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Fatty Acid Binding Protein FABP4 Mechanistically Links Obesity with Aggressive AML by Enhancing Aberrant DNA Methylation in AML Cells

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Abstract

Obesity is becoming more prevalent worldwide and is a major risk factor for cancer development. Acute myeloid leukemia (AML), the most common acute leukemia in adults, remains a frequently fatal disease. Here, we investigated the molecular mechanisms by which obesity favors AML growth and uncovered the fatty acid binding protein 4 (FABP4) and DNA methyltransferase 1 (DNMT1) regulatory axis that mediates aggressive AML in obesity. We showed that leukemia burden was much higher in high-fat diet-induced obese mice, which had higher levels of FABP4 and IL-6 in sera. Upregulation of environmental and cellular FABP4 accelerated AML cell growth in both a cell-autonomous and cell-non-autonomous manner. Genetic disruption of *FABP4* in AML cells or in mice blocked cell proliferation *in vitro* and induced leukemia regression *in vivo*. Mechanistic investigations showed that FABP4 upregulation increased IL-6 expression and STAT3 phosphorylation leading to DNMT1 overexpression and further silencing of the $p15^{INK4B}$ tumor suppressor gene in AML cells. Conversely, *FABP4* ablation reduced DNMT1-dependent DNA methylation and restored $p15^{INK4B}$ expression, thus conferring substantial protection against AML growth. Our findings reveal the FABP4/DNMT1 axis in the control of AML cell fate in obesity, and suggest that interference with the FABP4/DNMT1 axis might be a new strategy to treat leukemia.

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Author contributions

B.L. and S.J.L. conceived ideas and designed the experiments. F.Y., N.S., J.X.P., Y.W.Z., S.C.W. and E.Y.R. performed experiments; A.A. and M.R.L. provided the leukemia patient samples; D.E.Z provided the AML1/ETO9a mouse model and critically reviewed the paper; F.Y., A.M. B., A.A., M.R.L., B.L. and S.J.L. analyzed data and wrote the paper; F.Y. carried out the biostatistical analysis; S.J.L. oversaw the entire research project.

Introduction

Acute myeloid leukemia (AML) represents one of the most common acute leukemia in adults and remains a fatal disease for most patients, and yet the causes and risk factors for AML leukemogenesis are largely undefined. One factor that appears to play a prominent role in AML pathogenesis is aberrant DNA methylation, which is attributed to upregulation of DNA methyltransferases (DNMTs). Consistently, enforced *DNMT* expression induces genome-wide DNA hypermethylation,[1, 2] whereas *DNMT* deletion leads to a decrease of DNA methylation and re-expression of tumor suppressor genes (TSGs).[3–5] While DNMT-dependent DNA methylation is partially and cell-autonomously regulated by the Sp1/NFκB-*miR-29b* network,[3, 4] nucleolin[2] or AML1/ETO[6] in AML cells, mounting evidence indicates that epigenetic aberrations can arise as a consequence of environmental factors.[7, 8] This offers a plausible mechanism that environmental factors can modify cancer risk and tumor behaviors. However, the identification and mechanism as to how environmental factors alter the epigenetic landscape in AML cells remain elusive.

The excessive intake of saturated fatty acid (SFA) results in the development of obesity, a chronic disease that is strongly associated with alterations in the physiological function of adipose tissues. The high serum SFA in obesity has been shown to induce inflammation, a key factor in cancer development.[5, 9–12] Accordingly, obesity increases the incidence and mortality rate of many cancers, including AML.[13–15] However, the mechanisms underlying obesity-AML association are unclear. The fatty acid-binding proteins (FABPs) are highly conserved cytosolic intracellular receptors that can reversibly bind hydrophobic ligands, such as saturated and unsaturated fatty acids,[16, 17] thus coordinating lipid trafficking and responses in cells. Among the nine family members, FABP4 represents the best characterized metabolic biomarker and is the most strongly related to fat mass. It is highly expressed in adipocytes/macrophages of obese patients,[18] suggesting a role in metabolic deterioration.[19] FABP4 is also expressed at a higher level in cancer cells and its upregulation promotes tumor growth for largely unknown reasons.[20, 21] We speculated that in obesity, excess caloric intake results in excessive FABP4 production and subsequent DNA hypermethylation, leading to epigenetic silencing of TSGs thereby fueling rapid leukemia growth. We have now endeavored to test this hypothesis and demonstrated that a FABP4/IL-6/STAT3/DNMT1 cascade mechanistically links dietary-induced obesity to an aggressive AML.

Materials and Methods

Plasmids, reagents, cell lines and patient samples

Details are in Supplementary Materials and Methods. All patients signed an informed consent document approved by the Mayo Clinic Institutional Review Board before entering the study.

Cytospin/Wright-Giemsa staining, cell differentiation assays, immunosorbent analysis, DNA Dotblotting, bisulfite sequencing and reporter assays

Details are in Supplementary Materials and Methods.

Western blot, RNA isolation, cDNA preparation and qPCR

Details are in Supplementary Materials and Methods.

Animal studies

All animal experiments were approved by the Institutional Animal Care and Use Committees of the University of Minnesota and were in accordance with the U.S. National Institutes of Health (NIH) Guide for Care and Use of Laboratory Animals. Details are in Supplementary Materials and Methods.

Statistical analysis

All the graphs were generated using the Student's t test, but the Kaplan-Meier survival curves were created by the log-rank test. Correlation data were acquired using the Pearson correlation coefficients. Details are in Supplementary Materials and Methods.

Results

Dietary-induced obesity accelerates AML progression *in vivo*

To experimentally determine the impact of dietary-induced obesity on leukemia, we initially fed C57BL/6 mice (n=6) either HFD or LFD for 12 weeks. The mice fed a HFD became obese with significantly higher body weight (48.1 ±6.3 g versus 29.2 ±4.0 g, $P<0.05$) and a remarkable accumulation of white adipose tissue (not shown) compared to the mice fed a LFD. Then the lean and obese mice were challenged with C1498 cells (2×10^6), a mouse AML cell line that is syngeneic to C57BL/6 mice,[6] for the development of leukemic disease. Compared to their lean counterparts, the obese mice developed significantly larger spleens (44% increase; Supplementary Figure 1a) and had more aggressive leukemic growth on the lung surface evidenced by a 4.8-fold increase in metastatic foci (Supplementary Figure 1b,c). Giemsa-stained BM showed massive infiltration of blasts in leukemia-bearing obese mice with an 83% reduction of post-mitotic cells (Supplementary Figure 1d) compared to lean mice. The smaller number of mature BM was further evidenced by the lower levels of CD11b expression in obese compared to lean mice (23.5 ±4.4% versus 54.9 ±8.7%, $P<0.05$). In agreement with these pathological results, leukemia-bearing obese mice lived significantly shorter than lean controls (Supplementary Figure 1e). To establish the relevance of our discoveries to human leukemia, we analyzed 49 elderly patients (age ≥60) with AML and normal cytogenetics. Obesity was considered present if BMI was ≥30. We observed a trend towards shorter median overall survival in obese AML patients (Supplementary Figure 1f), consistent with a recent report[15] disclosing the strongest associations of AML patients with class II/III obesity (≥35 kg/m^2), but not in overweight patients. These findings support the idea that obesity facilitates leukemia cell proliferation and increases the risk of leukemia mortality.

Host FABP4 upregulation accounts for obesity-accelerated leukemia growth

Because FABP4 upregulation promotes tumorigenesis,[20, 22] we proposed that a more aggressive leukemia in obesity could result from FABP4 overproduction. To test this idea, we initially measured plasma Fabp4 levels in obese and lean mice. Western blot

quantification revealed an approximate 4-fold increase of serum Fabp4 in obese compared to lean mice (n=5, $P<0.05$), and serum ELISA results disclosed that Fabp4 levels were 20.6 ±0.54 ng/ml in lean, but 77.7 ±0.43 ng/ml in obese mice (n=5, $P<0.001$), supporting that FABP4 levels increase during obesity.[23–25] Further, ELISA results showed that serum FABP4 levels in AML patients (n=30) range from 12.25 to 112.58 ng/ml, displaying considerable variation that might be caused by multiple factors, such as BMI and/or the stage of leukemia at which the disease was diagnosed. Based on these results, MV4-11, C1498, Kasumi-1, THP-1 or SKNO-1 cells were treated with mouse or human FABP4 proteins at 10, 30, 100 and 200 ng/ml, to mimic the effects of increasing FABP4 expression in clinically relevant settings. Addition of FABP4 protein dose-dependently increased AML cell proliferation (Figure 1a). Importantly, treatment with FABP4 in AE9a mouse[26] or human AML patient primary cells led to a dramatic increase of colony number (Figure 1b,c), collectively, suggesting that the FABP4 protein alone is sufficient to augment leukemia growth.

Because many factors in obesity are possibly altered, we utilized a *Fabp4* knockout (*Fabp4*$^{-/-}$) mouse model to further determine the specificity of host FABP4 as an AML enhancer in obesity. Notably, under standard housing conditions with a chow diet, no significant differences in body weight, food intake or behaviors between *Fabp4*$^{-/-}$ and *Fabp4*$^{+/+}$ mice were observed. When engrafted with C1498 cells (2×10^6), *Fabp4* deficiency in mice greatly reduced leukemic disease burden, in contrast to the effects of treatment with the FABP4 protein. This reduction was supported by decreased white blood cell counts (432 ±83×10^6/ml versus 223 ±31×10^6/ml, $P<0.05$), deceased splenomegaly (756 ±76 versus 355 ±41 mg; Figure 1d and Supplementary Figure 2a), less aggressive leukemia growth in lung (18.3 ±2.5 versus 4.7 ±2.1; Figure 1e and Supplementary Figure 2b) and in liver (16.0 ±2.0 versus 5.3 ±1.5; Figure 1f and Supplementary Figure 2c), compared to *Fabp4*$^{+/+}$ sufficient mice. The BM histopathology from *Fabp4*$^{-/-}$ mice identified a higher degree of post-mitotic cells containing metamyelocytes, bands and segmented neutrophils compared to *Fabp4*$^{+/+}$ mice (89 ±11% versus 53 ±7%; Figure 1g and Supplementary Figure 2d). The better prognosis in *Fabp4*$^{-/-}$ compared to *Fabp4*$^{+/+}$ leukemia mice was further supported by a longer survival time (Figure 1h). These findings suggest that FABP4 has a cell nonautonomous role in obesity-promoted leukemia.

Ectopic FABP4 expression contributes to AML cell proliferation

While adipocytes/macrophages have been thought to be major resources of FABP4 production, our results revealed that leukemia cell lines, such as SKNO-1, MV4-11, K562, Kasumi-1, NB4, THP-1 and C1498, highly expressed FABP4, compared to U937 (macrophage-like), 293T or BM from *Fabp4*$^{-/-}$ mice (Supplemental Figure 3a,b). The relatively high FABP4 expression supports its impacts on leukemia cell proliferation. To further explore this idea, *FABP4* expression was knocked down in C1498, MV4-11 and Kasumi-1 cells. The colony-forming assays revealed that the *FABP4*-depleted cells formed significantly fewer colonies compared to scramble-transfected cells (Figure 2a). Such *in vitro* blockage of proliferation by cellular *FABP4* loss motivated us to pursue the growth potential of *FABP4*-depleted cells *in vivo*. Thus, C1498 cells (0.5×10^6) transfected with *Fabp4* or scrambled siRNA were intravenously injected into C57BL/6 mice (n=10).

Compared to the scramble-transfected group, C57BL/6 mice injected with *Fabp4*-depleted cells exhibited decreased splenomegaly (745 ±82 versus 387 ±52 mg; Figure 2b and Supplementary Figure 3c), and remarkable lessening of leukemic growth in lung (13.7 ±2.5 versus 2.7 ±1.5; Figure 2c and Supplementary Figure 3d) and in liver (15.0 ±3.6 versus 3.0 ±1.1; Figure 2d and Supplementary Figure 3e). Giemsa-stained BM from mice engrafted with *Fabp4*-depleted cells presented a greater percentage of post-mitotic cells than controls (56 ±6% versus 5 ±1%; Figure 2e and Supplementary Figure 3f). In addition, the mice inoculated with *Fabp4*-depleted cells had a significant increase in survival time (Figure 2f). To establish the clinical relevance of these findings, we analyzed AML patients from GSE12417[27] and GSE16432.[28] In contrast to the cellular *FABP4* loss, AML patients with higher *FABP4* had significantly shorter survival time (Figure 2g,h), supporting that FABP4 cell-autonomously regulates AML cell growth.

FABP4 upregulates *DNMT1* gene expression

DNMT1 overexpression frequently occurs in AML,[2, 29] which suggest a possible association of DNMT1 levels with AML pathogenesis. Indeed, the results from GPL570 of GSE12417[27] (Supplementary Table 2) suggested that *DNMT1* upregulation in AML patient cells determined by gene-expression arrays corresponded with short overall survival (Figure 3a), although DNMT1 expression measured by *in situ* hybridization did not identify *DNMT1* abundance as a prognostic marker.[30] Given that TSG silencing by DNA methylation confers a growth advantage to leukemia cells, we hypothesized that FABP4-promoted AML growth in obesity occurs through *DNMT1*-dependent DNA methylation in AML cells. First, we showed that *DNMT1* was higher in BM from obese than lean mice (Figure 3b, left), supporting that fat/dietary intake plays a part in controlling DNA methylation. Second, comparing *DNMT1* levels in *Fabp4*$^{-/-}$ mice (Figure 3b, right) or stable *Fabp4*-depleted macrophages (Supplementary Figure 4a) with their wild-type counterparts, *Fabp4* loss resulted in DNMT1 downregulation. We then mimicked FABP4 abnormality in obesity by adding the FABP4 protein to culture medium. FABP4 treatment increased *DNMT1* RNA and protein expression in C1498, MV4-11, Kasumi-1, SKNO-1 and THP-1 (Figure 3c, Supplementary Figure 4b), in human patient and mouse AML primary cells (Figure 3d) and in C1498 cells from obese and *Fabp4*$^{+/+}$ mice (Figure 3e). These results revealed the contribution of environmental FABP4 to *DNMT1* expression in AML cells. Further, siRNA-triggered depletion of *FABP4* in C1498, MV4-11 and Kasumi-1 led to a dramatic *DNMT1* downregulation at the RNA (not shown) and protein (Figure 3f) levels, which was independently confirmed by shRNA-abrogated *FABP4* expression (Supplementary Figure 4c). Importantly, *FABP4* knockdown caused *DNMT1* suppression in AML patient cells (Figure 3g). The positive role of FABP4 in *DNMT1* regulation was further supported by the results of GSE12417[27] analysis showing that *DNMT1* expression paralleled the *FABP4* expression in AML patients (Figure 3h). Thus, FABP4 mechanistically links obesity to DNMT1 aberrations in AML cells.

FABP4 regulates DNMT1 expression in an IL-6-dependent manner

No evidence has been produced showing that FABP4 is a transcriptional factor, suggesting that FABP4 indirectly governs the *DNMT1* gene. IL-6 has been found to be highly elevated in obesity,[31–33] which agrees with our result showing that obese mice had a 2.6-fold increase

in serum IL-6 compared to lean subjects (Figure 4a). However, the mechanisms underlying obesity-associated IL-6 overexpression remain elusive. Given that FABP4 is a master regulator of lipid homeostasis, and because GSE12417[27] analysis identified a positive correlation between *IL-6* and *FABP4* expression in AML patients (Figure 4b), FABP4 could be an activator of IL-6 signaling. In fact, treating C1498, MV4-11 and Kasumi-1 cells with the obesity-relevant FABP4 protein led to a dose-dependent increase in *IL-6* expression (Figure 4c). In addition, C1498 cells engrafted into obese mice exhibited higher levels of *IL-6* compared to levels in lean mice (Figure 4d). In contrast, IL-6 expression was significantly suppressed in *FABP4*-knockdown cell lines (Figure 4e), in BM of *Fabp4*$^{-/-}$ mice (67% ±6% reduction) and in C1498 cells from *Fabp4*$^{-/-}$ mice (Supplementary Figure 5a), compared to their respective controls. Mechanistic studies showed that exposure to the FABP4 protein increased NFκB phosphorylation at Ser536 (Figure 4f), supporting FABP4 as a positive NFκB modulator.[34] When *NFκB* was specifically depleted by siRNA, FABP4-mediated *IL-6* upregulation was attenuated (Figure 4g). Thus, FABP4 enhances IL-6 production through active NFκB signaling. Finally, we found that *IL-6* overexpression was associated with shorter survival of AML patients[27, 35] (Supplementary Figure 5b) and accompanied by *DNMT1* upregulation or vice versa (not shown). The IL-6 protein increased, whereas *IL-6* depletion suppressed, DNMT1 expression (Supplementary Figure 5c). Further, *IL-6* knockdown diminished FABP4-mediated *DNMT1* upregulation (Supplementary Figure 5d). Thus, the FABP4/IL-6 axis appears to be responsible for DNMT1 deregulation in AML cells.

An activated STAT3 pathway is required for IL-6-mediated DNMT1 upregulation

Given that STAT3 is an IL-6 signaling downstream mediator[36] and functions as a transcription factor by tyrosine phosphorylation, we reasoned that IL-6 regulates *DNMT1* through the STAT3 pathway. Because FABP4 positively regulates IL-6 production, we first assessed the levels of STAT3 phosphorylation in BM of *Fabp4*$^{-/-}$ and *Fabp4*$^{+/+}$ mice. Western blot results revealed a significant decrease in STAT3 phosphorylation in *Fabp4*$^{-/-}$ mice (Supplementary Figure 6a). Similarly, *FABP4* knockdown in C1498, MV4-11 and Kasumi-1 disrupted STAT3 phosphorylation (Supplementary Figure 6b), whereas FABP4 protein treatment increased it (Supplementary Figure 6c). When exposed to the IL-6 protein, STAT3 became hyperphosphorylated (Supplementary Figure 6d), suggesting that the FABP4-IL-6 cascade controls STAT3 phosphorylation and thereby its translocation to the nucleus where it binds target DNA. Using bioinformatic analysis, we identified two putative STAT3 binding sites in the 5'-flanking region 1 kb upstream of the transcription start site of the *DNMT1* promoter (not shown). We then cloned the *DNMT1* promoter region containing STAT3 binding sites into a luciferase reporter (pGL3-*DNMT1*) construct. Dual luciferase assays revealed that *STAT3* overexpression significantly increased *DNMT1* promoter-driven reporter activity (Supplementary Figure 6e). This promoter transactivation might require STAT3 phosphorylation, because the STAT3 inhibitor NSC74859 dose-dependently repressed pGL3-*DNMT1* activities (Supplementary Figure 6f). Moreover, ectopic *STAT3* expression increased (Supplementary Figure 6g), whereas STAT3 inactivation by siRNA (Supplementary Figure 6h) or NSC74859 (Supplementary Figure 6i) decreased, the endogenous DNMT1 expression. Given that depletion of IL-6 and STAT3 abrogated *DNMT1* upregulation mediated by the FABP4 protein (Supplementary Figure 6j), these

results suggest that the IL-6/STAT3 pathway bridges the gap between FABP4 upregulation and DNMT1 overexpression in obesity-related AML.

FABP4 is required to maintain the DNA hypermethylation profile

Because changes in *DNMT1* gene abundance are positively correlated with alterations in DNA methylation levels,[1, 3, 37] our finding that FABP4 upregulates *DNMT1* led to the hypothesis that FABP4 is a DNA methylation modulator. To test this, we used Dotblot analysis,[2, 6] where the specificity of the 5mC antibody and the quantitative feature of Dotblot were verified by Dot-blotting results from standard cytosine and 5-methylcytosine (Supplementary Figure 7a). In line with the observation of FABP4 and DNMT1 overexpression in obesity, the DNA methylation levels were significantly higher in BM of obese mice compared to lean mice (n=3; Figure 5a). FABP4 treatment resulted in a significant increase of global DNA methylation in C1498, MV4-11, Kasumi-1, THP-1 and SKNO-1 (Figure 5b, Supplementary Figure 7b), human patient or mouse AML primary cells (Figure 5c) and C1498 cells engrafted in obese or *Fabp4*$^{+/+}$ mice compared to those from lean or *Fabp4*$^{-/-}$ subjects (Supplementary Figure 7c). In contrast, a reduction of DNA methylation was observed in *Fabp4*$^{-/-}$ mice, in macrophages with stable *Fabp4* depletion (Figure 5d) and in AML cell lines with *FABP4* knockdown by siRNA (Figure 5e) or by shRNA (Supplementary Figure 7d). These results supported the regulatory role of FABP4 in DNA methylation. Because the IL-6/STAT3 pathway links FABP4 production to DNMT1 expression, we examined the impact of IL-6/STAT3 on DNA methylation. As expected, DNA methylation was significantly increased in the presence of the IL-6 protein (Figure 5f) or after *STAT3* overexpression (Supplementary Figure 7e), whereas dysfunction of STAT3 and IL-6 resulted in DNA hypomethylation (Figure 5g, Supplementary Figure 7f). Notably, *DNMT1* ablation by siRNA led to DNA demethylation and impaired leukemia clonogenic potential (Supplementary Figure 7g–i), verifying the contribution of *DNMT1* expression to DNA methylation and cell proliferation. Thus, the FABP4/IL-6/STAT3 axis determines DNMT1-dependent DNA methylation in AML cells.

TSGs are usually silenced by promoter DNA hypermethylation and their repression predicts poor prognosis in AML. The *p15*^{INK4B} gene, an inhibitor of cyclin-dependent kinases (INK), is a TSG that plays a critical role in cell growth inhibition and differentiation. Given that *p15*^{INK4B} expression is detectable in myeloid leukemia, although its level is low, we postulated that FABP4 upregulation in obesity could induce further silencing of *p15*^{INK4B}, thus enhancing AML cell proliferation. Indeed, treatment with the FABP4 protein led to *p15*^{INK4B} downregulation in AML cell lines (Supplementary Figure 8a), in human and mouse primary cells (Supplementary Figure 8b) and in C1498 cells from leukemic obese or *Fabp4*$^{+/+}$ mice (Supplementary Figure 8c), compared to their respective controls. In contrast, siRNA-triggered *FABP4* depletion restored *p15*^{INK4B} expression (Supplementary Figure 8d). Bisulfite sequencing revealed that the FABP4 protein increased (Supplementary Figure 8e), whereas *FABP4* depletion decreased (Supplementary Figure 8f), *p15*^{INK4} promoter DNA methylation, indicating that FABP4 inactivates *p15*^{INK4B} through promoter DNA hypermethylation. Functionally, FABP4-enhanced colony formation was significantly attenuated once the activities of NFκB, STAT3 or DNMT1 were genetically or pharmacologically disrupted in FABP4-treated cells (Supplementary Figure 9a–e).

Collectively, these data support that FABP4 mediates the association of obesity with more aggressive disease through the NFκB/STAT3/DNMT1 cascade in AML cells.

Discussion

Obesity is well known to significantly increase the likelihood and mortality of many cancers, but the existing literature regarding the obesity-AML association shows mixed results, including neutral, negative and positive relationships.[13, 15, 38–41] Although multiple factors could result in such contradictory findings, possible causes could be the limitations of BMI as a predictive tool, because many AML patients could be classified into the “overweight” group,[41] or the use of the BMI as a criterion for excluding patients from aggressive therapy.[42] Because epigenetic modifications appear more sensitive to environmental stimuli, we targeted the interplay between abnormal lipid metabolism and deregulated DNA methylation to explore potential molecular links of obesity to AML. While debate continues, our data strongly support that 1) dietary-induced obesity is a positive risk factor enhancing AML progression; 2) the lipid chaperone FABP4 mechanistically bridges the gap between obesity and aggressive AML; and 3) the FABP4/IL-6/STAT3/DNMT1 cascade links dietary intake to the DNA methylation program in AML cells (Supplementary Figure 10), thus serving as a novel therapeutic target in AML and, potentially, other types of cancer.

Although tremendous efforts have been made in understanding the obesity-cancer association, the underlying molecular mechanisms are still poorly understood. Previous studies showed that the microbe-related DCA–SASP axis[43] and the PI3K/Akt/mTOR cascade[44] possibly fill a gap between obesity and liver or breast cancer. Our discoveries propose FABP4 and its downstream IL-6/STAT3/DNMT1 signaling axis as an important mediator of obesity-accelerated AML, given that a key feature of obesity is the abnormal lipid hemostasis that is significantly regulated by FABP4 aberrations.[45] In support of this idea, we first demonstrated cell non-autonomous functions of the host FABP4 by showing that the overproduction of environmental Fabp4 in obese mice induced higher proliferation rate of AML cells compared to lean controls, but genetic deficiency of *Fabp4* in mice substantially attenuated AML cell expansion with impaired leukemic disease compared to *Fabp4* sufficient mice. By mimicking the obese microenvironment, we added the physiologically relevant FABP4 protein into the culture medium of AML cell lines, human patient and mouse primary cells, and found that FABP4 dose-dependently augmented AML cell proliferation. While numerous factors could be important, the aforementioned findings support FABP4 as a primary molecule in regulating AML cell fate in obesity. Additionally, leukemia cells highly expressed FABP4, consistent with its abundant expression in ovarian cancer[20] and oral squamous cell carcinoma.[21] *FABP4* ablation in AML cells blocked proliferation *in vitro* and induced leukemia regression *in vivo*. Importantly, AML patients having high FABP4 in primary cells had shorter lifespans than those with low FABP4, suggesting that FABP4 also executes cell-autonomous actions on leukemia cells. However, the determination of which action, cell-autonomous or cell-non-autonomous, of FABP4 plays a dominant role in regulating AML cell fate warrants further investigation. In addition, whether cellular FABP4 is released into the tissue microenvironment or whether FABP4 accounts for AML cell and host crosstalk with a focus on initial identification of secretory

peptide and/or cell surface receptor(s) also deserves further examination. The solutions to these puzzles will provide critical insights into the obesity-AML association.

DNA methylation is a hallmark and an early event of AML that potentially contributes to onset and progression of AML.[46] Although most investigations focus on its cell-autonomous regulation, the cell non-autonomous factors emerge as crucial methylation modulators, particularly in the context of obesity. Actually, our data demonstrated that the FABP4-governed IL-6/STAT3/DNMT1 cascade represents a molecular basis behind the environmentally-altered DNA methylation landscape, because changes in the environmental FABP4, including FABP4 protein treatment and *Fabp4* deficiency in mice, altered DNA methylation in AML cells. Mechanistically, the IL-6-STAT3 axis links environmental FABP4 to DNA hypermethylation in AML cells. This is based on the findings that the FABP4 protein upregulated IL-6 followed by an augmentation of STAT3 phosphorylation and *DNMT1* transactivation. In contrast, dysfunction of STAT3 and IL-6 suppressed DNMT1-dependent DNA methylation and attenuated the active function of FABP4 on DNA methylation. Notably, we cannot exclude the possibility that FABP4 regulates *DNMT1* expression partially through NFκB signaling, a *DNMT1* upregulator,[2, 4] because *FABP4* knockdown caused NFκB dephosphorylation and *Fabp4* deficiency in mice impaired NFκB activity.[34] Regardless, our discoveries reveal the FABP4/IL-6/STAT3/DNMT1 cascade as a molecular bridge of lipid abnormality and DNA hypermethylation, and a hereto unrecognized pathway behind FABP4-promoted tumorigenesis.[20, 22] They also demonstrated FABP4 as a new member of the epigenetic regulator family, in line with TET (ten-eleven-translocation) proteins,[47, 48] cytidine deaminases[49] and isocitrate dehydrogenase mutations.[50] Because DNA hypermethylation, particularly at CpG islands within the TSG promoters, is essential for many types of cancer, the crosstalk between FABP4 and DNA methylation machinery could be extrapolated to other types of human malignancies, such as liver, ovarian or breast cancer, where the aberrant lipid metabolism and deregulated DNA methylation coexist to control the cancer cell fate.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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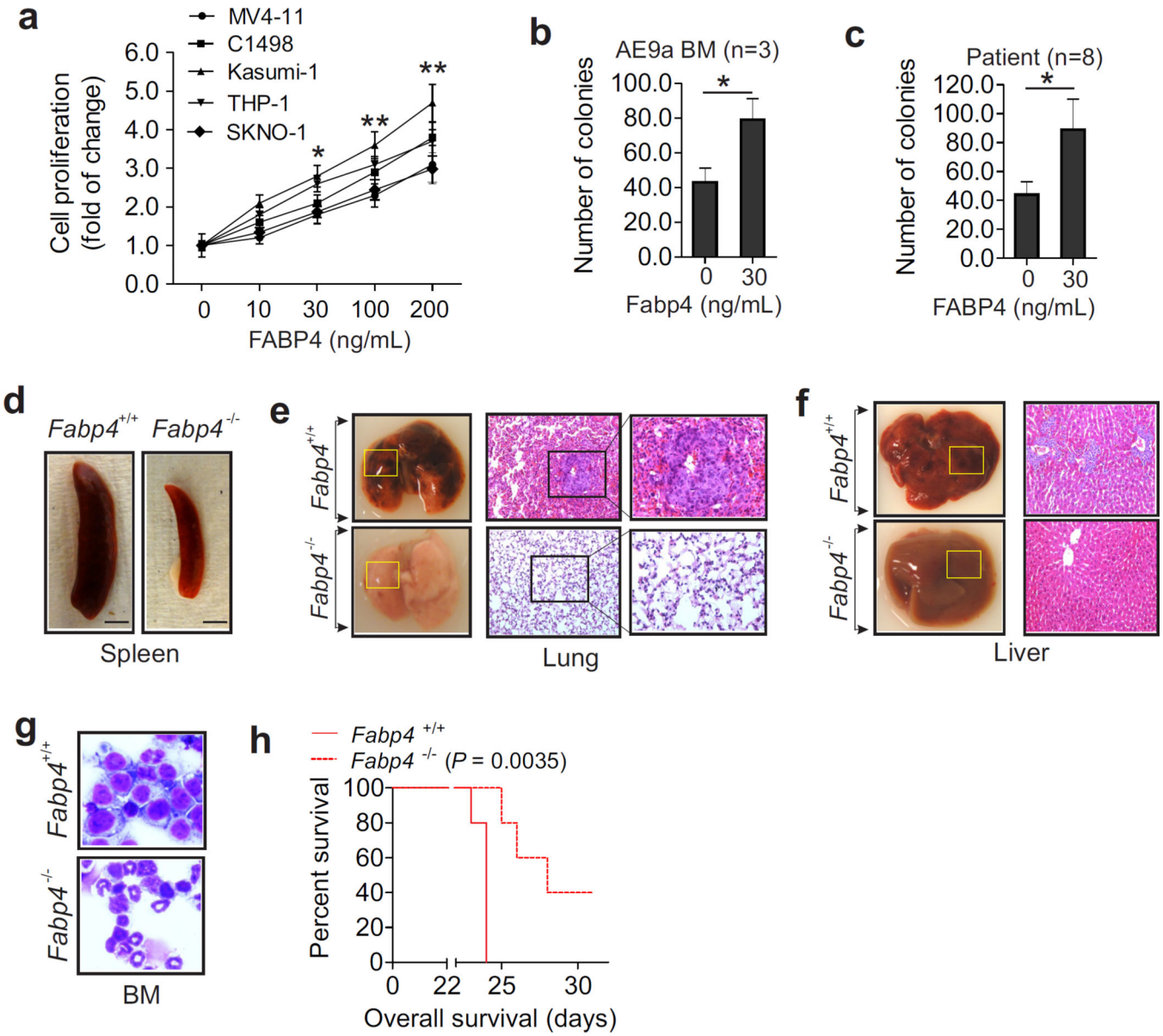

Figure 1. Obesity-related FABP4 upregulation accounts for aggressive leukemia

(**a**) AML cell lines were treated with the mouse or human FABP4 protein for 48 hours and subjected to MTS assays. Experiments were performed 3 times independently. Data are mean ±SD, **P*<0.05, ***P*<0.01. (**b,c**) Colony-forming assays for mouse (**b**, n=3) and human patient (**c**, n=8) AML primary cells treated with the FABP4 protein. **P*<0.05. (**d**) Photographs show representative external views of spleen. Scale bars, 5.0 mm. (**e**) Photographs show representative external views of the lung and the histological analysis of H&E-stained lung sections from boxed areas (original magnification ×200, ×400). (**f**) Photographs show representative external views of the liver and the histological analysis of H&E-stained liver sections from boxed lesions (original magnification ×200). (**g**) Wright-Giemsa-stained BM (original magnification ×400). (**h**) Effect of FABP4 on survival of

Fabp4$^{-/-}$ and *Fabp4*$^{+/+}$ mice injected with 2×10^6 C1498 cells (n=5). Estimated percent survival was calculated by the Kaplan-Meier method (log-rank test). In **d** to **g**, n=3.

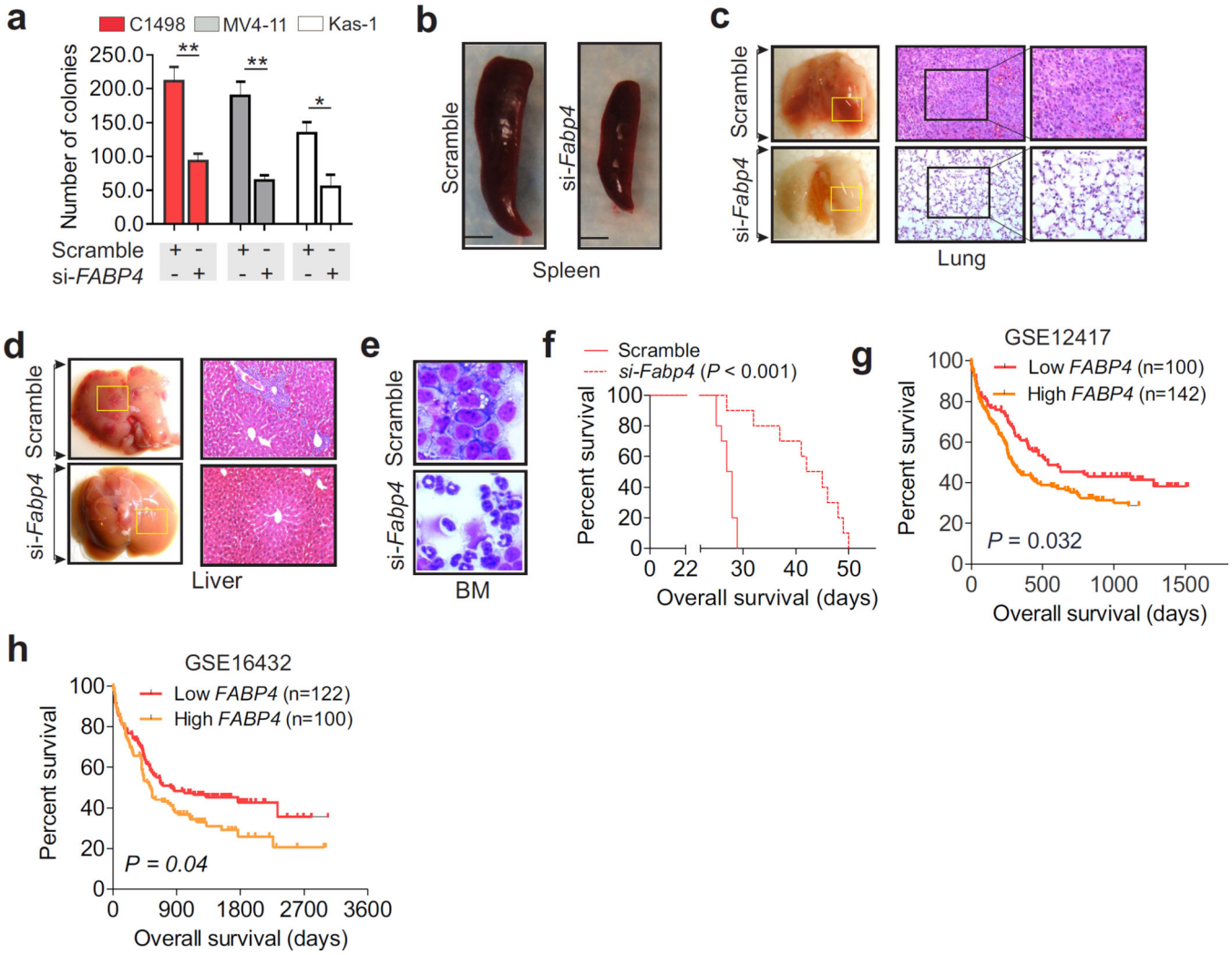

Figure 2. The cell intrinsic FABP4 protein promotes leukemia growth

(**a**) Colony-forming assays of C1498, MV4-11 or Kasumi-1 (Kas-1) transfected with *FABP4* siRNA. Graphs indicate the colony number from 3 independent experiments. Data are mean ±SD, **P*<0.05, ***P*<0.01. (**b**) Photographs show representative external views of spleen from leukemia-bearing mice. Scale bars, 5.0 mm. (**c**) Photographs show representative external views of lung and the histological analysis of H&E-stained lung sections from boxed areas (original magnification ×200, ×400). (**d**) Photographs show representative external views of the liver and the histological analysis of H&E-stained liver sections from boxed lesions (original magnification ×200). (**e**) Wright-Giemsa-stained BM cells. (**f**) The survival curve of leukemia-bearing mice calculated by Kaplan-Meier estimate (n=10). (**g,h**) The survival curve of the whole AML patient population was determined by Kaplan-Meier estimate, in which high *FABP4* was compared to low *FABP4*. Note: Leukemia-bearing mice were developed by the intravenous injection of C1498 cells (0.5×10^6) transfected with *Fabp4* siRNA into C57BL/6 mice; In **b** to **e**, n=3; In **f** to **h**, a log-rank test; si, siRNA.

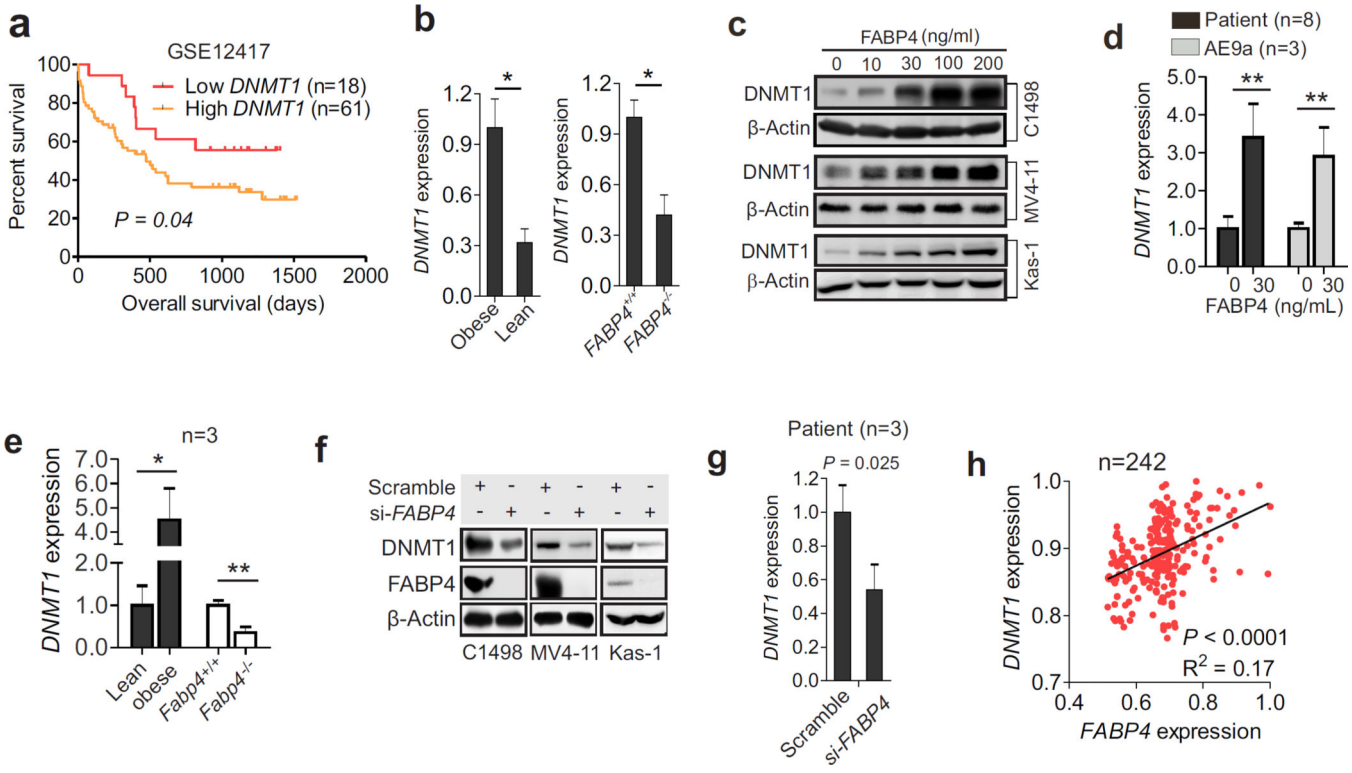

Figure 3. FABP4 regulates the *DNMT1* gene expression

(**a**) Kaplan-Meier estimate for overall survival (log-rank test) of AML patients. (**b**) qPCR of BM from obese and lean or *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice (n=3). (**c**) Western blotting of C1498, MV4-11 or Kasumi-1 (Kas-1) treated with the FABP4 protein. (**d**) qPCR of human patient (n=8) or mouse (n=3) primary cells treated with the FABP4 protein. (**e**) qPCR of C1498 cells from leukemia-bearing obese and lean or *Fabp4*$^{+/+}$ and *Fabp4*$^{-/-}$ mice. (**f**) Western blotting of C1498, MV4-11 or Kasumi-1 transfected with *FABP4* siRNA. (**g**) qPCR of AML patient (n=3) cells transfected with *FABP4* siRNA. (**h**) Spearman correlation analysis for the mRNA expression of *FABP4* and *DNMT1* in AML patients. The Y or X-axis represents the fold change in maximum expression.

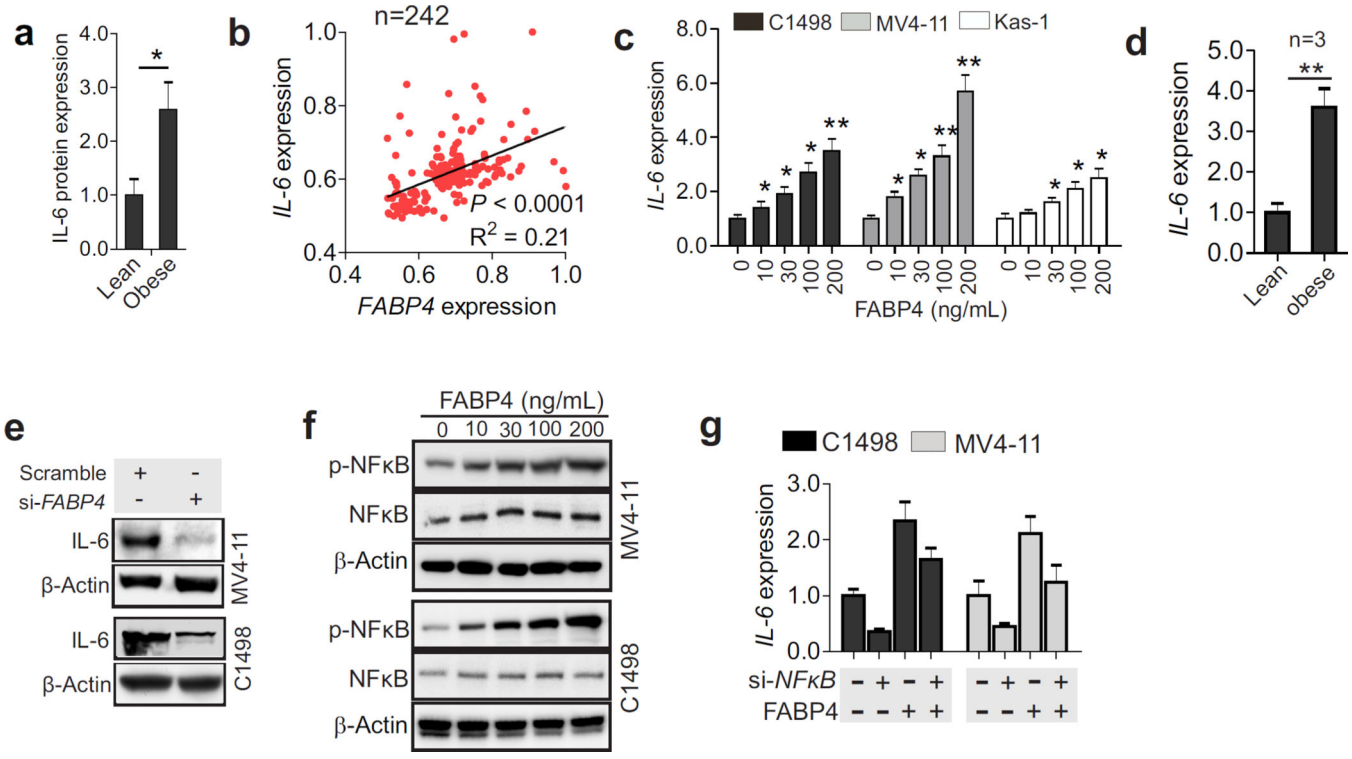

Figure 4. DNMT1 upregulation by FABP4 occurs through activated NFκB signaling

(a) Graph shows the quantification of Western blotting of sera from lean and obese mice (n=5). (**b**) Spearman correlation analysis for the mRNA expression of *FABP4* and *IL-6* in AML patients. The Y or X-axis represents the fold change in maximum expression. (**c**) qPCR of C1498, MV4-11 or Kasumi-1 (Kas-1) treated with the FABP4 protein. (**d**) qPCR of C1498 cells from leukemia-bearing obese and lean mice. (**e**)Western blotting of MV4-11 or C1498 transfected with *IL-6* shRNA for 48 hours. (**f**) Western blotting of MV4-11 or C1498 treated with the FABP4 protein for 48 hours. (**g**) qPCR of C1498 and MV4-11 transfected with *NFκB* siRNA for 24 hours followed by treatment with the FABP4 protein (30 mg/mL) for another 24 hours.

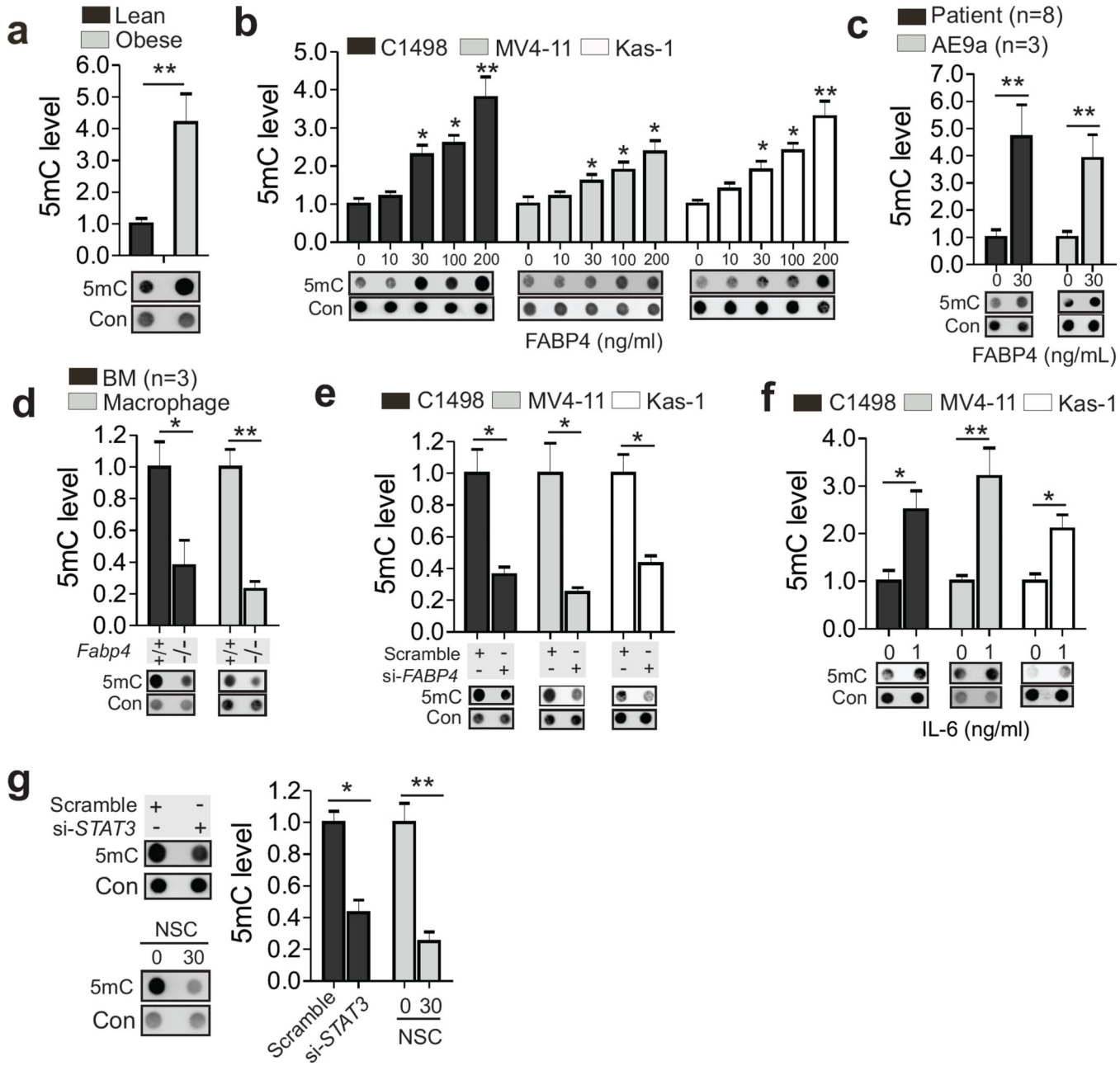

Figure 5. FABP4 modulates global and gene specific DNA methylation

(**a**) Dot-blotting of BM from obese and lean mice (n=3). (**b,c**) Dot-blotting of C1498, MV4-11 or Kasumi-1 (**b**) or AML human patient (n=8) and mouse (n=3) primary cells (**c**) treated with the FABP4 protein. (**d**) Dot-blotting of BM from *Fabp4*$^{-/-}$ and *Fabp4*$^{+/+}$ mice or BM-derived macrophages with stable *Fabp4* knockdown. (**e,f**) Dot-blotting of C1498, MV4-11 or Kasumi-1 transfected with *FABP4* siRNA (**e**) or treated with the IL-6 protein (**f**). (**g**) Dot-blotting of MV4-11 transfected with *STAT3* siRNA or treated with 30 μM NSC74895. In **a** to **g**, data are mean ±SD, **P*<0.05, ***P*<0.01; All treatments are 48 hours unless otherwise indicated; Con, loading control; si, siRNA; Kas-1, Kasumi-1.