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Monocyte chemoattractant protein-induced protein 1 impairs adipogenesis in 3T3-L1 cells

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ABSTRACT

Monocyte chemoattractant protein-induced protein 1 (MCPIP1) encoded by the *ZC3H12a* gene (also known as Regnase-1) is involved in the regulation of degradation of mRNA of inflammatory modulators and for processing of pre-miRNA. These functions depend on the presence of the PIN domain. Moreover, MCPIP1 was described as a negative regulator of NF-κB and AP-1 signaling pathways although mechanisms underlying such activity remain unknown. We aimed at determining the role of MCPIP1 in adipogenesis. Here, we present evidence that Mcpip1 transcription is transiently activated during 3T3-L1 transition from pre- to adipocytes. However Mcpip1 protein expression is also strongly decreased at day one after induction of adipogenesis. Knockdown of Mcpip1 results in an upregulation of C/EBPβ and PPARγ mRNAs, whereas overexpression of MCPIP1 reduces the level of both transcription factors and impairs adipogenesis. MCPIP1-dependend modulation of C/EBPβ and PPARγ levels results in a modulation of the expression of downstream controlled genes. In addition, decreased C/EBPβ, but not PPARγ, depends on the activity of the MCPIP1 PIN domain, which is responsible for RNase properties of this protein. Together, these data confirm that MCPIP1 is a key regulator of adipogenesis.

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1. Introduction

The adipose tissue is the largest organ in humans, representing approximately 10–30% of body weight. The classically attributed function of the adipose tissue is energy storage in the form of triglycerides. However, it is now known that the adipose tissue has also an endocrine role. Indeed it releases hundreds of different active molecules with multiple activities. Among such molecules are hormones and cytokines together named adipokines [1]. Adipokines provide an extensive network of communication both within the adipose tissue and with other organs. The adipose tissue can thus be regarded as an organ buffering environmental stimuli (i.e., diet, lifestyle, drugs) that are necessary for the maintenance of organism homeostasis. This view is supported by evolutionary and comparative studies, providing the observation that very early in mammalian evolution an association between the lymphoid and the adipose tissue has occurred [2]. A close association between these two tissues seems to be fundamental for the function of the innate immune system. There is an important similarity between foam cells, which are lipid-laden macrophages found in early atherosclerosis, and adipocytes, as well as between normal macrophages and pre-adipocytes so that preadipocytes can transdifferentiate into macrophages [3,4]. As a consequence, many signaling molecules and pathways are common to adipocytes and macrophages. For example, adipocytes display Toll-like receptors (TLRs) and cytokine receptors [5], thus responding to inflammatory mediators such as IL-1β and TNF-α. Conversely, metabolic hormones, such as leptin, modulate the inflammatory response [6]. This close functional and molecular integration of the immune and metabolic systems has emerged as a crucial homeostatic mechanism, the impairment of which underlies many chronic metabolic diseases, including type 2 diabetes, fatty liver disease, atherosclerosis, as well as asthma and some cancers [7]. A close relation between the adipose tissue and inflammation is clearly visible when the adipose tissue undergoes excessive expansion. A large body of literature indeed shows that obesity is often accompanied by a chronic inflammatory state, manifested by increased plasma levels of interleukin (IL)-1β, IL-6, tumor necrosis factor (TNF)-α, C-reactive protein, fibrinogen, as well as decreased levels of adiponectin [8,9].

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We have previously shown that the expression of monocyte chemoattractant protein-induced protein 1 (MCPIP1) is activated by pro-inflammatory stimuli, including IL-1β, TNF-α, phorbol myristate acetate (PMA) [10,11] and lipopolysaccharides in macrophages and hepatocytes [11,12]. MCPIP1 possesses ribonucleolytic activity and destabilizes a set of mRNAs involving IL-1β [11], IL-6 and IL-12b [13], IL-2, c-Rel and Ox40 transcripts [14], through cleavage of their 3′ untranslated region. Moreover, it was shown that MCPIP1 is engaged in the regulation of miRNA stability through a cleavage of the terminal loops of miRNA precursor (pre-miRNAs) [15]. Besides ribonucleolytic activity, MCPIP1 negatively regulates JNK, NF-κB and AP-1 transcription factor signaling [13,16,17]. Studies of MCPIP1 in relation with pre-adipocyte differentiation of murine 3T3-L1 cells, which are fibroblasts capable of adipocyte differentiation, have so far demonstrated a stimulatory effect [18,19]. Here, we present results, in the same previously used *in vitro* model, indicating that the roles of MCPIP1 in adipogenesis are much more complex.

2. Materials and methods

2.1. Materials and reagents

Human recombinant IL-1β was purchased from Promokine (Heidelberg, Germany). Restriction endonucleases were obtained from New England Biolabs (Ipswich, USA). Ligase was purchased from Promega (Madison, USA). Reagents for BCA test were obtained from Sigma (St. Louis, MO, USA) and actinomycin D was from Sigma Aldrich. Plastic materials were from BD Falcon (San Jose, CA, USA).

2.2. Cell culture

3T3-L1 and HepG2 cell lines were obtained from American Type Culture Collection (Manassas, VA). HEK-293 was a generous gift of Professor Jozef Dulak. HepG2 were cultured in DMEM containing 1 g/l of glucose and supplemented with 5% FBS. HEK-293 and 3T3-L1 cells were grown in high-glucose DMEM (4.5 g/l) (Lonza) containing 10% fetal bovine serum (FBS) (Sigma) in humidified, 5% CO_2 atmosphere. For differentiation 3.2×10^6 of 3T3-L1 cells were seeded on 12-well plate. After 48 h (day 0) cells were induced to differentiate in the presence of the culture medium containing 5 μg/ml insulin (Eli Lilly), 0.25 μM dexamethasone (Sigma) and 0.5 mM isobutylmethylxanthine (Sigma). After 48 h medium was replaced by growth medium supplemented with 10 μg/ml insulin. Medium was changed every 2 days up to day 8 when fully differentiated adipocytes have been developed (>90% of cells had adipocyte phenotype). For microscope visualization 3T3-L1 adipocytes and non-differentiated fibroblast were fixed with 10% paraformaldehyde and stained with Oil-Red Oil (Sigma). Stain was next extracted with 100% isopropanol and subjected to absorbance measurement. For HepG2 cells Actinomycin D was used in concentration 5 μg/ml.

2.3. Generation of cells with silenced MCPIP1 expression

From four different shRNAs designed toward *Zc3h12A* (SABiosciences) only one turned out to be effective and was used in later experiments to diminish MCPIP1 expression in 3T3-L1 cells. Transfection was carried out using Lipofectamine 2000 according to instructions of the supplier (Life Technologies). shRNA with a scrambled sequence was used as a control. Briefly, cells in 70% confluence were incubated with DNA complexes in FBS-deprived culture medium, split 4 h later in 1:4 ratio and seeded in complete culture medium. After 24 h gentamicin G418 (Sigma) was added to the medium in concentration 600 μg/ml. Gentamicin-resistant cells were selected for next two weeks. Silencing efficiency was assessed by measurement of the MCPIP1 mRNA level by real-time PCR and by Western blot.

2.4. Retroviral generation and infections

For overexpression experiments we used a pMX retrovirus system. Expression plasmids were as follows: pMX-MCPIP1 coding for wild-type human MCPIP1, pMX-MCPIP1ΔPIN coding for MCPIP1 without 137–296 region corresponding to an enzymatically active PIN domain [11], pMX-GFP, and empty pMX-puro. Retrovirus packaging was performed in modified HEK293 — Phoenix Amphotropic. The day before transfection cells were plated (2.5×10^6 in 10 cm plate) in high-glucose DMEM, supplemented with 10% FBS. Transfection was performed with PEI (Polyethylenimine). In brief, expressing vector was mixed with helper vector coding for gag and pol genes in 1:3 mass ratio. Then, PEI was added in 1:4 mass ratio (DNA:PEI). The mixture was added to the cells. After 16 h medium was changed for normal growth medium. After next 24 h supernatants were collected, centrifuged (1000 g, 10 min.), sterile filtered through 0.45 μm filters, divided into small portions and frozen for experiments. 3T3-L1 fibroblasts cells were infected with the mixture of a retroviral supernatant and appropriate complete growth medium (1:4 ratio) supplemented with 4 μg/ml of polybrene (Millipore). Titre of the vectors was estimated by real-time one step PCR analysis of the RNA isolated analytically from retroviral supernatant. Biological titre was assessed by flow cytometry analysis of HeLa cells transiently transduced with pMX-GFP generated at the same time with the other vectors. This pMX-GFP virus was also used for assessment of transduction efficiency of target cells in each experiment.

2.5. Real-time PCR

Total RNA was isolated using the modified Chomczynski–Sacchi method [20]. RNA concentration was measured with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA) and RNA integrity was verified on a 1% denaturing agarose gel. For the real-time PCR experiment, 1 μg of total RNA was reverse-transcribed using oligo(dT) primer and M-MLV reverse transcriptase (Promega). Following synthesis, cDNA was diluted 5 times and real-time PCR was carried out using Rotor-Gene 3000 (Corbett, Cambridge, UK) system and Sybr Green-based master mix (Finnzymes, Espoo, Finland). After an initial denaturation step (10 min at 95 °C) conditions for cycling were as follows: 40 cycles of 20 s at 95 °C, 20 s at 62 °C and 30 s at 72 °C. The fluorescence signal was measured right after the extension step. To verify specificity of the PCR product melting curve was generated. As an internal reference gene eukaryotic translation elongation factor 2 was used. All samples were run in duplicates.

The primers used in real-time PCR were as follows: eEF2 F: GACATCAC CAAGGGTGTGCAG, R: TCAGCACACTGGCATAGAGGC; C/EBPβ (mouse) F: CAAGCTGAGCGACGAGTACA, R: CAGCTGCTCCACCTTCTTCT; C/EBPβ (human); AGCGACGAGTACAAGATCCG, R: GCTGCTCCACCTTCTTCTGC; β-actin (human) F: CAAGAGATGGCCACGGCTGCTT, R: CAGGTCTTTGCG GATGTCCACG; PPARγ (mouse)F: AGGCCGAGAAGGAGAAGCTGTTG, R: TGGCCACCTCTTTGCTCTGCTC, MCPIP1(human) F: GGAAGCAGCCGTGT CCCTATG, R: TCCAGGCTGCACTGCTCACTC; Mcpip1(mouse) F: CAGCCT CGACCAGATGTGCC, R: CAGCCGCTCCTCGATGAAGC.

2.6. Western blotting

3T3-L1 cells after washing with PBS were harvested and lysed in RIPA buffer supplemented with Complete Protease Inhibitor Cocktail (Roche) and PhosSTOP Phosphatase Inhibitor Cocktail (Roche). Protein concentration in cell lysates was measured with the bicinchoninic acid assay. Cell lysates were separated on SDS/PAGE 10% polyacrylamide gel, electrotransfered to PVDF membrane (Millipore, Billerica, MA, USA) and blocked in 2% BSA (BioShop, Burlington, Canada) dissolved in Tris-buffered saline containing 0.1% Nonidet-P40 substitute (BioShop, Burlington, Canada). Membranes were then incubated with primary antibody overnight at 4 °C. After incubation (1 h, room temperature) with secondary antibody chemiluminescence was detected using Luminata

Crescendo (Millipore) substrate and MicroChemi chemiluminescence detector (DNR Bio-Imaging Systems, Jerusalem, Israel). Following antibodies were used: rabbit anti-MCPIP1 (1:2000, own production), goat anti-MCPIP1 (Santa Cruz) mouse anti-tubulin (1:1000; Sigma), rabbit anti p-p65 (1:1000; Cell Signaling), rabbit anti-PPARγ (1:500; Cell Signaling), rabbit anti-C/EBPβ (1:500; Cell Signaling), peroxidase-conjugated anti-rabbit (1:6000; Cell Signaling) and peroxidase-conjugated anti-mouse (1:2000, Sigma).

3. Results

3.1. MCPIP1 is induced at early stages of differentiation

To establish the involvement of Mcpip1 in adipogenesis, we used 3T3-L1 murine fibroblast cell line. Differentiation of these cells toward adipocytes was initiated by exposure of cells synchronized in phase G0 to the medium supplemented with dexamethasone, insulin and isobutylmethylxanthine (DMI). After 8 days, we observed accumulation of lipid droplets and development of fully differentiated cells (Fig. 1A). RNA and proteins were extracted from cells at indicated time points, starting from point 0 (beginning of stimulation) and ending at day 8. We observed strong augmentation of *Mcpip1* mRNA at early stages of differentiation (Fig. 1B). The highest transcript level was observed after 6 h from addition of adipogenic induction cocktail (DMI). We observed that the mRNA level of C/EBPβ increases in differentiating 3T3-L1 cells at the beginning of differentiation to descend on day 2. Then, the level of PPARγ mRNA and protein increases and is present at elevated level in mature adipocytes (Fig. 1B, C).

Different pattern of expression was observed at protein level. Mpip1 is visible 6 and 12 h after induction of adipogenesis and then at day 1 its level is significantly lower reaching again high level at day 3 and 6. Band corresponding to Mcpip1 on Western blot appears slightly lower than bands from samples collected at time points: 6 and 12 h and 3 and 6 days after induction of differentiation. What is interesting, strong decrease of Mcpip1 at day 1 is accompanied by significant increase of C/EBPβ (Fig. 1C).

3.2. Mcpip1 inhibition results in augmentation of C/ebpβ and Pparγ level

To study the influence of Mcpip1 on kinetics of the cell differentiation, we carried out differentiation of 3T3-L1 cells with a stably silenced Mcpip1. A plasmid encoding ZC3H12A-specific sh-RNA was used for silencing. After sh-RNA transfection, cells were subjected to antibiotic selection. A plasmid coding for unspecific sequence (sh-scrambled) was used as a control. Using real-time PCR we measured the expression level of mouse Mcpip1 in 3T3-L1 cells. We achieved a 70% decrease in Mcpip1 expression in comparison to control cells. Two populations of 3T3-L1 preadipocytes (sh-Mcpip1 and sh-scrambled) were then stimulated with differentiation medium. Differentiation efficiency was monitored visually and by assessment of C/EBPβ and PPARγ expression level (Fig. 2). The reduction of Mcpip1 expression leads to the increase in the basal level of mRNA coding for PPARγ and C/EBPβ. We confirmed also that C/EBPβ protein is also upregulated in when MCPIP1 is silenced (Fig. 2D). The rate and the efficiency of adipogenesis in sh2-Mcpip1 transfected cells were similar to the control cells (transfected with scrambled sh-RNA), as shown by Oil-red staining.

3.3. MCPIP1 overexpression leads to the decrease in C/EBPβ and PPARγ level

To confirm results obtained on 3T3-L1 cells with reduced Mcpip1 level we generated 3T3-L1 cells stably expressing wild type MCPIP1. Similarly to silencing approach, experiments were performed on a pool of cells stably expressing transgene. Microscopic observation of differentiation process revealed that differential potential of cells overexpressing MCPIP1 (pMX-MCPIP1) was much lower than control, transduced with an empty vector (pMX-puro). Cells overexpressing

MCPIP1 accumulated lipid droplets slower than control cells. Less efficient differentiation was reflected in Oil-red assay performed at 8th day of differentiation (Fig. 3A). We have observed in Western-blot that a high level of MCPIP1 is accompanied by a lower content of C/EBPβ and PPARγ (Fig. 3B). Furthermore, real-time analysis of mRNA content for C/EBPβ and PPARγ revealed that the level of transcripts of both transcription factors was relatively lower in cells over-expressing MCPIP1 (Fig. 3C). Next, we determined if MCPIP1 impairs expression of C/EBPβ and/or PPARγ — dependent genes. We have chosen two target genes for C/EBPβ (*C-fos* coding for C-fos protooncogene, *Il-6* coding for IL-6) and two for PPARγ (*Fabp4* coding for fatty acid binding protein 4, *AdipoQ* coding for adiponectin). We observed that elevated level of MCPIP1 was accompanied by a considerable decrease in their expression (Fig. 3D).

In order to find out the mechanism of this negative regulation, we overexpressed MCPIP1 devoid of PIN domain, responsible for RNase activity (MCPIP1ΔPIN). The mutant protein has a deletion of a region spanning amino acid residues from 137 to 296 [11]. The results show that overexpression of the protein without RNase domain results in a reduced rate of differentiation and reduced PPARγ mRNA level. However, in contrast to the wild form, MCPIP1ΔPIN does not decrease the mRNA for C/EBPβ (Fig. 4). This indicates that reduction of C/EBPβ mRNA involves RNase activity of MCPIP1.

3.4. Delayed MCPIP1 overexpression does not influence the C/EBPβ and PPARγ level

Retroviral system of transgene delivery in conjunction with the specificity of 3T3-L1 differentiation process gave us the opportunity to overexpress MCPIP1 in a delayed manner. Because pMX-MCPIP1 retrovirus was added together with differentiation cocktail only cells undergoing clonal expansion have been infected. However, expression of the transgene has occurred not earlier than 24 h after initiation of differentiation (DMI addition). In contrast to stably transduced 3T3-L1 cells where MCPIP1 has been already up-regulated at the moment of DMI addition, here we did not observe any influence of MCPIP1 on the level of C/EBPβ and PPARγ protein or mRNAs (Fig. 5).

3.5. MCPIP1 modulates C/EBPβ transcript stability

HepG2 cells have high basal expression of C/EBPβ therefore we use this model to check MCPIP1 influence on C/EBPβ transcript stability. Cells were transiently transfected with plasmids coding for MCPIP1, MCPIP1ΔPIN or empty plasmid. 24 h after transfection (time 0) actinomycin D was added to the medium and cells were harvested for RNA isolation 2 and 3 h later. Real-time analysis (Fig. 5D) shows fast decrease of C/EBPβ mRNA level after actinomycin D treatment. This decrease was faster when wild-type MCPIP1 was overexpressed. The transcript half-life was reduced also in the presence of MCPIP1 lacking RNase activity (MCPIP1ΔPIN), however no to the extent as for full protein. To exclude the possibility that the observed depletions result from unspecific effect of MCPIP1 on mRNAs we evaluate β-actin mRNA witch is known to not be a target for MCPIP1. We did not observe effect of MCPIP1 in this case. These results confirm observation made in 3T3-L1 model that MCPIP1 is involved in negative regulation of C/EBPβ.

3.6. Overexpression of MCPIP1 in preadipocytes does not influence ERK activation

C/EBPβ is necessary for mitotic clonal expansion (MCE) and induction of C/EBPα and PPARγ [21,22]. Binding of CREB protein controls transcription of C/EBP proteins, including C/EBPβ. Studies using the MEK inhibitor U0126 revealed that the CREB activity depends on activation of ERK1/2 [23]. Bost and collaborators [24] have shown that extracellular signal-regulated kinase (ERK1/2) pathway plays a pivotal role in proliferation and differentiation of adipocytes as adult preadipocytes
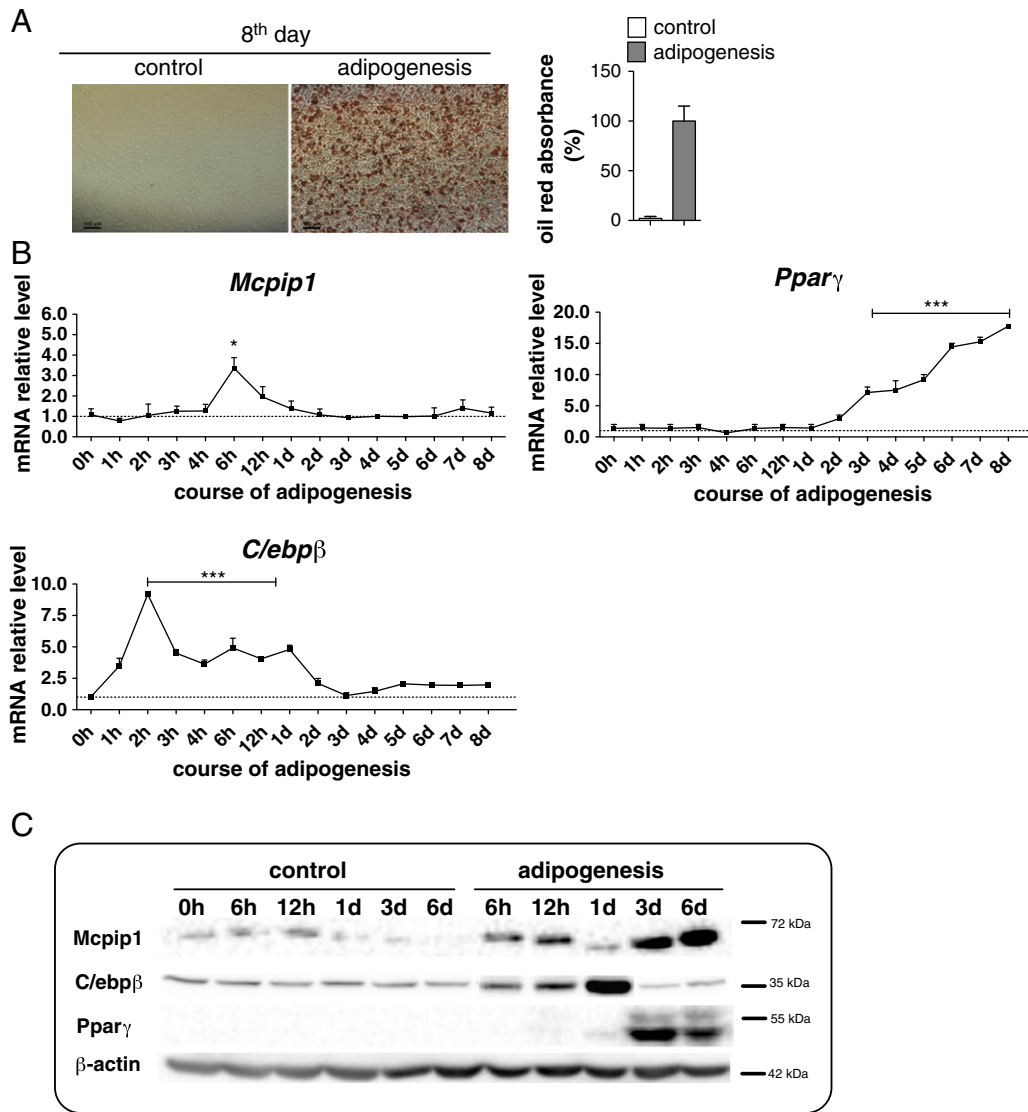
Fig. 1. Differentiation of 3T3-L1 preadipocytes involves induction of *Mcpip1* expression. Two days after reaching confluence (0 h) cells were differentiated by exposure to medium containing DEX, IBMX and insulin in the presence of FBS (MDI). Cells were harvested at indicated time points and subjected to RNA and protein isolation. A. Oil-red O staining of lipid droplets in control cells (no MDI) and differentiated 3T3-L1 (MDI) on the 8th day of differentiation (mean ± SD). B. Real time PCR analysis of mRNA content for MCPIP1, PPARγ and C/EBPβ normalized to unstimulated cells (set as one). Graphs represent the mean ± SEM of four independent experiments. C. Whole cell protein extracts were collected during differentiation and 30 μg of protein was subjected for western blot analysis using antibodies against MCPIP1, PPARγ, C/EBPβ and β-actin (loading control). Data is representative for three independent experiments. $^*p < 0.05$; $^{***}p < 0.002$ in comparison to unstimulated cells at the same time point. Scale bar represents 100 μm.

isolated from ERK1(−/−) adult animals exhibit impaired adipogenesis. Moreover, transient activation of ERK1/2 in 3T3-L1 cells during the initial 1–2 h after DMI exposure has also been shown to be essential for the induction of C/EBPβ and PPARγ [25]. We hypothesized that impairment of adipogenesis by MCPIP1 might influence activation of transcription of C/EBPβ by affecting MEK/ERK1/2. Therefore, we analyzed phosphorylation status a MEK/ERK1/2 in 3T3-L1 cells at selected time points within 2 h after addition of DMI. We did not observe any differences between control cells and cells expressing MCPIP1 (Suppl. 1). This indicates that the reduction in the level of C/EBPβ and PPARγ in cells overexpressing MCPIP1 does not depend on ERK1/2 activity.

3.7. MCPIP1 expression is activated by selected pro-inflammatory cytokines in adipocytes and preadipocytes

Pro-inflammatory cytokines such as IL-1β originated from adipocytes, preadipocytes and other cell types including macrophages can regulate adipose tissue metabolism. Thus, we analyzed whether IL-1β stimulates *Mcpip1* gene expression in 3T3-L1 adipocytes. Cells were

differentiated into mature adipocytes (8 days after DMI addition) and then subjected to stimulation. Using real-time PCR we analyzed the level of Mcpip1 mRNA at different time points − 4, 12, 24 h. IL-1β activated *Mcpip1* gene in adipocytes (6-fold increase after 4 h stimulation (Fig. 6A). This increase was reflected on protein level 24 h after treatment.

We observed that Mcpip1 transcript level in preadipocytes increases 17 times in comparison to unstimulated cells after 4 h of IL-1β addition. Later, the level of transcript significantly decreases but still is above the control (Fig. 6B). Western blot analysis shows increase in Mcpip1 protein level after 24 h of IL-1β treatment.

3.8. MCPIP1 expression increases with obesity

We analyzed samples of subcutaneous adipose tissue from 4 morbidly obese patients undergoing breast surgery (BMI > 30 kg/m^2) and analogous tissue specimens from 4 patients with normal BMI. Expression of *MCPIP1* mRNA in obese individuals was higher in comparison to low weight subjects, however we did not distinguish which cell type/types is responsible for this augmentation (Fig. 7A). We analyzed
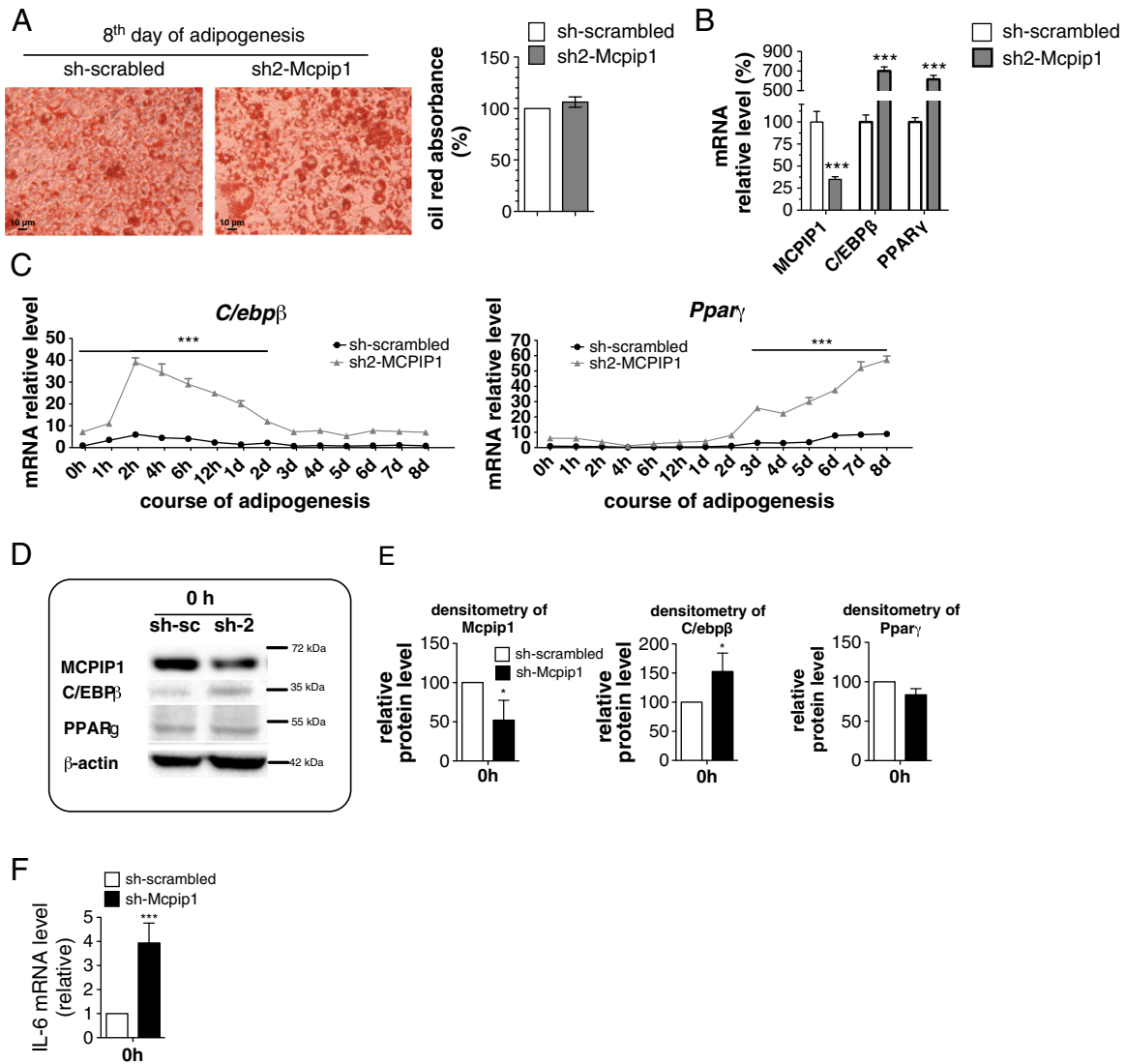
Fig. 2. Differentiation of 3T3-L1 preadipocytes with diminished *Mcpip1* expression. Two days after reaching confluence (0 h), cells with inhibited expression of *Mcpip1* gene (sh2-Mcpip1), and control cells (sh-scrambled) were differentiated in medium containing DEX, IBMX and insulin in the presence of FBS (MDI) or cultured in normal growth medium. Cells were harvested at indicated time points and subjected to RNA isolation. A. Oil-red O staining of differentiated control cells and cells with diminished *Mcpip1* at the 8th day of differentiation (mean ± SD). B. Real-time PCR analysis of *MCPIP1, C/EBPβ* and *PPARγ* transcript level at time 0 (before stimulation). C. Real-time PCR analysis of *C/EBPβ* and *PPARγ* transcript level during differentiation. D. Western blot analysis of whole cell protein extracts collected before induction of differentiation (time 0 h). Picture is representative for 2 independent experiments **E.** Densitometry analysis of the western blot results (mean ± SD). F. Real-time PCR analysis of Il-6 mRNA content before induction of differentiation (time 0 h). Graphs B, C and F show the mean ± SEM of three independent experiments. *p < 0.05, ***p < 0.002 in comparison to sh2-scrambled control at the same time point. Scale bar represents 100 μm.

expression level of *Mcpip1* and several inflammation-related genes also in subcutaneous fat tissue specimens from seven db/db mice and compare the results to their wild-type counterparts. Analysis revealed a positive correlation between *Mcpip1* gene expression and expression of CD14, a marker of monocytes/macrophages (Fig. 7B).

4. Discussion

Obesity has become a major and common problem in developed countries as a result of easy access to food and the associated reduction in caloric expenditure. New insights into adipogenic mechanisms are important from the perspective of prevention and/or treatment of obesity. MCPIP1 is a protein shown to trigger apoptosis and of promoting angiogenesis in response to the binding of chemokines. It possesses important RNase activity, and thus selectively degrades specific target mRNA species.

We studied a role for Mcpip1 in adipogenesis by applying an *in vitro* model of 3T3-L1 preadipocytes. This cell line has been so far used in numerous studies related to adipogenesis, and has greatly contributed to

the discovery of transcriptional events associated with this process. The first data about the role of Mcpip1 in 3T3-L1 differentiation have been published by Younce and co-workers [18]. Because of recent findings showing that MCPIP1 is involved in the degradation of mRNA coding for inflammatory modulators, miRNA processing and the negative regulation of NF-κB and AP-1 activities [11,13,15–17], we decided to re-evaluate its impact on pre-adipocyte differentiation and proliferation. We first confirmed that Mcpip1 transcript levels significantly increase during initiation of differentiation. This finding supports the hypothesis that Mcpip1 is involved in processes specific to the adipose tissue.

Trying to identify how Mcpip1 influences 3T3-L1 differentiation, we modulated the level of this protein. Here however, and contrary to our expectations, we obtained results opposite to those by Younce and co-workers [18]. Specifically, we found that endogenous transcripts encoding Mcpip1 sharply increase at the beginning of adipogenesis, and then decrease at the end of the process, as it was previously observed [18]. However, changes at the protein level are more dynamic, in that Mcpip1 protein is present in pre-adipocytes at a quite high level, and
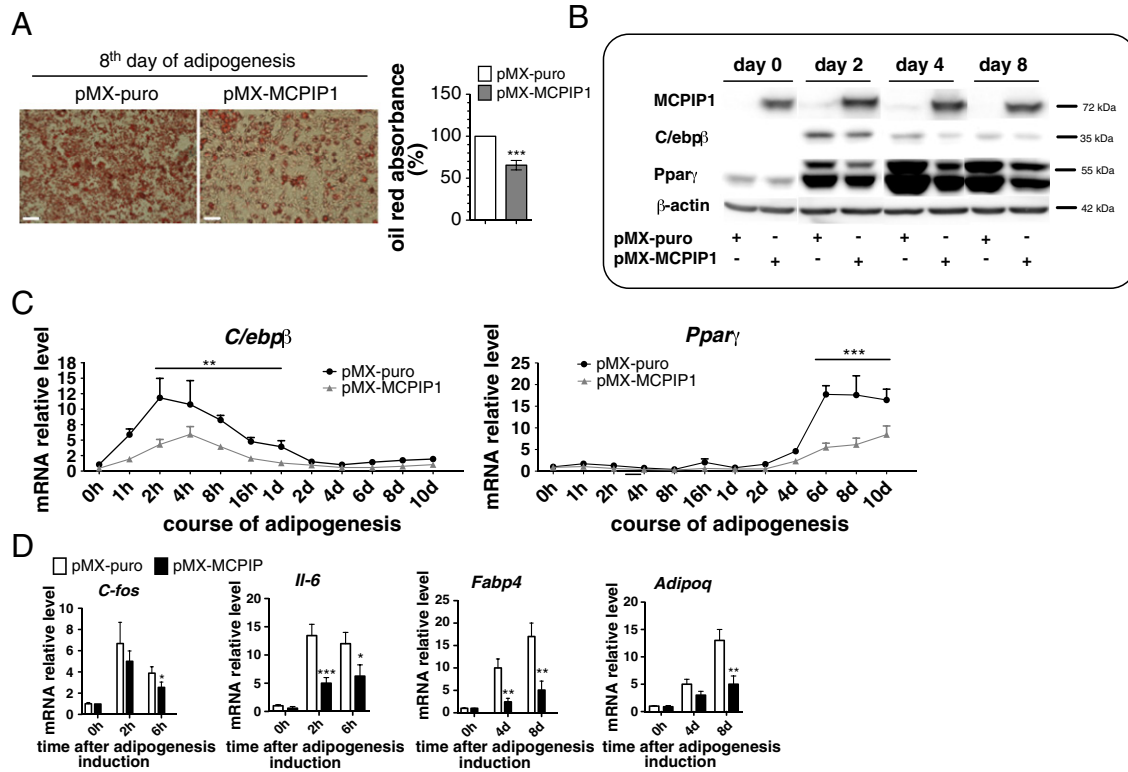
Fig. 3. Differentiation of 3T3-L1 preadipocytes overexpressing MCPIP1. Two days after reaching confluence (0 h), cells with overexpression of MCPIP1 (pMX-MCPIP1), and control cells (pMX-puro) were differentiated in medium containing DEX, IBMX and insulin in the presence of FBS (DMI) or cultured in normal growth medium. A. Oil-red O staining of lipid droplets within adipocytes stably over-expressing MCPIP1 (pMX-MCPIP1) and control plasmid (pMX-puro) at the end of differentiation protocol. B. Whole cell proteins were collected during differentiation at selected time points and 30 μg of proteins were subjected to Western blot analysis using antibodies against MCPIP1, PPARγ, C/EBPβ and β-actin (loading control). Picture shows representative data for 3 independent experiments. C. mRNA content of PPARγ and C/EBPβ assessed by real-time PCR. D. Real time analysis of PPARγ and C/EBPβ target genes expressed at selected time points during differentiation. *p < 0.05; **p < 0.02; ***p < 0.002 in comparison to pMX-puro at the same time point. Scale bar represents 100 μm. Data are representative of three independent experiments (±SEM).

then significantly decreases at day one after induction of adipogenesis, to reach again high levels at day 6. Furthermore, we found that the overexpression of MCPIP1 decreases the levels of at least two transcription factors essential during the initiation of adipogenesis — C/EBPβ and PPARγ.

Synthesis of C/EBPβ is initiated at the beginning of adipogenesis. During terminal adipogenesis, C/EBPβ, together with C/EBPδ, governs the synthesis of transcription factors essential for functions of mature adipocytes — PPARγ and C/EBPα [26]. Overexpression of MCPIP1

decreases mRNA and protein level of both transcription factors, and this phenomenon is accompanied by about a 40% reduction in adipogenesis. We confirmed the negative impact of MCPIP1 on C/EBPβ and PPARγ levels, whereby silencing of Mcpip1 expression was accompanied by enhanced mRNA expression of both transcription factors.

Trying to resolve the mechanism of C/EBPβ downregulation following MCPIP1 overexpression, we first looked at the phosphorylation status of ERK1/2, the kinase responsible for C/EBPβ activation. We did not
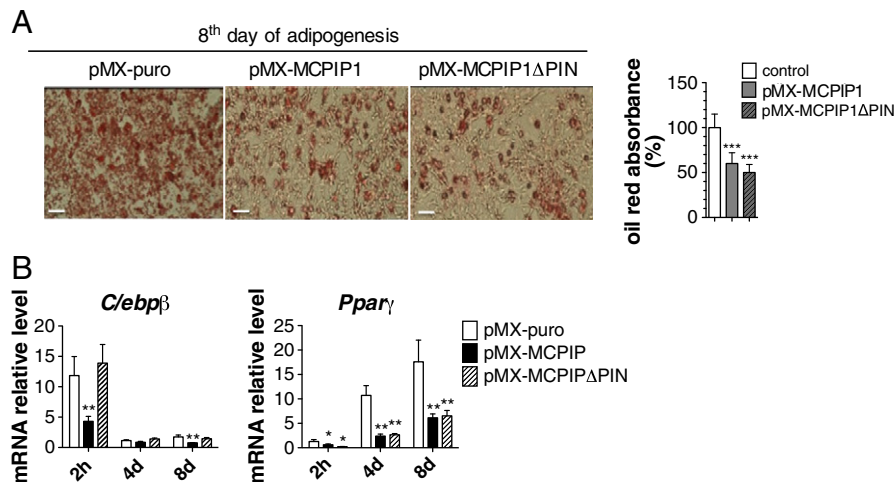

Fig. 4. Involvement of a PIN domain in C/EBPβ mRNA depletion. A. Oil-red O staining of lipid droplets within cells stably overexpressing MCPIP1 (pMX-MCPIP1), MCPIP1 lacking RNase properties (pMX-MCPIP1ΔPIN) and control cells (pMX-puro) performed at the end of differentiation protocol. B. Real time analysis of C/ebpβ, and Pparγ transcript levels within three sub-lines of adipocytes at selected time points. Data are representative of three independent experiments (±SEM; *p < 0.05; **p < 0.02; ***p < 0.002) in comparison to pMX-puro at the same time point.

observe any differences between control cells and cells overexpressing MCPIP1, and thus we checked the transcriptional activity of CREB, a known transcription factor activating C/EBPβ gene expression. However, MCPIP1 did not alter differentiation-induced CREB activation (Suppl. 2). Since MCPIP1 acts as RNase, controlling the half-life of transcripts coding for proinflammatory cytokines [such as IL-1, IL-6 [11,13]]

and the c-rel subunit of NF-κB [14], we decided to check whether lower levels of C/EBPβ transcripts in 3T3-L1 cells overexpressing MCPIP1 are the effect of the direct degradation triggered by MCPIP1. It has recently been shown that MCPIP1 promotes mRNA decay of IL-6 by targeting a stem-loop structure within IL-6 3′ UTR [13]. Furthermore, the hairpin structure has been shown to be also crucial for pre-miRNA degradation
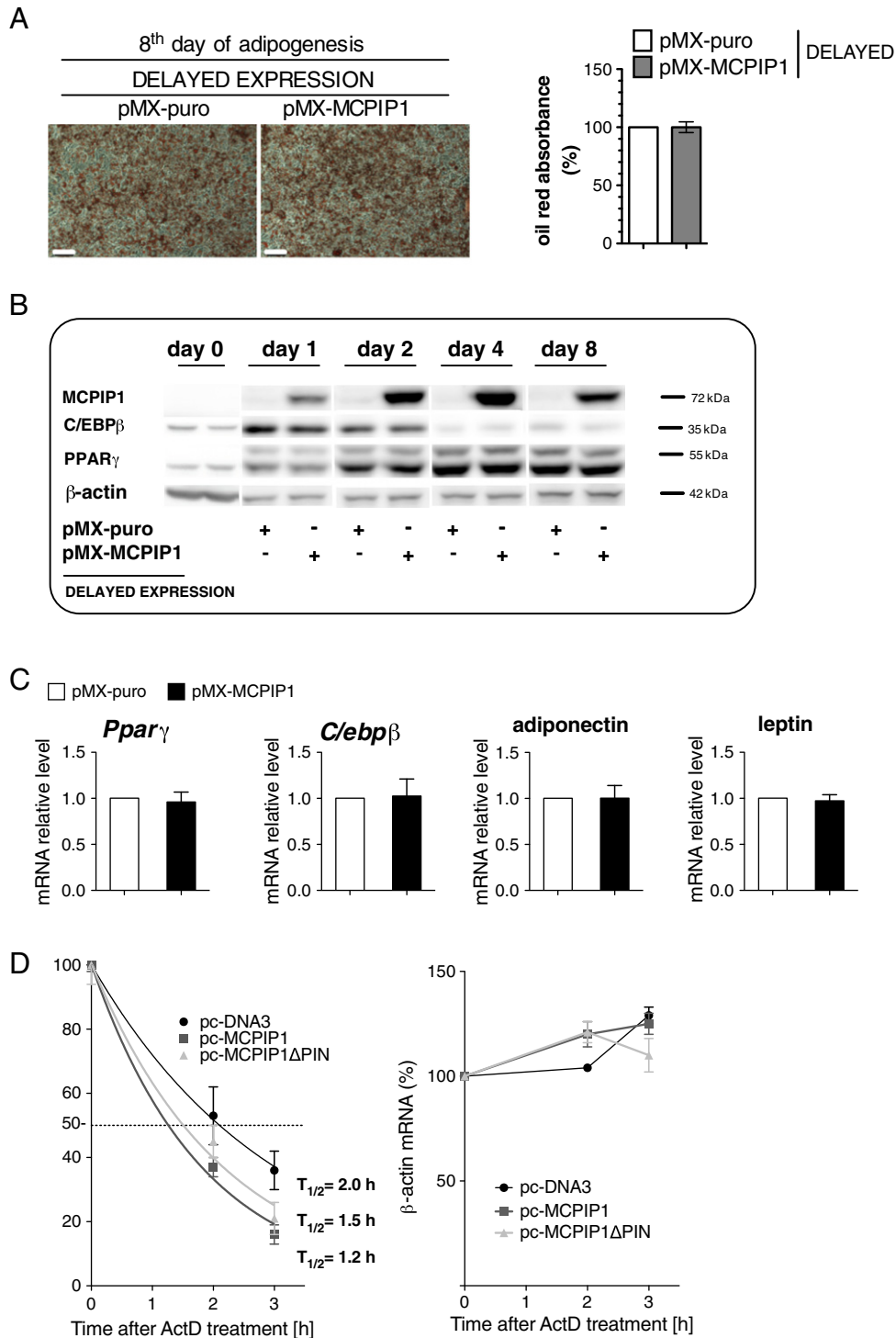

Fig. 5. Differentiation of 3T3-L1 preadipocytes with delayed MCPIP1 overexpression. At 2 days post-confluence (0 h), 3T3-L1 cells were infected with virus carrying MCPIP1 (pMX-MCPIP1), or control vector (pMX-puro). Infection was done together with initiation of differentiation. A. Oil-red O staining of lipid droplets within adipocytes overexpressing MCPIP1 (pMX-MCPIP1) and control plasmid (pMX-puro) at the end of differentiation protocol. B. Whole cell proteins were collected during differentiation at selected time points and 30 μg of proteins were subjected to Western blot analysis using antibodies against MCPIP1, PPARγ, C/EBPβ and β-actin (loading control). Picture shows representative data from 3 independent experiments. C. Real time PCR analysis of mRNAs content for selected genes at 8th day after initiation of differentiation. Data are representative of three independent experiments (±SEM). D. Real time PCR analysis of mRNAs encoding C/EBPβ and β-actin in HepG2 cells transfected with pc-DNA3.1, pc-MCPIP1 or pc-MCPIPΔPIN, then, 24 h later treated with actinomycin D. Graphs show mean ± SEM of three independent experiments performed in duplicates.
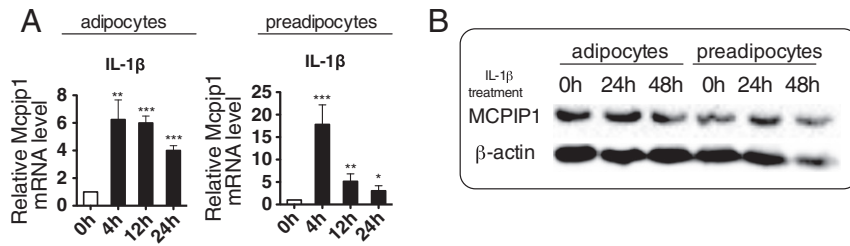
Fig. 6. Mcpip1 expression in 3T3-L1 adipocytes and preadipocytes treated with IL-1β. 3T3-L1 preadipocytes (A) and adipocytes (8th day of differentiation) (B) were treated with IL-1β (60 u/ml) for indicated time and then RNA was isolated. Real-time analysis was performed in order to measure the level of Mcpip1 mRNA. The graphs represent the mean ± SEM of three independent experiments. *$p < 0.05$; **$p < 0.02$; ***$p < 0.002$. Unstimulated cells served as a control (0 h).

[15]. Applying the M-fold web server tool [27], we analyzed the 3′ UTR of C/EBPβ mRNA, and found regions forming such structures (Suppl. 3). Together, these findings suggest that MCPIP1 may be directly involved in C/EBPβ mRNA degradation.

Analysis *in silico* was followed by a measurement of the mRNA half-life for this transcription factor in HepG2 cells overexpressing MCPIP1 and MCPIP1 devoid of the PIN domain. It appeared that C/EBPβ transcript half-life was lowered when MCPIP1 is overexpressed. Moreover, we found that the stability of transcripts encoding C/EBPβ was higher in cells overexpressing MCPIP1 lacking the PIN domain than in cells overexpressing the wild-type form of the protein.

Results of this experiment mimic changes of the level of endogenous Mcpip1 in adipocytes. A strong decrease of Mcpip1 level at day one after the induction of adipogenesis is essential to allow accumulation of transcript encoding C/EBPβ. Then, in further steps of adipogenesis, when PPARγ synthesis is induced, C/EBPβ is no longer needed, and its transcript is degraded by Mcpip1. Therefore, the increase of Mcpip1 level during the later period of adipogenesis is essential to remove C/EBPβ mRNA.

Iwasaki and collaborators [28] had already shown that MCPIP1 is phosphorylated by the IKK complex, then ubiquitinated and degraded in response to stimulation via the IL-1 receptor (IL-1R) or TLR. In this case, degradation is triggered by the proteasome [13,29]. A dynamic control of MCPIP1 level was also observed in T cells [14], where stimulation of the T cell receptor (TCR) leads to a cleavage of MCPIP1 at R111 by Malt1/paracaspase, thus liberating the cells from the Regnase-1-mediated suppression. Therefore, degradation of MCPIP1 allows for accumulation of mRNAs for a set of genes critical in the regulation of cellular processes such as initiation of inflammation and T cell activation [13,14].

Further studies are needed to determine whether disappearance of Mcpip1 at the beginning of adipogenesis is the result of degradation, and what mechanism underlies this process. The link between PPARγ downregulation and MCPIP1 overexpression also requires further

investigation. Since C/EBPβ is a known inducer of PPARγ expression [21,22], one may hypothesize that decreased level of the former simply results in decreased level of the latter. It is worth noting, however, that overexpression of MCPIP1 lacking RNase activity despite normal expression of C/EBPβ does not rescue the cells from decreased PPARγ level and adipogenesis impairment. Such result excludes MCPIP1 as a factor degrading PPARγ mRNA. This conclusion is supported by the finding that delayed expression of fully active MCPIP1 does not lead to PPARγ mRNA or protein reduction. Therefore, a negative influence of MCPIP1 on adipogenesis is restricted to the initial phase of this process, namely to regulation of C/EBPβ mRNA, and possibly other, yet undefined, transcripts. Here decreased PPARγ level is therefore likely to be the only consequence of events occurring during the initiation of differentiation.

The impairment of adipogenesis by MCPIP1 demonstrated here *in vitro* may have implications for lipid storage *in vivo*. New adipocytes are formed by the conversion of progenitor mesenchymal cells into adipogenic precursors. Results of experiments conducted by Wu and co-workers [22] on mouse NIH-3T3 showed that enhanced expression of C/EBPβ converts multipotent mesenchymal precursor cells into pre-adipocytes that respond to adipogenic inducers and are able to differentiate into adipocytes. Moreover, the efficiency of differentiation of NIH-3T3 cells into adipocytes depends on the amount of C/EBPβ protein. Our results indicate that MCPIP1 decreases C/EBPβ and prevents the formation of new adipocytes when upregulated in preadipocytes. The upregulation of MCPIP1 *in vivo* may result from cytokines secreted by macrophages residing in the adipose tissue, as well as by preadipocytes themselves. We showed that MCPIP1 mRNA level increases together with body mass index in humans, and correlates with CD14, a marker of monocytes/macrophages in the subcutaneous tissue of *db/db* mice. Although we did not distinguish whether elevated level of MCPIP1 comes from macrophages, adipocytes, or both, we show here that Mcpip1 mRNA expression can be activated in pre-adipocytes in response to IL-1β, one of the pro-inflammatory cytokines.
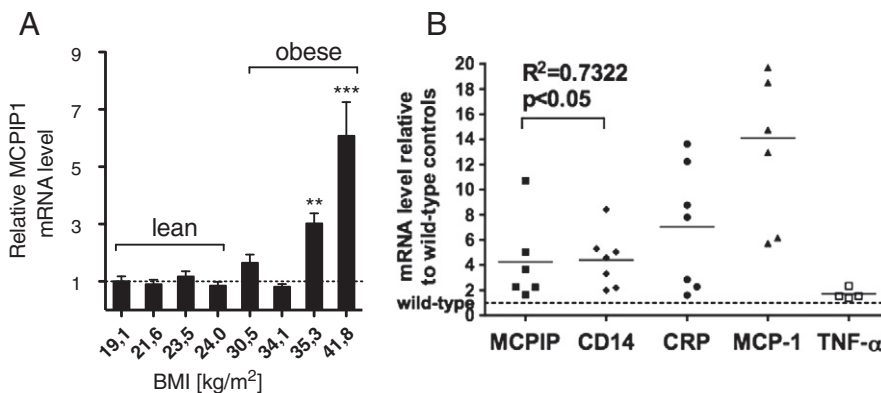

Fig. 7. MCPIP1 mRNA level in tissue specimen of obese subjects. A. Total RNA isolated from subcutaneous tissue samples was subjected to real-time PCR analysis with MCPIP1-specific primers. EF2 served as an internal reference gene. B. Total RNA isolated from subcutaneous tissue of db/db mice was subjected to analysis against Mcpip1 and selected genes related to inflammation. Pearson correlation coefficient for Mcpip1 versus other genes was performed with Prism 5. Significant values are marked on the chart. **$p < 0.02$, ***$p < 0.002$.

The group of Kolattukudy has published apparently different results. Younce and coworkers [18] observed that MCPIP1-GFP overexpressed in 3T3-L1 cells induces adipogenesis in the absence of DMI medium, and also that this protein can induce adipogenesis in PPARγ −/− cells [18]. Further studies of Younce and Kolattukudy [19] showed that overexpression of MCPIP1-GFP leads to the induction of reactive oxygen/nitrogen species (ROS/RNS) and endoplasmic reticulum (ER) stress, which result in the induction of the adipogenic factors C/EBPbeta, C/EBPδ, C/EBPα and PPARγ, and finally in the induction of autophagy. It is difficult to explain the reason of such discrepancies between ours and Younce's data [18,19]. We did not use GFP-tagged MCPIP1, but only the wild-type protein. We also found that cells transduced with a retroviral vector expressing MCPIP1 or GFP only slightly activate beclin 1, a marker of autophagy (data not shown). This marker was shown by Younce and Kolattukudy [19] to be induced in cells overexpressing GFP-tagged MCPIP1. It is possible that modifications of MCPIP1 by addition of tags at the ends of the protein change its properties.

In summary, we have found that MCPIP1 is directly responsible for decreasing the stability of C/EBPβ mRNA, but not for that of PPARγ. A PIN domain is essential for this activity. Decreased levels of C/EBPβ result in decreased levels of PPARγ and, in consequence, in the levels of other transcripts that are under the control of both transcription factors and are important for adipogenesis. In the light of these observations, we propose MCPIP1 as a factor responsible – at least in part – for the metabolic syndrome-related impairment of de novo adipogenesis. Further studies are however essential to understand the role of MCPIP1 in adipogenesis and explain discrepancies in results so far obtained by different groups.

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Acknowledgements

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