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# HMGA2 promotes adipogenesis by activating C/EBPβ-mediated expression of PPARγ

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ABSTRACT

Adipogenesis is orchestrated by a highly ordered network of transcription factors including peroxisome-proliferator activated receptor-gamma (PPARγ) and CCAAT-enhancer binding protein (C/EBP) family proteins. High mobility group protein AT-hook 2 (HMGA2), an architectural transcription factor, has been reported to play an essential role in preadipocyte proliferation, and its overexpression has been implicated in obesity in mice and humans. However, the direct role of HMGA2 in regulating the gene expression program during adipogenesis is not known. Here, we demonstrate that HMGA2 is required for C/EBPβ-mediated expression of PPARγ, and thus promotes adipogenic differentiation. We observed a transient but marked increase of *Hmga2* transcript at an early phase of differentiation of mouse 3T3-L1 preadipocytes. Importantly, *Hmga2* knockdown greatly impaired adipocyte formation, while its overexpression promoted the formation of mature adipocytes. We found that HMGA2 colocalized with C/EBPβ in the nucleus and was required for the recruitment of C/EBPβ to its binding element at the *Pparγ2* promoter. Accordingly, HMGA2 and C/EBPβ cooperatively enhanced the *Pparγ2* promoter activity. Our results indicate that HMGA2 is an essential constituent of the adipogenic transcription factor network, and thus its function may be affected during the course of obesity.



## 1. Introduction

Cellular differentiation is a highly ordered process, in which stepwise expression and/or activation of transcription factors (TFs) orchestrate the timely expression of a select set of genes [1–4]. During adipogenesis, preadipocytes undergo cell-cycle arrest followed by the reconstitution of a gene expression program, leading to the formation of lipid-accumulating mature adipocytes [5–7]. Upon adipogenic induction, key TFs including CCAAT-enhancer binding protein (C/EBP) family factors sequentially operate to activate the master adipogenic factor, peroxisome-proliferator activated receptor-gamma (PPARγ) [3]. Because the total adipocyte number has been intimately linked to obesity in humans, it is crucial to understand the precise mechanism of how preadipocytes cease proliferation and initiate the adipogenic program.

High mobility group protein AT-hook 2 (HMGA2) belongs to the non-histone chromosomal high mobility group (HMG) protein family and functions as an architectural transcriptional factor by recognizing AT-rich sequences in the minor groove of DNA and enhancing gene transcription [8–10]. *HMGA2* expression is absent or very low in adult tissues, while it is highly expressed in embryos and mesenchymal stem cells, implying that HMGA2 functions at an early developmental stage [9]. A previous study demonstrated that transgenic expression of *Hmga2* in mice caused a giant phenotype and hyperplasia of white adipose tissue (WAT) [11–13]. However, *Hmga2*-deficient mice exhibited a pygmy phenotype with drastically reduced fat tissue and resisted against diet-induced obesity [14,15]. Importantly, a recent study reported significantly elevated levels of *HMGA2* in human WAT of individuals with obesity and/or

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type 2 diabetes mellitus [16]. This evidence indicates the direct involvement of HMGA2 in adipose tissue development and obesity.

A previous study demonstrated that HMGA2 promotes cell-cycle progression by relieving retinoblastoma protein-mediated repression of E2F1 activity [17]. Thus, a plausible cause of adipose tissue atrophy in *Hmga2* knockout mice is impaired cell proliferation, which results in the reduction of the number of preadipocytes and consequent adipocytes. However, the precise function of HMGA2 during the adipogenic process is still poorly understood. Here, we report that a pulsatile expression of HMGA2 at the early phase of adipogenesis drives C/EBPβ-mediated induction of PPARγ and thus promotes differentiation. Our study suggests that HMGA2 plays an essential role in the transition of proliferative preadipocytes to differentiating adipocytes, which may be a key event during acquisition of the obese phenotype.

## 2. Materials and methods

### 2.1. Cell culture

Mouse 3T3-L1 cells were cultured in high glucose (4.5 g/L) DMEM supplemented with 10% heat-inactivated fetal bovine serum and penicillin/streptomycin. Adipogenic differentiation of 3T3-L1 preadipocytes was performed as previously described [18]. Briefly, cells were maintained for 3 days post-confluence and the medium was changed to DMEM containing 0.5 mM 3-isobutyl-1-methylxanthine (Sigma–Aldrich), 1.0 μM dexamethasone (Sigma–Aldrich), and 5.0 mg/mL bovine insulin (Sigma–Aldrich) for 2 days. Cells were then cultured in a growth medium for an additional 3–8 days before collection. Isolation and culture of stromal vascular fractions (SVFs) from inguinal WAT of C57BL/6 mice were performed as previously described [18]. Lipid accumulation was evaluated by oil red O staining, and the stained lipid droplets were visualized by light microscopy and photographed. Animal experiments were conducted in accordance with the guidelines of the Animal Care and Use Committee of Kumamoto University.

### 2.2. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNAs were extracted by TRIZOL (Qiagen). RNAs were reverse transcribed with the Transcriptor First Strand cDNA Synthesis Kit (Roche). For quantification, real-time PCR analysis was performed using LightCycler 480 SYBR Green I Master on a LightCycler 480 II (Roche) or ABI7500 Fast (Life Technologies). The relative fold changes were quantified using the comparative threshold cycle method, and β-actin was used as a control. Primers are listed in Table 1.

### 2.3. Western blotting

Total proteins were extracted and separated by SDS-PAGE as previously described [19]. Proteins were detected using antibodies against HMGA2 (#5269, Cell Signaling), PPARγ (sc-7273, Santa Cruz Biotechnology), C/EBPβ (sc-150, Santa Cruz Biotechnology), FABP4 (sc-18661, Santa Cruz Biotechnology), histone H3 (ab1791, Abcam), β-actin (A1978, Sigma–Aldrich), and FLAG (F3165, Sigma–Aldrich).

### 2.4. Plasmid and transfection

To prepare the HMGA2 expression construct, human *HMGA2* cDNA was cloned into the pcDNA3.1-FLAG vector (pcDNA3-FLAG-HMGA2). We also ligated *HMGA2* cDNA into the pCAG IRES-Puro vector to generate the GFP-HMGA2 fusion construct (pCAG-GFP-HMGA2), as described in our previous study [20]. Human *C/EBPβ* cDNA was cloned into the pcDNA3.1 vector (pcDNA3-C/EBPβ). DNA fragments harboring *C/EBPβ* and *Pparγ2* gene promoters were synthesized *in vitro* at Shanghai Novobio Biotechnology (Shanghai, China). *Pparγ2* promoter region containing the C/EBPβ binding sites from −363-bp to +17-bp (from the transcription start site) was cloned for analysis [21]. Newly synthesized sequences of *Pparγ2* promoter region were cloned into the pGL3 luciferase reporter vector for transient transfection studies: −363/+17-luc and mut-CEBP-363/+17-luc (C/EBPβ binding sites at −340/-336 and −327/-323 were mutated from GCAATTTTAAAAAGCAAT to GCGGTTTTAAAAAGCGGT; mutated sites are underlined). The pGL3 basic vector was used as a control. Plasmid transfection experiments were performed using Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen).

**Table 1**
A list of primers used in current study.

| | |
|---|---|
| **qRT-PCR primers** | |
| 36b4 S | 5′-CAACCCAGCTCTGGAGAAAC-3′ |
| 36b4 AS | 5′-CCAACAGCATATCCCGAATC-3′ |
| β-actin S | 5′-CCAACCGTGAAAAGATGACC-3′ |
| β-actin AS | 5′-CCAGAGGCATACAGGGACAG-3′ |
| Adipone S | 5′-GGAACTTGTGCAGGTTGGAT-3′ |
| Adipone AS | 5′-TCTCCAGGAGTGCCATCTCT-3′ |
| C/EBPβ S | 5′-ACAAGGCCAAGATGCGCAAC-3′ |
| C/EBPβ AS | 5′-TTCCGCAGGGTGCTGAGCT-3′ |
| Cd36 S | 5′-CCATTGGTGATGAAAAAGCA-3′ |
| Cd36 AS | 5′-ATCACCACTCCAATCCCAAG-3′ |
| Fabp4 S | 5′-CATCAGCGTAAATGGGGATT-3′ |
| Fabp4 AS | 5′-GTCGTCTGCGGTGATTTCAT-3′ |
| Hmga2 S | 5′-CATCAGCCCAGGGACAAC-3′ |
| Hmga2 AS | 5′-GAGAGGGCTCACAGGTTGG-3′ |
| Pparγ2 S | 5′-TGCAGGAGCAGAGCAAAGAG-3′ |
| Pparγ2 AS | 5′-CGGCTTCTACGGATCGAAAC-3′ |
| **ChIP primers** | |
| Pparγ2-58kbS | 5′-GCATTTCAGAAGATTGGGTTTCA-3′ |
| Pparγ2-58kb AS | 5′-CCAACTAAGGCATGACACTGAG-3′ |
| Pparγ2-556 S | 5′-TTACCTGTGTGGGTAACAAAATC-3′ |
| Pparγ2-468 AS | 5′-TGAAACTCCTAATCACACATCTG-3′ |
| Pparγ2-1756 S | 5′-TGAACAGCAGTTGGGACTAGC-3′ |
| Pparγ2-1892 AS | 5′-TGATAAACCAGATGACCATAAAAG-3′ |

### 2.5. Small interfering RNA (siRNA)-mediated knockdown (KD)

For the siRNA KD experiment, Hmga2 siRNAs (siHmga2-1: 5′-GACCCAAAGGCAGCAAAAATT-3′; siHmga2-2: 5′-GAAAGCAGAGACCAUUGGATT-3′) were synthesized and transfected into cells by RNAiMAX according to the manufacturer's protocol (Invitrogen). GL3 siRNA (siGL3: 5′-CUUACGCUGAGUACUUCGATT-3′) was used as a control.

### 2.6. Immunofluorescene analysis

After fixing cells with either 4% paraformaldehyde or 100% methanol, cells were incubated with primary antibodies against GFP (ab13970; Abcam) or C/EBPβ. Images were analyzed using Lumina Vision software.

### 2.7. Chromatin immunoprecipitation (ChIP) and quantitative PCR analysis

ChIP was performed as previously described [19]. Briefly, 3T3-L1 cells were cross-linked with 1% formaldehyde, and crude cell lysates were sonicated. ChIP was performed with anti-C/EBPβ and control rabbit IgG (Santa Cruz Biotechnology). DNA enrichment was determined by quantitative PCR, and was normalized to input. Primers are listed in Table 1.

### 2.8. Luciferase assay

Dual luciferase reporter assays were performed according to the manufacturer's protocol (Promega). For *Pparγ2* promoter analysis, the pGL3-Pparγ2 promoter reporter vector and the reference vector pRL-SV40 were co-transfected with pcDNA3-FLAG-HMGA2 and/or pcDNA3-C/EBPβ into 293T cells using Lipofectamine 2000 (Invitrogen). After 24 h, cells were lysed and collected for luciferase measurement. Firefly luciferase activities were normalized to renilla luciferase activities.

### 2.9. Statistic analysis

Data are presented as means ± S.D. A two-tailed Student's *t*-test was applied, and $p < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. HMGA2 is rapidly increased at the early phase of adipogenic differentiation

To understand the precise function of HMGA2 during adipogenesis, we first examined the time course of *Hmga2* expression during the differentiation of 3T3-L1 preadipocytes. As shown in Fig. 1A, the *Hmga2* mRNA level was rapidly elevated at 3 h after adipogenic induction and gradually declined to basal levels within 72 h. Thereafter, *Hmga2* mRNA remained at low levels until 168 h post-differentiation when a mature status of adipocyte was reached as indicated by the adipocyte marker proteins PPARγ and FABP4 (Fig. 1A and B). The protein level of HMGA2 exhibited a similar expression pattern with its mRNA level (Fig. 1B). While the *Hmga2* mRNA level peaked at 8 h after induction, the protein expression was highest at 24 h (Fig. 1A and B).

A further investigation was performed using *ex vivo* differentiation of SVF preadipocytes from mouse WAT, in which *Hmga2* mRNA expression rapidly increased after induction (Fig. 1C). Moreover, compared with SVF preadipocytes, the crude adipose tissue, which mainly consists of mature adipocytes, showed an extremely low level of *Hmga2* transcript (Fig. 1D). These data indicate that HMGA2 is transiently expressed at an early phase of adipogenesis both *in vitro* and *in vivo*.

### 3.2. HMGA2 promotes adipogenic differentiation

To test whether Hmga2 participates in the adipogenic program, we introduced *Hmga2* siRNAs into 3T3-L1 cells before differentiation induction (Fig. 2A–C). *Hmga2* KD resulted in the markedly decreased amount of lipid-containing mature adipocytes as revealed by oil red O staining (Fig. 2D). The same experiment was performed using *ex vivo* differentiation of SVF cells from mouse WAT (Fig. 2E–G). As shown in Fig. 2G, the frequency of mature adipocytes was reduced by *Hmga2* KD.

To further address whether HMGA2 could influence the progression of differentiation, we introduced human *HMGA2* cDNA, which shares a very high sequence similarity to mouse *Hmga2*, into 3T3-L1 preadipocytes (Fig. 2H). As shown in Fig. 2I, the overexpression of HMGA2 enhanced the intracellular lipid accumulation. These results strongly indicate that HMGA2 promotes the differentiation of preadipocytes.

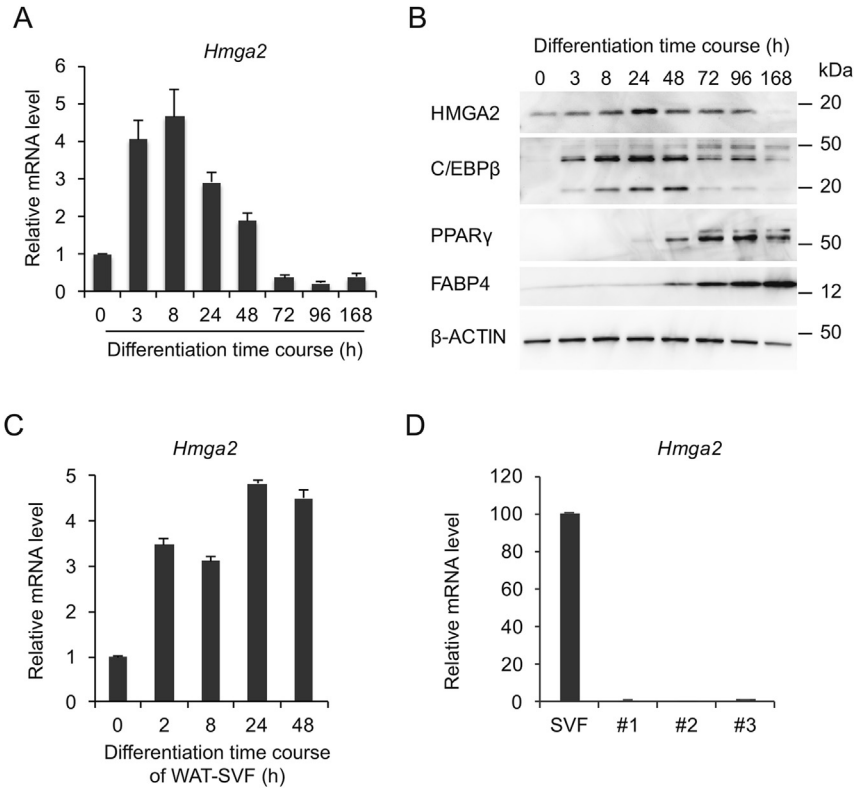


**Fig. 1.** Hmga2 is transiently increased during the early stage of adipogenic differentiation. (A) *Hmga2* mRNA expression during adipogenic differentiation of 3T3-L1 cells. Cells were harvested at indicated time points after adipogenic induction. *Hmga2* expression was determined by qRT-PCR using β-actin as a control. Values indicate mRNA levels relative to 0 h and are presented as means ± S.D. of three samples. (B) Protein levels of Hmga2, C/EBPβ, Pparγ, and FABP4 during adipogenic differentiation of 3T3-L1 cells were detected by western blot. (C) *Hmga2* mRNA expression during the adipogenic differentiation of stromal vascular fractions (SVF) from white adipocyte tissue (WAT) of C57BL/6 mouse. SVF from WAT was cultured and harvested at indicated time points after adipogenic induction. (D) *Hmga2* mRNA expression in mouse SVF and crude adipocytes.

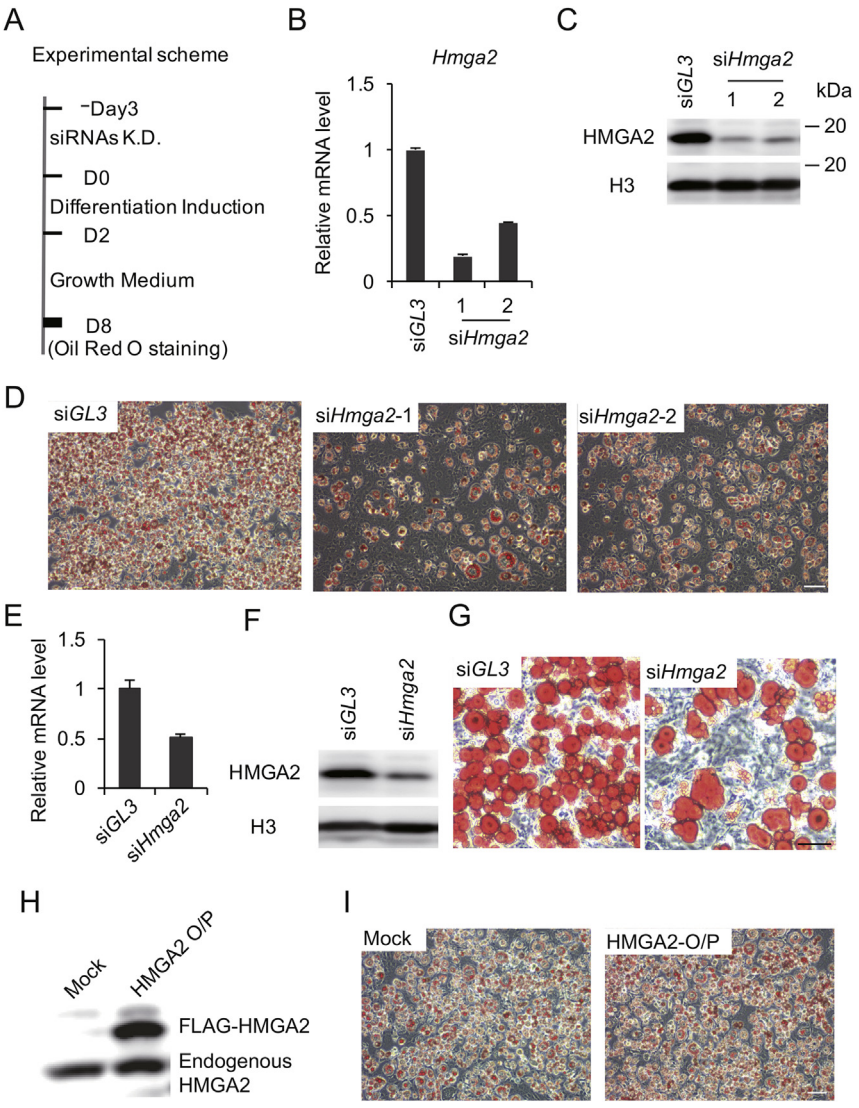


**Fig. 2.** HMGA2 is promotes adipogenesis in 3T3-L1 cells. (A) Schematic of the experiment. (B and C) siRNA-mediated knockdown of Hmga2. Reduction of Hmga2 was confirmed at mRNA (B) and protein (C) levels at 96 h after siRNA introduction. siGL3 was used as a non-targeting control. (D) Oil red O staining of siRNA-transfected 3T3-L1 cells at 8 days of differentiation. Scale bar indicates 10 μm. (E and F) siRNAs-mediated knockdown of *Hmga2*. Reduction of HMGA2 was confirmed at mRNA (E) and protein (F) levels at 96 h after siRNA introduction. siGL3 was used as a non-targeting control. (G) Oil red O staining of siRNAs treated cells at eight days of differentiation. (H) Verification of human HMGA2 expression by western blot in 3T3-L1 cells. (I) Oil red O staining of 3T3-L1 cells transfected with human HMGA2 after 5 days of differentiation. Scale bar indicates 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

### 3.3. *HMGA2 promotes adipogenic expression*

PPARγ2, a master adipogenic transcription factor, is absolutely necessary for adipogenesis. We observed that during adipogenesis, Pparγ2 expression increased after the pulsatile expression of HMGA2 (Fig. 1B and Fig. 3A), suggesting the possible regulation of *Pparγ2* gene by HMGA2. Indeed, Pparγ2 expression at both mRNA and protein levels were decreased by *Hmga2* KD at 24 and 168 h (Fig. 3B–E). In agreement with this, the expression of downstream target genes of PPARγ, such as *Fabp4*, *Adiponectin*, and *Cd36*, were dramatically decreased (Fig. 3B and D). These results suggest that HMGA2 promotes the expression of PPARγ2 and its downstream genes during adipogenesis.

### 3.4. *HMGA2 cooperates with C/EBPβ to facilitate PPARγ2 expression*

Activation of *Pparγ2* gene expression is mainly controlled by C/EBPβ, which is an early-induced transcription factor during adipogenesis [1]. Interestingly, we observed a synchronous expression pattern between C/EBPβ and Hmga2 (Fig. 1B), raising the possibility that these TFs work cooperatively. To test this, we performed an immunocytochemical experiment and found that HMGA2 and C/EBPβ were co-localized in the nucleus of differentiating adipocytes (Fig. 4A). We next performed ChIP assays to evaluate the recruitment of C/EBPβ at the *Pparγ2* gene promoter. We observed an enrichment of C/EBPβ at its known binding region [21], which was abolished by *Hmga2* KD (Fig. 4B). To gain a direct insight into whether HMGA2 is involved in C/EBPβ-mediated expression of *Pparγ2*, we carried out a reporter assay using a luciferase gene under control of the *Pparγ2* promoter that harbors a C/EBPβ binding site (Fig. 4B & C) [21]. As displayed in Fig. 4D, either overexpression of HMGA2 or C/EBPβ significantly increased the reporter expression, which was further augmented by the combination of two factors. Our results strongly suggested that HMGA2 positively affected C/EBPβ regulation on *Pparγ2* promoter activity. Moreover, when the reporter construct bearing a mutated C/EBP

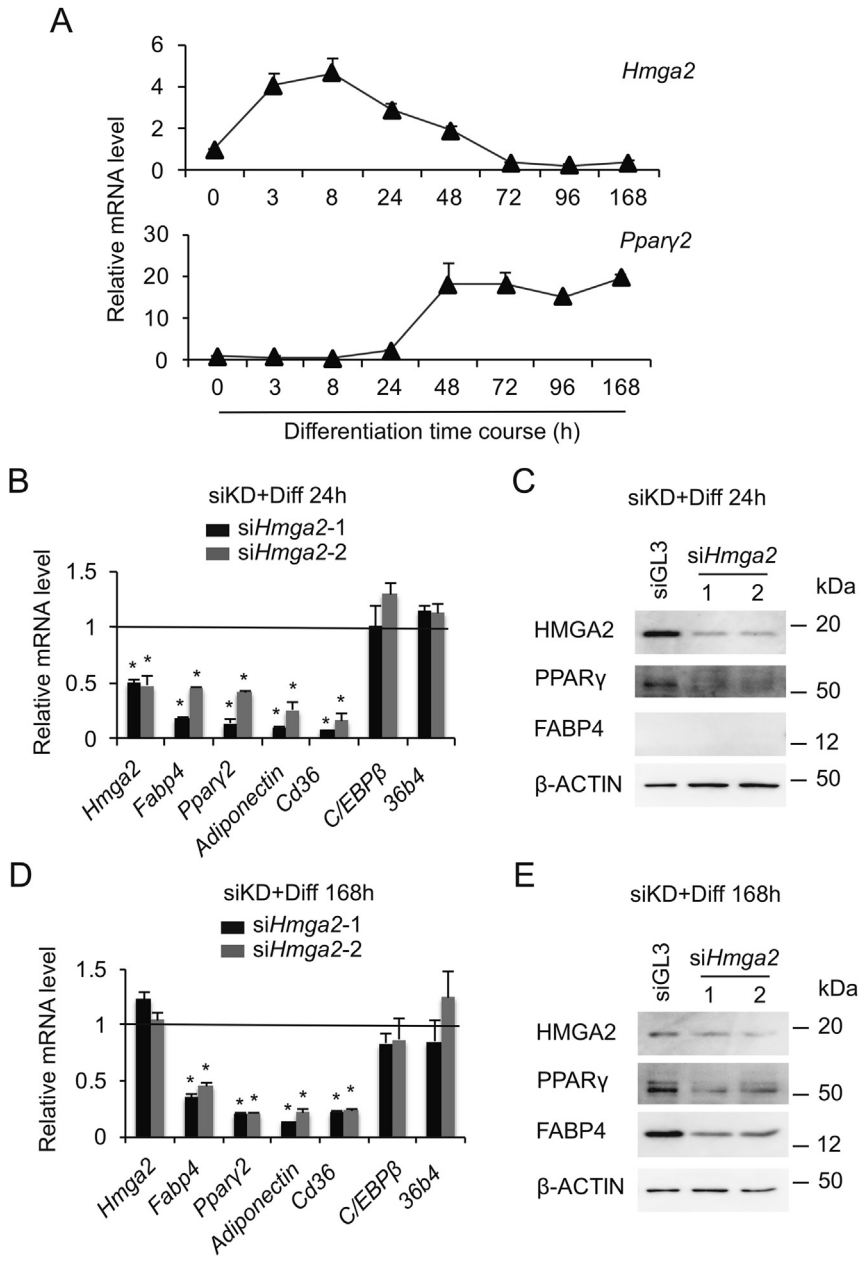


**Fig. 3.** Decreased expression of *PPARγ2* and its downstream target genes by *Hmga2* knockdown. (A) 3T3-L1 cells were harvested at indicated time points after adipogenic induction. Expression of *Hmga2* and *Pparγ2* was determined by qRT-PCR using β-actin as a control. Values are presented as means ± S.D of three samples. (B, D) qRT-PCR analysis of mRNA expression of *Pparγ2* and its target genes after *Hmga2* siRNA introduction for 72 h, followed by differentiation induction of 24 h (B) and 168 h (D). β-Actin was used as a control. Values indicate the mRNA levels relative to siGL3 and are presented as means ± S.D. of three samples. $^{*}P < 0.05$. (C, E) Western blot analyses of PPARγ2 and its targets after *Hmga2* siRNA treatment for 72 h followed by a differentiation induction of 24 h (C) and 168 h (E). β-Actin was used as a protein loading control.

site was used, HMGA2 did not enhance the reporter expression, suggesting that the effect of HMGA2 on *Pparγ2* promoter activity was mediated mostly by C/EBPβ (Fig. 4D).

Collectively, our data clearly indicate that HMGA2 promotes *Pparγ2* gene expression by facilitating the C/EBPβ binding at the promoter.

## 4. Discussion

Previous studies have demonstrated that HMGA2 is an essential component of the adipogenic program with a possible link to human obesity [16]. In addition, Hmga2 was preferentially expressed in embryonic and undifferentiated cells compared with mature cells [22], raising the possibility that HMGA2 might regulate the proliferation and/or differentiation of adipogenic progenitor cells. In the present study, we found that Hmga2 is expressed exclusively at the very early phase of adipogenesis and promotes differentiation. HMGA2 has been implicated in the regulation of cell-cycle progression [12,15,17]. However, we did not observe a proliferative defect in *Hmga2* KD preadipocytes, suggesting that the severe lipodystrophic phenotype of *Hmga2* knockout mice was mainly due to the impaired differentiation potency of preadipocytes [14].

C/EBPβ is an early adipogenic factor that increases shortly after differentiation induction [7]. C/EBPβ induces the expression of PPARγ2, which in turn activates downstream targets involved in adipocyte functions such as triglyceride storage [6,23]. Previous

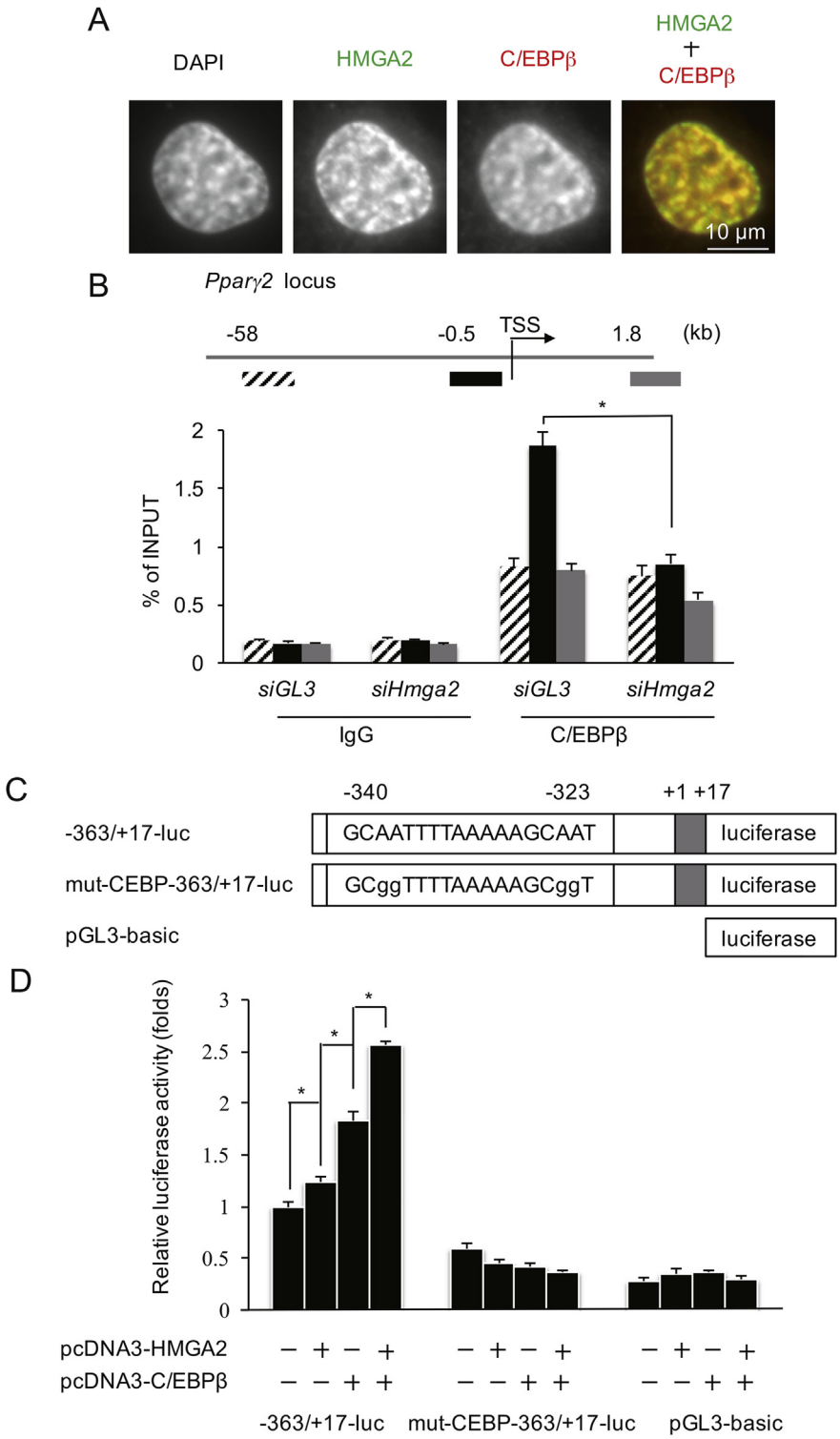


**Fig. 4.** HMGA2 co-localized with C/EBPβ and was necessary for C/EBPβ binding at the *Pparγ2* promoter for C/EBPβ-mediated transcriptional activation of the *Pparγ2* gene. (A) Co-localization analysis of HMGA2 and C/EBPβ by immunofluorescence. Human HMGA2 was overexpressed in 3T3-L1 cells for 72 h and immunofluorescence analysis was performed, followed by a differentiation induction for 24 h. Scale bar indicates 10 μm. (B) Analysis of C/EBPβ binding at the *Pparγ2* promoter by ChIP. ChIP was performed using C/EBPβ antibodies after *Hmga2* siRNA treatment for 72 h followed by a differentiation induction for 24 h. C/EBPβ enrichment was evaluated using the primer sets against three genomic regions as indicated. Pre-immune IgG was used as a control. Values indicate the percentage against input DNA and are presented as means ± S.D. of three samples. $^{*}P < 0.05$. (C) Schematic of luciferase reporter construct with cloned *Pparγ2* promoter region containing a C/EBPβ binding site. The C/EBPβ binding site is originally located at −340 to −323 from transcription start site of the *Pparγ2* gene. Small characters indicate the mutated nucleotides of the C/EBPβ-binding site. (D). Effects of exogenous HMGA2 and/or C/EBPβ on transcriptional activity of *Pparγ2* by luciferase reporter assay. Overexpression plasmids pcDNA3-HMGA2 and/or pcDNA3-C/EBPβ were co-transfected either with −363/+17-luc, mut-CEBP-363/+17-luc, or pGL3basic into 293T cells for 24 h. Bar graphs represent means ± SE (%) of luciferase activity (the activity of −363/+17-luc without pcDNA3-HMGA2 and pcDNA3-C/EBPβ as 100%). $^{*}P < 0.05$.

reports demonstrated that C/EBPβ occupies the promoter of the *Pparγ* gene prior to the transcriptional activation during the early phase of adipogenesis [4,5]. We found that HMGA2 enhances C/EBPβ-mediated transcription of the *Pparγ2* gene. HMGA2, as an architectural TF, changes chromatin conformation through binding to AT-rich sequences and bending DNA, which consequently provides docking sites for other TFs [9,22,24,25]. Therefore, HMGA2 may facilitate the DNA binding of C/EBPβ by controlling the local chromatin structure at the *Pparγ2* promoter. Moreover, recent high-throughput sequencing approaches have revealed that the chromatin structure is dramatically remodeled at a genome-wide level during adipogenesis [26]. Thus, an intriguing possibility is that HMGA2 might work at many gene loci to build an epigenetic foundation that allows the access of adipogenic TFs.

A recent study showed a significant association of obesity with an increase in the expression of *HMGA2* [16]. Moreover, higher *HMGA2* expression in adipose tissue in patients with type 2 diabetes was detected [16]. Interestingly, high expression of *HMGA2* was reported to be induced by a high-fat diet, which was associated with the acquisition of obesity in mice [15]. Here, we found that HMGA2 plays a key role in the initiation of the adipogenic program, which may be a critical event that links nutrition to obesity and related metabolic diseases.

## Competing financial interests

The authors have no competing financial interests.

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