Altered MENIN expression disrupts the MAFA differentiation pathway in insulinoma

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Abstract

The protein MENIN is the product of the multiple endocrine neoplasia type I (\textit{MEN1}) gene. Altered MENIN expression is one of the few events that are clearly associated with foregut neuroendocrine tumours (NETs), classical oncogenes or tumour 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 the expression of v-MAF musculoaponeurotic fibrosarcoma oncogene homologue A (MAFA), a master glucose-dependent transcription factor in differentiated \(\beta\)-cells. Immunohistochemical analysis of a series of human insulinomas revealed a correlated decrease in both MENIN and MAFA. Decreased MAFA expression resulting from targeted \textit{Men1} ablation was also consistently observed in mouse insulinomas. \textit{In vitro} analyses using insulinoma cell lines showed that MENIN regulated MAFA protein and mRNA levels, and bound to \textit{Mafa} promoter sequences. MENIN knockdown concomitantly decreased mRNA expression of both \textit{Mafa} and \(\beta\)-cell differentiation markers (\textit{Ins1/2}, \textit{Gck}, \textit{Slc2a2} and \textit{Pdx1}) 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 \textit{MEN1} lost the WT 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.

Key Words

- 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 & Wiedenmann 2012). The multiple endocrine neoplasia type 1 (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 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 NETs (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 amount 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 & Jensen 2008, Shin et al. 2010). In mouse models, targeted Men1 ablation in pancreatic β-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 & 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 & Hua 2007). It has been shown in several cellular contexts that decreased MENIN expression is associated with increased proliferation and modified apoptotic sensitivity (Schnepp et al. 2004, Hussein et al. 2007, Bazzi et al. 2008, Wu & 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 β-cell differentiation and of the TFs involved are intensively studied. MENIN has been implicated in the control of adaptive β-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 homologue 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 β-cells (Pouponnot et al. 2006). In addition, MAFA is a master glucose-regulated TF that contributes to the maintenance of β-cell differentiation and controls, either directly or indirectly, the expression of target genes implicated in β-cell functions, including Ins1/2 (encoding insulin), Slc2a2 (encoding the glucose transporter GLUT2), Gck (encoding glucokinase) and Pdx1 (Aramata et al. 2007, Yang & Cvekl 2007). The level of MAFA in β-cells is tightly controlled mainly by glucose, the major growth and differentiation regulator of β-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 β-cell function and survival (Martens & Pipeleers 2009). MAFA is thus considered a barometer of β-cell health (Hang & Stein 2011).

In this study, we investigated whether MENIN can regulate the expression of MAFA, a major TF that
maintains β-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 β-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 (two males and 13 females) had a mean age of 47.5 years (range 25–79 years). Of these 15 tumours, ten were functional at the time of removal and could be termed insulinomas, whereas the remaining five 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 ten functional tumours were classified as neuroendocrine neoplasms G1 and the other five 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<sup>F/F</sup>-RipCre<sup>-</sup> and Men1<sup>F/F</sup>-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-μm-thick sections. The whole sections, containing both tumour and adjacent peritumoural tissues, were used. The tissue sections were incubated with MENIN (Epitomics, Burlingame, CA, USA) and MAFA antibodies (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-rβ (Wang & Iynedjian 1997). These cell lines were originally independently established from radio-induced rat insulinoma INS1 cells (Asfari et al. 1992) and are glucose responsive (Merglen et al. 2004). We also used MafA47 cells, which were previously established from INS-rβ-cells; they can conditionally overexpress MAFA protein under doxycyclin treatment (Wang et al. 2007). All three cell lines used expressed 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-rβ-cells that conditionally overexpress WT MENIN or MEN1-related variants have been previously described (Bazzi et al. 2008). For MENIN induction of INS-rβ-cells,
1000 ng/ml doxycyclin (Sigma–Aldrich) 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 μg/ml cycloheximide (CHX, Sigma–Aldrich) or 4 μg/ml actinomycin D (ActD, Sigma–Aldrich) for various periods of time.

Immunoblot analysis

Cells were lysed in RIPA buffer (Santa Cruz Biotechnology), and proteins were resolved on 10% precast Criterion XT polyacrylamide gels (Bio-Rad Laboratories). 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) and 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 HRP-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-TARGETplus SMART pool siRNAs # L-090784-02 and L-081264-02 for Men1 and Mafa genes, respectively; Dharmacon, Lafayette, CO, USA) and the loading control tubulin (Sigma–Aldrich). 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).

Chromatin immunoprecipitation assays

Chromatin immunoprecipitation (ChIP) was performed using the enzymatic ChIP-IT Express kit (Active Motif, La Hulpe, Belgium). Protein–DNA cross linking 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-PDX1 antibody (sc-14664, Santa Cruz Biotechnology). Anti-PolII antibodies were included in the kit. The recovered chromatin was subjected to 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/RT-quantitative PCR analysis

A RT-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). RT of 1 μg each RNA was performed using the QuantiTect Reverse Transcription kit (Qiagen SA), which includes elimination of genomic DNA contamination in RNA samples. The qPCR analyses were performed using the Maxima SYBR 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 ΔΔC(t) method (Livak & 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 β-cells. In this study, we evaluated the expression of these two proteins by IHC in 15 insulin-expressing PETs (14 sporadic cases and one MEN1 related). Different MENIN expression patterns were observed in the 14 sporadic cases. In five of these cases, MENIN was detectable in all tumour cells, with a high expression level similar to that detected in non-tumoural 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-tumoural 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 MEN1-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 eight of the ten 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 β-cells in mice. Specific Men1 disruption mediated by rat insulin promoter-driven Cre in floxed Men1 mice led to full penetrance insulinoma of development at about 10–12 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 β-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-rβ. 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-rβ-cells.

We therefore tested the effect of MENIN inhibition on MAFA expression during acute glucose stimulation. In INS-1E and INS-rβ-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-rβ-cells (Fig. 2C and D; Supplementary Figure S1, see section on supplementary data given at the end of this article).

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-rβ-cells (Supplementary Figure S1B) and was unchanged after MENIN downregulation.

MENIN inhibition alters MAFA transcription
We then tested whether downregulation of MENIN expression 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 Figure S2A, see section on supplementary data given at the end of this article). Comparable results were obtained using INS-rβ-cells (Supplementary Figure S2B). To determine if MENIN levels
Figure 1
Analysis of MENIN and MAFA expression in PETs by IHC and RT-qPCR. (A) Left: representative data on human insulin-expressing PETs. Tumour cells (T), adjacent non-tumoural tissue (N) and normal islets of Langerhans (isl) are indicated. Scale bar, 50 μm. 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 μm. Right: RT-qPCR analysis of Men1 and Mafa mRNA levels inagematched control islets from Men1<sup>F/F</sup>-RipCre<sup>−</sup> mice (open triangle, n = 10) and insulinomas developed in Men1<sup>F/F</sup>-RipCre<sup>+</sup> mice (closed triangle, n = 10). All experiments were performed in triplicate. The bars represent the median.
were affecting $\text{Mafa}$ mRNA stability, RNA synthesis was blocked by ActD treatment in control and anti-Men1 siRNA-transfected INS-1E cells. The half-life (i.e. about 2 h) of $\text{Mafa}$ mRNA was unaffected by MENIN knockdown in either INS-1E cells (Fig. 3A, right) or INS-β-cells (Supplementary Figure S2C).

To analyse if MENIN directly modulates $\text{Mafa}$ transcription, we evaluated the effect of altering MENIN expression level on the activity of various 5'-flanking $\text{Mafa}$ promoter-driven luciferase reporter genes. The $\text{Mafa}$ 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).
inability to detect binding to R3 was not due to a technical problem, as PDX1 was observed as described previously (Raum et al. 2006). Unexpectedly, no signal was detected with anti-PolII antibody using R6 primers, in contrast to the b-actin primers provided as a positive ChIP control in the kit. The related technical reasons for this inability to detect PolII binding to R6 remain unclear.

MENIN inhibition impairs expression of b-cell differentiation markers

We next analysed the effect of either MENIN or MAFA inhibition on several MAFA-regulated genes critical to b-cell differentiation and function, including Ins1/Ins2, Slc2a2, Gck and Pdx1 (Matsuoka et al. 2007, Wang et al. 2012).
Figure 4
Effect of MENIN vs MAFA inhibition on differentiation genes. (A) Under basal 5 mM glucose culture conditions, the expression levels of the Men1, Mafa, Ins1/Ins2, Slt2a2, Gck and Pdx1 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 $P < 0.05$. (B) 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 earlier. 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 $P < 0.05$. 

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2007). The expression of these differentiation markers was differentially affected by either Men1 or Mafa siRNA-mediated knockdown in the 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, Slc2a2, Gck and Pdx1 (to about 50% of control). Interestingly, if the MENIN level was kept stable, direct inhibition of Mafa affected Ins1/Ins2 in an identical manner to that described above but had a reduced effect on Slc2a2 and Gck (> 80% of control) and no effect on Pdx1 (Fig. 4A). Similar results on regulation of genes were obtained following stimulation of INS-1E cells by 11-mM glucose (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-β-cells in which MENIN knockdown had been found to affect Mafa levels in response to glucose stimulation (Supplementary Figure 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 Slc2a2 levels were decreased (Figs 5 and 6). No reproducible data were obtained with the Ins1 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 β-cell mitogen activity both in vitro and in vivo (Martens & Pipeleers 2009), hence short-term stimulation (up to 24 h) can stimulate the β-cell proliferation rate as measured by bromodeoxyuridine (BrdU) incorporation. As expected, the basal proliferation rate of INS-1E and INS-β-cells greatly increased at 24 h after variation of glucose concentrations (the concentration of foetal 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-β-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-β-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 β-cell proliferation in response to either glucose stimulation, MENIN expression level or both (Cozar-Castellano et al. 2006, Yesil & Lammert 2008, Yang et al. 2010). 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-β-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 β-cell differentiation and function. Consequently, loss of MENIN alters both MAFA expression and the endocrine β-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’s region-dependent fashion. First, MENIN could act through a direct mechanism, as suggested by its binding to the R1, R2, R4, R5 and R6 promoter regions, and second, it could also operate through an indirect mechanism, by regulating the expression of other TFs that themselves bind to the R3 region. PDX1 might constitute such a candidate partner; it binds specifically to the R3 region (Raum et al. 2006), and we observed here that Pdx1 mRNA levels were decreased by MENIN inhibition.

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 Fig. 5. The expression levels of Mafa, Ins2 and Slc2a2 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 $P<0.05$. 
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 INS1 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 (Yaguchi et al. 2004, Canaff et al. 2012) and that of its mRNA is around 3 h in the INS1 cells (C Vercherat & M Cordier-Bussat, 2012, unpublished observations).

Our second relevant observation is that MENIN expression is crucial for MAFA-mediated β-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 Pdx1. Indeed, MENIN inhibition had greater deleterious effects compared 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 (Yang & Hua 2007, Dreijerink et al. 2009). 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 β-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 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 β-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 (Lejonkloo 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 β-cells, with their levels being in inverse proportion to one another (Artner et al. 2010) and MAFB expression in mice being restricted to immature β-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 β-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 β-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 MAFB also stimulated the proliferation rate, suggesting that MAFB could possess an unsuspected tumour suppressor function in β-cells. MENIN inhibition affected candidate proliferation genes that had previously been implicated in rodent β-cell proliferation, but interestingly, MAFB repression did not have any effect on these genes, suggesting the implication of other MAFA-regulated proliferation pathways. Large MAFB proteins were previously shown to act as tumour suppressors or oncogenes, depending on the cell type, although nothing is known concerning this aspect in β-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...
β-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 (Li & Edlund 2001, Hang & Stein 2011) 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 Pit1 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 β-cell differentiation factors following MENIN loss. Whether such a cooperative oncosuppressive mechanism exists in other foregut NETs deserves to be explored.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-13-0164.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
M Cordier-Bussat 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. Z Hamze, C Vercherat, A Bernigaud-Lacheretz, W Bazzi, J Lu and R Bonnavion contributed to the experiments. C X Zhang, C Pouponnot, P Bertolino, C Roche, J Y Scoazec and A Calender contributed to valuable discussions. M Cordier-Bussat, C Vercherat and Z Hamze wrote the manuscript. C Pouponnot and R Stein reviewed and edited the manuscript R Stein provided MAFA promoter and luciferase plasmids.

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