Epigenetic modifications of the Zfp/ZNF423 gene control murine adipogenic commitment and are dysregulated in human hypertrophic obesity

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Abstract

Aims/hypothesis Subcutaneous adipocyte hypertrophy is associated with insulin resistance and increased risk of type 2 diabetes, and predicts its future development independent of obesity. In humans, subcutaneous adipose tissue hypertrophy is a consequence of impaired adipocyte precursor cell recruitment into the adipogenic pathway rather than a lack of precursor cells. The zinc finger transcription factor known as zinc finger protein (ZFP) 423 has been identified as a major determinant of pre-adipocyte commitment and maintained white adipose cell function. Although its levels do not change during adipogenesis, ectopic expression of Zfp423 in non-adipogenic murine cells is sufficient to activate expression of the gene encoding peroxisome proliferator-activated receptor γ (Pparγ; also known as Pparg) and increase the adipogenic potential of these cells. We investigated whether the Zfp423 gene is under epigenetic regulation and whether this plays a role in the restricted adipogenesis associated with hypertrophic obesity.

Methods Murine 3T3-L1 and NIH-3T3 cells were used as fibroblasts committed and uncommitted to the adipocyte lineage, respectively. Human pre-adipocytes were isolated from the stromal vascular fraction of subcutaneous adipose tissue of 20 lean non-diabetic individuals with a wide adipose cell size range. mRNA levels were measured by quantitative real-time PCR, while methylation levels were analysed by bisulphite sequencing. Chromatin structure was analysed by micrococcal nuclease protection assay, and DNA-methyltransferases were chemically inhibited by 5-azacytidine. Adipocyte differentiation rate was evaluated by Oil Red O staining.

Results Comparison of uncommitted (NIH-3T3) and committed (3T3-L1) adipose precursor cells revealed that Zfp423 expression increased ($p < 0.01$) in parallel with the ability of the cells to differentiate into mature adipocytes owing to both decreased promoter DNA methylation ($p < 0.001$) and nucleosome occupancy (NUC1: $p < 0.01$; NUC2: $p < 0.001$) in the 3T3-L1 compared with NIH-3T3 cells. Interestingly, non-adipogenic epigenetic profiles can be reverted in NIH-3T3 cells as 5-azacytidine treatment increased Zfp423 mRNA levels ($p < 0.01$), reduced DNA methylation at a specific CpG site ($p < 0.001$), decreased nucleosome occupancy (NUC1: $p < 0.01$; NUC2: $p < 0.001$) and induced adipocyte differentiation ($p < 0.05$). These epigenetic modifications can also be initiated in response to changes in the pre-adipose cell microenvironment, in which bone morphogenetic protein 4 (BMP4) plays a key role. We finally showed that, in human adipocyte precursor cells, impaired epigenetic regulation of zinc nuclear factor (ZNF)423 (the human orthologue of murine Zfp423) was associated with inappropriate subcutaneous adipose cell hypertrophy. As in NIH-3T3 cells, the normal
**Introduction**

The worldwide increase in obesity is a major cause of the current epidemic of type 2 diabetes [1, 2]. However, obesity is not a homogeneous condition. Approximately 10–30% of obese individuals do not show metabolic complications. These individuals typically have an increased number of small adipocytes in their subcutaneous adipose tissue (SAT) and low visceral and other ectopic fat depots [3–5]. In addition, a similar proportion of non-obese individuals exhibit reduced insulin sensitivity and altered glucose metabolism [6]. At the molecular level, these human phenotypes remain incompletely characterised, generating uncertainties on how fat tissue expansion impacts the trajectory to type 2 diabetes.

Adipose tissue expansion is usually caused by an increase in adipocyte size (hypertrophy) and/or recruitment of new adipocytes from multipotent mesenchymal stem cells (MSCs) already in the stromal vascular compartment (hyperplasia) [7, 8]. Limited expandability and recruitment of new cells in SAT leads to prominent adipocyte hypertrophy, which is associated with ectopic accumulation of fat, functional dysregulation of SAT, low-grade chronic inflammation, decreased insulin sensitivity and enhanced oxidative stress [9–11]. In humans, SAT hypertrophy appears to be a consequence of impaired adipocyte precursor cell recruitment into the adipogenic pathway rather than a lack of precursor cells [12–15]. Although the underlying molecular mechanisms have only been partially elucidated, current evidence indicates that restricted adipogenesis in SAT predicts future development of type 2 diabetes independent of obesity [16]. The present understanding of SAT expansion in human obesity and diabetes is limited by incomplete understanding of the molecular basis of pre-adipocyte determination [17]. Recently, the zinc finger transcription factor known as zinc finger protein (ZFP) 423 was identified as a major determinant of pre-adipocyte commitment [17] and maintained white adipose cell function [18]. Zfp423 expression is enriched in a number of adipogenic fibroblast cell lines compared with fibroblasts uncommitted to the adipocyte lineage. Although Zfp423 levels are essentially unchanged during adipogenesis, ectopic expression of Zfp423 in non-adipogenic murine cells is sufficient to activate expression of the gene encoding peroxisome proliferator-activated receptor γ (Pparγ; also known as Pparg) and increase the adipogenic potential of these cells [17, 19]. Zfp423 knockout mice feature impaired development of both white and brown adipose tissue [17, 19].

The activity of ZFP423 in adipose precursor cells is repressed by the intracellular and secreted mediator Wnt-inducible secreted protein 2 (WISP2). WISP2 production is significantly upregulated in the SAT of individuals with hypertrophic obesity, and is positively correlated to adipose cell size [20]. In the cytoplasm, WISP2 protein forms a complex with ZFP423 and prevents its translocation into the nucleus. Bone morphogenetic protein 4 (BMP4), a secreted protein and key regulator of the commitment of multipotent MSCs to the adipocyte lineage, dissociates this complex, allowing nuclear entry of ZFP423, thereby activating Pparγ transcript and commitment of precursor cells into the adipocyte lineage [12, 20].

Several studies have reported that epigenetic regulatory mechanisms are involved in the determination of multipotent precursor cells to form committed pre-adipocytes and the differentiation of pre-adipocytes to mature adipocytes [21]. Bioinformatic analysis of CpG islands in the promoter regions of obesity-related genes has identified regions with a high density of CpGs implicated in adipogenesis and inflammation, such as Pparγ, phosphatase and tensin homologue, leptin and tumour necrosis factor-α [22, 23]. Methylation of these CpG islands influences local chromatin structure and function, and participates in regulation of transcriptional activation of genes [24, 25].

Elucidation of the molecular mechanisms responsible for transcriptional regulation of Zfp423 may improve the understanding of restricted adipogenesis in hypertrophic obesity. Here, we investigated whether Zfp423 is epigenetically regulated and whether these events are involved in the restricted adipogenesis seen in humans with expanded subcutaneous adipose cells.
Methods

Media, sera, insulin, TRIZol and SuperScript III were obtained from Invitrogen (San Diego, CA, USA), rosiglitazone from Alexis (Grüningen, Germany) and 5-azacytidine, 3-isobutyl-1-methylxanthine and dexamethasone from Sigma-Aldrich (St Louis, MO, USA). pCpGfree-Lucia, Escherichia coli GT115 cells, and Luciferase reporter assay kit were from InvivoGen (San Diego, CA, USA), SYBR Green from Bio-Rad (Hercules, CA, USA) and the DNA Methylation Kit from Zymo Research (Orange, CA, USA). Micrococcal nuclease (MNase), Dam̅/Dcm̅ Escherichia coli cells and HpyCH41V, M.SsII, HhaI and HpaII enzymes were obtained from New England Biolabs (Ipswich, WI, USA). The DNA Purification Kit and pGEM-T Easy Vector were from Promega (Madison, WI, USA), the PCR Purification kit from Qiagen (Hilden, Germany), and the Big Dye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems (Foster City, CA, USA).

Cell culture and adipocyte differentiation Mouse embryonic fibroblasts (3T3-L1, NIH-3T3) were obtained from the American Type Culture Collection (Manassas, VA, USA). These mycoplasma-free cell lines were grown in DMEM with 10% FCS. For adipocyte differentiation, see electronic supplementary material (ESM) Methods.

Participants This study is a secondary analysis of participants from the European network on Functional Genomics of Type 2 Diabetes (EUGENE2) consortium [26]. Adipose tissue-derived stromal vascular fraction (SVF) cells were obtained from 20 healthy, non-obese individuals whose recruitment and clinical phenotyping has previously been described [26]. The study was approved by the appropriate Institutional Review Boards. All participants gave informed consent.

Adipose tissue biopsies were obtained from abdominal SAT. Following careful dissection, adipose cells were digested with collagenase for 45 min at 37°C. After digestion, the suspension was centrifuged to obtain two phases: an upper (mature adipocytes) and a lower (SVF cells) phase. Adipocyte size was measured according to previously described procedures [13, 16]. SVF cells, in which we analysed ZNF423 expression, were cultured in DMEM and Ham’s F-12 supplemented with 10% FBS and 0.002 mol/l glutamine as previously reported [13], in order to remove erythrocytes and inflammatory cells.

RNA isolation and quantitative real-time PCR RNA was isolated by TRIZol reagent according to the manufacturer’s protocol. RT-PCR of 1 μg of RNA was performed using SuperScript III. The cDNA obtained was used as a template for quantitative real-time PCR (qPCR), performed in triplicate using iQ SYBR Green Supermix on an iCycler real-time detection system (Bio-Rad). Relative quantification of gene expression was relative to the control (equal to 1) and was calculated according to the comparative 2−ΔΔCt method based on the cycle threshold (Ct) values of the target and housekeeping genes. The primers used for Ppia (also known as Cypa), Pparγ2, Fabp4 (also known as Ap2), Glut4 (also known as Slc2a4), Adipoq, Zfp423 and ZNF423 gene expression in qPCR are reported in the ESM Table 1.

MNase protection assay Nuclei were isolated from both NIH-3T3 (5 × 10^5 cells) and 3T3-L1 (5 × 10^5 cells) and digested with 200 U of MNase for 20 min at 37°C. The purified DNA was subsequently amplified by qPCR. The percentage of nucleosome occupancy across the analysed regions was quantified by the 2−ΔΔCt method using the undigested input as a normalising control, using NuPoP software (available from https://rdrr.io/bioc/NuPoP/; accessed November 2015).

DNA methylation assessment Genomic DNA was extracted using a DNA Purification Kit (Promega). Bisulphite treatment of extracted genomic DNA was performed using the EZ DNA Methylation Kit (Zymo Research). The bisulphite-converted genome was amplified by PCR using bisulphite-specific primers for Zfp423 and ZNF423 (see ESM Table 1 for the primers used). Bisulphite genomic sequencing was performed as previously reported [27]. DNA sequencing was performed on an ABI 3500 Automatic Sequencer using Big Dye Terminator v3.1 (Applied Biosystems). Bioinformatic analysis was carried out using EMBOSs CpGPlot (available from www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot/; accessed November 2015).

In vitro methylation and luciferase reporter assay The 5′-flanking region of Zfp423 (~1324 to ~764) was amplified by PCR and cloned into pCpGfree-Lucia (InvivoGen) luciferase reporter vector. Amplification of the reporter construct was performed using Dam̅/Dcm̅ E. coli cells. These cells were purchased from the New England Biolabs and are mycoplasma-free. The luciferase reporter vector was in vitro methylated by incubation with 1 unit/μg of M.SsIl enzyme (which methylates all CpGs) or 1 unit/μg of Hhal (which methylates the cytosines of the sequence GCGC) and HpaII enzymes (which methylate the cytosines in the sequence CCGG) at 37°C for 1 h. Fully methylated, unmethylated and partially methylated Zfp423 reporter vectors were transfected in NIH-3T3 cells. To normalise luciferase activity, a control plasmid encoding a Renilla luciferase gene was cotransfected into the cells. After 48 h, firefly and Renilla luciferase activity were assayed using a luciferase reporter assay kit (InvivoGen), according to the manufacturer’s instructions.

Site-directed mutagenesis and luciferase reporter assay Zfp423 promoter (~1037/~1002) was amplified by PCR and
cloned into the firefly luciferase reporter pCpGfree-promoter-Lucia vector (InvivoGen). A one-step PCR-based mutagenesis technique was used to generate site-specific mutation [28] and produce a mutated construct. One complementary pair of primers was designed that contained the desired mutation, replacing the cytosine at position −1016 with adenine. The wild-type and mutated constructs were transformed into E. coli GT115 cells. These cells were purchased from InvivoGen and are mycoplasma-free. In vitro methylation was performed using M.SssI methyltransferase following the manufacturer’s protocol (New England Biolabs). Unmethylated wild-type and mutated constructs were obtained in the absence of M.SssI. Methylation was confirmed by resistance to HpyCH4IV digestion (New England Biolabs). After 48 h, firefly and Renilla luciferase activity were assayed using a luciferase reporter assay kit, as reported in the previous paragraph.

Statistical analysis All experiments were performed three times for each determination, and results are shown as mean ± SD. Values for p between datasets were determined by two-tailed, unpaired Student’s t test. Significant p values are indicated as ***p < 0.001, **p < 0.01 and *p < 0.05. Correlation analysis was calculated using Pearson’s correlation coefficient.

Results

Promoter methylation reduces Zfp423 expression in NIH-3T3 cells Using qPCR, we compared Zfp423 mRNA expression in NIH-3T3 and in 3T3-L1 cells and found it to be barely detectable in the former and high in the latter (p < 0.01) (Fig. 1a). Importantly, there was no sequence variation of the Zfp423 promoter in NIH-3T3 and 3T3-L1 cells (data not shown), suggesting that the differential expression observed had to be attributed to other mechanisms, including different epigenetic profiles. Furthermore, the expression of other key adipogenic marker genes was strongly silenced in NIH-3T3 cells (ESM Fig. 1).

To explore this, we subjected the Zfp423 promoter region to bioinformatic analysis. EMBOSS CpGplot revealed a large 560 bp CpGi upstream Zfp423 transcription start site, providing a potential basis for methylation control of Zfp423 expression. We analysed the methylation status of the Zfp423 CpGi by bisulphite sequencing in NIH-3T3 and 3T3-L1 cells, and found massive demethylation in the latter (methylation:15.3% vs 90.2% in NIH-3T3 cells; p < 0.001; Fig. 1b, c).

We then cloned the Zfp423 promoter into the luciferase reporter vector (pCpGfree-basic-Lucia), which was either treated with M.SssI methylase and fully methylated, or partially methylated using HhaI and HpaII methylases. Digestion with the methylation-sensitive restriction enzyme HpyCH4IV enabled control of methylation level in the two conditions (data not shown). Luciferase activity in the constructs harbouring the fully and the partially methylated Zfp423 promoters declined by 80% and 40%, respectively, compared with the unmethylated promoter (p < 0.001) (Fig. 1d). These results demonstrate that methylation regulates Zfp423 promoter function in vitro.

Nucleosome occupancy of Zfp423 promoter is increased in NIH-3T3 compared with 3T3-L1 cells Based on NuPoP analysis, the Zfp423 promoter exhibited several potential regions where nucleosome positioning featured a high prediction score (Fig. 2a), suggesting that differential Zfp423 expression in NIH-3T3 and 3T3-L1 cells is also accompanied by a variation in nucleosome occupancy. To validate this and assess nucleosome occupancy at the best predicted regions, an MNase protection assay was performed and nucleosome positioning checked in mono-nucleosomal DNA by qPCR (Fig. 2b). The percentage of nucleosome occupancy at two such regions of the Zfp423 promoter was significantly higher in NIH-3T3 cells (nucelose [NUC]1% occupancy: 72.2 vs 51.5 in 3T3-L1 cells, p < 0.01; NUC2% occupancy: 94.6 vs 46.4 in 3T3-L1 cells, p < 0.001; Fig. 2c). No significant difference was observed in the CTRL R (negative control) region, where nucleosome positioning featured a low bioinformatic prediction score. Thus, in these cells, nucleosome occupancy of the promoter inversely correlates with Zfp423 expression, suggesting that dynamic chromatin remodelling may also contribute to transcriptional regulation.

5-Azacytidine enhances Zfp423 expression and allows differentiation of non-adipogenic NIH-3T3 cells To assess whether DNA methylation also regulates Zfp423 expression in intact cells, we investigated the ability of the DNA methyltransferase inhibitor 5-azacytidine to remove the transcriptional block imposed on Zfp423 in the NIH-3T3 cells. Incubating the cells with 5-azacytidine mainly affected methylation level at CpG position −1016 (40% methylation in exposed vs 90% in unexposed cells, p < 0.01; Fig. 3a, b); this was associated with a sixfold increase in Zfp423 mRNA expression (p < 0.01; Fig. 3c). However, apart from the −1016 CpG, the overall methylation profile at the Zfp423 promoter did not change in 5-azacytidine-treated cells (data not shown), providing a potential explanation for why mRNA expression levels are still lower than in 3T3-L1 cells (Fig. 3c). MNase protection studies revealed that 5-azacytidine significantly reduced nucleosome occupancy at the NUC1 and NUC2 regions (p < 0.001; Fig. 3d), further underlining the potential role of chromatin remodelling of the Zfp423 regulatory region in transcriptional regulation.

In parallel, 5-azacytidine robustly enhanced expression of Pparγ and the differentiation markers Fabp4, Adipoq and Glut4 after induction of differentiation (Fig. 4a), accompanied
by greater than twofold increased cytoplasmic accumulation of Oil Red O (Fig. 4b, c). Thus, in parallel with transcriptional activation, Zfp423 promoter demethylation by 5-azacytidine also promoted differentiation of the non-adipogenic NIH-3T3 cell line.

**BMP4 promotes Zfp423 expression by inducing promoter demethylation**

BMPs are members of the transforming growth factor superfamily that play a key role in inducing adipocyte precursor cell commitment towards the adipogenic lineage; however, the molecular details of their action have been only partially elucidated. Adding BMP4 to the culture medium of NIH-3T3 cells enhanced the expression of both Zfp423, by almost fivefold ($p < 0.001$; Fig. 5a), and its downstream target gene Ppara ($p < 0.01$; Fig. 5b), while Zfp423 expression was only slightly reduced, albeit not reaching significance, by BMP2 treatment ($p = 0.118$; Fig. 5a). Bisulphite sequencing revealed that this BMP4-dependent change was accompanied by an almost threefold reduction in methylation at position $-1016$ in the Zfp423 promoter ($p < 0.01$), reminiscent of that seen with 5-azacytidine treatment (Fig. 5c, d).

We therefore aimed to establish the functional significance of the $-1016$ dinucleotide for Zfp423 promoter function by site-directed mutagenesis. After replacing the cytosine at position $-1016$ with adenine, the 35 bp fragment ($-1037$ to $-1002$) of the Zfp423 promoter was cloned in a luciferase reporter construct. Relative luciferase activity was normalised against the activity of a cotransfected internal vector. Results are the mean ± SD of three independent experiments. Statistical significance was tested by two-tailed Student’s $t$ test (**$p < 0.01$ vs unmethylated vector, $p < 0.05$ vs partially methylated vector).
reporter vector (pCpGfree-promoter-Lucia). Luciferase activity was assayed in NIH-3T3 cells transfected with either the in vitro methylated or the unmethylated promoter. As shown in Fig. 5e, the −1016 mutation did not affect the activity of the unmethylated Zfp423 promoter. However, it abolished the effect of methylation on promoter silencing, indicating that the −1016 dinucleotide modulates Zfp423 transcription in vitro.

Pre-adipocyte ZNF423 expression correlates with mature subcutaneous adipose cell size in humans To explore the significance of Zfp423 expression in the development and function of human adipose tissue, we analysed transcription of human ZNF423 (the human orthologue of Zfp423) [29] in pre-adipocytes from the SVF of 20 healthy, non-obese individuals. Participants were recruited as previously described [26] (see Table 1 for their clinical characteristics). The size of the subcutaneous adipose cells varied over a broad range even in these non-obese individuals, consistent with different adipogenic potential of the precursor cells. ZNF423 mRNA was detectable in cells from all donors, and, importantly,
expression in pre-adipocytes exhibited a significant negative correlation with the size of mature subcutaneous adipose cells from the same individuals (Fig. 6; \( r = -0.5258, p < 0.05 \)). Thus, low ZNF423 expression in adipose precursor cells is a marker of the donor’s subcutaneous adipocyte cell size, and thus adipogenesis and the development of inappropriate adipose cell hypertrophy, and associated with an insulin-resistant phenotype.

The central enhancer CpGi at the human ZNF423 locus features \( >80\% \) homology in mammals [29] and, based on site-specific mutagenesis studies in leukaemia cells, has been shown to be relevant for the functional regulation of both \( \alpha \) and \( \beta \) ZNF423 promoters [29]. In human pre-adipocytes, we observed that ZNF423\( \alpha \) was the predominant isoform, while ZNF423\( \beta \) was barely detectable (ESM Fig. 2).

To further explore the mechanisms determining ZNF423 expression in SVF pre-adipocytes, we performed bisulphite sequencing of the entire enhancer CpGi in three individuals who had the smallest sized subcutaneous adipocytes, and an equal number of individuals exhibiting the largest adipocyte size. All individuals were non-obese and had a similar BMI. This revealed massively increased methylation levels at two subregions of the CpG enhancer island in the latter individuals (Fig. 7a). The R2 subregion showed \( >90\% \) methylation at CpG dinucleotides 30 and 33 in participants with subcutaneous adipocyte hypertrophy, compared with \(<20\% \) in those with smaller adipocytes (Fig. 7b). The R3 subregion revealed that methylation at CpG nucleotides 54, 55, 56, 57, 60 and 62 is \( >90\% \) and \(<45\% \) respectively, in individuals with and without adipose cell hypertrophy. In addition, 5-azacytidine treatment of pre-adipocytes from individuals with adipose cell hypertrophy led to a greater than twofold increase in expression of ZNF423 (Fig. 7c) and its downstream target gene PPARG (Fig. 7d). 5-Azacytidine strongly decreased the methylation level at CpG positions 54, 55, 56, 57, 60 and 62 of the R3 subregion, consistent with the important role of these CpGs in regulating ZNF423 expression (Fig. 7e). Methylation at regions R1 and R2 was not affected by 5-azacytidine (data not shown).

Taken together, these data indicate a non-permissive transcriptional state at the ZNF423 locus in SVF precursor cells from individuals who develop inappropriate subcutaneous adipocyte cell hypertrophy, a marker of impaired adipogenesis.

**Discussion**

Previous studies have demonstrated the importance of the Ppar\( \gamma \) transcriptional activator ZFP423 in regulating pre-adipocyte determination [17], and shown that Zfp423 expression identifies committed pre-adipocytes [19]. Thus, Zfp423 is crucial for the initial formation of white adipocytes and, importantly, also plays a later role in maintaining the energy-storing phenotype of white adipose cells [18]. Epigenetic mechanisms have been linked to the transcriptional regulation of Zfp423 exerted by ZFP521. ZFP521 binds the promoter and intronic regions of Zfp423 and represses its expression by promoting histone modifications. These findings support growing evidence that lineage determination of multipotent
MSCs to the adipocyte lineage is also epigenetically regulated [30]. Consistent with this, we show here that Zfp423 was transcribed in 3T3-L1 pre-adipose cells but not in NIH-3T3 non-pre-adipose fibroblasts. Furthermore, we identified a large CpGi at the Zfp423 promoter and report, for the first time, that Zfp423 expression in 3T3-L1 cells is accompanied by an extensive demethylation of this Zfp423 region, followed by decreased nucleosome occupancy.

This implicated a causal relationship between the different epigenetic profile and Zfp423 transcription in 3T3-L1 and NIH-3T3 cells. Consistent with this, we found no DNA sequence variation at the Zfp423 promoter in the two cell types. Luciferase assays provided formal proof that methylation directly represses Zfp423 promoter function in vitro. In addition, exposure to the demethylating agent 5-azacytidine simultaneously caused Zfp423 promoter demethylation and rescued Zfp423 transcription in intact NIH-3T3 fibroblasts. Thus, promoter methylation is an important regulator of the differential transcription of Zfp423 in non-pre-adipose and pre-adipose fibroblasts.

DNA methylation inhibits gene expression by at least two mechanisms. First, cytosine methylation may directly inhibit
the association of DNA-binding factors [31, 32]. Second, proteins that recognise methylated CpG sites may recruit transcriptional corepressor molecules, including histone modification and chromatin remodelling enzymes, and cause a transcriptionally repressed chromatin state [31, 32]. Here, MNase digestion assays revealed that, in the pre-adipose 3T3-L1 fibroblasts, CpG demethylation of the Zfp423 promoter was accompanied by nucleosome repositioning in an open chromatin state, which may contribute to Zfp423 active transcription. The ability of 5-azacytidine to induce this nucleosome repositioning suggests that, in NIH-3T3 cells, demethylation of the Zfp423 promoter may trigger chromatin remodelling in a transcriptionally active conformation, thereby inducing Zfp423 expression. Therefore, although we did not investigate a direct association of transcriptionally relevant DNA-binding factors to methylated cytosines, CpG methylation-triggered chromatin condensation appears to be an important mechanism for maintaining methylated Zfp423 promoter silencing.

Previous studies have demonstrated that Zfp423 transcription is essential for pre-adipocyte commitment, enabling further adipogenic differentiation [17–19]. In line with this, we showed that the effect of 5-azacytidine on Zfp423 promoter epigenetics and active gene transcription was followed by rescue of the differentiation capacity of the NIH-3T3 fibroblasts, as revealed by a robust rise in Pparγ, Fabp4, Adipoq and Glut4 levels. Oil Red O accumulation in NIH-3T3 cytoplasm was also increased following exposure to 5-azacytidine. Therefore, in the model we now propose, commitment of an adipocyte precursor cell is accompanied by acquisition of a specific chromatin epigenetic signature of the Zfp423 locus [17, 33]. Importantly, as shown here, these events appear to be reversible. Indeed, exposure of the uncommitted NIH-3T3 cell to an epigenetic agent, i.e. 5-azacytidine, partly reprogrammed the epigenetic signature at the Zfp423 promoter, favouring commitment and adipogenesis. However, 5-azacytidine does not make the NIH-3T3 cells an in vitro model of spontaneous adipogenesis (data not shown). This is not surprising because Zfp423, identified as a major determinant of pre-adipocyte commitment, is not responsible for the early phase of adipogenesis. At this stage, only ectopic expression of CCAAT/enhancer-binding protein β provides a surrogate for the requirement for 3-isobutyl-1-methylxanthine in the adipogenic differentiation of NIH-3T3 cells [34]. It is possible that further work will generate novel opportunities to overcome the restricted subcutaneous adipogenesis that is predictive of type 2 diabetes.

Studies by Bowers and co-workers [35] have demonstrated stem cell commitment to the adipocyte lineage by 5-azacytidine inhibition of DNA methylation. They also provided evidence supporting the role of BMP4 signalling in adipocyte lineage determination induced by 5-azacytidine [36]. Additional studies have revealed that increased expression and secretion of BMP4, a key molecule in the adipogenic microenvironment as it is also secreted by mature adipose cells [37], correlate with the capacity of MSCs to undergo adipogenic differentiation [36, 38]. Importantly, BMP4 has been shown to enable nuclear entry of ZFP423 by dissociating the cytoplasmic WISP2–ZFP423 protein complex, which retains ZFP423 in the cytosol [20], thereby activating Pparγ transcription. Silencing of Zfp423 completely prevents the induction of Pparγ and other adipogenic marker genes in BMP4-treated cells, showing that ZFP423 is crucial for Pparγ activation and for the ability of BMP4 to induce Pparγ transcription [20].

Here, we demonstrate, for the first time, that BMP4 also causes demethylation of the Zfp423 promoter, which is sufficient to commit otherwise non-adipogenic cells to the adipogenic lineage. Thus, convergence of BMP4 signalling on Zfp423 enables its action on pre-adipocyte determination.
through multiple mechanisms, including epigenetic modifications at key genes and nuclear import of ZFP423. Interestingly BMP2, a BMP4 homologue, only slightly reduces Zfp423 expression in NIH-3T3 cells, probably because, as previously reported [39, 40], the BMP2 target and Zfp423 inhibitor ZFP521 is expressed in these cells. Addison et al have reported that BMP2-induced commitment of MSCs to the adipose lineage is likely to be suppressed by ZFP521 through direct inhibition of Zfp423, providing a potential explanation for why BMP2 responses are predominantly osteogenic [30].

Detailed analysis of BMP4 action on Zfp423 transcription revealed that BMP4-induced demethylation selectively involved the CpG dinucleotide at position −1016 from the Zfp423 transcription start site. Interestingly, methylation at this site was invariably inhibited after 5-azacytidine treatment of NIH-3T3 cells. These novel findings suggest a functional relevance of the −1016 dinucleotide. Subsequent mutagenesis experiments demonstrated that, while not affecting the in vitro function of the demethylated Zfp423 promoter, the introduction of a point mutation at the −1016 CpG position prevented the methylation-dependent silencing of Zfp423 transcription. Accordingly, we suggest that the regulatory effect of methylation at the Zfp423 promoter is dependent not only on the quantitative dimension of the methylation events, but also on the specific promoter region that is affected.

Restricted adipogenesis in human SAT is determined by impaired adipocyte precursor cell commitment [12, 13, 16] and results in hypertrophy of adipocytes in the SAT [17, 41]. Our recent studies have shown that markers of restricted subcutaneous adipogenesis with inappropriate adipocyte cell hypertrophy are associated with a family history of type 2 diabetes.

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**Fig. 7** DNA methylation at the ZNF423 promoter in SVF cells from non-obese individuals with subcutaneous adipocyte hypertrophy. Genomic DNA from pre-adipose SVF cells was obtained from three individuals analysed in Fig. 6 who exhibited the most extreme subcutaneous adipocyte hypertrophy (Hypertrophy) and three individuals with the smaller adipocyte size (Ctrl). Individual DNA preparations were exposed to bisulphite as described in Methods and individually analysed by PCR amplification of the CpGi at the ZNF423 enhancer region (−12.1 kb; −11.4 kb from ZNF423 gene transcription start site). PCR products were then individually cloned and sequenced. (a) Methylation of 65 CpGs at the three ZNF423 promoter regions (R1–R3) for 15 replica clones. White circles, unmethylated CpGs; black circles, methylated CpGs. A representative experiment with one individual with SAT hypertrophy and one control individual is shown (n = 3). (b) Quantification of the methylation levels at eight specific CpGs in individuals with the smallest size of subcutaneous adipocytes (white bars) or the largest adipocyte size (black bars). Error bars represent SD from three individuals per group. Statistical significance was tested by two-tailed Student’s t tests (***p < 0.01, ****p < 0.001 vs individuals with smallest adipocytes). (c, d) Expression of ZNF423 and PPARγ mRNA was measured by qPCR. Results are the mean ± SD from three independent experiments. Statistical significance was analysed by two-tailed Student’s t tests (***p < 0.01, ****p < 0.001 vs SVF cells). (e) Methylation at the R3 region on ZNF423 promoter for ten replica clones. White circles, unmethylated CpGs; black circles, methylated CpGs; representative of three individual experiments with 5-azacytidine (AZA)-treated or untreated from one individual with SAT hypertrophy.
specificity [16]. However, as demonstrated in the leukaemia adipocyte precursor cells, this is different, probably reflecting tissue which are targeted by methylation events in leukaemia and cells [29]. The position of the regulatory CpG dinucleotides, previously been shown to silence the gene in human leukaemia ZNF423 cells, methylation at the pre-adipocyte ZNF423 central enhancer or signalling). Expansion of this work may generate tissue microenvironment (e.g. changes in BMP4 abundance). Pertrophy rescues both the hypomethylated and the permisive state at specific CpG enhancer region dinucleotides, as well as ZNF423 expression.

Taken together, our present results suggest that the restricted subcutaneous adipogenesis associated with insulin resistance [6] and a family history of type 2 diabetes [16, 42, 43] may be due to dysfunctional epigenetic regulation rather than conventional DNA risk genes.

Secretion of BMP4 by mature adipose cells is positively correlated with adipose cell size, and we have suggested that this is part of positive feedback in the tissue to enhance the commitment and differentiation of new precursor cells to prevent inappropriate hypertrophy [37, 44]. Here, we provide a molecular basis for the effect of BMP4 in enhancing adipogenesis, although secretion of BMP4 antagonists, in particular Gremlin 1 in humans [37], is increased in hypertrophic obesity and prevents the expected positive effect of BMP4 on adipogenesis.

The overall structure of the regulatory regions of human ZNF423 and mouse Zfp423 is quite different [29]. However, we observed massive hypermethylation at distinct CpG dinucleotides in the central island serving as a promoter enhancer in human ZNF423. Hypermethylation of this island has previously been shown to silence the gene in human leukaemia cells [29]. The position of the regulatory CpG dinucleotides, which are targeted by methylation events in leukaemia and adipocyte precursor cells, differ, probably reflecting tissue specificity [45]. However, as demonstrated in the leukaemia cells, methylation at the pre-adipocyte ZNF423 central enhancer island may also feature a repressive function as its presence closely correlated with reduced ZNF423 expression in the adipocyte precursor cells. We propose, therefore, that changes in the methylation profile at the regulatory region account for the reduced ZNF423 expression observed in hypertrophic adipose tissue. Indeed, 5-azacytidine treatment of pre-adipocytes isolated from individuals with adipose cell hypertrophy rescues both the hypomethylated and the permissive state at specific CpG enhancer region dinucleotides, as well as ZNF423 expression.

Thus, based on our findings, methylation at the ZNF423 regulatory region and its expression can be targeted both pharmacologically (e.g. 5-azacytidine) and by changes in the adipose tissue microenvironment (e.g. changes in BMP4 abundance or signalling). Expansion of this work may generate attractive and novel opportunities to overcome the restricted subcutaneous adipogenesis and prevent inappropriate adipose tissue hypertrophy and its negative consequences on metabolism and risk of type 2 diabetes.

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Data availability Data supporting the findings of this study are available upon request from the corresponding author.

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Contribution statement ML and FZ designed, performed, analysed, interpreted and described the experiments; they also contributed to writing the manuscript. GAR was in charge of daily supervision of the work and contributed to discussion and data analysis, along with ML, LP, AD, RS, CN, PM and FF contributed to the acquisition of data. AH, SH and JMH were in charge of the human studies, acquisition of adipose tissue biopsies, isolation of SVF pre-adipocytes and acquisition of human data. CM and PF contributed to discussion and data analysis. US and FB conceived and designed the study, supervised the work and contributed to the writing of the paper. All authors critically revised and approved the final version the manuscript. FB is the guarantor of the manuscript and takes responsibility for the integrity of the work as a whole.

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