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Article

Retinoic acid inhibits white adipogenesis by disrupting GADD45A-mediated *Zfp423* DNA demethylation

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Retinoic acid (RA), a bioactive metabolite of vitamin A, is a critical mediator of cell differentiation. RA blocks adipogenesis, but mechanisms remain to be established. ZFP423 is a key transcription factor maintaining white adipose identity. We found that RA inhibits *Zfp423* expression and adipogenesis via blocking DNA demethylation in the promoter of *Zfp423*, a process mediated by growth arrest and DNA-damage-inducible protein alpha (GADD45A). RA induces the partnering between retinoic acid receptor (RAR) and tumor suppressor inhibitor of growth protein 1 (ING1), which prevents the formation of GADD45A and ING1 complex necessary for locus-specific *Zfp423* DNA demethylation. *In vivo*, vitamin A supplementation prevents obesity, downregulates *Gadd45a* expression, and reduces GADD45A binding and DNA demethylation in the *Zfp423* promoter. Inhibition of *Zfp423* expression due to RA contributes to the enhanced brown adipogenesis. In summary, RA inhibits white adipogenesis by inducing RAR and ING1 interaction and inhibiting *Gadd45a* expression, which prevents GADD45A-mediated DNA demethylation.

Keywords: vitamin A, retinoic acid, *Gadd45a*, adipogenesis, demethylation, *Zfp423*

Introduction

Due to the global epidemic of obesity, there is an urgency to understand mechanisms regulating adipose development. Adipogenesis is initiated by the expression of ZFP423, which induces the expression of peroxisome proliferator-activated receptor γ (PPARGγ) and CCAAT-enhancer-binding proteins (C/EBPs), and genes specific for adipocytes (Gupta et al., 2010, 2012). Recently, ZFP423 was found to maintain white adipocyte identity by suppressing beige cell thermogenic gene program (Shao et al., 2016). This exciting discovery provides a new target for obesity prevention.

Retinoic acid (RA), a derivative of vitamin A, plays major roles in adipogenesis. In embryonic stem cells, RA induces preadipocyte commitment (Dani et al., 1997). In 3T3-L1 cell line, RA blocks adipocyte maturation by enhancing preadipocyte gene expression (Berry et al., 2012), as well as inhibiting C/EBPβ-mediated transcription (Schwarz et al., 1997). All-trans-RA serves as a ligand for retinoic acid receptors (RARs), which partner with retinoid X receptors (RXRs) (Chawla et al., 2001), and the complex binds to retinoic acid response elements (RAREs) on target genes (de The et al., 1990). Depending on the availability of RA, RAR-RXR heterodimers interact with either nuclear co-repressor proteins including silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) and nuclear receptor co-repressor (NCoR), or coactivators such as p160 steroid receptor co-activator (SRC/p160) family and p300/CREB binding protein (CBP) (Kashyap and Gudas, 2010). RA also binds to peroxisome proliferator-activated receptor β/Δ (PPARβ/Δ) to induce the expression of uncoupling protein 1 (*Ucp1*), which promotes energy dissipation (Noy, 2013).

Growth arrest and DNA damage protein 45a (*Gadd45a*) is a member of a stress response gene family, which encodes 18-kDa acidic histone fold proteins (Zhan et al., 1994). GADD45A mediates DNA demethylation by recruiting the nucleotide excision repair (NER) and base excision repair machineries (Niehrs and Schafer, 2012), as well as through thymine DNA glycosylase (TDG)-induced oxidative demethylation (Li et al., 2015). However, GADD45A lacks a DNA-binding domain and depends on tumor suppressor inhibitor of growth protein 1 (ING1) to recruit to promoters enriched with H3K4me3, which then triggers locus-specific DNA demethylation (Schafer et al., 2013). ING1 has several isoforms; the human $p33^{ING1b}$ (p37 for mouse)

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induces hyperacetylation of histones H3 and H4 through forming complexes with histone acetyltransferase, whereas human $p47^{ING1a}$ (p31 for mouse) binds histone deacetylases (HDACs) and inhibits histone acetylation (Gong et al., 2005).

Up to now, there is only indirect evidence supporting the role of epigenetic regulation in adipogenesis. One important evidence is derived from the preventive effect of dietary methyl donors on obesity (Waterland et al., 2008), while deficiency of methyl donors, which are required for DNA and histone methylations, induces obesity and metabolic diseases (Pogribny et al., 2009). In this study, we found that GADD45A is involved in *Zfp423* demethylation during adipogenesis. RA induces RAR and ING1 partnering and downregulates *Gadd45a* expression, which reduces GADD45A recruitment to ING1. Consequently, RA inhibits DNA demethylation in the *Zfp423* promoter and adipogenesis.

Results

RA blocks adipogenesis of preadipocyte via downregulating Zfp423

C3H10T1/2, adipose tissue-derived stromal vascular fraction cells (SVCs), and 3T3L1 cells were treated with RA at the first 2 days of adipogenesis. Consistent with previous studies (Schwarz et al., 1997; Berry et al., 2012), RA strongly inhibited lipid accumulation in these cells (Figure 1A). To explore the effects of RA on *Zfp423* expression during preadipocyte commitment and adipocyte maturation stages, C3H10T1/2 cells were pre-treated with 10 ng/ml BMP4 for 2 days, then induced adipogensis using adipogenic cocktails (MDI) containing IBMX, dexamethasone (DEX), and insulin. Cells were treated with or without RA throughout the experiment. During both commitment and terminal adipogenic differentiation of C3H10T1/2 cells, in the absence of RA, *Zfp423* expression was upregulated. With RA treatment, at 4 h, *Zfp423* mRNA expression was slightly increased, but then profoundly reduced after 12 h (Figure 1B). ZFP423 and PPARG protein content were also reduced by RA at 4 days after MDI treatment (Figure 1C). Similarly, RA inhibited the expression of *Zfp423* in 3T3-L1 cells and SVCs (Supplementary Figure S1). ZFP423 is a key transcription factor which initiates adipogenesis (Gupta et al., 2010, 2012). To determine whether RA inhibits adipogenesis via *Zfp423*, C3H10T1/2 cells were transfected with *Zfp423* or eGFP plasmid. RA downregulated the expression of adipose genes including *Pparg*, *Cebpa*, *Cebpb*, and *Fabp4* (Figure 1D) and completely inhibited lipid accumulation (Figure 1E), whereas *Zfp423* overexpression rescued it. In summary, RA blocks adipogenesis of preadipocytes via downregulating *Zfp423*.

GADD45A is involved in Zfp423 demethylation during adipogenesis of C3H10T1/2 cells

Our lab previously reported that *Zfp423* DNA is demethylated during fetal adipose development (Yang et al., 2013), then we further explored the mechanism of *Zfp423* demethylation. GADD45A was reported to play an essential role in active DNA

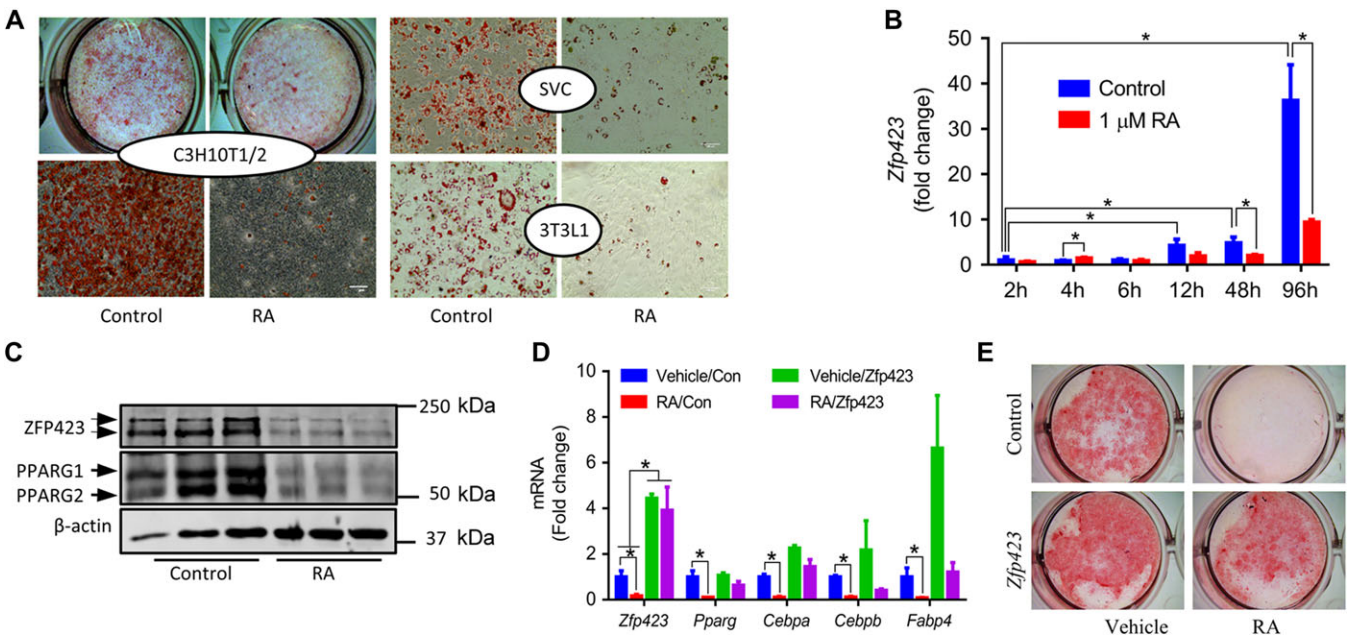

Figure 1 RA blocks adipogenesis of preadipocyte via downregulating *Zfp423*. (**A**) C3H10T1/2 cells, stromal vascular fraction cells (SVC), and 3T3L1 cells were treated with or without RA at the first 2 days of adipogenesis. Lipid droplets were stained by Oil-Red-O after 6 days of differentiation. (**B** and **C**) C3H10T1/2 cells were pre-treated with 10 ng/ml BMP4 for 2 days. Cells were treated with or without 1 μm RA at 48 h when adipogenesis was induced using MDI. The mRNA levels of *Zfp423* at different time point (**B**) and protein contents of ZFP423 and PPARG after 4 days of adipogenesis (**C**) were analyzed. (**D** and **E**) C3H10T1/2 cells were transfected with *Zfp423* or eGFP (control) plasmid, and treated with BMP4 for 2 days. Then adipogenesis was induced by MDI with or without RA. Adipose gene expressions were analyzed after 4 days of differentiation (**D**). Lipid droplets were stained by Oil-Red-O after 6 days of differentiation (**E**). Results are presented as mean ± SEM (*$P < 0.05$, $n = 3$).

demethylation during osteogenic differentiation of adipose-derived cells (Zhang et al., 2011). We found that *Gadd45a* (Figure 2A), but not *Gadd45b* (Figure 2B) or *Gadd45g* (Figure 2C), was upregulated during C3H10T1/2 adipogenesis. The C3H10T1/2-derived preadipocytes, which were pre-treated with BMP4, had the highest *Gadd45a* expression after MDI treatment (Figure 2D). To determine the role of *Gadd45a* in adipogenesis, we overexpressed or knocked down *Gadd45a* in C3H10T1/2 cells and found that *Gadd45a* played an indispensable role in adipogenesis (Figure 2E and F) by regulating the expression of adipose genes including *Zfp423*, *Pparg*, *Cebpa*, *Cebpb*, and *Fabp4* (Figure 2G). Consistently, *Gadd45a* overexpression increased ZFP423 and PPARG protein contents (Figure 2H). Using pyrosequencing analysis, we found that *Gadd45a* overexpression strongly demethylated the CpG sites located at −584, −580, and −570 bp from TSS (Figure 2I). Collectively, we found that GADD45A mediates DNA demethylation of *Zfp423* and plays an essential role in adipogenesis.

RA blocks GADD45A-induced adipogenesis by preventing Zfp423 demethylation

We further determined the interaction of RA and GADD45A during adipogenesis. While *Gadd45a* overexpression promoted adipogenesis, RA strongly inhibited lipid accumulation in control (eGFP) and *Gadd45a*-overexpressing C3H10T1/2 cells (Figure 3A and B). Western blot (Figure 3C) and qRT-PCR (Figure 3D–F) data showed that although GADD45A protein level was not significantly changed by RA, RA strongly downregulated *Zfp423*, *Pparg* (Figure 3D), *Cebpa*, *Cebpb*, and *Fabp4* (Figure 3E) expression. Consistently, RA prevented the GADD45A-mediated DNA demethylation on *Zfp423* (Figure 3G). However, RA or GADD45A overexpression had no influence on DNA methylation levels of *Pparg* (Supplementary Figure S2), showing that the demethylation was locus-specific. Interestingly, *Gadd45a* overexpression downregulated the expression of *Pref-1* (Figure 3F), indicating that GADD45A promoted the differentiation of preadipocytes into mature adipocytes. Consistent with a previous study (Berry et al., 2012), RA upregulated the expression of *Pref-1*, *Sox9*,

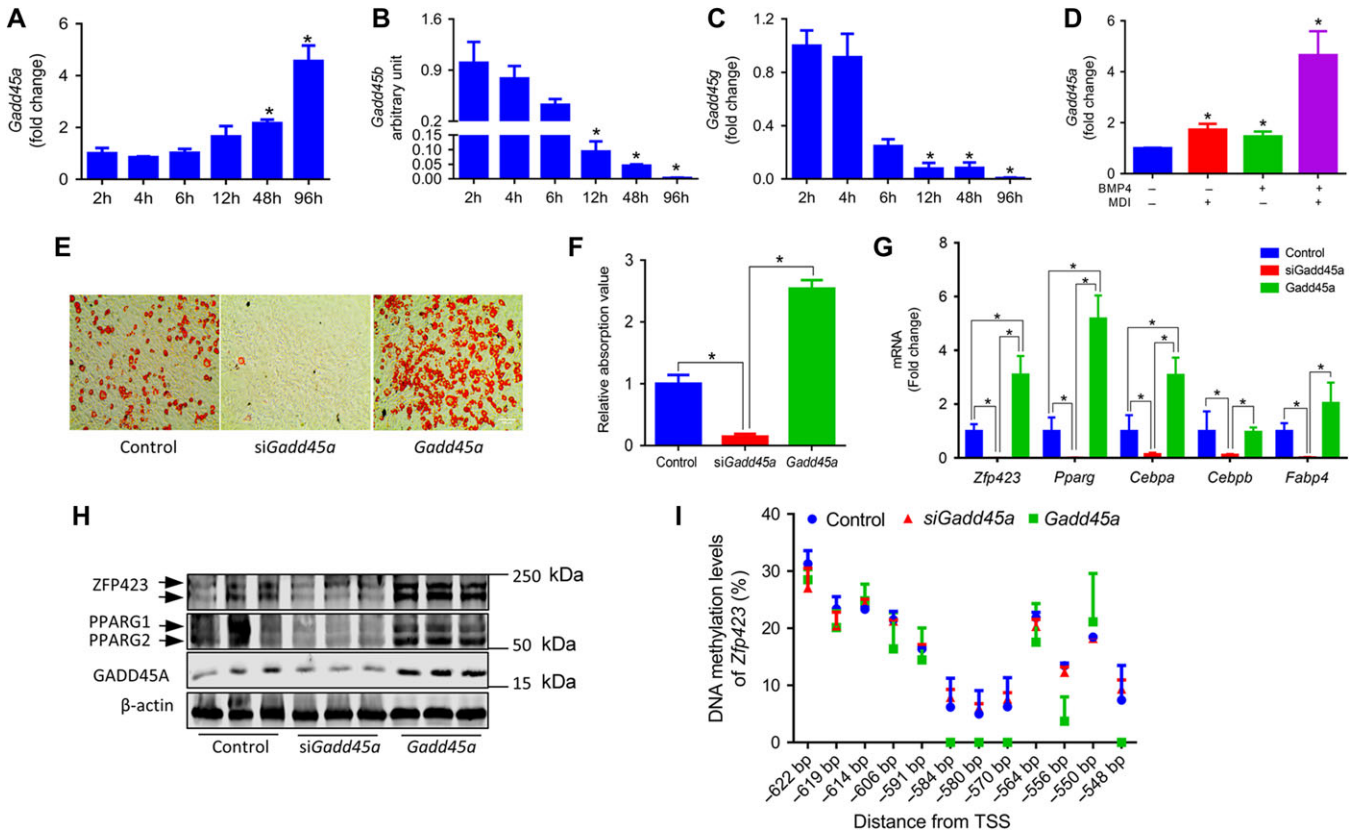

Figure 2 GADD45A is involved in *Zfp423* demethylation during adipogenesis of C3H10T1/2 cells. (**A**–**C**) C3H10T1/2 cells were pre-treated with 10 ng/ml BMP4 for 2 days. Adipogenesis was induced using MDI. *Gadd45a* (**A**), *Gadd45b* (**B**), and *Gadd45g* (**C**) expression was analyzed at different time points. (**D**) C3H10T1/2 cells were treated with/without BMP4 or vehicle for 2 days followed with/without MDI for 2 days, and *Gadd45a* expression was analyzed. (**E**–**I**) C3H10T1/2 cells were transfected with *Gadd45a*, eGFP (control) plasmids, or si*Gadd45a*, and treated with BMP4 for 2 days. Then adipogenesis was induced by MDI with or without RA. Lipid droplets were stained by Oil-Red-O after 6 days of differentiation (**E**) and the absorption values were quantified (**F**). The mRNA (**G**) and protein (**H**) levels of adipose genes were analyzed after 4 days of differentiation. (**I**) DNA methylation levels in the promoter of *Zfp423* were analyzed by bisulfite pyrosequencing. Results are presented as mean ± SEM (*$P < 0.05$, $n = 3$).

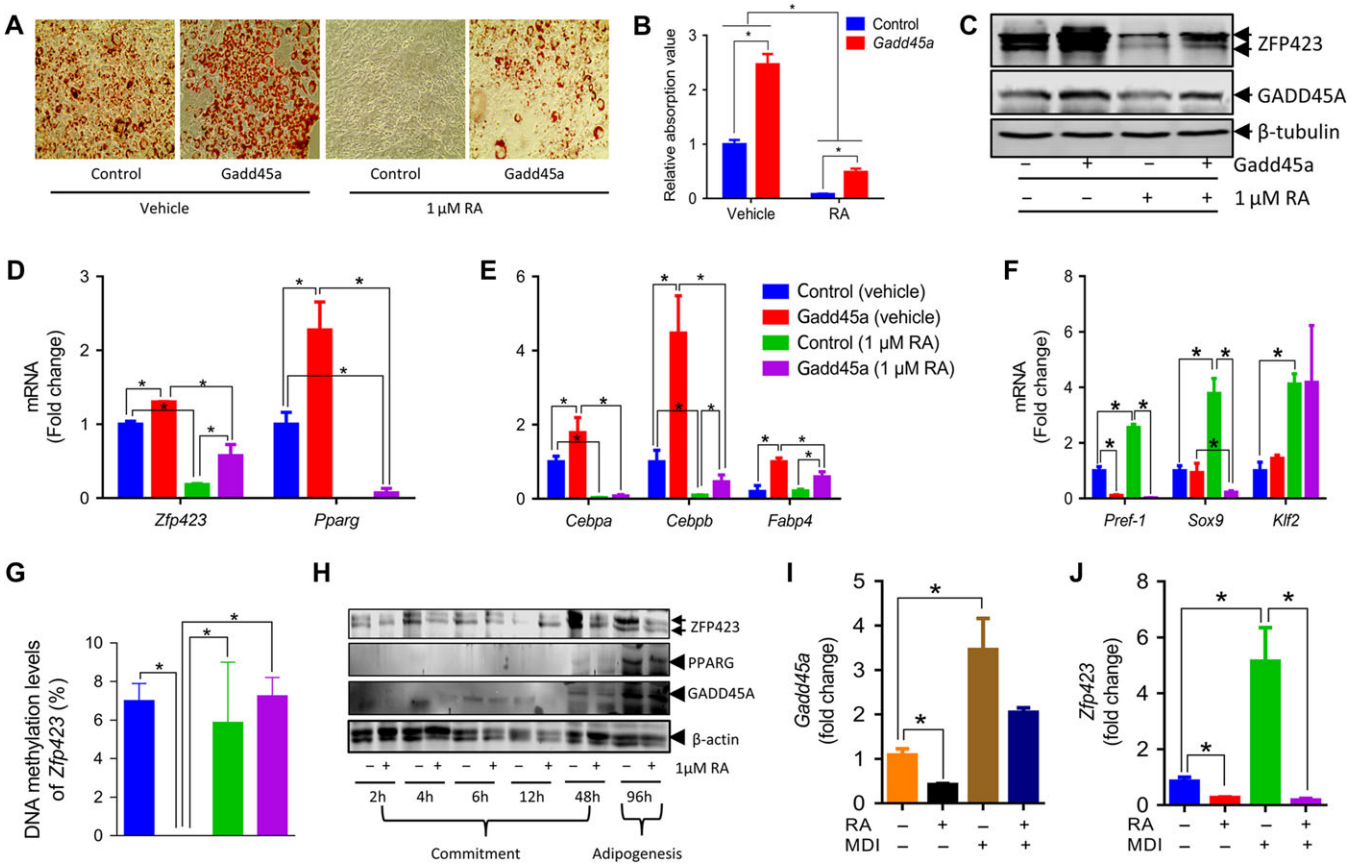

Figure 3 RA blocks GADD45A-induced adipogenesis by preventing *Zfp423* demethylation. (**A**–**F**) C3H10T1/2 cells were transfected with Gadd45a or eGFP (control) plasmid, and treated with 10 ng/ml BMP4 for 2 days. Adipogenic differentiation was then induced, with 1 μM RA treatment during the first 2 days of adipogenesis. After 6 days of adipogenesis, lipid droplets were stained with Oil-Red-O (**A**) and the absorption values were quantified (**B**). ZFP423 and GADD45A protein contents were analyzed at 4 day after RA addition (**C**). The expression of adipogenic genes including *Zfp423*, *Pparg* (**D**), *Cebpa*, *Cebpb*, and *Fabp4* (**E**) and preadipose genes including *Pref-1*, *Sox9*, and *Klf2* (**F**) at 4 days after RA was added. (**G**) C3H10T1/2 cells were transfected with *Gadd45a* or eGFP (control) plasmid. After treated with 10 ng/ml BMP4 for 2 days, cells were then treated with 1 μM RA or vehicle (DMSO) for 2 days. Methylation levels of CpG sites located at −570, −580, and −584 bp from TTS of *Zfp423* were analyzed by bisulfite pyrosequencing. (**H**) C3H10T1/2 cells were pre-treated with 10 ng/ml BMP4 for 2 days. Cells were treated with or without 1 μm RA for 48 h and then adipogenesis was induced using MDI. ZFP423, PPARG, and GADD45A protein contents were analyzed at different time points. (**I** and **J**) C3H10T1/2 cells were pre-treated with 10 ng/ml BMP4 for 2 days, then treated with RA and/or MDI as indicated for 2 days, and the expression of *Gadd45a* (**I**) and *Zfp423* (**J**) were analyzed by qRT-PCR. Results are presented as mean ± SEM (*$P < 0.05$, $n = 3$).

and *Klf2* (Figure 3F) to keep cells in the preadipocyte stage. We further found that the protein levels of ZFP423, PPARG, and GADD45A were strongly induced by MDI (Figure 3H). RA inhibited *Gadd45a* expression when cells were not induced by MDI, but had no effect after cells underwent adipogenesis (Figure 3I). However, RA constantly inhibited *Zfp423* expression regardless of MDI treatment (Figure 3H and J). Collectively, RA blocks adipogenesis by preventing GADD45A-mediated *Zfp423* demethylation without affecting *Gadd45a* expression.

RAR activation induces dissociation of GADD45A from the Zfp423 promoter

As mentioned above, RA interacts with other transcription factors and regulates gene expression through RAR-RXR heterodimer. However, RA also activates PPARβ/δ to enhance lipid oxidation and energy dissipation (Berry and Noy, 2009; Berry et al., 2010). To determine the involvement of RARs or PPARβ/δ in the inhibitory effects of RA on adipogenesis, we further treated C3H10T1/2 cells with the RAR-selective agonist TTNPB (Minucci et al., 1996), the RARα-selective antagonist ER50891 (Ren et al., 2005), the RXR antagonist PA452 (Takahashi et al., 2002), the RXRα-selective agonist CD3254 (Nahoum et al., 2007), the PPARβ/Δ-selective antagonist GSK0660 (Shearer et al., 2008), or the PPARβ/Δ-selective agonist GW0742 (Smith et al., 2004). TTNPB downregulated the expression of *Zfp423* and *Pparg*, while ER50891 upregulated *Zfp423* (Figure 4A). TTNPB treated cells remained spindle-shaped, and few visible lipid droplets were detected after 6 days of adipogenesis (Figure 4B). The

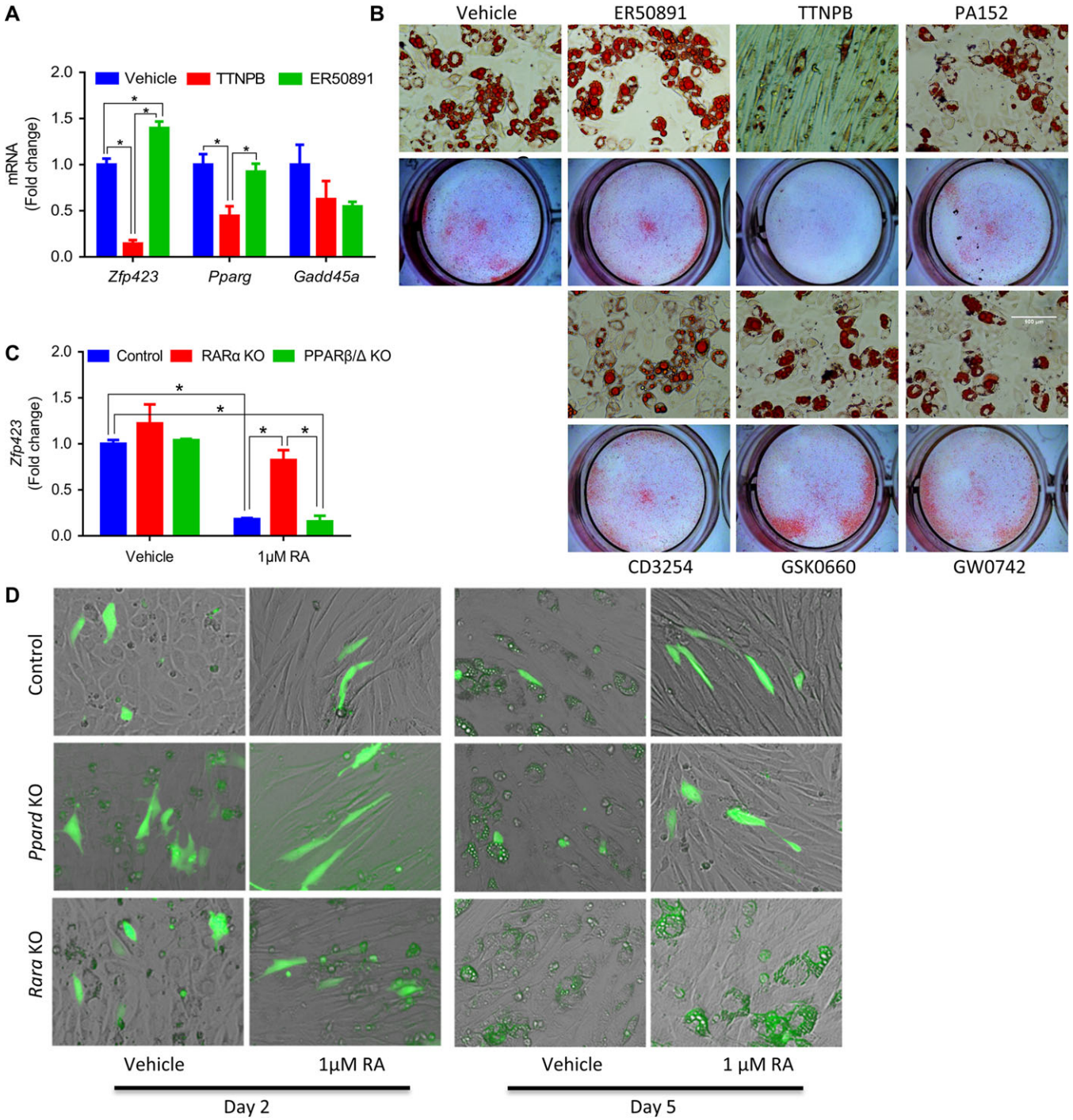

Figure 4 RAR activation mediates the inhibitory effect of RA on *Zfp423* expression and adipogenesis. (**A** and **B**) BMP4-pre-treated C3H10T1/2 cells were treated with DMSO (Vehicle), 1 μM TTNPB, 0.1 μM ER50891, 1 μM PA452 (a RXR antagonist), 1 μM CD3254 (a RXRα-selective agonist), 1 μM GSK0660 (a PPARβ/Δ-selective antagonist), or 0.05 μM GW0742 (a PPARβ/Δ-selective agonist). The mRNA expression of *Zfp423*, *Pparg*, and *Gadd45a* was analyzed by qRT-PCR at 4 days after adding MDI (**A**). Adipocytes were stained with Oil-Red-O at 6 days after inducing adipogenic differentiation (**B**). (**C** and **D**) *Rara* or *Ppard* in C3H10T1/2 cells was knocked out using CRISPR/Cas9 system, and the control CRISPR activation plasmid encodes a non-specific gRNA. Adipogenesis and RA treatment were performed as described previously. Cells were sampled 2 days after adding the cocktail. *Zfp423* expression was analyzed by qRT-PCR (**C**). Representative images at 2 and 5 days after adding cocktail (**D**). Results are presented as mean ± SEM (*$P < 0.05$, $n = 3$).

agonists and antagonists targeting RXR or PPARβ/Δ showed no visible effects on the adipocyte formation (Figure 4B). Furtherly, *Rara* and *Ppard* were knocked out in C3H10T1/2 cells using CRISPR/Cas9 system (gene-null cells were marked by GFP, which was carried by the transfected plasmid). *Rara* knockout abolished the inhibitory effect of RA on *Zfp423* expression, while *Ppard* knockout showed no effect (Figure 4C), indicating that RA inhibited *Zfp423* expression through RARα. After 6 days of adipogenesis, *Ppard* KO and control cells treated with RA were spindle-shaped and failed to accumulate lipid, while *Rara* KO cells accumulated lipid regardless of RA treatment (Figure 4D). Overall, these data show that RA inhibits *Zfp423* expression and adipogenesis via activation of RAR.

To further explore the mediatory role of RAR, we identified 6 potential RAREs on the *Zfp423* promoter (Figure 5A, Supplementary Table S4). Using chromatin immunoprecipitation (ChIP)–PCR, we found that region #1 had high binding affinity to both RAR (Figure 5B) and RXR (Figure 5C) upon RA activation. We analyzed the binding of GADD45A to the RARE region and the previous identified region (D-region) that can be demethylated by GADD45A, and an unrelated region was selected as a control (Figure 5D). GADD45A had high binding affinity to the D-region in the absence of RA. In the presence of RA, GADD45A dissociated from the D-region (Figure 5E). ING1 recruits GADD45A to DNA loci enriched with H3K4me3 to induce DNA demethylation (Schafer et al., 2013), so we further analyzed ING1 binding to the *Zfp423* promoter. Interestingly, we found more ING1 binding to the RARE region and less binding to the D-region (Figure 5F), indicating that ING1 switched from D-region to the RARE region. Furtherly, we found enrichment of H3K27me3, a repressive histone marker, at D-region (Figure 5G), while reduction of H3K4me3 on these sites (Figure 5H). To further confirm that the changes in binding pattern of factors to *Zfp423* is induced by RAR, we treated cells with TTNPB or ER50891. TTNPB led to GADD45A (Figure 5I) and ING1 (Figure 5J) dissociation from D-region. TTNPB recruited ING1 to the RARE region while ER50891 prevented it (Figure 5J).

RAR activation reduces GADD45A binding to the Zfp423 promoter by competing for ING1 ($p37^{ING1b}$)

The expression of *Ing1* was increased during adipogenesis of C3H10T1/2 cells (Supplementary Figure S3A). *Ing1* overexpression had no effects on *Zfp423* expression, but *Ing1* knockout inhibited *Zfp423* expression (Supplementary Figure S3B and C). Moreover, *Ing1* overexpression could not rescue *Zfp423* expression which was inhibited by RA (Supplementary Figure S3D and E). To further investigate the interaction among ING1, GADD45A and RAR, we performed co-immunoprecipitation (Co-IP) assays.

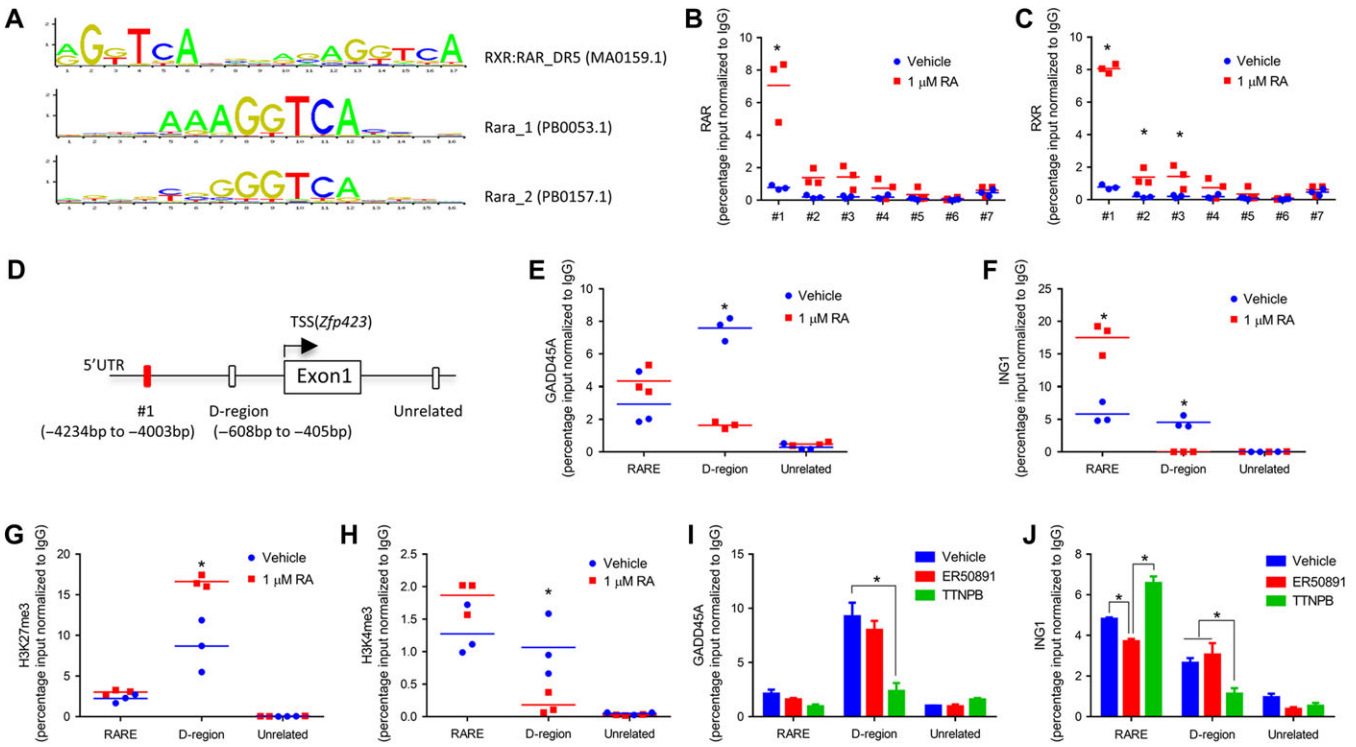

Figure 5 RAR activation induces dissociation of GADD45A from the *Zfp423* promoter. (**A**) Sequences for RARE prediction. (**B**–**H**) C3H10T1/2 cells were induced for adipogenic differentiation and treated with or without 1 μM RA for 2 days. ChIP analysis for the binding of RAR (**B**), RXR (**C**), GADD45A (**E**), ING1 (**F**), H3K27me3 (**G**), and H3K4me3 (**H**) to the specific regions in the *Zfp423* promoter. An unrelated region was selected as a control (**D**). (**I** and **J**) BMP4-pre-treated C3H10T1/2 cells were treated with DMSO (Vehicle), 1 μM TTNPB, or 0.1 μM ER50891 together with the adipogenic cocktail, and cells were sampled after 2 days of adipogenesis. ChIP analysis was performed to determine the binding of GADD45A (**I**) and ING1 (**J**) to the specific regions in the *Zfp423* promoter. Results are presented as mean ± SEM (*$P < 0.05$, $n = 3$).

Using anti-GADD45A as the IP antibody, we found RA inhibited the binding between $p37^{ING1b}$ and GADD45A (Figure 6A), while the binding between $p31^{ING1a}$ and GADD45A was undetectable. On the contrary, RA enhanced the binding between RAR and $p37^{ING1b}$ (Figure 6B). We further delivered plasmids overexpressing DDK-ING1 and HA-GADD45A to C3H10T1/2 cells, and found that RA reduced the binding of ectopically expressed $p37^{ING1b}$ to GADD45A (Figure 6C). When using anti-ING1 antibody as the IP antibody, we found more RAR but less GADD45A bound to ING1 (Figure 6D). Overall, these data demonstrate that RAR activation recruits ING1, which antagonizes the interaction between GADD45A and ING1 in the *Zfp423* promoter.

Vitamin A inhibits Zfp423 expression and prevents obesity induced by high-fat diet

To explore the effect of vitamin A on diet-induced obesity, 12-week-old C57BL/6 male mice were fed with either control diet or high-fat diet (HFD) supplemented without or with 30 IU/ml vitamin A (HFDVA, retinyl acetate) through water. Diet-induced obesity was prevented by vitamin A supplementation (Figure 7A–D). Although vitamin A supplemented mice had higher feed consumption (Supplementary Figure S4B). Compared to mice fed with control diet, the diameter of adipocytes (Figure 7B and C) and the total fat mass (Figure 7D) were increased in mice with HFD, while supplementation of vitamin A prevented this increase. These data showed that vitamin A supplementation inhibited fat accumulation when mice were challenged with HFD.

We did glucose tolerance test and found that mice fed with HFD plus vitamin A (HFDVA) had strong ability to clear blood glucose when compared to HFD without vitamin A (Figure 7E). HFD also increased serum triglyceride level, while vitamin A reduced it (Figure 7F). Similarly, vitamin A reduced the increase in serum insulin that was induced by HFD (Figure 7G). We then analyzed the expression of adipogenic genes in epididymal white adipose tissue (eWAT) and found that HFD dramatically upregulated the expression of *Gadd45a*, *Zfp423*, and downstream genes including *Pparg*, *Cebpa*, *Cebpb*, and *Fabp4*, which were abolished due to vitamin A supplementation (Figure 7H–M). Because *Zfp423* is a critical gene initiating white adipogenesis, its upregulation might promote white adipogenesis instead of brown adipogenesis in HFD mice. Consistently, it was recently reported that *Zfp423* deficient mice had higher expression of brown adipose genes in WAT (Shao et al., 2016). Here we furtherly found that vitamin A supplementation upregulated the expression of brown adipose genes (Supplementary Figure S4C) including peroxisome proliferative activated receptor, gamma, coactivator 1 alpha (*Pgc1a*), PR domain containing 16 (*Prdm16*), uncoupling protein 1 (*Ucp1*), cell death-inducing DNA fragmentation factor, alpha subunit-like effector A (*Cidea*), and cytochrome c oxidase subunit VIIa 1 (*Cox7a1*), consistent with the reduction of fat accumulation in mice.

In alignment with *in vitro* data, HFD dramatically increased the binding of GADD45A to the D-region, and vitamin A supplementation decreased GADD45A (Figure 7N) and ING1 (Figure 7O) binding to the D-region but increased ING1 (Figure 7O) binding to the

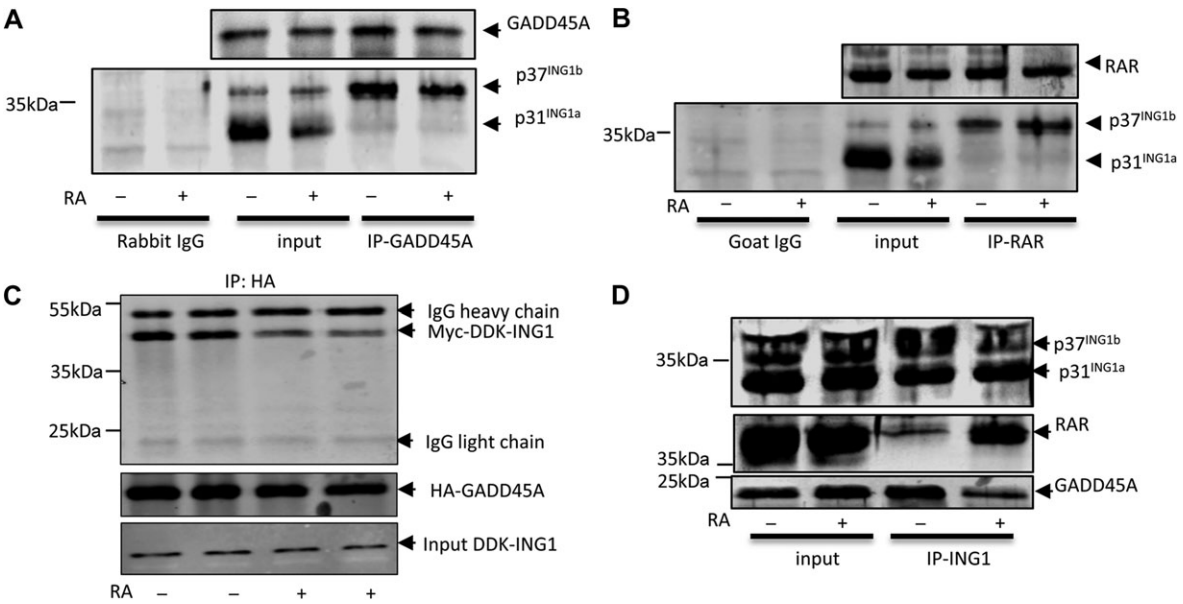

Figure 6 RAR activation reduces GADD45A binding to the *Zfp423* promoter by competing for ING1. (**A**, **B**, and **D**) C3H10T1/2 cells were pretreated with 10 ng/ml BMP4 for 2 days, and then adipogensis was induced using adipogenic cocktail with or without RA. Cells were sampled 2 days after adding the cocktail for Co-IP analysis. Antibodies used for IP and proteins analyzed by western blotting were indicated, and non-specific IgG antibody was used as negative control. (**C**) C3H10T1/2 cells were transfected with Myc-DDK-ING1 or pcDNA3-HA-GADD45A plasmid with/without 1 μM RA for 24 h. Co-IP analysis was performed using anti-HA antibody to pull down the protein complex, and western blot analysis was performed using the indicated antibodies. Results are presented as mean ± SEM ($*P < 0.05$, $n = 3$).

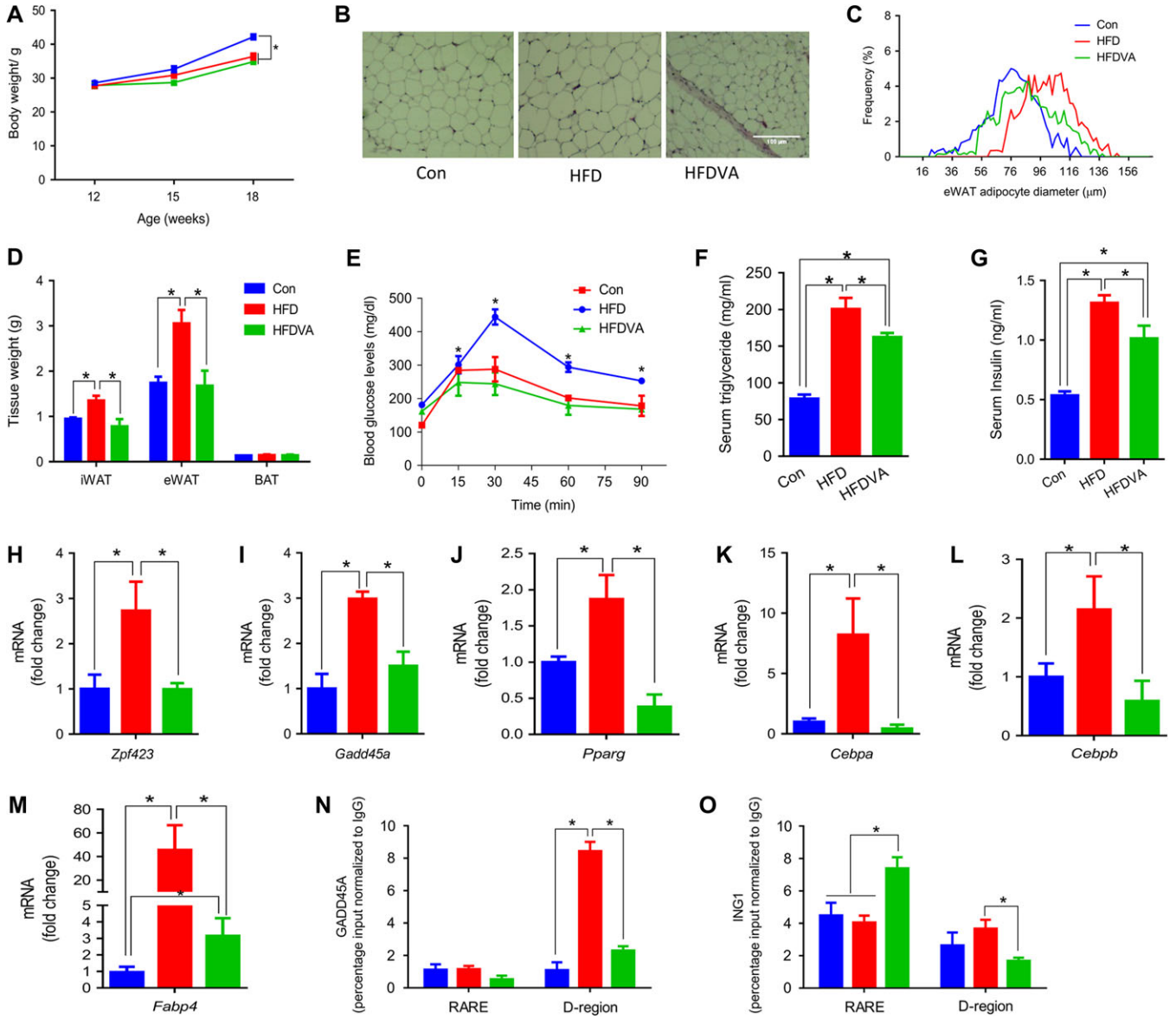

Figure 7 Vitamin A supplementation prevents diet-induced obesity. Three groups of C57BL/6 male mice were treated with control diet (Con), HFD, or HFDVA (with 30 IU/ml vitamin A) for 6 weeks. (**A**) Body weight. (**B**) Representative H&E stained paraffin sections showing eWAT adipocytes. (**C**) Adipocyte size distribution in eWAT. (**D**) Weight of inguinal WAT (iWAT), eWAT, and brown adipose tissue (BAT). (**E**) Glucose tolerance test. (**F**) Serum triglyceride content. (**G**) Serum insulin content. (**H**–**M**) Expression of *Zfp423*, *Gadd45a*, *Pparg*, *Cebpa*, *Cebpb*, and *Fabp4* in eWAT analyzed by qRT-PCR. (**N** and **O**) Genomic DNA was extracted from eWAT, and ChIP analysis was performed for binding of GADD45A (**N**) and ING1 (**O**) to the specific regions in the *Zfp423* promoter. Results are presented as mean ± SEM (*$P < 0.05$, $n = 6$).

RARE region. To determine the effects of vitamin A supplementation on the methylation status of *Zfp423* promoter, we extracted genomic DNA from eWAT and analyzed the methylation level using methylation-specific PCR covering sites with differential DNA methylation identified previously (Figure 8A). HFD strongly reduced the methylation of *Zfp423* promoter, while vitamin A supplementation increased the methylation level (Figure 8B and C). We found the similar DNA methylation pattern of *Zfp423* in SVCs separated from adipose tissue of these mice (Supplementary Figure S4D), but the methylation levels were higher in SVCs than the whole tissue lysate. Overall, these data show that diet-induced obesity promotes *Zfp423* DNA demethylation and gene expression that is mediated by GADD45A. Vitamin A inhibits *Zfp423* expression and prevents diet-induced obesity *in vivo* by downregulating *Gadd45a* expression and dissociating GADD45A from the *Zfp423* promoter.

Discussion

RA is known to regulate adipose development and metabolism. Ablation of retinol dehydrogenase 1 (*rdh1*) in mice leads to

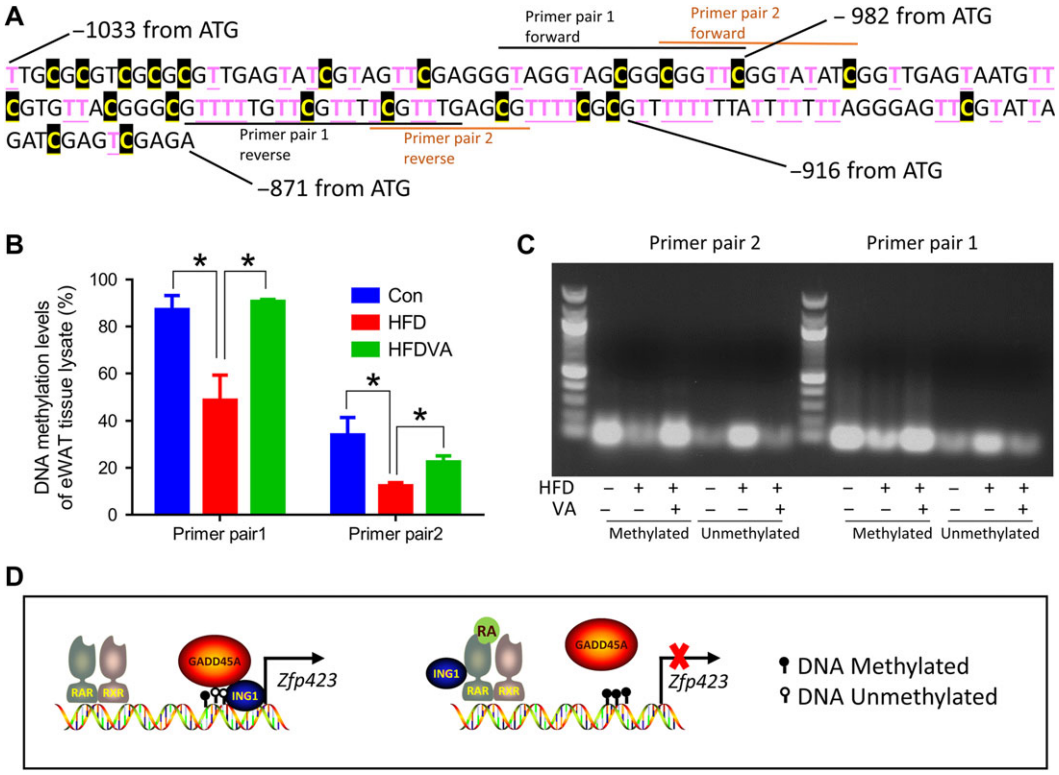

Figure 8 Vitamin A inhibits *Zfp423* demethylation in adipose tissue of obese mice. (**A**) Location of bisulfate-converted *Zfp423* nucleotide sequence and methylation-specific PCR (MSP) primers. (**B** and **C**) Genomic DNA was extracted from eWAT of the treated mice in Figure 6. Methylation level of the *Zfp423* promoter was analyzed by MSP and quantified by qRT-PCR (**B**). Results are presented as mean ± SEM (*$P <$ 0.05, $n = 6$). MSP products shown by agarose gel electrophoresis (**C**). (**D**) Diagram showing that RA inhibits GADD45A-induced DNA demethylation and expression of *Zfp423*.

increased adipocyte size and adiposity (Zhang et al., 2007). On the contrary, RA treatment results in weight loss and improves glucose tolerance (Berry and Noy, 2009). In embryonic stem cells, RA promotes adipogenic program at the early stage of adipogenesis, before PPARG functions (Dani et al., 1997); RA activates RA receptors to upregulate the expression of adipogenic inhibitors *Pref-1*, *Sox9*, and *Klf2* (Berry et al., 2012). In mature adipocytes, RA activates both RARs and PPARβ/δ to enhance lipid oxidation and energy dissipation (Berry and Noy, 2009; Berry et al., 2010), strongly reducing lipid accumulation (Schwarz et al., 1997; Berry et al., 2012). The accessibility of RA to different receptors is mediated by lipid-binding proteins, cellular retinoic acid binding protein II (CRABP2) and fatty acid binding protein 5 (FABP5) (Berry et al., 2010). CRABP2 delivers RA to RARs, while FABP5 transports it to PPARβ/Δ (Tan et al., 2002; Schug et al., 2007). The expression of CRABP2 dominates in progenitor cells and preadipocytes, which delivers RA to RARs (Berry et al., 2010); as a result, the biological function of RA in progenitor cells is primarily mediated by RARs.

Despite these progresses, the mechanisms responsible for the inhibitory effect of RA on adipogenic differentiation remains undefined. During white adipogenesis, ZFP423 induces preadipocyte determination, as well as the expression of PPARG and adipocyte differentiation (Gupta et al., 2010; Hudak et al., 2014). In this study, we used C3H10T1/2 cells that pre-treated with BMP4 as a white adipogenesis model (Huang et al., 2009). Upregulation of *Zfp423* was observed during the white adipogenesis of C3H10T1/2 cells. RA blocked terminal white adipogenesis by downregulating the expression of *Zfp423* and its downstream adipogenic genes including *Pparg*, *Cebpa*, and *Cebpb*, but upregulated brown adipose genes. Consistently, ZFP423 was recently identified as a white/brown switcher, which induces white adipogenesis whereas inhibits beige/brown adipogenesis (Shao et al., 2016). Thus, inhibition of ZFP423, at least partially, contributed to the upregulation of brown/beige adipose genes.

DNA methylation is indispensable for determining cell fates during development (Sheaffer et al., 2014). Recent evidence, however, highlights the critical role of DNA demethylation in initiating lineage-specific gene expression and cell differentiation, including the differentiation of osteocytes (Zhang et al., 2011), adipocytes (Palacios-Ortega et al., 2014), and monocytes (Karpurapu et al., 2014). During adipogenesis, though alteration of epigenetic patterns of *Pparg* (Zhao et al., 2013) and caveolin 1 (*Cav1*) (Palacios-Ortega et al., 2014) has been observed, DNA demethylation of key adipogenic genes has not been examined. For the first time, we found that GADD45A is involved in adipogenesis of C3H10T1/2 by promoting *Zfp423* demethylation in the promoter region.

As previously discussed, ING1 directs GADD45A to the locus enriched with H3K4me3 (Schafer et al., 2013). Here, we discovered that RA liganded RARs bind with ING1, which deprives ING1 and hampers the partnership between GADD45A and ING1. ING1 also forms complexes with both HATs and HDACs that regulate gene expression through remodeling chromatin structure (Gong et al., 2005), which may explain the accumulation of HDAC5 on *Zfp423* potential RARE sites. Interestingly, RARs also physically interact with HDACs upon RA ligation, which regulates epigenetic changes in embryonic stem cells (Urvalek and Gudas, 2014). Thus, ING1 functions as a hub for a regulatory complex involving RARs or GADD45A to regulate DNA demethylation and the expression of key developmental genes. Such mechanism is expected to not only function during adipogenesis, but also be applicable for other stem cell differentiation.

In summary, we discovered a new mechanism by which vitamin A and RA prevent fat accumulation via blocking active DNA demethylation in the promoter of a key adipogenic gene, *Zfp423*; RA switches the partnership of ING1 from GADD45A to RAR, reducing GADD45A available for catalyzing *Zfp423* DNA demethylation. The inhibition of *Zfp423* expression due to RA may contribute to the enhanced brown adipogenesis. Due to the easiness to manipulate vitamin A intake, our studies have important clinical applications for the prevention of obesity.

Materials and methods

Tissue culture, adipogenesis induction, plasmid, and siRNA transfection

C3H10T1/2 cells were grown at 37°C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM), 10% fetal calf serum (FBS), and a 1% antibiotic mixture. Unless declared, cells were pre-treated with 10 ng/ml BMP4 at 40%–60% confluence, in order to induce adipogenic commitment (Tang et al., 2004). At 100% confluence, culture medium was added the adipogenic cocktail including insulin (1 μg/ml), dexamethasone (0.1 μg/ml), and IBMX (27.8 μg/ml) for 3 days, then switched to insulin (1 μg/ml) for 3 days. Plasmids pcDNA3-HA + WT GADD45A (Addgene plasmid #24929) and ING1 (Myc-DDK-tagged) (ORIGENE, #MR225175), CRISPR plasmids (synthesized by GenScript) targeting *Rara*, *Ppard*, and *Ing1*, and *Gadd45a* siRNA (BioNEER, #1360555) were transfected using a Neon Transfection System for Electroporation (ThermoFisher Scientific).

Oil-Red-O staining of differentiated adipocytes and the quantification of Oil-Red-O dye were conducted as described previously (Yang et al., 2013).

Antibodies and chemicals

Antibodies against ZFP423 (sc-48785), GADD45A (sc-797), PPARG (sc-7273), RAR (sc-773), RXR (sc-774), ING1 (sc-7566), and HA-probe (sc-7392) were purchased from Santa Cruz Biotech. Antibodies against β-tubulin (#2146), H3K4me3 (#9751 s), H3K27me3 (#9756 s), HDAC5 (#2082 s), H3K27ac (#4353 s), and DYKDDDDK Tag (#8146 s) were purchased from Cell Signaling. TTNPB (#0761), ER50891 (#3823), PA452 (#5086), CD3254 (#3302), GSK0660 (#3433), and GW0742 (# 2229) were purchased from Tocris Bioscience.

Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol (Life technologies), and cDNA was synthesized using iScript™ cDNA Synthesis Kit (Bio-Rad). qRT-PCR was performed using CFX RT-PCR detection system (Bio-Rad) with SYBR green RT-PCR kit from Bio-Rad. Primer sequences are listed in Supplementary Table S1. All values were normalized to the level of a housekeeping gene 18S rRNA.

Immunoblotting analyses

Immunoblotting analysis was performed as previously described (Yang et al., 2013) using an Odyssey Infrared Imaging System (LiCor Biosciences). Density of bands was quantified and then normalized according to the β-actin or β-tubulin content.

Bisulfite pyrosequencing

Genomic DNA was isolated from treated cells using a genomic DNA isolation kit (Qiagen). Bisulfite conversion of DNA was performed using a Zymo Research EZ DNA methylation-direct kit (Cat. No. D5021). Then, converted genomic DNA was used as the template to amplify target sequence. The Biotinylated PCR primers for *Zfp423* were designed to cover 12 CpG sites located at −982 to −908 bp from ATG and −622 to −548 bp from TSS (Ensembl Transcript ID: ENSMUST00000109655). After PCR amplification, amplicons were sent to EpigenDx for pyrosequencing.

Methylation-specific PCR

Genomic DNA was extracted from eWAT and bisulfite conversion was performed. Primers amplifying modified & methylated DNA and modified & unmethylated DNA were designed using Methylprimer Express Software 1.0 (ThermoFisher Scientific) and listed in Supplementary Tables S2 and S3. qRT-PCR was performed and the sizes of PCR amplicons were verified using 1.5% agarose gel electrophoresis.

Co-IP and ChIP

Co-IP and ChIP were conducted as previously described (Yang et al., 2013). The putative RAREs in *Zfp423* promoter were predicted using the JASPAR database (http://jaspardev.genereg.net/). Primers covering these sites are listed in Supplementary Table S4.

Mice

Animal studies were conducted in AAALAC-approved facilities and according to protocols approved by the Institutional Animal Use and Care Committee (IAUCC). Mice were fed with control diet (Con, D12450H, Research Diets) containing 4.7 IU/g vitamin A and 10% energy from fat, HFD (D12492, Research Diets) containing 4.7 IU/g vitamin A and 60% energy from fat, or HFDVA supplemented with 30 IU/ml vitamin A (Retinyl acetate which is

water dispersible, Cat. No. 02103257, MP Biomedicals) through water for 6 weeks. Water was changed every other day to avoid degradation of vitamin A. The dose of vitamin A was chosen based on previous reports (Granados et al., 2013) and our previous study (Wang et al., 2017). The mice drank about 3 ml/day (Supplementary Figure S4A), thus 90 IU/day/mouse vitamin A was provided through water. At the end of experiments, mice were anaesthetized by fluorine inhalation before euthanization by cervical dislocation, and fat samples were collected.

Glucose tolerance test

Following 6 h-fasting, mice were administered with 2 g/kg BW D-glucose. Blood samples were collected from the tail veil at 0, 15, 30, 60, and 90 min post injection and glucose concentration was measured using a glucose meter (Bayer Contour).

Triglyceride measurement

Serum triglyceride was measured using a triglyceride colorimetric assay kit purchased from Cayman (Cat. 10010303).

Statistics

Data were analyzed using unpaired two-tailed Student's *t*-test or one-way ANOVA (for multiple comparison) where appropriate, using SAS 9.0 (SAS Institute Inc.). $P < 0.05$ was considered significant. All data are expressed as mean ± standard errors of the mean (SEM).

Supplementary material

Supplementary material is available at *Journal of Molecular Cell Biology* online.

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