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EVI1 Mediated Stimulation of 3T3-L1 Preadipocyte Differentiation Is CtBP Dependent

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Abstract

Myelodysplasia syndrome 1 (*MDS*1) and Ecotropic viral integration site 1 (*EVI*1) complex (*MECOM*) locus encode multiple isoforms of the EVI1 protein that are essential for normal vertebrate development and when inappropriately expressed play a significant role in malignancy and in particular leukaemias. However, the function of individual EVI1 isoforms is not fully understood. Recently, EVI1 or PRDM3, which is structurally closely related to the brown adipose tissue determining factor PRDM16, was shown to be required for differentiation of adipocytes. In this study, we show that 3T3-L1 preadipocytes sustain expression of all Evi1 isoforms examined, including Mds1-Evi1, Evi1FL, Evi1Δ324, Evi1FL + 9 and Evi1Δ105 throughout the adipogenesis differentiation programme. We also show that differentiation markers are enhanced by enforced expression of either Evi1, Evi1FL + 9 or Evi1Δ105 isoforms. Interestingly 3T3-L1 differentiation markers are also moderately enhanced by enforced expression of Evi1Δ324, which lacks part of the N-terminal zinc finger domain (ZF1), demonstrating a biological activity for this particular isoform. Enforced expression of an Evi1 mutant lacking C-terminal binding protein (CtBP) co-repressor protein binding activity fails to stimulate 3T3-L1 differentiation markers and may have dominant negative activity, causing partial inhibition of this developmental programme. These studies show that multiple EVI1 isoforms are expressed in adipocytes and can stimulate adipogenic markers in a manner that is partially independent of the ZF1 DNA binding domain but fully dependent upon interaction with co-repressor CtBP proteins.

Keywords

MECOM, PRDM3, EVI1 Isoforms, C-Terminal Binding Proteins, Adipogenesis

1. Introduction

Myelodysplasia syndrome 1 (*MDS*1) and Ecotropic virus integration site 1 (*EVI*1) complex (*MECOM*) locus gene transcripts include *MDS*1, *EVI*1 and a fusion of part of *MDS*1 with *EVI*1 [1] and their inappropriate expressions are associated with poor prognosis leukaemias and other malignancies [2] [3]. Those transcripts containing *EVI*1 encode transcription factors with multiple *cys*2*his*2 zinc finger DNA binding motifs [4] and are required for mammalian development [5]. EVI1 has been shown to contribute to a number of developmental programmes including maintenance of haemopoietic stem cells and various committed progenitor cells in haemopoiesis [6], neuroectodermal cell differentiation [7], nephrogenesis [8] and cardiac development [9].

EVI1 is also known as positive regulatory domain I-binding factor 1, retinoblastoma protein-binding zinc finger protein (PR) domain protein 3 (PRDM3) and the structurally similar PRDM16 is a key regulator of brown adipose tissue development [10]. Recent studies show that EVI1 also participates in adipogenesis [11] [12]. These studies show that EVI1 converts nonadipogenic cells to adipocytes and knockdown (KD) suppresses preadipocyte differentiation by impairing CCAAT/Enhancer-binding protein-beta (CEBPβ) assisted induction of peroxisome proliferator-activated receptor-gamma 2 (PPARγ_2).

There are multiple naturally occurring isoforms of EVI1 but it is not known which are expressed in preadipocytes and which might participate in adipogenesis as all are potentially affected in the knockdown (KD) study of Ishibashi *et al.*, 2012. The isoforms include MDS1-EVI1, EVI1FL, EVI1Δ324 [13], EVI1RP+ and EVI1Δ105 (murine specific) [14]. MDS1-EVI1 comprises intergenic transcripts containing coding exons of both the *MDS*1 and the *EVI*1 genes and encodes an EVI1 protein with an N-terminal PR domain. EVI1FL is the original full length murine protein encoded by the cDNA first isolated from leukaemia cells [4]. EVI1RP+ is similar to EVI1FL but has an additional 9 amino acids inserted within the repressor domain (RP). EVI1Δ324 lacks 324 amino acids, including part of the first zinc finger domain up to, but excluding, RP and EVI1Δ105 has 105 C-terminal amino acids deleted. Various properties have been attributed to some of these isoforms and in some instances they have been shown to have opposing activities. For example, MDS1-EVI1 has been associated with tumor suppressing activity whereas EVI1FL is oncogenic. MDS1-EVI1 activates AGATA motif promoters whereas EVI1FL represses [1], EVI1FL inhibits 32Dcl3 cell response to granulocyte-colony stimulating factor (G-CSF) and transforming growth factor beta (TGFβ_1) whereas MDS1-EVI1 has no effect on G-CSF response and enhances TGFβ_1 signalling [15] and EVI1FL enhances proliferation of haemopoietic colonies from differentiating embryonal stem (ES) cells whereas MDS1-EVI1 represses these activities [16]. The MDS1-EVI1 isoform has a PR domain [17] which confers intrinsic histone H3 lysine 9 monomethyltransferase catalytic activity [18] which is absent from other EVI1 isoforms.

The significance of the remaining isoforms remains unclear. Studies show ex-

pression of each isoform in all tissues examined but little difference in DNA binding, CtBP protein binding, transcriptional repression or cell transformation activities between EVI1FL, EVI1RP+ or EVI1Δ105 [14]. EVI1Δ324 however lacks 3 N-terminal zinc fingers (ZF1), neither binds nor represses transcription via ZF1 DNA binding sites, does not transform fibroblasts [19] and to date no biological activity has been assigned to this isoform.

In this study we investigate the profile of expression and biological activity of EVI1 isoforms in 3T3-L1 preadipocytes and throughout the adipocyte differentiation programme.

2. Materials and Methods

2.1. Cell Culture

Plat-E (Cambridge Bioscience, Cambridge, UK, RV-101) and 3T3-L1 (ATCC® CL-173™) cells were cultured in complete medium (CM) comprising Dulbecco's Modified Eagle's Medium (Lonza Group Ltd., Basel, Switzerland, BE12-604F) supplemented with 10% (v/v) newborn calf serum (3T3-L1) (Sigma-Aldrich, Poole, UK, N4637) or 10% (v/v) foetal calf serum (Plat-E cells) (FCS, Lonza, DE14-801F) and 2.5 mM glutamine, 50 μg/ml penicillin, 50 units/ml streptomycin (Lonza Group Ltd., BE17-605E and BE17-603E), 37˚C, 5% CO_2. For differentiation 3T3-L1 were cultured with induction medium 1 (IM1), comprising CM with 10% (v/v) FCS, 5 μg/ml insulin (Sigma-Aldrich, I9278), 0.25 μM dexamethasone (Sigma-Aldrich, D4902), 0.5 mM Isobutylmethylxanthine (IBMX, Sigma-Aldrich I5879), for 48 h followed by a further 48 h incubation with induction medium 2 (IM2) comprising CM supplemented with 10% FCS and 5 μg/ml insulin. Culture medium was subsequently replaced with fresh IM2 every 48 h for up to 10 days. For retrovirus production, Plat-E cells were transiently transfected with retroviral plasmid DNA using Fugene6® (Roche Diagnostics GmbH, Mannheim, Germany, 11815091001); virus was harvested and used to infect 3T3-L1 as described before [20].

2.2. Preparation of Total Cellular RNA, cDNA Synthesis and Quantitative Real-Time Polymerase Chain Reaction QPCR

RNA was prepared from cultures of cells by the TRI Reagent® method (Sigma-Aldrich, 93289). Total cellular RNA (1 μg) was used to synthesise cDNA using Maxima reverse transcriptase (Thermo Fisher Scientific Inc., St. Leon-Rot, Germany, EP0742) with random hexamer (Thermo Fisher Scientific, S0142) and oligo dT (Thermo Fisher Scientific, S0131) primers according to the manufacturer's instructions. The cDNA reaction (5%) was used for real time quantitative polymerase chain reaction using QPCR SYBR Green mix (Thermo Fisher Scientific, 11873913), gene specific oligonucleotide primers (Integrated DNA Technologies, Leuven, Belgium), 95˚C, 15 min followed by 40 cycles 95˚C, 30 s, 60˚C, 30 s in a CFX96 C1000 Thermal cycler (BIO-RAD Laboratories Ltd., Hemel Hempstead, UK).

The efficiency of the Q-PCR reactions were calculated by using the formula Efficiency = $-1 + 10^{(-1/\text{slope})}$ against the standard curve of each assay over a gra-

dient of template concentration with each gene. The efficiency of primers are *Ca*3 (88%), *C/ebpα* (75%), *Pparγ*$_2$ (92%), *Fabp*4 (91%), *Evi*1 (101%) and *Gapdh* (90%). Relative expression levels between target and *Gapdh* were determined using the arithmetic comparative $2^{-\Delta\Delta Ct}$ method [21] and were determined relative to the target gene in MX infected 3T3-L1 cells (calibrator). Oligonucleotide primers were supplied by Integrated DNA Technologies (Leuven, Belgium) *Pparγ*$_2$*FP*: GCCCACCAACTTCGGAATC, *Pparγ*$_2$*RP*: TGCGAGTGGTCTTCC ATCAC, *C/ebpαFP*: GAGCTGAGTGAGGCTCTCATTCT, *C/ebpαRP*: TGGGA GGCAGACGAAAAAAC, *Fabp4FP*: GGGCGTGGAATTCGATGAAATCA, *Fab p4RP*: CCCGCCATCTAGGGTTATGAT, *Evi1FP*: CGCTTGAAGCTTTGAAAG AAAAATA, *Evi1RP*: TGTTCTCAATTGCTGACATTTGC, *Evi*1 *probe* (*HEX*): TTGAGACCTTCTCCAGGATTCTTGTTTCACC, *Ca3FP*: CCGGGACTATTGG ACCTATCAC, *Ca3RP*: TTGAGCAGCAGCCACACAA, *Ca*3 *probe* (*FAM*): CTCC TTCACCACGCCGCCCTG, *GapdhFP*: GGGCTGCCCAGAACATCA, *GapdhRP*: CCGTTCAGCTCTGGGATGAC, *Gapdh probe* (*FAM*): CCCTGCATCCACTG GTGCTGCC.

2.3. Endpoint PCR

cDNA (0.5 µl) was amplified by PCR with 140 ng/µl forward and reverse primers using ReddyMix PCR master mix [1.5 mM $MgCl_2$] (Thermoscientific) 95˚C, 5 min followed by 40 cycles 95˚C, 15 s, 60˚C, 60 s in a PTC-100™ Thermal cycler (MJ Research, Inc.). Products were analysed by 3% (w/v) agarose gel electrophoresis in 40 mM Tris-acetate, 1 mM EDTA (pH 8.0) buffer (1XTAE). Oligonucleotide primers were supplied by Integrated DNA Technologies. Mds1/Evi1 and Evi1 specific primers were *EF*, *MF*1 and *GSP*3 [22], RP+ primers were *ME*1/*ME*3 and Δ105 primers were *ME*2/*ME*4 [14] and Δ324 primers were *Δ*324*F*: CGTCA GGGCCTCAAACAGC, *Δ*324*R*: GGGTACATTGATTGAGAGAATGAGA. CtBP 1 and 2 primers were: *CtBP*1*FP*; CACACAGGAGATCCATGAGAAG, *CtBP*1*RP*; CTCTGGTCAGTGTGATGGTATG, *CtBP*2*FP*; GCACAGTCCACTCAGGAAAT, *CtBP*2*RP*; CCTTGAACTTCTCCAGGTCTTC.

2.4. Western Blot Analysis

Protein extracts, SDS polyacrylamide gel electrophoresis and western blotting were performed as described previously [23] with either *α*-EVI1 (1806) or *α*-GAPDH (Fitzgerald Industries, North Acton, MA, USA, 6C5) diluted 1/1000 (1806) or 1/5000 (6C5) respectively. Appropriate IRDye® 800CW conjugated anti-rabbit (Li-Cor Biosciences, 926-32211) or IRDye® 680RD conjugated anti-mouse (Li-Cor Biosciences, 926-68072) IgG secondary antibodies were used at 1/15000 dilutions and detection was performed by fluorescence using an Odyssey Fc Imaging System (Li-Cor Biosciences).

2.5. Statistical Analysis

Unpaired Student's t-test was used to determine the significance of data using

Graphpad Prism® 6.0 software. $P \leq 0.05$ was considered significant. *$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$, ns not significant.

3. Results

3.1. Enforced Expression of EVI1 in 3T3-L1 Preadipocytes

In order to investigate the effect of EVI1 expression on adipogenesis it was expressed in 3T3-L1 cells. Initially Plat-E cells were transiently transfected with the previously described Evi1FL encoding p50M5.6-neo retroviral vector [20] and the resulting virus containing supernatants used to infect varying numbers of 3T3-L1 cells (Materials and Methods). The 3T3-L1 cells were re-infected with virus containing supernatant again 24 hrs later. After virus infection (48 h) cells were examined for Evi1 expression by western blot analysis with α-EVI1. The results show production of the 145 kd Evi1 protein in cells infected with the 5.6 retroviral vector (**Figure 1(a)**). Even loading of samples was confirmed by western blot analysis with α-GAPDH (**Figure 1(a)**). Highest Evi1 expression is observed when either 2×10^4 or 5×10^4 cells were used for virus infection and therefore 5×10^4 cells were chosen for further experiments.

3.2. EVI1 Enhances 3T3-L1 Adipocyte Differentiation

To investigate the impact of enforced EVI1FL expression on adipogenesis it was expressed in 3T3-L1 cells using the transient retroviral infection scheme and subsequent induction of adipocyte differentiation programme outlined in **Figure 1(b)**. Cells were transiently infected with either p50MX-neo (MX, empty vector control) or p50M5.6-neo (5.6) virus, induced to differentiate and RNA prepared from cell extracts at various time points. Initially, expression of the adipocyte differentiation marker gene *Fabp*4 at days 0 and 10 were examined. The results show induction of this marker for both control infected cells as well as cells with enforced expression of Evi1 (**Figure 1(c)**, MX, 5.6), however the induction of *Fabp*4 is significantly increased in cells with enforced Evi1 expression at day 10 compared to MX infected cells on the same day (**Figure 1(c)**, 5.6).

We next examined expression of key regulators of adipocyte differentiation *Ppar*γ_2 and *C/ebp*α in the presence (5.6) or absence (MX) of Evi1 at days 0, 1, 2, 3 and 4 of induction. The results show that both markers are induced during the 4 day period but accumulate to significantly higher levels in the presence of Evi1 (**Figure 2(a)**, **Figure 2(b)**, 5.6) when compared with empty vector infected cells at each point examined (**Figure 2(a)**, **Figure 2(b)**, MX). *Ppar*γ_2 expression initially declines between day 0 and days 1 and 2 in MX and 5.6 cells but one-way ANOVA and Dunnett's multiple comparison post-test using MX day 0 or 5.6 day 0 as control group confirms significant increases by day 4 [$P \leq 0.001$ (MX), $P \leq 0.05$ (5.6)]. Other studies have shown that the enzyme carbonic anhydrase III (Ca3) is induced during adipocyte differentiation [24] and is either a marker or regulator of this process. *Ca*3 gene expression increases significantly (D1 $p \leq 0.01$, D2, D3 and D4, $P \leq 0.001$) in control MX cells compared to levels at D0 in-

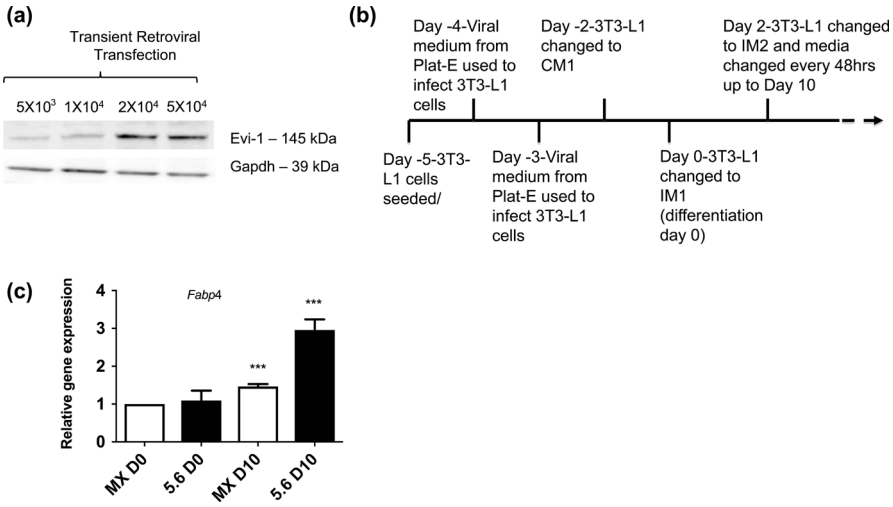

Figure 1. (a) Western blot analysis of whole cell protein extracts derived from 3T3-L1 cells transiently infected with p50M5.6neo retrovirus. The number of cells exposed to retrovirus is shown at the top of each lane. The size of Evi-1 and Gapdh proteins observed with α-Evi1 and α-Gapdh are indicated; (b) Strategy for transient retroviral infection of 3T3-L1 cells and timeline for induction of differentiation. Complete media (CM1), induction media 1 (IM1) and 2 (IM2) are described in materials & methods; (c) Histogram showing relative gene expression of *Fabp*4 in empty vector control (MX, clear bars) and *Evi*1 vector (5.6, black bars) infected 3T3-L1 cells at days 0 (D0) and 10 (D10) of differentiation. Error bars are the standard deviation of 3 (n = 3) independent virus infection and differentiation experiments. ***P ≤ 0.001 indicates statistical significance of MXD10 vs. MXD0 and 5.6D10 vs. MXD10.

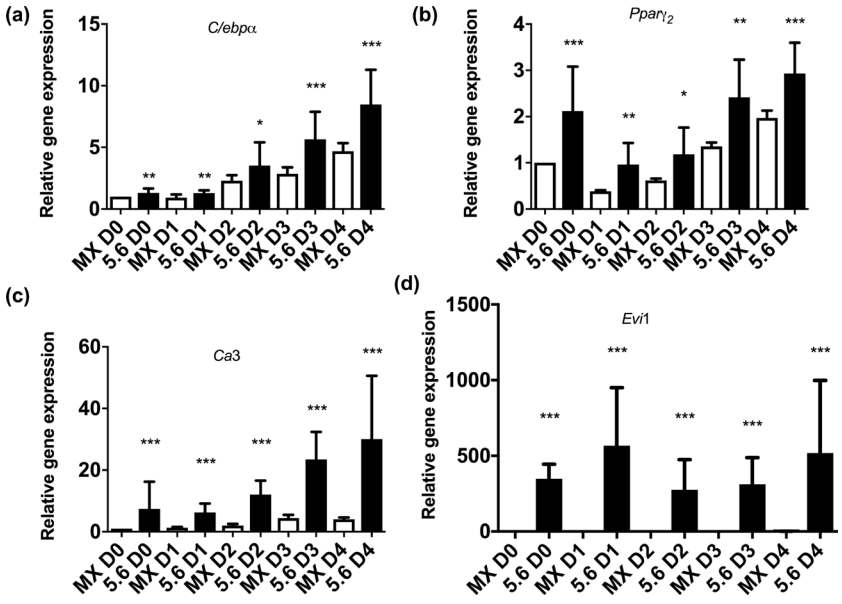

Figure 2. Histograms showing relative gene expression of *C/ebpα* (a), *Pparγ*$_2$ (b), *Ca*3 (c) and *Evi*1 (d) in empty vector control (MX, white bars) and Evi1 vector (5.6, black bars) virus infected 3T3-L1 cells at days 0 (D0), 1 (D1), 2 (D2), 3 (D3) and 4 (D4) of differentiation. Error bars are the standard deviation of 3 (n = 3) independent virus infection and differentiation experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 indicates statistically significant differences in expression of the indicated gene for EVI1 expressing cells (5.6) compared to MX infected cells on the same day.

dicating progression through the differentiation programme. Comparison of *Ca*3 gene expression in MX and 5.6 cells shows its expression is significantly elevated in 3T3-L1 cells with enforced expression of Evi-1 (**Figure 2(c)**, 5.6) compared with cells examined at the same time point that were infected with the empty vector (**Figure 2(c)**, MX). Finally, Evi1 transgene expression was maintained for at least the 4 day duration of the transient expression and differentiation system as its mRNA expression is significantly higher in 5.6 infected cells compared with low, but detectable, endogenous Evi1 expression observed in MX infected 3T3-L1 cells at each time point examined (**Figure 2(d)**, MX, 5.6).

3.3. Naturally Occurring EVI1 Splice Variants, RP+, Δ105 and Δ324 Stimulate 3T3-L1 Adipocyte Differentiation

These data suggest enforced expression of Evi1 accelerates adipocyte differentiation of induced 3T3-L1 cells. Multiple, naturally occurring Evi1 splice variants exist in murine cells [14]. A schematic representation of the isoform shown to stimulate adipocyte differentiation (**Figure 2**) is shown in **Figure 3**, designated EVI1FL, along with other splice variants MDS1/EVI1, EVI1RP+ (RP+), EVI1Δ324 (Δ324) and EVI1Δ105 (Δ105). Endogenous expression of each of these in 3T3-L1 cells, in preadipocytes (**Figure 4(a)**) and throughout 10 days of differentiation (data not shown), was confirmed using isoform specific oligonucleotide primers (Materials and Methods) by end point PCR.

Since all isoforms examined are expressed in 3T3-L1 cells we investigated which can induce adipocyte differentiation. Previously described retroviral vectors [14] [19] were used to transiently express each isoform (RP+, Δ324 and Δ105) in 3T3-L1 cells. Infected cells were induced to differentiate and similar levels of

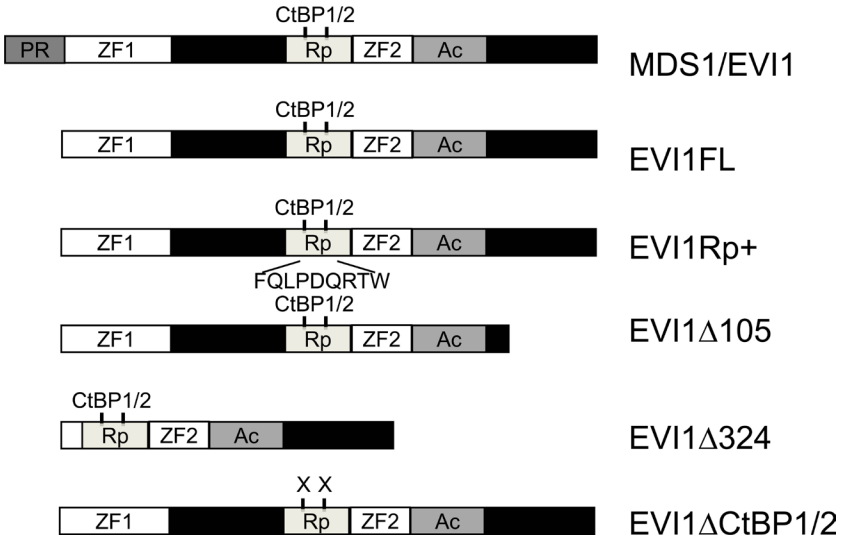

Figure 3. Schematic representation of the domain structure of the indicated EVI1 splice variant encoded proteins showing the PR domain (PR), 1st and second 2nd zinc finger domains (ZF1 & ZF2), repressor domain (Rp), acidic domain (Ac), CtBP binding sites 1 & 2 and the additional 9 amino acids (single letter amino acid code) found in the repressor domain of Rp+. X indicates CtBP binding inactivating point mutations.

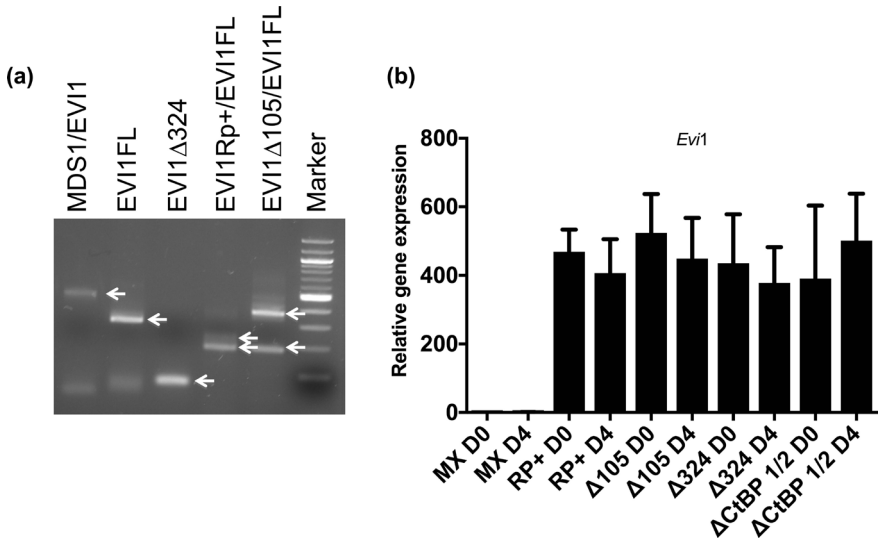

Figure 4. (a) Agarose gel (3% NuSieve® 3-1 agarose, Lonza) electrophoresis of end point PCR products for EVI1 splice variants at day 0 of 3T3-L1 cell differentiation. White arrows indicate amplified DNA fragments of the expected size for each splice variant: MSD1/EVI1 460 bp; EVI1FL 298 bp; EVI1Δ324 76 bp; EVI1RP+/EVI1FL 216 bp/189bp; EVI1Δ105/EVI1FL 187 bp/371bp. Marker is 100 bp ladder (quickload, New England Biolabs); (b) Histogram showing relative gene expression of *Evi*1 in empty vector control (MX, white bars) and indicated Evi1 splice variant vector infected 3T3-L1 cells at days 0 (D0) and 4 (D4) of differentiation. Error bars are the standard deviation of 3 (n = 3) independent virus infection and differentiation experiments. There is no statistically significant difference in expression of each mutant form relative to EVI1RP+ (RP+) at day 0.

ectopic expression of *Evi*1 splice variants was achieved over the 4 days examined (**Figure 4(b)**).

Cells were then examined for expression of *Fabp*4, *Ca*3 (day 0 and 10), *Pparγ*$_2$ and *C/ebpα* (day 0, 1, 2, 3 and 4). In each case gene expression in *Evi*1 isoform expressing cells was compared to MX infected cells on the same day. Surprisingly, the results show a significant increase in induction of *Fabp*4 (**Figure 5(a)**) and *Ca*3 (**Figure 5(b)**) expression in induced 3T3-L1 cells at day 10 with each isoform examined. Furthermore, both RP+ or Δ105 isoform expression results in a significant increase in *C/ebpα* and *Pparγ*$_2$ (**Figure 6(a)**, **Figure 6(b)**, RP+ D3 and 4, Δ105 D3 and 4 vs. MXD3 and 4) gene expression. Δ324 transgene expression results in a significant increase in both *C/ebpα* and *Pparγ*$_2$ gene expression at day 3 (**Figure 6(a)**, **Figure 6(b)**, Δ324D3 vs. MXD3) but no significant change at day 4 (**Figure 6(a)**, **Figure 6(b)**, Δ324D4 vs. MXD4).

3.4. Interaction with CtBP Proteins Is Required for EVI1 Mediated Stimulation of 3T3-L1 Adipogenesis

The results suggest that enforced expression of each naturally occurring Evi1 isoform tested can stimulate adipogenesis in induced 3T3-L1 cells. Evi1 interacts with CtBP proteins to mediate some biological activities and so we investigated if this interaction is required to stimulate adipocyte differentiation markers as well. A retroviral vector encoding a CtBP binding mutant EVI1ΔCtBP1/2 (ΔCtBP1/2)

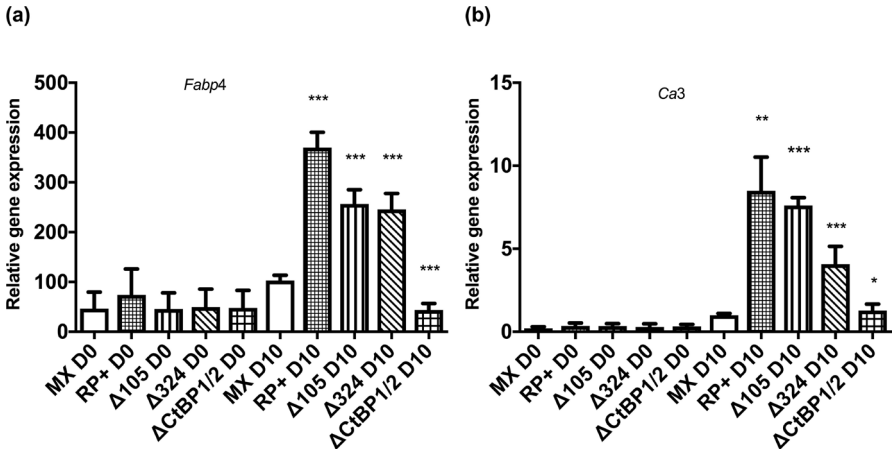

Figure 5. Histograms showing relative gene expression of *Fabp*4 (a) and *Ca*3 (b) in empty vector control (MX) and EVI1RP+ (RP+), EVI1Δ105 (Δ105), EVI1Δ324 (Δ324) and EVI1ΔCtBP1/2 (ΔCtBP1/2) virus infected 3T3-L1 cells at days 0 (D0) and 10 (D10) of differentiation. Error bars are the standard deviation of 3 (n = 3) independent virus infection and differentiation experiments. *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001 indicates statistically significant differences in expression of the indicated gene for each form of EVI1 relative to MX infected cells at day 10.

(**Figure 3**) that is unable to bind CtBP proteins [25] was transiently expressed in 3T3-L1 cells. The cells were induced to differentiate and examined for expression of the same molecular markers as before. ΔCtBP1/2 mutant transgene expression was observed at similar levels to the other Evi1 isoforms studied (**Figure 4(b)**, ΔCtBP1/2 D0 and D4). The results show that instead of an increase, there is a significant decrease in *Fabp*4 (**Figure 5(a)**, **Figure 5(b)**, ΔCtBP1/2 D10), *C/ebpα* and *Pparγ*$_2$ (**Figure 6(a)**, **Figure 6(b)**, ΔCtBP1/2 D4) gene expression in ΔCtBP1/2 expressing cells when compared to cells infected with the empty vector (MX) control on the same days. Only *Ca*3 gene expression shows a small increase in expression in cells with enforced ΔCtBP1/2 expression (**Figure 5(b)**, ΔCtBP1/2 D10). Expression of both *CtBP*1 and *CtBP*2 genes are observed throughout the 3T3-L1 cell differentiation programme (**Figure 6(c)**). These data show that Evi1 mediated stimulation of 3T3-L1 cell differentiation markers is dependent on interaction with CtBP binding proteins.

4. Discussion

In this study a transient retroviral infection system was developed to investigate the effect of enforced EVI1 expression on 3T3-L1 pre-adipocyte cell differentiation to adipocytes. Results show that under these conditions EVI1 enhances chemically induced 3T3-L1 differentiation as measured by characteristic gene markers and mediators of this process (*Fabp*4, *Ca*3, *C/ebpα* and *Pparγ*$_2$). Furthermore, we show that all previously described and naturally occurring EVI1 splice variants are expressed in 3T3-L1 preadipocytes as well as throughout the differentiation programme and that enforced expression of splice variants EVI1RP+, EVI1Δ105 and EVI1Δ324 similarly enhance the process. Finally, we demonstrate

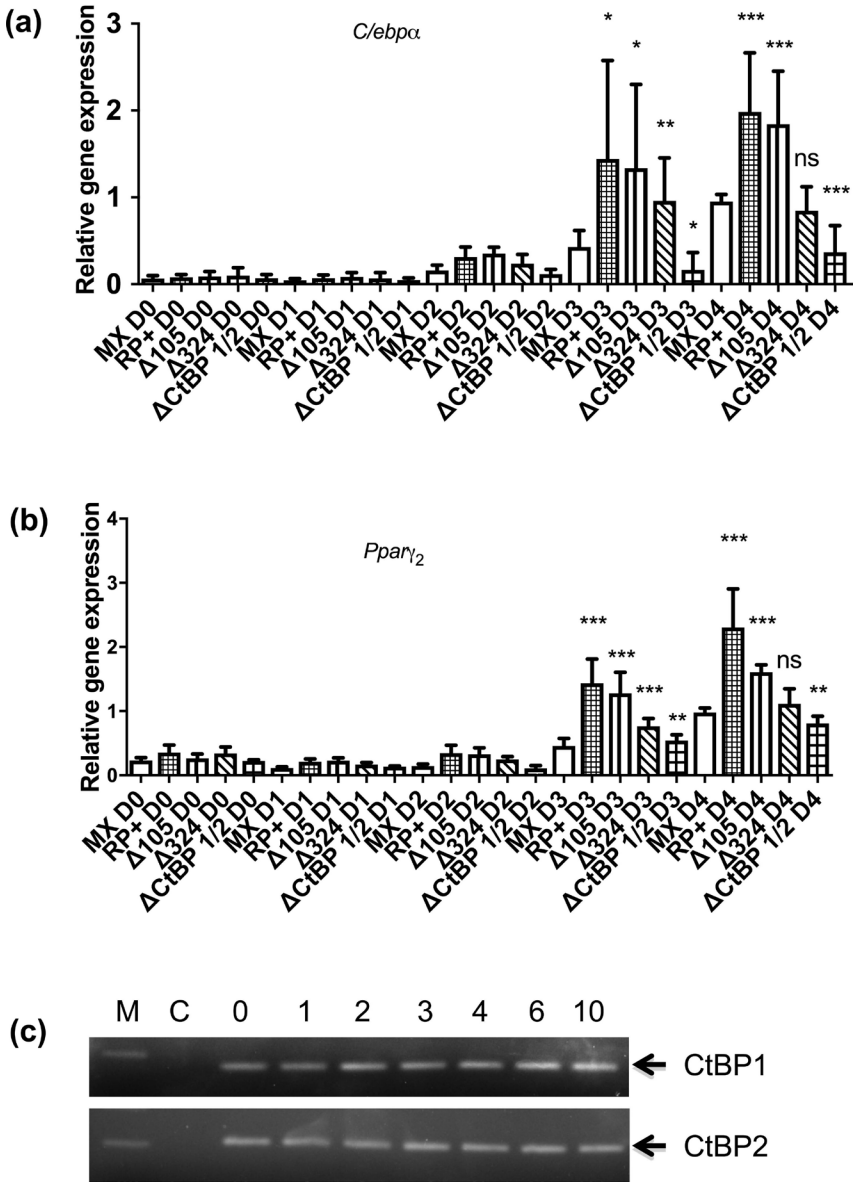

Figure 6. Histograms showing relative gene expression of *C/ebpa* (a) and *Pparγ*$_2$ (b), in empty vector control (MX) and EVI1Rp+9 (Rp+), EVI1Δ105 (Δ105), EVI1Δ324 (Δ324) and EVI1ΔCtBP1/2 (ΔCtBP1/2) virus infected 3T3-L1 cells at days 0 (D0), 1 (D1), 2 (D2), 3 (D3) and 4 (D4) of differentiation. Error bars are the standard deviation of 3 (n = 3) independent virus infection and differentiation experiments. Statistical analysis of days 3 (D3) and 4 (D4) data only are shown. **P ≤ 0.01, ***P ≤ 0.001 indicates statistically significant differences in expression of the indicated gene for each form of EVI1 relative to MX infected cells on the same day. ns indicates no significant difference in expression relative to MX infected cells on the same day. (c) Agarose gel (3% NuSieve® 3-1 agarose) electrophoresis of end point PCR products for *CtBP*1 and *CtBP*2 gene expression at indicated days 0, 1, 2, 3, 4, 6 and 10 of 3T3-L1 cell differentiation. M indicates 100 bp ladder marker (quickload) and C the negative control.

that a mutant of EVI1, which no longer binds CtBP proteins, is unable to stimulate the 3T3-L1 differentiation markers that are observed with wild type variants.

The efficiency of differentiation of empty vector control 3T3-L1 cells is suboptimal as indicated by relatively small changes in molecular marker gene expression shown in **Figure 1(c)**; **Figures 2(a)-(c)**; **Figure 5(a)** & **Figure 5(b)** and **Figure 6(a)** & **Figure 6(b)**. In the virus infection and differentiation scheme used here (**Figure 1(b)**) 3T3-L1 cells are unlikely to be confluent for 48 hrs prior to induction of differentiation as is normally the case [26] because of the need to optimize retroviral infection in dividing cells [27]. However, our results clearly show that differentiation is significantly enhanced by enforced expression of EVI1 under these conditions, based on the molecular markers examined. Following growth arrest, efficient induction of 3T3-L1 cell differentiation is accompanied by mitotic clonal expansion (MCE) [28]. Studies have shown that EVI1 stimulates cell proliferation [29] and this property may stimulate MCE, contributing to the enhanced expression of adipogenic markers observed here, which are consistent with previous observations [11].

All known naturally occurring EVI1 splice variants are expressed in preadipocytes and throughout differentiation of 3T3-L1 cells. The relative abundance of splice variants has not been determined in this study but others have shown that general EVI1 expression is low in proliferating preadipocytes, transiently peaks during chemical stimulation of differentiation then is low again for the remaining programme [11].

Enforced expression of splice variants EVI1FL, EVI1RP+, EVI1Δ105 and EVI1Δ324 are each capable of stimulating adipocyte differentiation based on relative increases in programme mediator (*Cebpa*, *Pparγ*$_2$) and marker (*Fabp*4, *Ca*3) gene expression (**Figure 2**, **Figure 5**, **Figure 6**). It is interesting that EVI1Δ324 can stimulate adipogenic markers as this represents one of the few biological activities associated with the isoform to date. This splice variant lacks part of zinc finger 6 and all of zinc finger 7 of the ZF1 domain as well as 275 intervening amino acids to the Rp domain [30]. Recent studies show EVI1FL and EVI1Δ324 co-regulate largely the same genes in cells and that EVI1Δ324 can induce anchorage independent growth in HeLa cells [31]. However, our results show EVI1Δ324 cannot fully complement the activity of the other EVI1 splice variants studied as stimulation of gene expression of the markers examined is less in most cases when compared with the other isoforms. This indicates the missing amino acids, including the ZF1 domain, are important for optimal EVI1 mediated stimulation of adipogenesis.

The interaction of EVI1 with CtBP proteins has previously been shown to be essential for biological activities including cell transformation [25] and inhibition of TGFβ signaling [32]. This study shows EVI1 mediated stimulation of adipogenic markers is also CtBP binding dependent. Interestingly, EVI1ΔCtBP1/2 not only fails to stimulate adipogenic markers in 3T3-L1 cells but it actually appears to repress them when compared with MX infected cells. Gene expression of *Fabp*4, *C/ebpa* and *Pparγ*$_2$ are all significantly repressed in EVI1ΔCtBP1/2 expressing cells (**Figure 5** and **Figure 6**) which suggests it has dominant negative

activity with regard to adipocyte differentiation. Other regulators of adipogenesis are also dependent upon CtBP complexes including Klf3 [33], Fog1 and Fog2 [34]. Both CtBP1 and CtBP2 are expressed throughout adipocyte differentiation (**Figure 6(c)**) and their binding is required for both negative (Klf3, Fog1 and Fog2) and positive (EVI1) regulation. Furthermore, the EVI1 related protein PRDM16 also binds CtBP proteins to repress white fat specific genes and are displaced to promote brown adipose tissue development [35]. CtBP proteins bind NAD+ and NADH with higher affinity for the latter which promotes interaction with partner proteins [36]. CtBP proteins have been proposed to have a role in metabolic sensing [37]. High calorie intake is associated with increased levels of NADH. Based on our study this would be predicted to promote association of EVI1 and CtBP and stimulate adipogenesis.

Obesity, the expansion of adipose tissue depots, is one underlying cause of major health conditions worldwide including both type 2 diabetes mellitus and cardiovascular disease, but the mechanisms involved are not fully understood. Understanding the molecular mechanisms regulating adipogenesis might identify novel targets for therapeutic intervention. Regulation of the adipogenesis developmental programme is controlled by a complex network of transcription factors and EVI1 has only recently been identified to be involved in this process. These studies show for the first time that multiple EVI1 isoforms are expressed in adipocytes and can stimulate adipogenic markers in a manner that is partially independent of the ZF1 DNA binding domain but fully dependent upon interaction with co-repressor CtBP proteins. Blocking EVI1/CtBP interaction may be a target for drug development controlling obesity.

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