aramata *et al.* 2007). We hypothesised that decreased MENIN expression or impaired MENIN function might result in altered MAFA expression and function, along with disruption of the differentiation/proliferation balance. In this study, we used both *in vivo* and *in vitro* insulinoma models to determine whether MENIN could regulate MAFA expression. We also evaluated the functional consequences of MENIN mutations present in patients with MEN1 after MAFA regulation. Our data show for the first time, that MENIN interferes with the  $\beta$ -cell-specific MAFA pathway, thus opening a new field of investigation into the molecular mechanisms involved in pancreatic endocrine tumorigenesis.

## Materials and methods

### Human tissues

A series of samples from human insulin-expressing PETs were obtained from a tissue biobank (Tumorothèque des Hospices Civils de Lyon, supported by the Institut National du Cancer and the French Ministry of Health), which adheres to French ethical rules and regulations. The tissue in this bank is either from patients who have given their informed consent for their tissue to be used or is from samples that qualify for research use according to French regulations. The research project was approved by the biobank steering committee.

All tissue samples ( $n = 15$ ) used in this study were obtained from surgical resections. The patients from whom the tissues were obtained (2 males and 13 females) had a mean age of 47.5 years (range 25 to 79 years). Of these 15 tumours, 10 were functional at the time of removal and could be termed insulinomas, whereas the remaining 5 were non-functional tumours, despite insulin expression being detected in most or all tumour cells by immunohistochemistry (IHC). According to the 2010 World Health Organization (WHO) classification, the 10 functional tumours were classified as neuroendocrine neoplasms G1, and the other 5 were neuroendocrine neoplasms G2. One tumour was associated with MEN1 syndrome, while the others were sporadic. Three patients had distant metastases.

### Murine tissues

Pancreatic islets and insulinomas were isolated from 12-month-old  $Men1^{F/F}$ -RipCre<sup>-</sup> and  $Men1^{F/F}$ -RipCre<sup>+</sup> mice, respectively, by hand-picking the tissues from dissociated pancreases on dark-field dishes under a dissecting microscope, as previously described (Fontaniere, *et al.* 2006).

### IHC analysis

For the IHC study, formalin-fixed, paraffin wax-embedded tissue was cut into 4- $\mu$ m-thick sections. Whole sections, containing both tumour and adjacent peritumoral tissues, were used. Tissue sections were incubated with antibodies to MENIN (Epitomics, Burlingame, CA, USA) and to MAFA (Bethyl Laboratory Inc., Montgomery, TX, USA) at appropriate dilutions (1:50 and 1:250, respectively), and an indirect streptavidin-biotin/peroxidase technique was used to visualise the labelling. The apparent expression levels of MENIN and MAFA in tumour cells were assessed by comparison with internal controls in the same section, i.e. normal endocrine cells of Langerhans islets for MAFA and normal pancreatic epithelial cells for MENIN. If the apparent expression level in all or the majority of tumour cells was equal to or higher than that observed in normal internal controls, it was considered “high” expression, while if it was lower than that observed in normal internal controls, it was considered “low” expression. If the expression level was comparable in different tumour cells, the tumour was considered homogeneous, whereas if the expression level varied among different tumour cells, the tumour was considered heterogeneous, and the expression level reported was that of the majority of the tumour cells.

## Cell lines

The two insulinoma cell lines used were INS-1E (Merglen *et al.* 2004) and INS-r $\beta$  (Wang and Iynedjian 1997). These cell lines were originally independently established from radio-induced rat insulinoma INS-1 cells (Asfari *et al.* 1992) and are glucose-responsive (Merglen *et al.* 2004). We also used MafA47 cells, which were previously established from INS-r $\beta$  cells; they can conditionally overexpress MAFA protein under doxycyclin treatment (Wang *et al.* 2007). All three cell lines used express wild-type (WT) MENIN protein.

Cells were maintained in a standard culture medium as previously described (Asfari *et al.* 1992) but that contained only 5 mM glucose, which allows a greater sensitivity to physiological glucose stimulation (11 mM). INS-r $\beta$  cells that conditionally overexpress WT MENIN or MEN1-related variants have been previously described (Bazzi *et al.* 2008). For MENIN induction of INS-r $\beta$  cells, 1000 ng ml<sup>-1</sup> doxycyclin (Sigma-Aldrich, St. Louis, MO, USA) was added to the culture medium at the time of seeding. To determine the half-life of the MENIN protein and mRNA, cells were treated with 20  $\mu$ g ml<sup>-1</sup> cycloheximide (CHX, Sigma-Aldrich) or 4  $\mu$ g ml<sup>-1</sup> actinomycin D (ActD, Sigma-Aldrich) for various periods of time.

## Immunoblot analysis

Cells were lysed in RIPA buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and proteins were resolved on 10% pre-cast Criterion XT polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA, USA). The proteins were transferred to polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) which were first treated with blocking solution (Tris-buffered saline with 0.1% Tween 20 and 5% milk), then incubated with a primary antibody directed against the C-terminal region of the MENIN protein (A300-105A, Bethyl Laboratory Inc.), the C-terminal region of the MAFA protein (A300-611A, Bethyl Laboratory Inc.) or the loading control tubulin (Sigma-Aldrich). A species-matched horseradish peroxidase-labelled secondary antibody (Jackson Laboratory, Bar Harbor, ME, USA) was used to visualise the antibody-antigen complexes by chemiluminescence, using an ImmunStar kit (Bio-Rad).

## Gene expression knockdown by RNA interference

Cells were seeded in standard culture medium to ensure about 50% confluence and then transfected with a final concentration of 100 nM small interfering RNAs (siRNAs) using a mixture of four siRNAs provided as a single reagent with high specificity and low off-target effects (ON-TARGET plus SMART pool siRNAs # L-090784-02 and L-081264-02 for *Men1* and *MafA* genes, respectively; Dharmacon, Lafayette, CO, USA and Thermo Fisher Scientific Inc., Waltham, MA, USA). The non-targeted (si*Ctrl*), anti-*Men1* or anti-*MAFA* siRNA pools were incorporated into cells using the DharmaFECT4 transfection reagent in accordance with the manufacturer's instructions. At 48 h following transfection, the cells were starved in media containing 0.1 mM glucose and no pyruvate. This medium was then replaced by the stimulation medium containing 5 or 15 mM glucose and no pyruvate. The cells were harvested at the indicated time points and stored at -80°C until protein or RNA extraction was performed.

### Plasmid transfection/luciferase assays

The *MAFA* promoter-luciferase (LUC) (Raum et al. 2006) and the cytomegalovirus-driven WT *MEN1* (Bazzi et al. 2008) constructs have been described previously. Plasmids were co-transfected using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA), while the DharmafectDuo reagent (Dharmacon) was used to co-introduce plasmids and siRNAs. The cells were harvested at 48 h after transfection. A co-transfected Renilla (REN) reporter plasmid served as an internal transfection efficiency control. The cells were lysed in the provided PLB buffer, and LUC/REN activity was measured using the Dual-Luciferase Reporter Kit (Promega, Madison, WI, USA).

### Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed using the enzymatic ChIP-IT<sup>®</sup> Express kit (Active Motif, La Hulpe, Belgium). Protein-DNA crosslinking was performed using formaldehyde (Thermo Scientific Pierce) and chromatin was sheared by enzymatic digestion following the manufacturer's instructions. Immunoprecipitations were performed using the same anti-MENIN and anti-MAFA antibodies described above, as well as anti-PDX-1 antibody (sc-14664, Santa Cruz Biotechnology). Anti-PolII antibodies were included in the kit. The recovered chromatin was subjected to polymerase chain reaction (PCR) analysis, and the PCR products were separated by gel electrophoresis in an agarose gel. DNA fragments were visualised with a Chemidoc instrument (Bio-Rad).

### RNA extraction/reverse transcription-quantitative PCR analysis

A reverse transcription-quantitative PCR (RT-qPCR) protocol was developed to meet the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) criteria (Bustin *et al.* 2009). Total RNA from frozen cell line samples and frozen tissues was extracted using the TRIzol Plus RNA Purification kit (Invitrogen). Reverse transcription of 1 µg each RNA was performed using the QuantiTect<sup>®</sup> Reverse Transcription kit (Qiagen SA, Venlo, The Netherlands) which includes elimination of genomic DNA contamination in RNA samples. The qPCR analyses were performed using the Maxima SYBR<sup>®</sup> Green/ROX qPCR Master Mix (Stratagene; Agilent Technologies, Santa Clara, CA, USA) on a CFX96 qPCR instrument (Bio-Rad). Data were analysed using CFX Manager software and the  $C_t$  method (Livak and Schmittgen 2001). For normalisation, five housekeeping genes were tested, and at least two of the genes with the lowest M-values (<0.150) were chosen as reference: *β-actin* and *Rplp0* for rat cell lines; *β-ACTIN*, *TFCP2* and *GAPDH* for human tumours and *β-actin* and *Hprt* for mouse tumours. Primers for *Ins1/2* and *Gck* were purchased from Qiagen; the other primer sequences are available on request.

### Statistical analysis

Mann-Whitney U tests were used for statistical analysis, and  $P < 0.1$  was considered significant. Statistical analyses were performed on at least four independently generated datasets.

## Results

### MENIN and MAFA expression levels correlate in human PETs

MENIN and MAFA are co-expressed in native  $\beta$  cells. In this study, we evaluated the expression of these two proteins by IHC in 15 insulin-expressing PETs (14 sporadic cases and 1 MEN1-related). Different MENIN expression patterns were observed in the 14 sporadic cases. In 5 of these cases, MENIN was detectable in all tumour cells, with a high expression level similar to that detected in non-tumoral tissue; in parallel, MAFA was detected in all tumour cells with the same apparent high expression level as in adjacent normal Langerhans islets (Fig. 1A, Type 1). In seven additional sporadic cases, the majority of tumour cells displayed low but detectable MENIN expression levels compared with the non-tumoral tissue, and the MAFA expression level was also low (Fig. 1A, Type 2). In the final two sporadic cases, MENIN was low or heterogeneously expressed in tumour cells, and MAFA expression was heterogeneous in one case and high in the other (Fig. 1B, “unclassified”). In the MEN-1 related case, MENIN was undetectable, whereas MAFA was detectable in a minority of tumour cells, with low and heterogeneous intensity (Fig. 1A, MEN1).

There was no correlation between the functional status of the tumour and MENIN/MAFA expression; the proportion of cases with abnormal MENIN/MAFA expression was comparable in both functioning and non-functioning tumours (7/10 and 3/5, respectively). Interestingly, the three malignant cases were all associated with low MENIN/MAFA expression. In addition, the Ki67 proliferation index was <2% in all cases with normal MENIN/MAFA expression but >2% in 8 of the 10 cases with abnormal MENIN/MAFA expression. RT-qPCR analysis on a series of four human insulinoma confirmed a strong correlation between the MENIN and MAFA mRNA levels (Fig. 1A, right panel).

### MAFA expression is reduced in mouse insulinoma

We next determined if MAFA expression was also compromised after *Men1* inactivation in islet  $\beta$  cells in mice. Specific *Men1* disruption mediated by Rat insulin promoter (RIP)-driven Cre in floxed *Men1* mice led to full-penetrance insulinoma development at about 10 to 12 months, as we described previously (Bertolino *et al.* 2003). All tested mouse *Men1* insulinomas (n=10) displayed marked reduction in MAFA staining compared with normal islet  $\beta$  cells. This result was confirmed by RT-PCR analysis of MAFA mRNA levels, with >50% reduction found in islets from the mutant mice (Fig. 1C).

### MENIN level affects MAFA protein expression following acute glucose stimulation

We next analysed the effects of MENIN downregulation on MAFA protein expression in two insulinoma cell lines, INS-1E and INS-r $\beta$ . We found that siRNA-mediated inhibition of MENIN induced a slight decrease in MAFA levels detectable by immunoblotting (IB) in INS-1E, but this protein decrease was not always reproducible in basal culture conditions, as shown in Fig. 2A in INS-r $\beta$  cells.

We therefore tested the effect of MENIN inhibition on MAFA expression during acute glucose stimulation. In INS-1E, and INS-r $\beta$  cells, acute stimulation with 11 mM glucose

medium resulted in rapid and robust accumulation of MAFA protein, with maximum protein levels reached after 4 h (Fig. 2B). A reduction in MENIN level greatly reduced MAFA accumulation, whereas MAFB protein level increased in parallel, both in INS-1E and in INS-r $\beta$  cells (Fig. 2C, D; Supplementary Fig. S1).

To determine if MENIN knockdown might be accelerating MAFA degradation, nascent protein synthesis was blocked by CHX treatment in control and MENIN siRNA-treated INS cells. The half-life of the MAFA protein was about 4 h in INS-1E (Fig. 2D) and INS-r $\beta$  cells (Supplementary Fig. S1B) and was unchanged after MENIN down-regulation.

### MENIN inhibition alters MAFA transcription

We then tested whether down-regulation of expression of MENIN might alter MAFA mRNA levels. Transfection of INS-1E cells with anti-*Men1* siRNA was found to reduce both endogenous *Men1* and *MafA* mRNA levels (by about 92% and 50%, respectively) under basal culture conditions with 5 mM glucose (Fig. 3A, left) and also during glucose stimulation (Supplementary Fig. S2A). Comparable results were obtained using INS-r $\beta$  cells (Supplementary Fig. S2B). To determine if MENIN levels were affecting *MafA* mRNA stability, RNA synthesis was blocked by actinomycin D treatment in control and anti-*Men1* siRNA-transfected INS-1E cells. The half-life (i.e. about 2 hours) of *MafA* mRNA was unaffected by MENIN knockdown in either INS-1E cells (Fig. 3A, right) or INS-r $\beta$  cells (Supplementary Fig. S2C).

To analyse if MENIN directly modulates *MafA* transcription, we evaluated the effect of altering MENIN expression level on the activity of various 5'-flanking *MafA* promoter-driven luciferase reporter genes. The *MafA* promoter fragments examined spanned the six conserved regions controlling islet  $\beta$ -cell-specific expression (termed R to R6 (Raum *et al.* 2006)). MENIN level modulated the activity of all these regions in both underexpression and overexpression studies (Fig. 3B).

Using ChIP experiments in INS-1E cells, we could recover MENIN bound to the R1, R2, R4, R5 and R6 regions, but it did not appear to bind to R3 (Fig. 3C). This inability to detect binding to R3 was not due to a technical problem, as PDX-1 was observed as described previously (Raum *et al.* 2006). Unexpectedly, no signal was detected with anti-PolIII antibody using R6 primers, in contrast to the  $\beta$ -actin primers provided as a positive ChIP control in the kit. The related technical reasons for this inability to detect PolIII binding to R6 remain unclear.

### MENIN inhibition impairs expression of $\beta$ -cell differentiation markers

We next analysed the effect of either MENIN or MAFA inhibition on several MAFA-regulated genes critical to  $\beta$ -cell differentiation and function, including *Ins-1/Ins-2*, *Slc2a2*, *Gck* and *Pdx-1* (Matsuoka *et al.* 2007; Wang *et al.* 2007). The expression of these differentiation markers was differentially affected by either *Men1* or *MafA* siRNA-mediated knockdown in INS-1E cells, although *MafA* levels were inhibited to a similar level in both cases (Fig. 4). Under basal culture conditions, MENIN inhibition resulted in a decrease not only in *MafA* but also in *Ins1/Ins2*, *Scl2a*, *Gck* and *Pdx-1* (to about 50% of control). Interestingly, if the MENIN level was kept stable, direct inhibition of *MafA* affected *Ins-1/*



*Ins-2* in an identical manner to that described above but had a reduced effect on *Slc2a2* and *Gck* (>80% of control) and no effect on *Pdx-1* (Fig. 4A). Similar gene-regulation results were obtained following 11-mM glucose stimulation of INS-1E cells (Fig. 4B).

### **MEN1-related missense MENIN proteins lose their regulatory effects on MAFA expression**

Two different missense versions of MENIN found in patients with MEN1 syndrome were next analysed for their ability to influence glucose-activated MAFA expression. We used INS-r $\beta$  cells in which MENIN knockdown had been found to affect *MafA* levels in response to glucose stimulation (Fig. S1A). As expected, conditional WT MENIN overexpression amplified MAFA protein and mRNA stimulation in INS\_WT/B7 cells (Figs. 5A and 6A), but overexpression of the MENIN p.A160T and p.A541T variants did not increase MAFA expression (Figs. 5B and 6B, C). In addition, the effects of these two variants on MAFA were different. The p.A160T protein actually decreased MAFA protein accumulation (Fig. 5B) and had the same effect on *Ins2* and *Slc2A2* mRNA levels (Fig. 6B). By contrast, MENIN p.A541T did not have either an activating or a repressive effect on glucose-induced *MafA* or *Ins2* mRNA expression, although *Slc2A* levels were decreased (Figs. 5 and 6). No reproducible data were obtained with the *Ins-1* gene in these experiments.

### **MENIN and MAFA modulate the glucose-evoked proliferation rate in insulinoma cell lines**

To test whether downregulation of differentiation markers might be associated with dysregulation of the differentiation/proliferation balance, we analysed the effects of MENIN inhibition on proliferation in insulinoma cell lines. Glucose has potent  $\beta$ -cell mitogen activity both *in vitro* and *in vivo* (Martens and Pipeleers 2009), hence short-term stimulation (up to 24 h) can stimulate the  $\beta$ -cell proliferation rate as measured by bromodeoxyuridine (BrdU) incorporation. As expected, the basal proliferation rate of INS-1E and INS-r $\beta$  cells greatly increased at 24 h after variation of glucose concentrations (the concentration of fetal calf serum remaining constant) from 0.1 to 5 and 15 mM (Fig. 7A). Interestingly, inhibition of MENIN expression resulted in greater BrdU incorporation after 5- and 15-mM glucose stimulation in both INS-1E and INS-r $\beta$  cells.

Because MENIN inhibition decreased MAFA expression, we then tested whether direct siRNA-mediated inhibition of MAFA could modify the proliferation rate in insulinoma cell lines. Direct MAFA knockdown was correlated with increased BrdU incorporation rate following glucose stimulation both with 5 and 15 mM glucose (Fig. 7B, left panel). Similar results were obtained using INS-r $\beta$  cells (data not shown). In a parallel set of experiments, we used MAFA47 cells (Wang et al. 2007) that overexpressed MAFA after doxycyclin treatment (Fig. 7B, right panel) and observed that, remarkably, the glucose-induced BrdU incorporation rate decreased as MAFA expression increased.

Finally, we measured the expression of candidate genes whose expression had previously been found to be related to  $\beta$ -cell proliferation in response to either glucose stimulation, MENIN expression level or both (Cozar-Castellano *et al.* 2006; Yang *et al.* 2010; Yesil and Lammert 2008). We found that following glucose stimulation, the basal mRNA level of the start-cyclin genes *Ccnd1* and *Ccnd2* had a significant increase not modified after MENIN inhibition (Fig. 7C). By contrast, MENIN inhibition significantly stimulated expression of

the cyclin kinase inhibitor genes *p18* and *p27*, of the mitotic cyclins a2 and b2 (*Ccna2* and *Ccnb2*) and of the mitotic PDZ-binding kinase (*Pbk*), whose expression was not affected after glucose stimulation. Similar results were obtained in INS-1 $\beta$  cells (data not shown). Unexpectedly, expression of these proliferation genes was not modified following direct MAFA inhibition by anti-MAFA siRNA (data not shown). Double knockdown by anti-*Men1* and anti-MAFA siRNA reproduced the anti-*Men1* siRNA effect (data not shown).

## Discussion

Our data show for the first time that MENIN directly regulates the expression of MAFA, a TF that is vital for maintaining endocrine  $\beta$ -cell differentiation and function. Consequently, loss of MENIN alters both MAFA expression and the endocrine  $\beta$ -cell differentiation/proliferation balance. These data indicate a potentially new oncosuppressive mechanism in insulinoma that is mediated through the direct deregulation of the terminal differentiation pathway.

Our first relevant observation is that MENIN directly regulates MAFA, both *in vivo* and *in vitro*. A prevalent co-repression of these two proteins was evident in human insulinomas. Owing to the scarcity of these tumours, large series are difficult to obtain. Nevertheless, a convincing confirmation came from our *Men1*-mice insulinoma analysis, which reproduced these observations. *In vitro* analyses showed that MENIN directly regulated *MafA* transcription, thus affecting MAFA protein and mRNA levels. We show that MENIN modulate the transcriptional activity of the large *MafA* R1-6 promoter fragment, which ensures specific pancreatic expression and mediates glucose-induced MAFA expression *in vivo* (Raum *et al.* 2006). MENIN also regulated all the promoter sub-fragments tested. We found that MENIN bound to multiple DNA sequences in this R1 to R6 promoter fragment, including the R1, R2, R4, R5 and R6 regions, but not R3. Thus, MENIN may regulate *MafA* transcription via distinct molecular mechanisms in a promoter region-dependent fashion. Firstly, MENIN could act through a direct mechanism, as suggested by its binding to the R1, R2, R4, R5 and R6 promoter regions, and secondly, it could also operate through an indirect mechanism, by regulating expression of other TFs that themselves bind to the R3 region. PDX-1 might constitute such a candidate partner; it binds specifically to the R3 region (Raum *et al.* 2006), and we observed here that *Pdx-1* mRNA levels were decreased by MENIN inhibition.

It is known that MAFA protein expression is tightly controlled in response to glucose stimulation, but the mechanisms of this are poorly understood. Numerous post-translational modifications modulate MAFA stability and activity (Eychene *et al.* 2008). Our data show that MENIN is essential for MAFA protein accumulation in response to glucose, partly through transcriptional mechanisms, but additional mechanisms cannot be excluded. MENIN levels were previously reported to be significantly reduced by long-term high-glucose exposure of both INS-1 cells and rat primary islets (Zhang *et al.* 2012). In our experiments, we did not find any modification of endogenous MENIN protein or mRNA levels following glucose stimulation. These discrepancies could be attributable to different experimental conditions. In the current study, only acute (<6 h) stimulation with 11 mM glucose was tested; the half-life of the WT MENIN protein is >8 h (Canaff *et al.* 2012;

Yaguchi *et al.* 2004), and that of its mRNA is around 3 h in INS-1 cells (personal observations).

Our second relevant observation is that MENIN expression is crucial for MAFA-mediated  $\beta$ -cell differentiation, as its downregulation had a dramatic negative effect on the expression of major MAFA-regulated differentiation markers such as *Slc2a2*, *Gck* and especially *Pdx-1*. Indeed, MENIN inhibition had greater deleterious effects than found with MAFA inhibition on these markers. The transcriptional properties of MENIN could be directly or indirectly responsible for these observations. MENIN can regulate transcription by interacting directly with TFs or through several epigenetic mechanisms (Dreijerink *et al.* 2009; Yang and Hua 2007). We cannot exclude that MENIN could participate through such mechanisms to regulate transcription not only of *MafA* but also of a larger series of differentiation-related  $\beta$ -cell genes, including those studied here. Consequently, differentiation functions must be altered. In particular, MENIN expression affected *Slc2a2* gene transcription and missense MENIN proteins were unable to simulate the *Slc2a2* mRNA level, emphasising a potential key functional link. Thus, because *Slc2A2* is the gene encoding for the glucose transporter GLUT2, modulation of the number of GLUT2 molecules at the cell surface could directly affect glucose uptake and consequently downstream differentiation/proliferation signalling in  $\beta$  cells. More generally, MENIN inhibition seems to block maintenance of the differentiation process; such a mechanism could contribute to tumorigenesis, in parallel with re-expression of early markers (MAFB and HLXB9) and stimulation of proliferation/survival (Lejonklou *et al.* 2009; Lu *et al.* 2012; Shi *et al.* 2013). A very tightly controlled and interconnected regulation of MAFA and MAFB has been described in developing  $\beta$  cells, with their levels being in inverse proportion to one another (Artner *et al.* 2010) and MAFB expression in mice being restricted to immature  $\beta$  cells (Nishimura *et al.* 2006). These previous observations suggest that the effect of MENIN on MAFA expression level can directly or indirectly influence MAFB expression. In line with this, we found that MENIN knockdown simultaneously downregulated MAFA and upregulated MAFB in both cell lines analysed here. These observations corroborate our previously published data showing that, in adult  $\beta$  cells, decreased expression level of MENIN is correlated with an increased expression of MAFB in insulinoma and in the tested cell lines (Lu *et al.* 2012). By regulating the MAFA/MAFB ratio, MENIN could thus influence the differentiation/proliferation balance in  $\beta$  cells. In line with this, our data showed an increase in proliferation rate following MENIN inhibition in the insulinoma cells analysed. A relevant observation is that isolated inhibition of MAFA also stimulated the proliferation rate, suggesting that MAFA could possess an unsuspected tumour suppressor function in  $\beta$  cells. MENIN inhibition affected candidate proliferation genes that had previously been implicated in rodent  $\beta$ -cell proliferation, but interestingly, MAFA repression did not have any effect on these genes, suggesting the implication of other MAFA-regulated proliferation pathways. Large MAF proteins were previously shown to act as tumour suppressors or oncogenes depending on the cell type, although nothing is known concerning this aspect in  $\beta$  cells (Eychene *et al.* 2008).

In addition, increases in both Ki67 expression and malignancy in human insulinomas with concomitant MENIN and MAFA repression support this hypothesis. Finally, the relevance of the MENIN/MAFA/MAFB axis in tumorigenesis is supported by our data, showing that

in insulinoma cell lines, the p.A160T or p.A541T protein counterparts of MENIN found in patients with MEN1 were not able to exert a similar influence to WT MENIN on MAFA regulation. We found different functional consequences depending on the type of mutation; p.A160T had a dominant-negative effect that mimicked the effect of anti-*Men1* siRNA, while p.A541T mutation abolished the stimulatory effect of WT MENIN. Unexpectedly, the p.A541T variant (dBSNP: rs 2959656), the only abnormality identified in a French cohort of patients with MEN1, is also present in a percentage of healthy individuals. Because the frequency of p.A541T in the normal population is higher than expected for a rare disease, it is thus considered a polymorphism. We previously reported that this controversial p.A541T variant did display a disease-related mutant behaviour in a series of functional and biochemical experiments (Bazzi *et al.* 2008). The data from the current study are therefore in agreement with our previous observations.

In conclusion, we show for the first time that MENIN loss is associated with downregulation of MAFA, a crucial TF for maintaining endocrine differentiation in mature  $\beta$  cells. Interestingly, the previously identified TF MAFB and HLXB9 are reportedly upregulated with MENIN loss (Lu *et al.* 2012; Shi *et al.* 2013). They are known to play crucial roles during endocrine pancreatic development (Hang and Stein 2011; Li and Edlund 2001) and to control proliferation/survival in specific cells (Lu *et al.* 2012; Shi *et al.* 2013). The downregulation of MAFA associated with MENIN loss has a dramatic effect on the differentiation/proliferation balance in insulinoma cells. Previous studies reported the crucial role of MENIN in the regulation of Pit-1 TF, a major regulator of terminal differentiation in pituitary cells, but without elucidating the molecular basis (Lacerte *et al.* 2004). Tissue specificity of tumours may thus result from a complex network of complementary effects on  $\beta$ -cell differentiation factors following MENIN loss. Whether such a cooperative oncosuppressive mechanism exists in other foregut NETs deserves to be explored.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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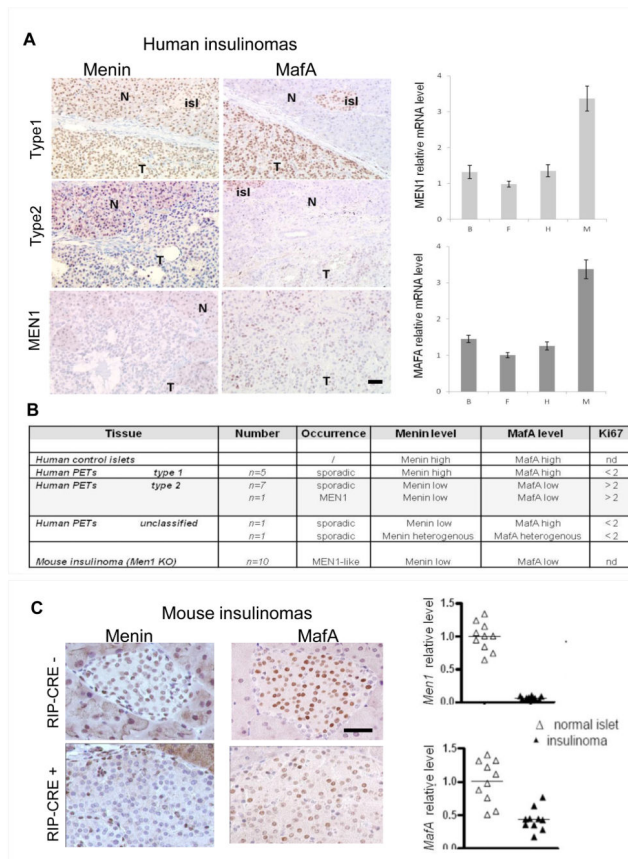
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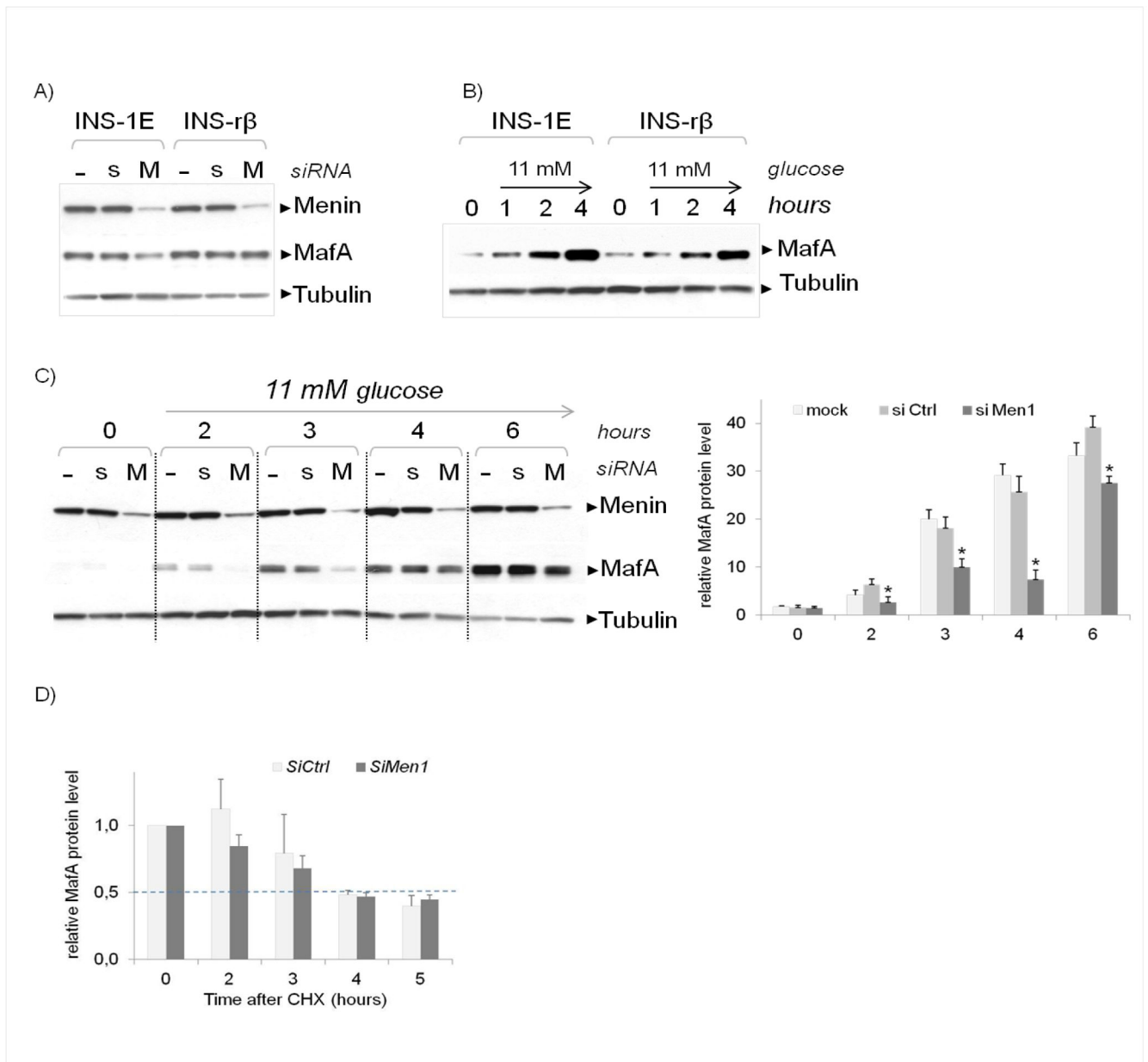
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**Figure 1. Analysis of MENIN and MAF A expression in PETs by IHC and RT-qPCR**  
 (A) Left: Representative data on human insulin-expressing PETs. Tumour cells (T), adjacent non-tumoral tissue (N) and normal Langerhans islets (isl) are indicated. Scale bar, 50  $\mu$ m. Right: RT-qPCR analysis of *MEN1* and *MAFA* mRNA levels in a series of four human insulinomas (B, F, H and M). (B) Table with recapitulative data on tumours. (C) Representative data on mouse insulinomas. Left: Normal islets are from a control *Men1<sup>F/F</sup>RipCre<sup>-</sup>* mouse and an insulinoma from a 12-month-old mutant *Men1<sup>F/F</sup>-RipCre<sup>+</sup>* mouse. Scale bars, 50  $\mu$ m. Right: RT-qPCR analysis of *Men1* and *MafA* mRNA levels in age-matched control islets from *Men1<sup>F/F</sup>-RipCre<sup>-</sup>* mice ( $\Delta$ , n = 10) and insulinomas developed in *Men1<sup>F/F</sup>-RipCre<sup>+</sup>* mice ( $\blacktriangle$ , n = 10). All experiments were performed in triplicate. The bars represent the median.

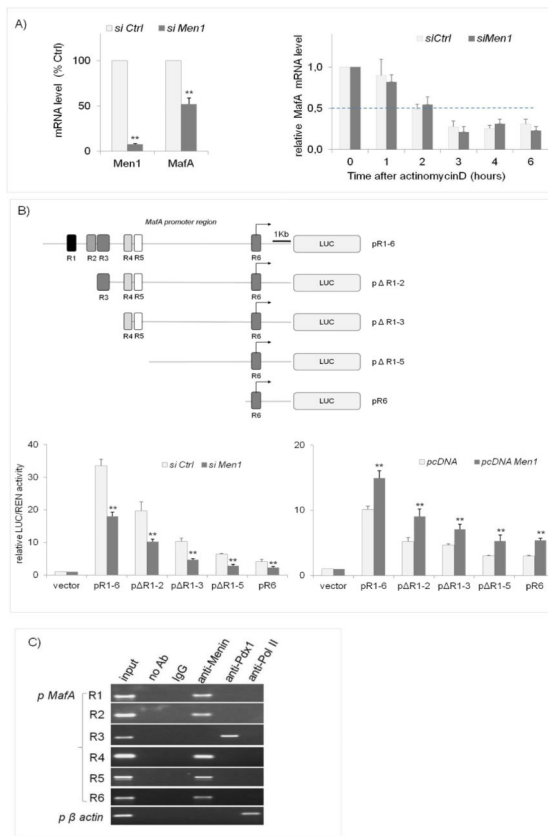




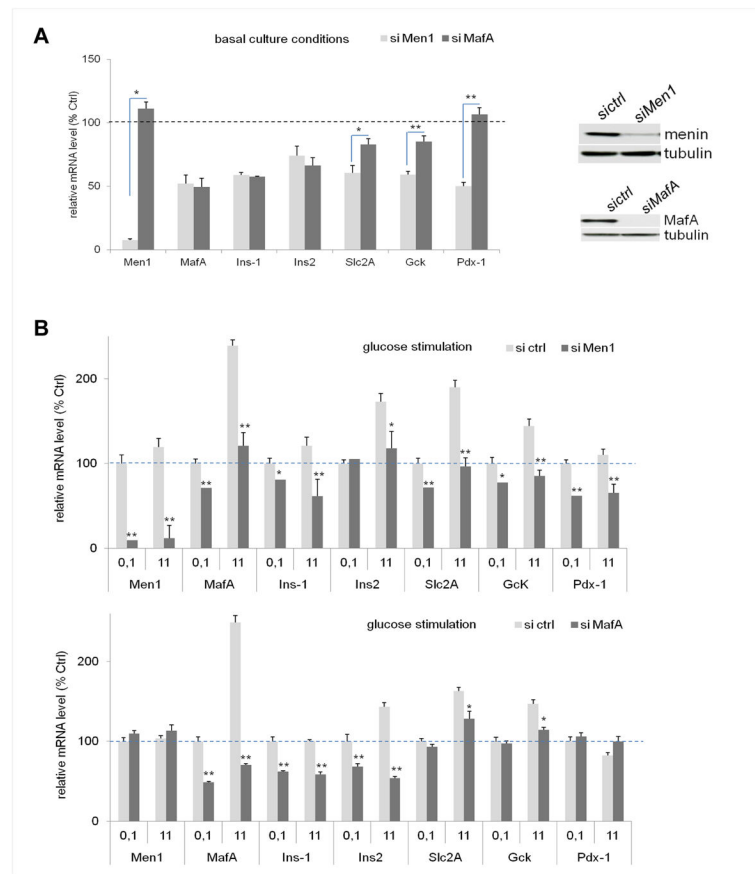
**Figure 2. Effect of MENIN inhibition on MAFA protein levels and stability in cell lines**

(A) IB of MAFA expression in basal culture conditions: INS-1E and INS-r $\beta$  cells were transfected with either non-targeted (s) or anti-*Men1* (M) siRNA pools or with transfection agent only (-), then maintained in 5 mM glucose medium and harvested 48 h after transfection. Each analysis was performed with 30  $\mu$ g of total protein. Each membrane was cut in half at the 60-kDa ladder band; the upper section was used to detect MENIN, and the lower section was used to detect MAFA (between 45 and 55 kDa) and the loading control tubulin (around 55 kDa). The image is representative of at least four experiments. (B) IB of MAFA following acute glucose stimulation. Two days after seeding, the cells were starved overnight in 0.1 mM glucose and then stimulated with 11 mM glucose medium before being harvested at several time points. As mentioned in Fig. 2A, 30  $\mu$ g total protein was analysed

Lane 0: after overnight 0.1 mM glucose starvation before glucose stimulation. C) Left: IB of MAFA following MENIN inhibition and acute glucose stimulation. Two days after seeding, cells were transfected as described for Fig. 2A; after 36 h, they were starved overnight in 0.1 mM glucose and then stimulated in 11 mM glucose medium as described for Fig. 2B, before being harvested and analysed by IB as described above. The image is representative of at least four experiments. Right: Quantitative analysis was performed using Bio-Rad Quantity One software (version 4.6.7). The results represent the relative MAFA signal normalised to the tubulin signal at each time point. \* $p < 0.05$ . (D) Effect of MENIN inhibition on MAFA protein stability. INS-1E cells were transfected as described above. After 48 h, cells were treated with CHX and harvested at the indicated time points before quantitative analysis of the IB was performed as described above. The results represent the MAFA signal normalised to the tubulin signal at each time point. To better visualise any differences or similarities, we arbitrarily set 1 as the value for MAFA mRNA level at T0 both in *siCtrl* and *siMen1* conditions. The dotted lines represent half of the protein quantity. The experiments were repeated in triplicate.

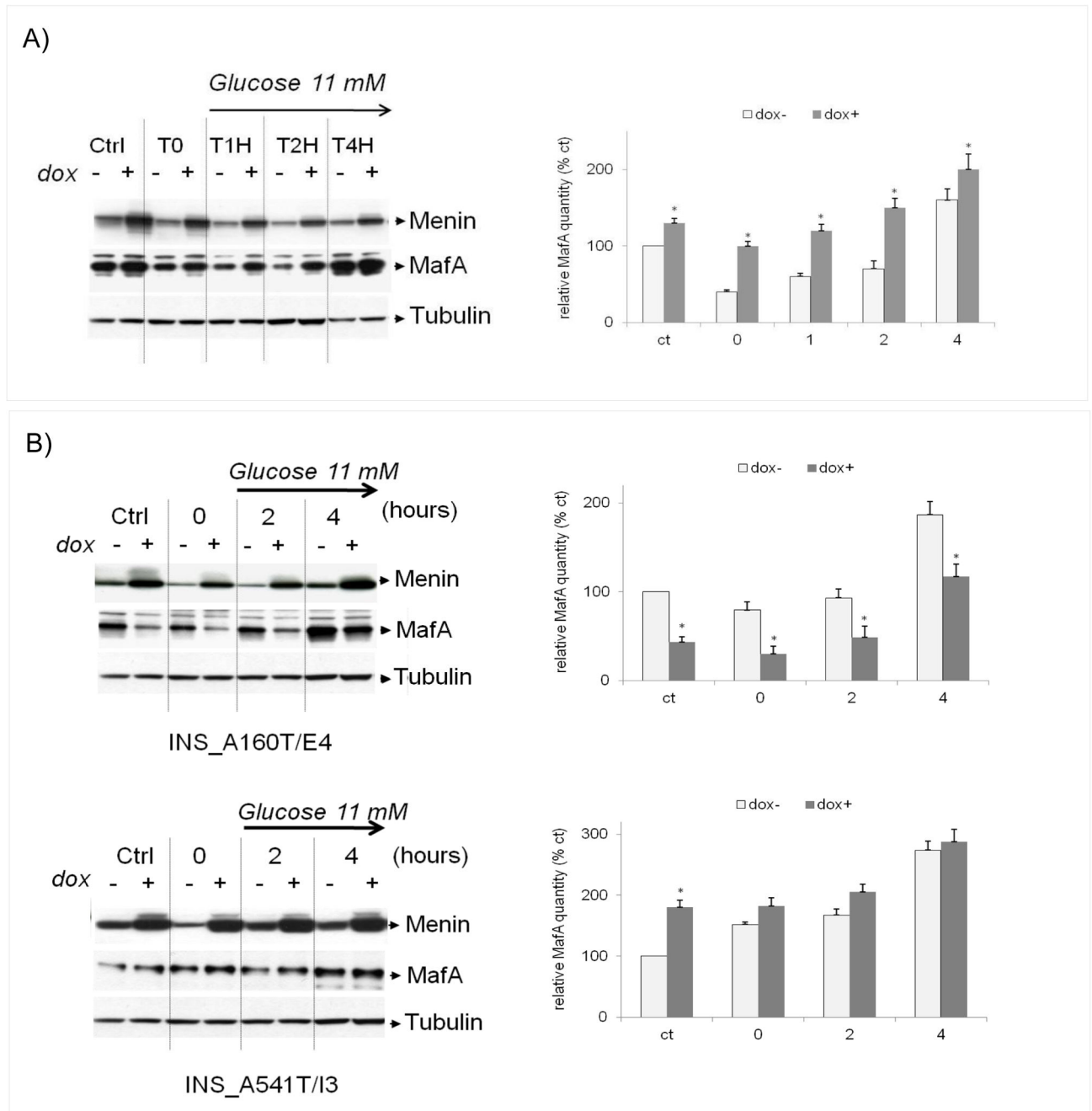


**Figure 3. Effect of MENIN on MAFA gene transcription under basal culture conditions**  
 (A) Left panel: INS-1E cells were transfected with either non-targeted (siCtrl) or anti-*Men1* siRNA pools as described in Figure 2A. After 48 h, total RNA was extracted. Gene expression was measured by RT-qPCR. The relative quantity of mRNA in INS-1E cells is indicated as a percentage. \*\*Statistical significance was defined as  $p < 0.05$ . Right panel: The effect on MAFA mRNA stability. INS-1E cells were transfected as described above. After 48 h, the cells were treated with actinomycin D and harvested at the indicated time points. The relative levels of MAFA mRNA were measured by RT-qPCR as described above. The time point T0 was arbitrarily set to 1, independently of each condition. (B) Top: Schematic representation of the MAFA promoter regions inserted upstream of a luciferase-coding sequence (LUC) as previously described (Raum *et al.* 2006). Bottom: The effect of MENIN on MAFA promoter activity. INS-1E cells were co-transfected with the plasmids described above and with either anti-*Men1* siRNA pool (left panel) or the WT MENIN expression vector (right panel). LUC and REN activity were measured in the cell lysate 48 h after transfection. The relative LUC/REN activity shown is the result of three independent experiments. \*\*Statistical significance was defined as  $p < 0.05$ . (C) *MENIN* binding to the MAFA promoter region. ChIP was performed from INS-1E cells using anti-MENIN, anti-Pdx-1 and anti-PolIII antibodies. The recovered chromatin was subjected to PCR analysis using primers directed against the R1 to R6 MAFA promoter regions to identify the bound promoter fragments. The image is representative of at least three experiments. Anti-polIII antibodies and  $\beta$ -actin primers were provided in the ActiveMotif kit to serve as a positive control for ChIP experiments.



**Figure 4. Effect of MENIN versus MAFA inhibition on differentiation genes**

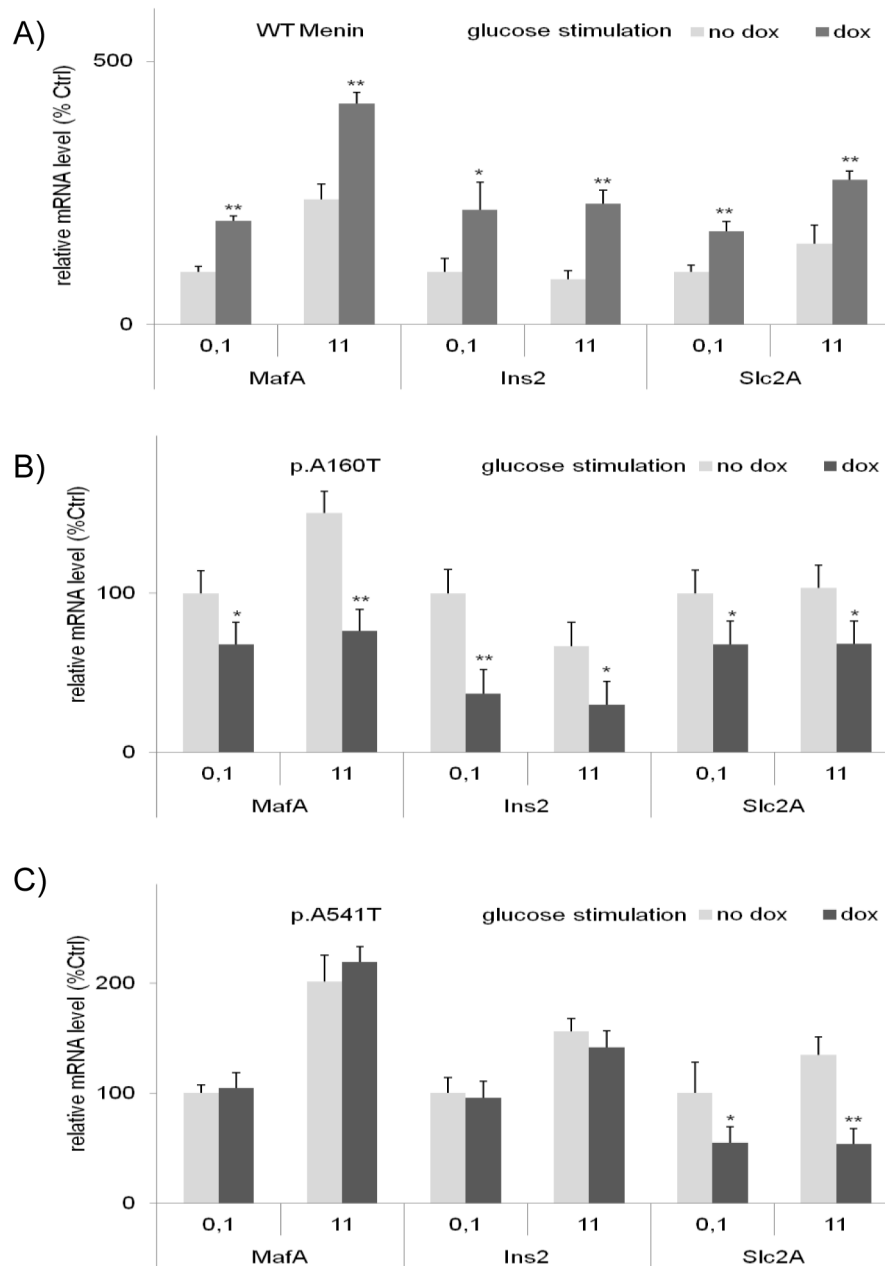
(A) Under basal 5 mM glucose culture conditions, the expression levels of the *Men1*, *MAFA*, *Ins1/Ins2*, *Slc2A*, *Gck* and *Pdx-1* genes were measured using RT-qPCR in INS-1E cells after transfection with either anti-*Men1* or anti-*MAFA* siRNA pools. Cells were maintained for 48 h in standard culture conditions. Inset: effective downregulation of MENIN and MAFA was determined by IB as in Fig. 2A. The relative quantity of each mRNA is indicated as a percentage of the respective control values (i.e. mRNA value, arbitrary fixed to 100%, of cells transfected with non-targeted siRNA and represented here by the dotted line). \*Statistical significance was defined as  $p < 0.1$ . \*\*Statistical significance was defined as  $p < 0.05$ . (A) Following 11 mM glucose stimulation, the cells were transfected with either anti-*Men1* (top panel) or anti-*MAFA* (bottom panel) siRNA pools as described above and then starved overnight in 0.1 mM glucose (delineated as 0.1 on diagram) and stimulated in 11 mM glucose medium (11 on diagram). RT-qPCR analysis was performed as described above. The relative quantity of each mRNA is indicated as a percentage of the respective control value fixed to 100% (i.e. the mRNA value for cells transfected with non-targeted siRNA maintained in 0.1 mM glucose). \*Statistical significance was defined as  $p < 0.1$ . \*\*Statistical significance was defined as  $p < 0.05$ .



**Figure 5. The effect of overexpression of either WT MENIN or MEN1 disease-related variants on MAFA protein expression**

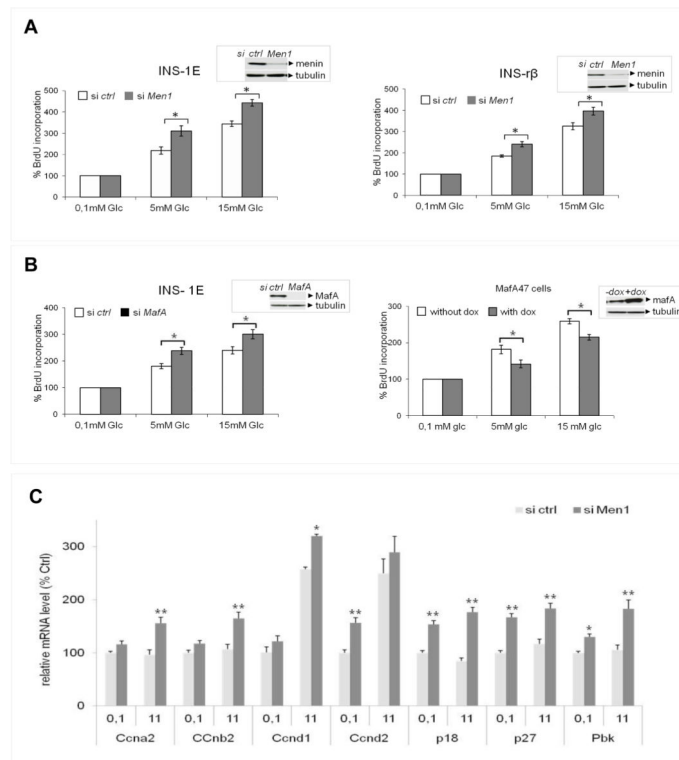
Following overnight glucose starvation (hour 0), INS- $\beta$  cells overexpressing WT or variant MENIN after a doxycyclin treatment (dox  $-/+$ ) were incubated in 11 mM glucose for 1 to 4 h as described in Fig. 2B. Under the Ctrl conditions, the cells were maintained in 5 mM glucose medium for the entire experiment. (A) IB of MAFA in INS\_WT/B7 cells, a representative cell clone overexpressing WT MENIN. (B) IB of MAFA in INS\_A160T/E4 and INS\_A541T/I3 cells, representative cell clones overexpressing variant MENIN. The

image is representative of at least four experiments on two independent cell clones for WT and variant MENIN proteins.



**Figure 6. The effect of overexpression of either WT MENIN or disease-related variants on target genes mRNA levels**

Total RNA was extracted in parallel with the experiments shown in Figure 5. The expression levels of *MafA*, *Ins2* and *Slc2A* mRNA were measured by real-time RT-qPCR as described above in *INS\_WT/B7 cells* (A), *INS\_A160T/E4* (B) and *INS\_A541T/I3* (C) cells. The experiments were repeated four times. The relative quantity of each mRNA is indicated as a percentage of the respective control value fixed to 100% (i.e mRNA value for cells without overexpression of MENIN (no doxycyclin treatment) maintained in 0.1 mM glucose). \*Statistical significance was defined as  $p < 0.1$ . \*\*Statistical significance was defined as  $p < 0.05$ .



**Figure 7. Alteration of MENIN and MAFA expression modified cell proliferation rate**  
NewFigure

(A, B) BrdU incorporation was measured by a colorimetric assay following manufacturer's instructions (Roche Diagnostics GmbH, Mannheim, Germany) after incubation in 0.1, 5 and 15 mM glucose in cells transfected with non-targeting (*siCtrl*) or anti-*Men1* (*siMen1*) siRNA (A) or anti-MAFA siRNA (B, left) or in cells overexpressing MAFA after doxycyclin treatment (B, right). Results are expressed as a percentage of the reference value, arbitrarily chosen as the optical density (OD) value obtained for cells incubated in 0.1 mM glucose medium without siRNA transfection. Results represent the mean of at least four experiments. \*Statistical significance was defined as  $p < 0.05$ , *Insets*: Efficiency of siRNA transfection or doxycyclin induction was controlled by IB. (C) The cells transfected with anti-*Men1* were starved overnight in 0.1 mM glucose (delineated as 0.1 on diagram) and stimulated in 11 mM glucose medium (11 on diagram) as described in Fig. 2C. Expression of proliferation-related genes was analysed by RT-qPCR as described above. The relative quantity of each mRNA is indicated a percentage of the respective control value fixed to 100% (i.e. the mRNA value for cells maintained in 0.1 mM glucose). \*Statistical significance was defined as  $p < 0.1$ . \*\*Statistical significance was defined as  $p < 0.05$ .