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RESEARCH ARTICLE

MLL3/MLL4-Associated PAGR1 Regulates Adipogenesis by Controlling Induction of C/EBPβ and C/EBPδ

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ABSTRACT Transcription factors C/EBPβ and C/EBPδ are induced within hours after initiation of adipogenesis in culture. They directly promote the expression of master adipogenic transcription factors peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα and are required for adipogenesis *in vivo*. However, the mechanism that controls the induction of C/EBPβ and C/EBPδ remains elusive. We previously showed that histone methyltransferases MLL3/MLL4 and associated PTIP are required for the induction of PPARγ and C/EBPα during adipogenesis. Here, we show MLL3/MLL4/PTIP-associated protein PAGR1 (also known as PA1) cooperates with phosphorylated CREB and ligand-activated glucocorticoid receptor to directly control the induction of C/EBPβ and C/EBPδ in the early phase of adipogenesis. Deletion of *Pagr1* in white and brown preadipocytes prevents the induction of C/EBPβ and C/EBPδ and leads to severe defects in adipogenesis. Adipogenesis defects in PAGR1-deficient cells can be rescued by the ectopic expression of C/EBPβ or PPARγ. Finally, the deletion of *Pagr1* in Myf5$^+$ precursor cells impairs brown adipose tissue and muscle development. Thus, by controlling the induction of C/EBPβ and C/EBPδ, PAGR1 plays a critical role in adipogenesis.

KEYWORDS C/EBPβ, C/EBPδ, PAGR1, adipogenesis

The differentiation of preadipocytes toward adipocytes (adipogenesis) is regulated by a transcriptional network of sequentially expressed transcription factors (TFs) (1–3). Peroxisome proliferator-activated receptor γ (PPARγ) and C/EBPα are two master regulators of adipogenesis (3, 4). The ectopic expression of either PPARγ or C/EBPα in fibroblasts can induce adipogenic conversion (5, 6). During adipogenesis in culture, the expression of *Pparg* and *Cebpa* in preadipocytes is induced at relatively late phase, 1 to 2 days after exposure to a standard adipogenic cocktail of isobutylmethylxanthine (IBMX), dexamethasone (DEX), and insulin (collectively known as MDI), and are maintained at high levels throughout the differentiation (7, 8).

C/EBPβ and C/EBPδ are induced within hours of initiation of adipogenesis, preceding the induction of PPARγ and C/EBPα (7, 9). C/EBPβ and C/EBPδ promote PPARγ and C/EBPα expression and are required for adipogenesis (9, 10). The induction of these early adipogenic TFs in preadipocytes is largely dependent on stimulation by the adipogenic cocktail MDI. C/EBPβ and C/EBPδ are induced by IBMX and DEX, respectively, in the absence of protein synthesis (11). Although many studies have expanded our knowledge on the transcriptional regulation of adipogenesis, little is known about the factors that link hormone signaling to the coordinated induction of C/EBPβ and C/EBPδ (12).

Unlike many adipogenic TFs that are induced during adipogenesis, cyclic AMP

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(cAMP) response element-binding protein (CREB) is constitutively expressed prior to and throughout adipogenesis, suggesting that CREB is one of the initiating adipogenic TFs (13). CREB is phosphorylated at the Ser-133 residue upon stimulation by cAMP inducers IBMX and forskolin (14). Phospho-CREB (pCREB), the active form of CREB, binds to CRE-like elements in the *Cebpb* promoter, activates *Cebpb* expression, and promotes adipogenesis. Conversely, expression of a dominant-negative CREB, A-CREB, blocks *Cebpb* expression and adipogenesis, indicating that CREB acts upstream of C/EBPβ during adipogenesis (15). DEX, a synthetic ligand for glucocorticoid receptor (GR), induces *Cebpd* expression, suggesting that GR directly activates *Cebpd* expression in the early phase of adipogenesis (11). Studies have shown that GR, when bound and activated by DEX, associates with transiently open chromatin regions during the early phase of adipogenesis (16, 17). Although GR is dispensable for adipose development *in vivo*, it accelerates adipogenesis in cells (18).

Studies have identified essential roles of epigenomic regulators in promoting the expression of adipogenic TFs (12). We previously showed that histone H3K4 methyltransferases MLL3/MLL4 and associated PTIP are required for the induction of PPARγ and C/EBPα and adipogenesis (19, 20). Along with PTIP, a novel protein, PAGR1 (also known as PA1), is a unique component of the MLL3/MLL4 complex (21). PAGR1 and PTIP also form a separate lower molecular weight complex of ~200 kDa, which is independent of the MLL3/MLL4 complex of ~2 MDa (22, 23). PAGR1 interacts directly with PTIP but has no recognizable domains. PAGR1 has been implicated in DNA damage response (22). However, the biological functions of PAGR1 under normal physiological conditions remain almost completely unknown, except that PAGR1 is essential for mouse embryonic development (24). Therefore, we sought to determine whether PAGR1 plays a role in regulating adipogenesis.

In this study, we show that PAGR1 is dispensable for cell proliferation but is required for adipogenesis in cells and in mice. PAGR1 cooperates with phosphorylated CREB and ligand-activated GR to directly control the induction of early adipogenic TFs C/EBPβ and C/EBPδ within hours of the initiation of adipogenesis.

RESULTS

Reduced expression of C/EBPβ and C/EBPδ in PAGR1 knockout mouse embryonic fibroblasts (MEFs). To understand the biological functions of the novel protein PAGR1, we generated conditional knockout (KO) *Pagr1*$^{lox/lox}$ mice as well as whole-body KO *Pagr1*$^{-/-}$ mice (Fig. 1A to D). The mouse *Pagr1* gene contains 3 exons. Exons 1 and 2 encode the first 187 amino acids of the 253-amino-acid-long PAGR1 protein and were flanked by two *loxP* sites in the conditional KO (lox) allele. The targeted allele contains a *loxP* site in front of exon 1 and a neomycin (neo) selection cassette flanked by 2 *loxP* sites located in intron 2. Deletion of the neo selection cassette from the targeted allele by Cre recombinase generates the lox allele. Deletion of the entire loxed region, including exons 1 and 2 from the targeted allele, generates the null allele (Fig. 1A). *Pagr1*$^{-/-}$ mice showed embryonic lethality and died at around embryonic day 8.5 (E8.5) to ~E9.5 (24).

To identify PAGR1-regulated genes, we isolated and immortalized *Pagr1*$^{lox/lox}$ mouse embryonic fibroblasts (MEFs). Cells were infected with retroviruses expressing Cre recombinase (Fig. 1E). Deletion of *Pagr1* was confirmed by Western blotting and quantitative reverse transcriptase PCR (qRT-PCR) (Fig. 1F and I). KO of PAGR1 in MEFs did not affect cell growth (Fig. 1G). Microarray analysis using mouse whole-genome expression arrays revealed that deletion of *Pagr1* led to a more than 2.5-fold decreased expression of 61 genes and increased expression of 3 genes (see Table S1 in the supplemental material). Gene ontology analysis of the downregulated genes identified *Bcl3*, *Bmp4*, *Cebpb*, *Gap43*, *Igfbp5*, *Ntrk3*, *Sfrp1*, and *Col11a1* as the general cell differentiation genes and *Cebpb*, *Cebpd*, *Klf4*, and *Krox20* as the fat cell differentiation genes (Fig. 1H). PAGR1-regulated genes identified from the microarray analysis were further confirmed by qRT-PCR. Specifically, deletion of *Pagr1* in MEFs led to a more than 4-fold decreased expression of genes encoding the pioneering adipogenic TFs C/EBPβ and

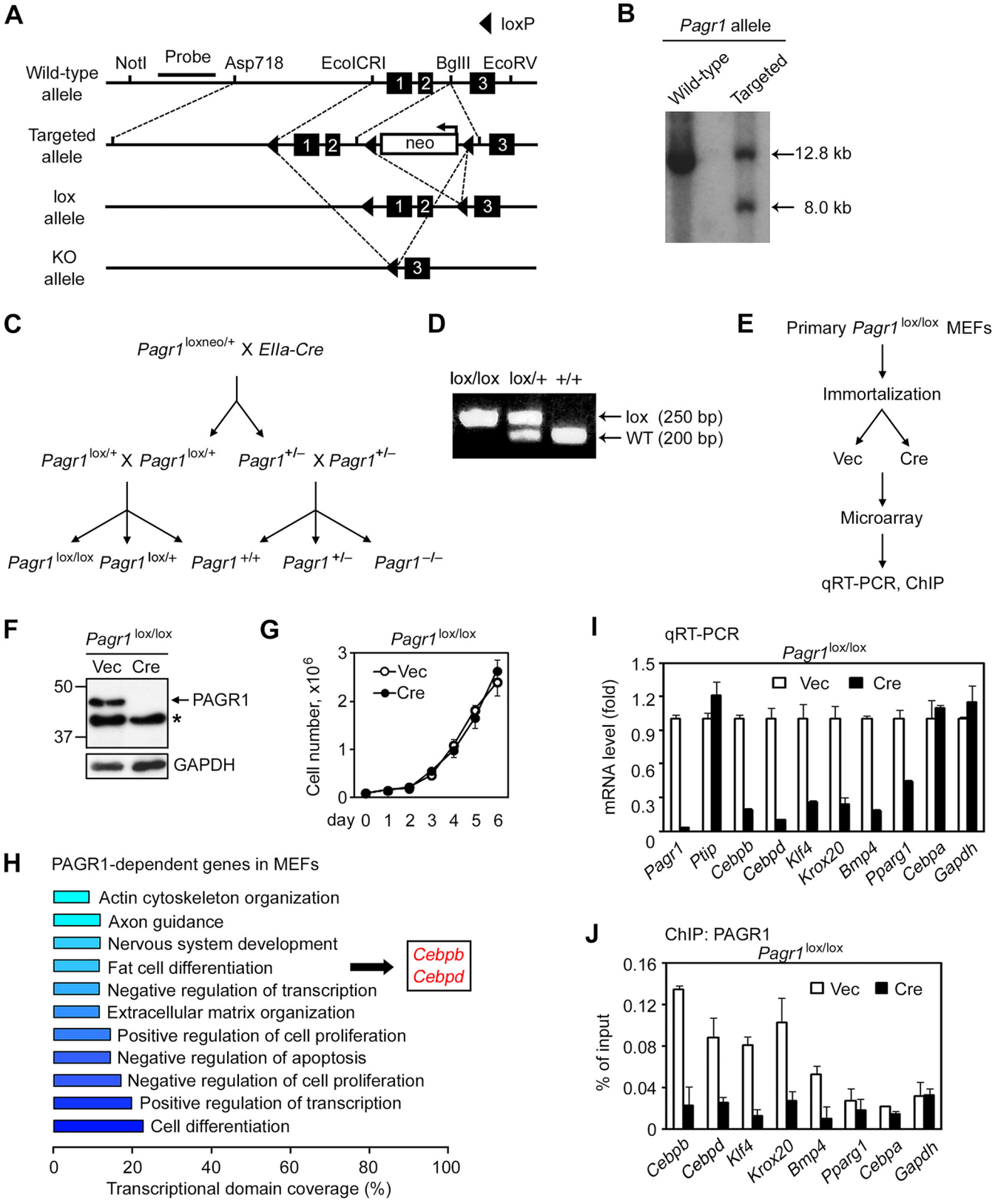

FIG 1 PAGR1 is required for *Cebpb* and *Cebpd* expression in MEFs. (A) Schematic representation of mouse *Pagr1* genomic locus (wild-type allele), targeted allele, *lox* allele, and knockout (KO) allele. Restriction endonuclease sites and Southern blot probe are indicated. The *Pagr1* gene contains 3 exons, represented by the 3 black rectangles. (B) Genomic DNA prepared from ES cells was digested with NotI and EcoRV and hybridized with 5′ flanking probe in Southern blotting. Southern blotting detected an expected 12.8-kb band from the wild-type allele and an expected 8.0-kb band from the targeted allele. (C) Mating strategies for generating PAGR1 conditional KO and whole-body KO mice. (D) Confirmation of 3-week-old *Pagr1*$^{lox/lox}$ mice by PCR genotyping. (E) Schematic of experimental

(Continued on next page)

C/EBPδ (Fig. 1I). By chromatin immunoprecipitation (ChIP) assays in Vec- and Cre-infected *Pagr1*$^{lox/lox}$ MEFs, we observed specific enrichment of endogenous PAGR1 protein on the promoters of *Cebpb* and *Cebpd* but not those of *Pparg1* and *Cebpa*, which work in the later phase of adipogenesis (Fig. 1J). Taken together, these data indicate that PAGR1 directly controls the expression of early adipogenic TFs C/EBPβ and C/EBPδ in MEFs.

PAGR1 is required for adipogenesis in culture. Since PAGR1 was required for expression of C/EBPβ and C/EBPδ in MEFs, we tested whether PAGR1 was required for adipogenesis. For this purpose, we isolated primary white and brown preadipocytes from *Pagr1*$^{lox/lox}$ mice. Cells were infected with adenoviral Cre followed by adipogenesis assays. Cre-mediated *Pagr1* deletion was confirmed by qRT-PCR (Fig. 2A and E). Deletion of *Pagr1* had little effect on cell growth but led to severe adipogenesis defects in primary white and brown preadipocytes, as determined by oil red O staining (Fig. 2B, C, and F). Accordingly, induction of adipogenesis markers *Pparg1*, *Pparg2*, *Cebpa*, and *Fabp4* was severely impaired in PAGR1 null white preadipocytes (Fig. 2D). Induction of adipogenesis markers and brown adipocyte markers was also inhibited by *Pagr1* deletion in primary brown preadipocytes (Fig. 2G). Consistent with data obtained from primary preadipocytes, short hairpin RNA (shRNA)-mediated knockdown of Pagr1 inhibited adipogenesis in immortalized 3T3-L1 preadipocytes (Fig. 2H and I). Deletion of *Pagr1* in immortalized brown preadipocytes also resulted in adipogenesis defects (Fig. 2J to L). These data demonstrate that PAGR1 is dispensable for cell growth but is required for adipogenesis in culture.

PAGR1 is required for induction of C/EBPβ and C/EBPδ during adipogenesis. We examined expression patterns of adipogenic TFs during differentiation of primary white preadipocytes (Fig. 3A). *Creb* expression did not change during differentiation. *Cebpb* and *Cebpd* were immediately and transiently induced upon exposure to the adipogenic cocktail MDI, with the expression levels peaking at 4 h. *Klf5* and *Klf15*, two downstream targets of C/EBPβ and C/EBPδ, were induced at 8 h. *Pparg*, *Cebpa*, and *GR* levels started to increase at day 1 and continued to increase throughout the differentiation. These results are highly consistent with what has been reported in the immortalized 3T3-L1 white preadipocyte cell line (2, 7, 13, 25).

Consistent with the requirement of PAGR1 for *Cebpb* and *Cebpd* expression in MEFs, *Pagr1* deletion by Cre in primary white preadipocytes prevented the robust induction of *Cebpb* and *Cebpd* but had no effect on *Creb* and *Pparg* levels in the early phase of differentiation (0 to 8 h) (Fig. 3B). Further, ChIP assays revealed that endogenous PAGR1 was recruited to the promoters of *Cebpb* and *Cebpd* in a manner that correlated with their expression patterns in the early phase of adipogenesis (Fig. 3C). In contrast, no enrichment of PAGR1 was detected on *Creb* and *Pparg1* promoters. PAGR1 mRNA and protein levels remained unchanged in the early phase of adipogenesis (Fig. 3D and E), suggesting that PAGR1 controls its target gene expression through its binding to specific genomic regions. Together, these results indicate that PAGR1 directly controls the induction of *Cebpb* and *Cebpd* in the early phase of adipogenesis.

PAGR1 regulates CREB- and GR-dependent induction of C/EBPβ and C/EBPδ in culture. Next, we asked which TFs recruit PAGR1 to the promoters of *Cebpb* and *Cebpd* in the early phase of adipogenesis, since PAGR1 itself does not contain any recognizable DNA-binding domain. Because induction of *Cebpb* depends on IBMX while induction of *Cebpd* requires DEX (8), we focused on CREB and GR, which are activated by

FIG 1 Legend (Continued)
approach to identify PAGR1-regulated genes in MEFs. Immortalized *Pagr1*$^{lox/lox}$ MEFs were infected with retrovirus MSCVpuro expressing Cre or vector (Vec) alone. Total RNA was extracted for microarray analysis (Table S1). The resulting putative PAGR1-regulated genes were further confirmed by qRT-PCR and ChIP assays. (F) Western blot confirmation of *Pagr1* deletion in MEFs. The asterisk indicates a nonspecific band. (G) Cell growth curves of Vec- and Cre-infected *Pagr1*$^{lox/lox}$ MEFs. (H) Gene ontology analysis of genes that show over 2.5-fold decrease in PAGR1 null MEFs. Transcriptional domain coverage shows the most significant biological functions of annotated genes. (I) qRT-PCR confirmation of putative PAGR1-regulated genes in *Pagr1*$^{lox/lox}$ MEFs. (J) ChIP assays of PAGR1 recruitment to target gene promoters in Vec- and Cre-infected *Pagr1*$^{lox/lox}$ MEFs. All results are representative of two to four independent experiments. Quantitative PCR data in all figures are presented as means ± SD.

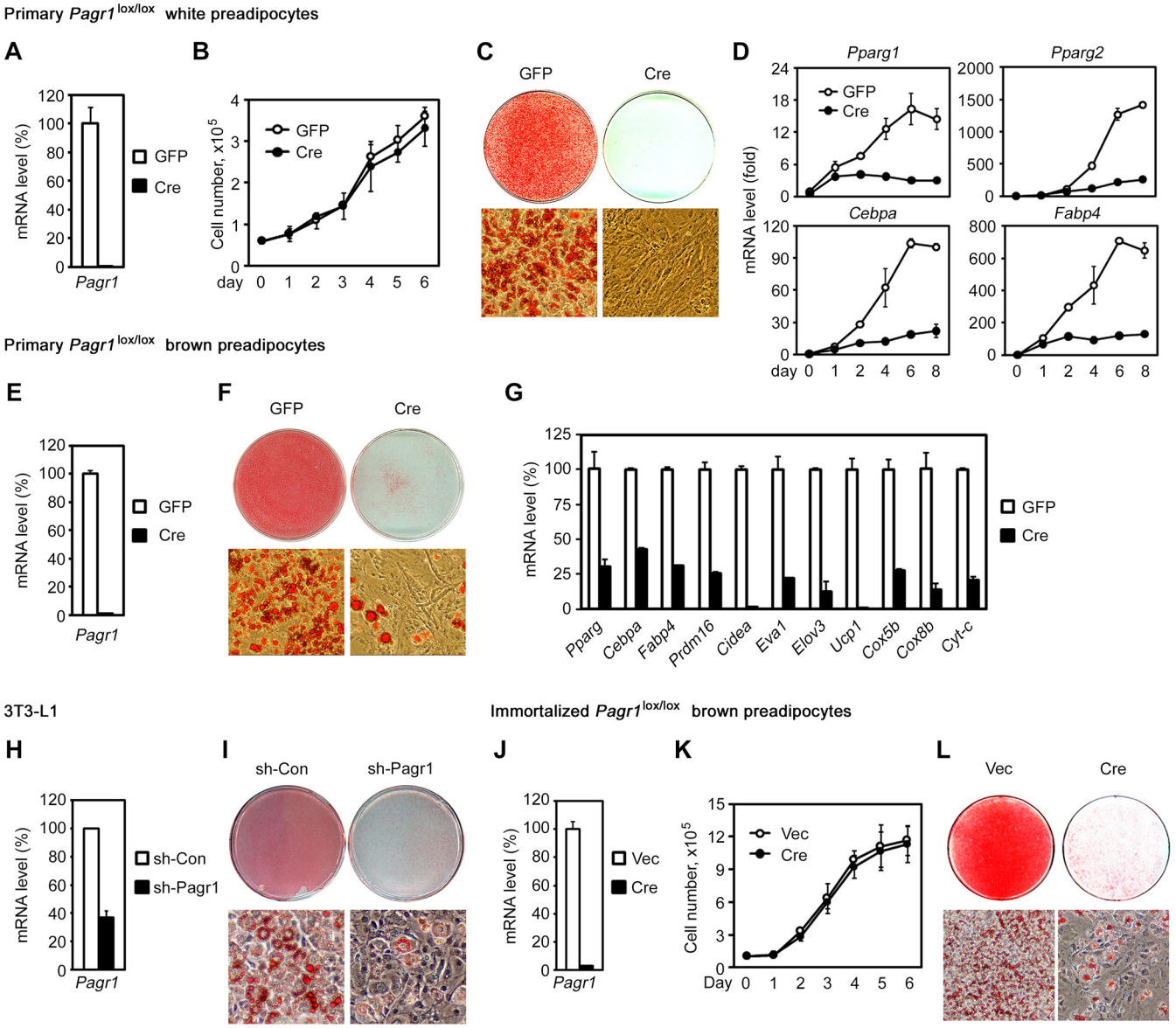

FIG 2 PAGR1 is required for adipogenesis in culture. (A to G) Primary white (A to D) and brown (E to G) preadipocytes isolated from *Pagr1*$^{lox/lox}$ mice were infected with adenoviruses expressing Cre or GFP, followed by adipogenesis assay. (A and E) qRT-PCR analysis of Cre-mediated *Pagr1* gene deletion before adipogenesis. (B) Cell growth curves of GFP- and Cre-infected primary white preadipocytes. (C and F) Morphological differentiation with oil red O staining (top, stained dishes; lower, representative fields under phase-contrast microscope). (D) During differentiation of primary white preadipocytes, total RNA was isolated at indicated time points for qRT-PCR analysis of induction of adipocyte markers. (G) After differentiation of primary brown preadipocytes, total RNA was isolated for qRT-PCR analysis of expression of genes common to white and brown adipocytes (*Pparg*, *Cebpa*, and *Fabp4*), genes specific for brown adipocytes (*Prdm16*, *Cidea*, *Eva1*, and *Ucp1*), and mitochondrial components (*Cox5b*, *Cox8b*, and *Cyt-c*). All results are representative of two to four independent experiments. (H and I) 3T3-L1 preadipocytes were infected with lentiviral shRNA targeting *PAGR1* (sh-Pagr1) or control virus alone (sh-Con), followed by adipogenesis assay. (H) qRT-PCR analysis of *Pagr1* knockdown efficiency. (I) Oil red O staining of differentiated adipocytes. (J to L) SV40T-immortalized *Pagr1*$^{lox/lox}$ brown preadipocytes were infected with retroviruses expressing Cre or Vec, followed by adipogenesis assay. (J) qRT-PCR analysis of Cre-mediated *Pagr1* gene deletion before adipogenesis. (K) Cell growth curves of Vec- and Cre-infected immortalized brown preadipocytes. (L) Oil red O staining of differentiated adipocytes.

IBMX and DEX, respectively, and are already expressed before the induction of adipogenesis.

Phospho-CREB (pCREB) directly binds to the *Cebpb* proximal promoter after induction of adipogenesis and activates endogenous *Cebpb* expression (15). By time course ChIP assays, we confirmed the enrichment of pCREB on the *Cebpb* promoter in the early phase of primary white preadipocyte differentiation. pCREB enrichment on the *Cebpb* promoter could be inhibited by adenovirus-mediated overexpression of a dominant-negative CREB (A-CREB) (Fig. 4A and B). A-CREB impaired the induction of *Cebpb*

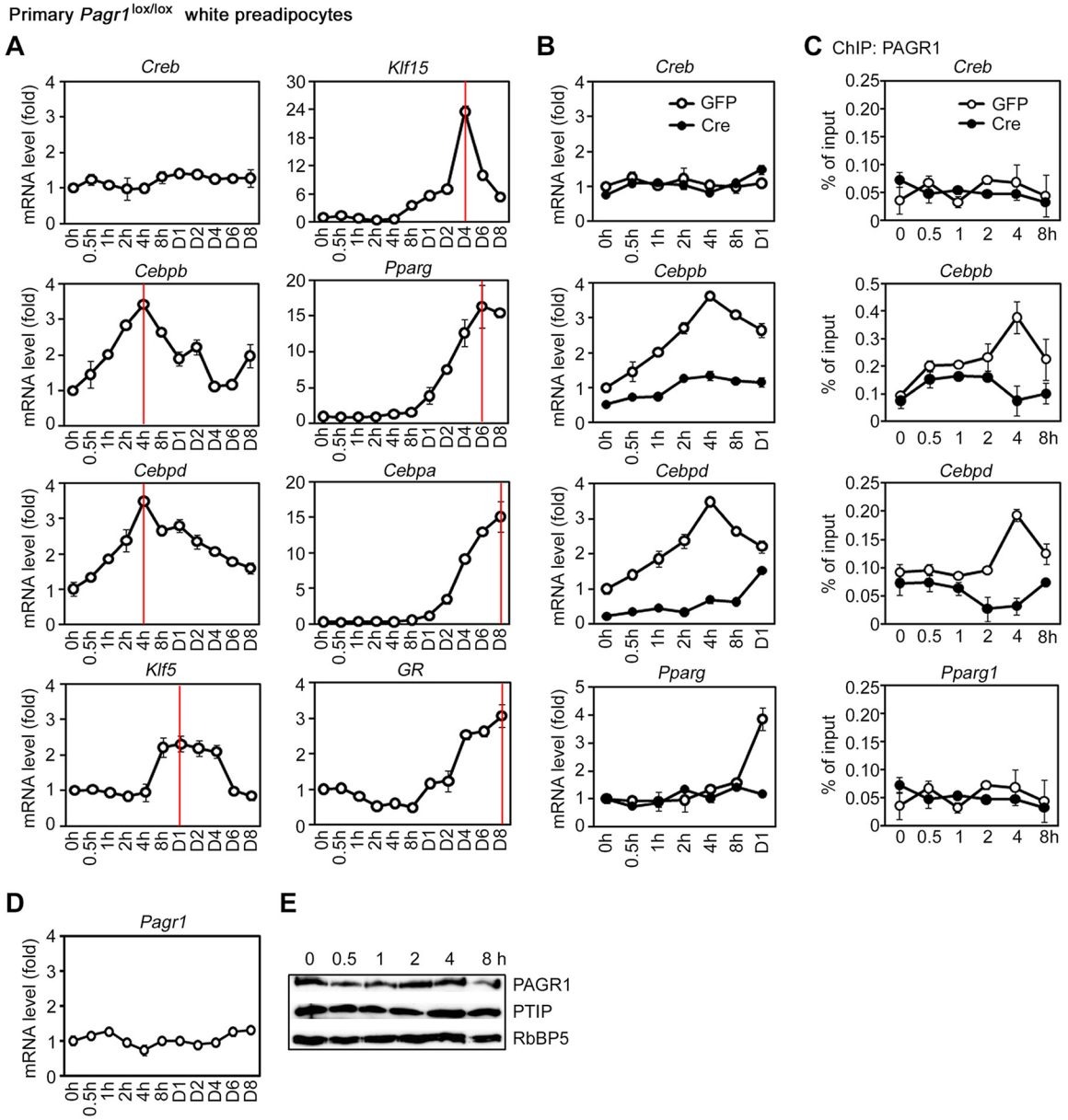

FIG 3 PAGR1 is required for induction of *Cebpb* and *Cebpd* during adipogenesis. (A) Expression of TFs implicated in adipogenesis during primary white preadipocyte differentiation. During adipogenesis of primary *Pagr1*$^{lox/lox}$ white preadipocytes, total RNA was isolated at indicated time points and subjected to qRT-PCR analysis. The red lines indicate the time points at which each TF reaches its peak expression level. (B and C) PAGR1 controls the induction of *Cebpb* and *Cebpd* in the early phase of adipogenesis. Primary *Pagr1*$^{lox/lox}$ white preadipocytes were infected with adenoviruses expressing Cre or GFP. Cells were replated and induced to undergo adipogenesis. Cells were collected at indicated time points for qRT-PCR (B) and ChIP (C) assays using PAGR1 antibody. (B) PAGR1 is required for induction of *Cebpb* and *Cebpd* but not *Creb* and *Pparg* in the early phase of adipogenesis (0 to 8 h). (C) Time course ChIP assays of endogenous PAGR1 enrichments on promoters of adipogenic TFs. (D) *Pagr1* mRNA levels during primary white preadipocyte differentiation. (E) Western blot analysis of PAGR1 and PTIP in the early phase of adipogenesis of primary white preadipocytes. The nuclear protein RbBP5 served as a control. All results are representative of two to four independent experiments.

(Fig. 4C), indicating that pCREB directly controls *Cebpb* induction in the early phase of adipogenesis. Interestingly, A-CREB also blocked PAGR1 enrichment on the *Cebpb* promoter (Fig. 4D). Conversely and surprisingly, deletion of *Pagr1* inhibited pCREB binding to the *Cebpb* promoter (Fig. 4E), suggesting an interdependent binding of pCREB and PAGR1 on the *Cebpb* promoter. Deletion of *Pagr1* did not affect pCREB levels induced by the adipogenic cocktail MDI (Fig. 4F). To directly assess the role of PAGR1 in CREB-mediated *Cebpb* induction, primary white preadipocytes and simian virus 40T

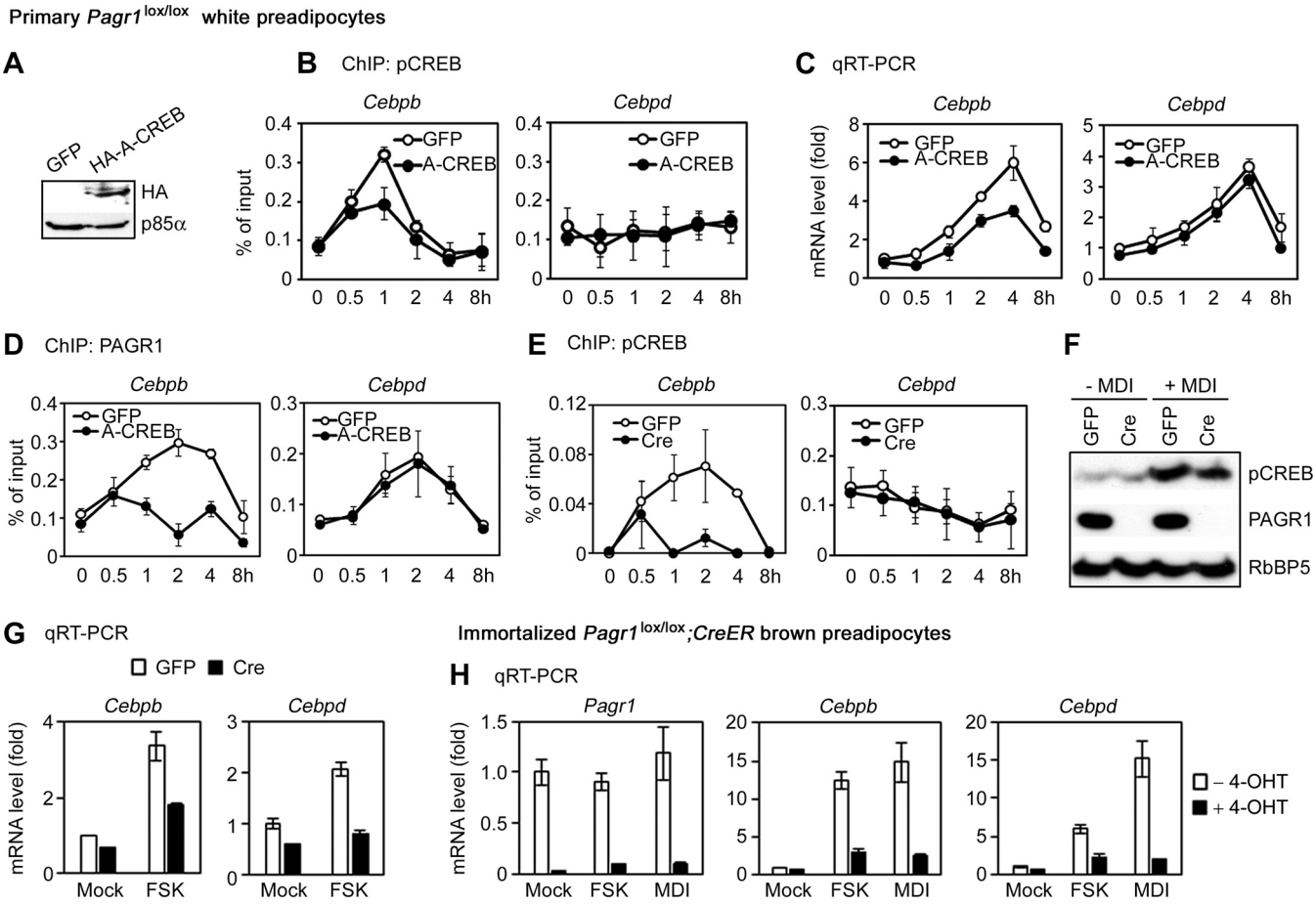

FIG 4 pCREB and PAGR1 cooperate to induce *Cebpb* expression in culture. (A to C) Phospho-CREB (pCREB) directly controls the induction of *Cebpb* in the early phase of adipogenesis. Primary *Pagr1*$^{lox/lox}$ white preadipocytes were infected with adenoviruses expressing the dominant-negative CREB (A-CREB) or GFP, followed by induction of adipogenesis. Cells were collected at the indicated time points for ChIP assays and qRT-PCR. (A) Expression of HA-tagged A-CREB before adipogenesis was confirmed by Western blotting with an HA antibody. (B) ChIP assays using a pCREB antibody. (C) qRT-PCR analysis of *Cebpb* and *Cebpd*. (D) pCREB recruits PAGR1 to *Cebpb* promoter in the early phase of adipogenesis. (E) PAGR1 is required for stable enrichment of pCREB on *Cebpb* promoter in the early phase of adipogenesis. Primary *Pagr1*$^{lox/lox}$ white preadipocytes were infected with adenoviruses expressing Cre or GFP, followed by induction of adipogenesis. Samples were collected at indicated time points for ChIP assays using a pCREB antibody. (F) Deletion of *Pagr1* does not affect overall pCREB levels induced by adipogenic cocktail MDI. (G and H) PAGR1 is required for *Cebpb* induction by activated CREB. (G) Primary *Pagr1*$^{lox/lox}$ white preadipocytes infected with adenoviruses expressing Cre or GFP were treated with 10 μM forskolin (FSK) for 1 h and collected for qRT-PCR analysis. (H) Immortalized *Pagr1*$^{lox/lox}$; *CreER* brown preadipocytes were treated with 2 μM 4-OHT for 2 days to delete *Pagr1*. Cells were then treated with 10 μM forskolin (FSK) or MDI for 1 h and collected for qRT-PCR analysis.

(SV40T)-immortalized brown preadipocytes were treated with forskolin, which induces phosphorylation and activation of endogenous CREB (14). Deletion of *Pagr1* impaired the induction of *Cebpb* by activated CREB (Fig. 4G and H). Together, these results suggest that pCREB and PAGR1 cooperate to induce *Cebpb* expression during adipogenesis.

By ChIP assays, we found that GR was enriched on *Cebpb* and *Cebpd* promoters at around 2 h after induction of adipogenesis in primary white preadipocytes. Deletion of *Pagr1* impaired GR binding to the promoter of *Cebpd* but not that of *Cebpb* (Fig. 5A). In contrast, knockdown of GR in 3T3-L1 white preadipocytes did not affect PAGR1 recruitment (Fig. 5B). GR knockdown in 3T3-L1 cells impaired the induction of *Cebpd* but not *Cebpb* in the early phase of adipogenesis (Fig. 5C). Similarly, knockdown of GR inhibited DEX-induced *Cebpd* expression in 3T3-L1 (Fig. 5D). Deletion of *Pagr1* in SV40T-immortalized brown preadipocytes blocked DEX-induced *Cebpd* expression, indicating that PAGR1 is required for GR-mediated induction of *Cebpd* (Fig. 5E). Taken together, these data suggest a model in which PAGR1 cooperates with IBMX-activated pCREB and DEX-activated GR to control the induction of *Cebpb* and *Cebpd*, respectively, in the early phase of adipogenesis (Fig. 5F).

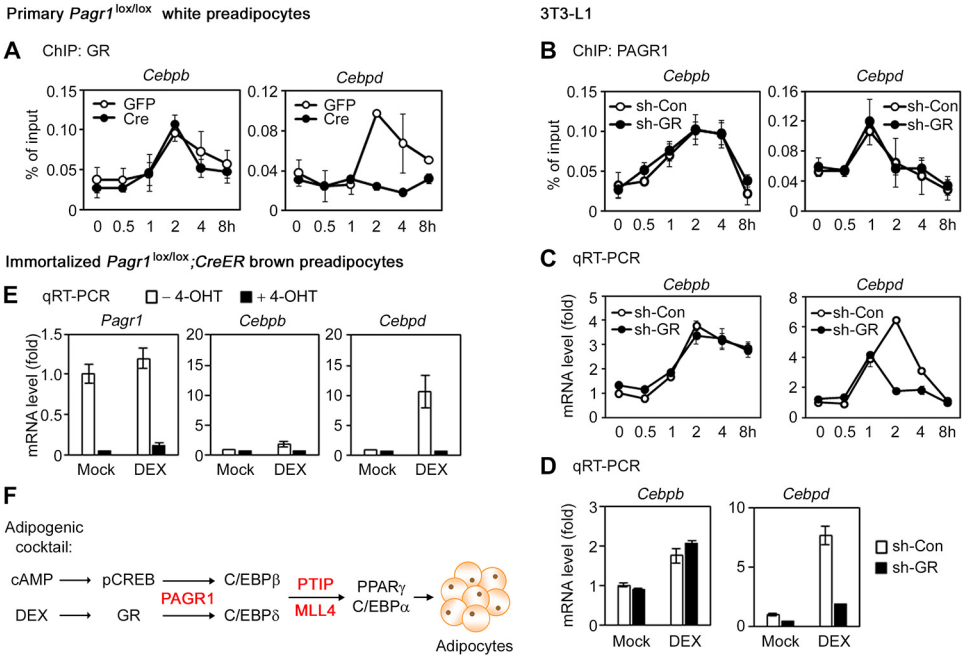

FIG 5 PAGR1 regulates GR-dependent induction of *Cebpd* in culture. (A) PAGR1 is required for GR enrichment on *Cebpd* promoter. Experiments were done as described for Fig. 4E. Samples were collected at the indicated time points for ChIP assays using a GR antibody. (B and C) Knockdown of GR inhibits the induction of *Cebpd* but has little effect on PAGR1 recruitment on *Cebpd* promoter in the early phase of adipogenesis. 3T3-L1 cells were infected with lentiviral shRNA targeting GR (sh-GR) or control virus alone (sh-Con), followed by induction of adipogenesis. Cells were collected at indicated time points for ChIP assays using a PAGR1 antibody (B) and qRT-PCR (C). All results are representative of two to four independent experiments. (D) Knockdown of GR inhibits DEX-induced *Cebpd* expression. 3T3-L1 cells infected with lentiviral sh-GR or sh-Con were treated with 1 μM DEX for 1 h and collected for qRT-PCR analysis. (E) PAGR1 is required for *Cebpd* induction by activated GR. Immortalized *Pagr1*$^{lox/lox}$; *CreER* brown preadipocytes were treated with 2 μM 4-OHT for 2 days to delete *Pagr1*. Cells were treated with 1 μM DEX for 1 h and collected for qRT-PCR analysis. (F) Model depicting the role of PAGR1 in transcriptional regulation of adipogenesis in culture. PAGR1 regulates adipogenesis by cooperating with IBMX-induced pCREB and DEX-activated GR to control the induction of C/EBPβ and C/EBPδ in the early phase of adipogenesis.

Ectopic C/EBPβ or PPARγ can rescue adipogenesis defect in PAGR1-deficient cells. Since PAGR1 is required for *Cebpb* gene induction in the early stage of adipogenesis, we tested if the adipogenesis defects in PAGR1-deficient cells could be rescued by forced expression of C/EBPβ or PPARγ. We isolated and immortalized primary brown preadipocytes from inducible PAGR1 KO mice (*Pagr1*$^{lox/lox}$; *CreER*). Cells were treated with 4-hydroxytamoxifen (4-OHT) to delete *Pagr1*, followed by retroviral vector-mediated ectopic expression of C/EBPβ or PPARγ (Fig. 6A). Consistent with the data obtained from viral Cre-infected PAGR1 null cells, 4-OHT-induced deletion of *Pagr1* resulted in adipogenesis defects. Such defects can be rescued by ectopic expression of C/EBPβ or PPARγ (Fig. 6B and C), suggesting that PAGR1 functions upstream of C/EBPβ during adipogenesis.

PAGR1 is essential for brown adipose tissue and muscle development in mice. To investigate PAGR1 function in adipogenesis *in vivo*, we crossed *Pagr1*$^{lox/lox}$ mice with Myf5-Cre mice, in which Cre expression is under the control of the *Myf5* promoter (26). Myf5-Cre is active in precursor cells that can develop into brown adipocytes and skeletal muscle cells in the back (27). The KO mice died within minutes after birth due to breathing defects caused by reduced muscles in the rib cage. Therefore, we removed E18.5 embryos by cesarean section. KO embryos exhibited abnormal cervical curvature and died instantly after dissection (Fig. 7A and B). Sagittal sections along the midline or ~400 μm lateral to the midline of E18.5 embryos were used for hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) using antibodies specifically recognizing brown adipose tissue (BAT)-specific UCP1 and muscle-specific myosin (Fig. 7C and D). Compared with their littermate controls, KO mice showed a dramatic decrease of

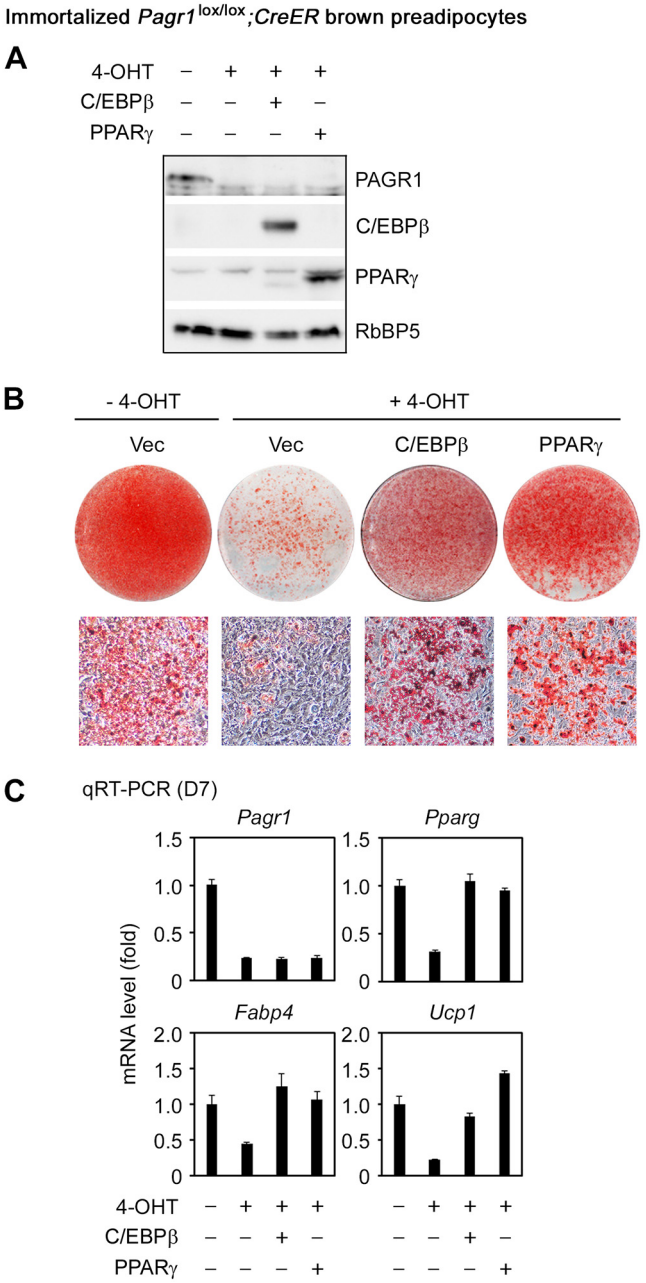

FIG 6 Ectopic C/EBPβ or PPARγ can rescue adipogenesis defect in PAGR1-deficient cells. Immortalized *Pagr1*$^{lox/lox}$; *CreER* brown preadipocytes were treated with 2 μM 4-OHT for 2 days to delete *Pagr1* and then infected with retroviruses expressing C/EBPβ or PPARγ, followed by adipogenesis assay. (A) Western blot analyses of PAGR1 deletion and ectopic expression of C/EBPβ and PPARγ before adipogenesis. (B) Oil red O staining. (C) qRT-PCR analysis of *Pagr1*, *Pparg*, *Fabp4*, and *Ucp1*.

interscapular BAT and muscle. These data are consistent with the essential role for PAGR1 in adipogenesis in culture and indicate that PAGR1 is required for adipogenesis *in vivo*.

DISCUSSION

Early adipogenic TFs C/EBPβ and C/EBPδ are induced within hours after initiating adipogenesis and promote expression of the master adipogenic TFs PPARγ and C/EBPα (25). We previously showed that PTIP and MLL3/MLL4 control the induction of PPARγ and C/EBPα during adipogenesis (19, 20). In this paper, we report that the MLL3/MLL4/

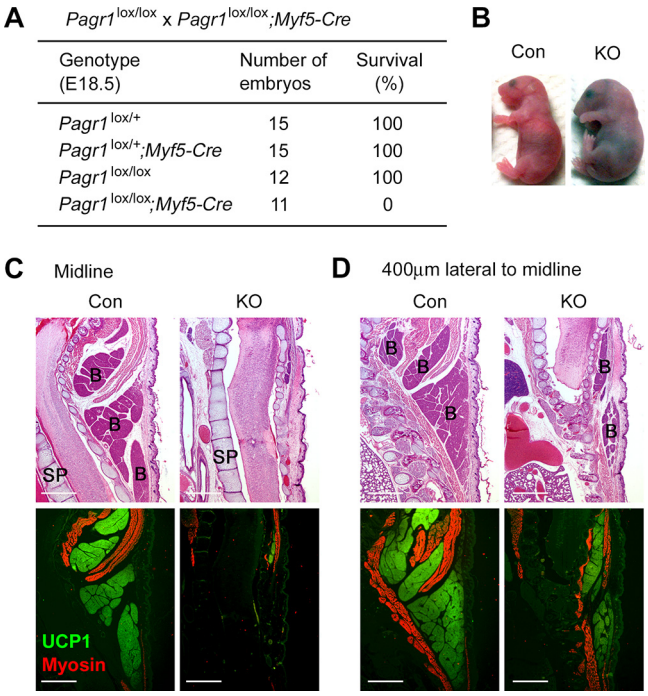

FIG 7 PAGR1 is required for brown adipose tissue and muscle development. (A) Genotype of E18.5 embryos from crossing *Pagr1*$^{lox/+}$; *Myf5-Cre* with *Pagr1*$^{lox/lox}$ mice. The expected ratios of the four resulting genotypes are 1:1:1:1. (B) Representative pictures of control (Con) and PAGR1 KO (*Pagr1*$^{lox/lox}$; *Myf5-Cre*) E18.5 embryos. KO embryos died instantly after cesarean section because of breathing defects. (C and D) Loss of interscapular brown adipose tissue (BAT) in E18.5 PAGR1 KO embryos. E18.5 embryos were sagitally sectioned along the midline (C) or ~400 μm lateral to the midline (D). The sections were subjected to H&E staining (top) as well as IHC using antibodies recognizing BAT-specific UCP1 protein (green) and skeletal muscle-specific myosin (red) (bottom). SP indicates spine. B indicates BAT. Ventral is on the left side, dorsal is on the right side. Scale bar, 800 μm.

PTIP-associated PAGR1 protein controls the induction of C/EBPβ and C/EBPδ and, thus, is required for adipogenesis. Together, components of the MLL3/MLL4 complex regulate a key transcriptional cascade that governs adipogenesis.

Regulation of gene expression by PAGR1. Deletion of *Ptip* in MEFs leads to a more than 2.5-fold decrease of 60 genes, including *Pparg*. PTIP directly controls *Pparg* and *Cebpa* expression in MEFs as well as during adipogenesis (19). On the other hand, *Pagr1* deletion in MEFs led to a more than 2.5-fold decrease of 61 genes, including *Cebpb*, *Cebpd*, *Klf4*, and *Krox20* (see Table S1 in the supplemental material), which were listed as fat cell differentiation genes in the gene ontology analysis. Because KLF4 and Krox20 have been shown to be dispensable for adipogenesis (28), we focused on PAGR1 regulation of C/EBPβ and C/EBPδ expression. Although *Pparg* showed an ~2-fold decrease in PAGR1 null MEFs, PAGR1 is undetectable on the *Pparg* promoter in wild-type MEFs, suggesting the decreased *Pparg* expression in PAGR1 null MEFs is a secondary effect. Despite a direct physical interaction between them, PTIP and PAGR1 shared only 10 overlapping target genes in MEFs. These include *Adamts2*, *Ahr*, *Ak5*, *Bmp4*, *Col11a1*, *Fbn2*, *Fmod3*, *Inhbb*, *Klf4*, and *Tnfrsf11b*. Collectively, these results suggest that while PAGR1 and PTIP directly regulate several common target genes, they regulate adipogenesis by controlling the expression of distinct sets of adipogenic TFs. Despite the physical association of PAGR1 with the MLL3/MLL4-containing histone H3K4 methyltransferase complex (21), our data suggest that PAGR1 regulation of some of its target genes is independent of the MLL3/MLL4 complex. Indeed, induction of C/EBPβ is not altered in *Mll3*$^{-/-}$*Mll4*$^{-/-}$ brown preadipocytes during adipogenesis (20).

PAGR1 cooperates with pCREB and GR to induce early adipogenic transcription factors. Although PAGR1 protein levels show little change in the early phase of adipogenesis, PAGR1 is recruited to *Cebpb* and *Cebpd* promoters in a manner that

highly correlates with the transient induction of these early adipogenic TFs by adipogenic cocktail. IBMX induces phosphorylation and activation of CREB while DEX binds and activates GR (14, 16). IBMX induces *Cebpb* expression while DEX induces *Cebpd* (8). Consistent with these previous reports, we show that PAGR1 cooperates with pCREB and GR to induce *Cebpb* and *Cebpd* in the early phase of adipogenesis.

CREB family members associate with the *Cebpb* promoter even before induction of adipogenesis, whereas pCREB is detected only after induction (15). Therefore, CREB may already sit on target gene promoters but IBMX-induced CREB phosphorylation triggers the recruitment of PAGR1 to the *Cebpb* promoter. PAGR1 recruitment may stabilize CREB complex assembly, because pCREB enrichment on the *Cebpb* promoter is severely impaired in PAGR1 null cells.

Our data suggest an updated model on transcriptional regulation of adipogenesis. After preadipocytes are treated with adipogenic cocktail MDI, IBMX induces phosphorylation and activation of CREB while DEX binds and activates GR. PAGR1 cooperates with pCREB and GR to directly induce *Cebpb* and *Cebpd* expression in the early phase of adipogenesis. The elevated levels of these early adipogenic TFs are critical for the induction of the master adipogenic TFs PPARγ and C/EBPα, which directly activate adipocyte gene expression and lead to phenotypic conversion of preadipocytes to adipocytes (Fig. 5F). Interestingly, physically interacting PAGR1 and PTIP control the induction of distinct sets of adipogenic TFs during adipogenesis. Together, PAGR1 and PTIP control the induction of the cascade of adipogenic TFs that governs adipogenesis.

Further studies are needed to understand the role of PAGR1 in undifferentiated preadipocytes. We cannot rule out the possibility that before induction of adipogenesis, PAGR1 interacts with preadipocyte TFs, such as ZFP423 (29), TCF7L1 (30), KAISO (31), and ATF4 (32), to regulate the expression of genes involved in maintaining an undifferentiated state or committing cells to the preadipogenic state. This possibility is supported by the fact that in MEFs, PAGR1 recruitment to its target genes occurs in the absence of an adipogenic cocktail and does not require activated CREB or GR.

Essential role of PAGR1 in adipose tissue development *in vivo*. PAGR1 is required for IBMX- and DEX-induced expression of early adipogenic TFs and is essential for adipogenesis of both white and brown preadipocytes in culture. However, IBMX and DEX are synthetic chemicals that do not exist *in vivo*. To investigate the physiological importance of PAGR1 in adipose tissue development, we utilized Myf5-Cre mice. Myf5-expressing progenitor cells give rise to both skeletal muscle and BAT but not WAT (27). Myf5-Cre starts to be expressed at early embryonic stages (E10.5 or earlier), which is much earlier than the initial appearance of BAT (around E14.5 to E15.5) (33, 34). Therefore, Myf5-Cre mice enable us to study the roles of PAGR1 in the early stage of BAT development. We show that the deletion of *Pagr1* leads to severe defects in BAT development. Myf5-Cre-mediated deletion of *Ptip* also leads to severe defects in BAT development, confirming the critical role of PTIP in adipogenesis *in vivo* (data not shown). Together, our animal data are highly consistent with the cell culture results and strongly suggest that, by controlling the induction of C/EBPβ and C/EBPδ, PAGR1 is essential for adipogenesis *in vivo*.

In MEFs, PAGR1 also controls the expression of BMP4, a cytokine that commits the multipotent mesenchymal cells to the adipocyte lineage (35). It will be interesting to determine the roles of PAGR1 in earlier developmental events, in particular the lineage commitment of mesenchymal stem cells. Future work will be needed to fully understand the molecular mechanism by which PAGR1 regulates induction of C/EBPβ and C/EBPδ as well as other target genes.

PAGR1 as a developmental regulator. The facts that *Pagr1*$^{-/-}$ mice are embryonic lethal and that Myf5-Cre-driven deletion of *Pagr1* reduces BAT and muscle suggest that PAGR1 is important for both embryonic and tissue development. *Pagr1*$^{-/-}$ mice die before embryonic day 10.5 due to the abnormal development of extraembryonic tissues, including amnion, chorion, and visceral yolk sac (24). At the molecular level, *Pagr1*$^{-/-}$ embryos show reduced expression of BMP2, which is critical for extraembry-

onic development. While *Cebpb*$^{-/-}$*Cebpd*$^{-/-}$ mice do not exhibit embryonic death, approximately 85% of them die at the early neonatal stage (10). The impaired expression of other PAGR1-regulated genes may also contribute to the embryonic lethality of *Pagr1*$^{-/-}$ mice. For instance, whole-body KO of *Bmp4*, a PAGR1-regulated gene in MEFs, leads to early embryonic lethality due to defective mesoderm formation and patterning (36). Targeted deletion of many PAGR1-regulated genes, such as *Klf4* (37) or *Krox20* (38), also leads to defects in the postnatal development of various tissues and causes neonatal death. It remains to be investigated whether PAGR1 is required for development of other tissues.

MATERIALS AND METHODS

Generation of *Pagr1*$^{lox/lox}$ mice. PAGR1 conditional KO mice, *Pagr1*$^{lox/lox}$ mice, were generated by following the published protocol (39). The mouse *Pagr1* gene, also known as *2900092E17Rik*, contains 3 exons. The targeting vector introduced a *loxP* site into the EcolCRI restriction enzyme site upstream of exon 1 and a neomycin cassette flanked by two *loxP* sites into the BglII site in intron 2 to generate the targeted allele (loxneo) in embryonic stem (ES) cells (Fig. 1A). Among the 160 ES cell clones that we screened using the Expand Long Template PCR system (Roche), clone 138 was identified as a *Pagr1*$^{loxneo/+}$ clone carrying a targeted allele and a wild-type allele. The correct targeting in clone 138 was confirmed by Southern blotting using a 5′ flanking probe generated by PCR amplification of the NotI-EcoRV genomic DNA fragment (Fig. 1B).

Pagr1$^{loxneo/+}$ ES cell clone 138 was injected into blastocysts to obtain male chimera mice, which were crossed with wild-type C57BL/6J females to screen for germ line transmission. Mice bearing germ line transmission (*Pagr1*$^{loxneo/+}$) were crossed with *EIIa-Cre* mice (no. 003724; The Jackson Laboratory) to generate both *Pagr1*$^{lox/+}$ and *Pagr1*$^{+/-}$ mice by following a procedure described earlier (Fig. 1C) (40). *Pagr1*$^{lox/+}$ mice were crossed with C57BL/6J mice for 7 generations to obtain congenic mice. *Pagr1*$^{lox/lox}$ mice were obtained by intercrossing between *Pagr1*$^{lox/+}$ mice. For genotyping the lox allele, allelic PCR was developed using forward primer 5′-TGGCCCAAACCTAAACATTAG-3′ and reverse primer 5′-TTATGG CGCTTCATGTCTGAG-3′. PCR amplified 200 bp from the wild-type allele and 250 bp from the lox allele (Fig. 1D).

Plasmids, antibodies, and chemicals. The retroviral plasmids MSCVpuro-Cre, MSCVhygro-PPARγ2, and WZLhyg-C/EBPβ have been described already (20, 41). The lentiviral shRNA plasmid pLKO.1 targeting *Pagr1* (clone ID TRCN0000177775) was purchased from Sigma. The following antibodies have been described: anti-PTIP#3, anti-PAGR1#2, anti-GAPDH, anti-p85α, anti-RbBP5, and all histone modification antibodies (19, 42). Anti-phospho-CREB (Ser133) (D1G6) was from Cell Signaling Technology. Anti-GR (sc-1004x) was from Santa Cruz Biotechnology. Rosiglitazone was from Cayman (71740). Other chemicals were from Sigma: IBMX (I-7018), DEX (D4902), T3 (T-2877), insulin (I-6634), indomethacin (I-7378), and forskolin (F3917).

Cell culture, adipogenesis, and virus infection. Primary MEFs were isolated from two E13.5 *Pagr1*$^{lox/lox}$ embryos. One was immortalized by transfecting cells with a SV40T-expressing plasmid, and the other was immortalized by following the 3T3 protocol (43). The isolation, culture, and adipogenesis of primary white and brown preadipocytes, the immortalization of brown preadipocytes, and the retroviral infection of the immortalized MEFs were done as described previously (19). Briefly, the adipogenesis of primary white preadipocytes was induced with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 0.5 mM IBMX, 1 μM DEX, 5 μg/ml insulin, 0.2 nM T3, and 0.5 μM rosiglitazone. For adipogenesis of primary and immortalized brown preadipocytes, cells were induced with DMEM supplemented with 10% FBS, 0.02 μM insulin, 1 nM T3, 0.5 mM IBMX, 2 μg/ml DEX, and 0.125 mM indomethacin. Adenovirus expressing the dominant-negative CREB (A-CREB) was described previously (44). Adenoviral infection of preadipocytes was done at a multiplicity of infection of 50 (19). 4-OHT treatment was done at 2 μM concentration for 2 days.

Microarray, gene ontology analysis, qRT-PCR, and ChIP. Total RNA extraction, microarray analysis on the Mouse Genome 430 2.0 array (Affymetrix, Santa Clara, CA), and qRT-PCR were done as described previously (19). Gene ontology analysis of downregulated genes in PAGR1-deficient MEFs was done using the FunNet bioinformatics tool (www.funnet.info) (45). A total of 61 genes with a more than 2.5-fold decrease in PAGR1-deficient cells were subjected to gene ontology analysis. Of the 61 downregulated genes, the 51 annotated genes were analyzed with a 5% false discovery rate (FDR). qRT-PCR primer sequences are shown in Table S2 in the supplemental material. TaqMan probes for *Pparg1* and *Pparg2* have been described already (19).

ChIP was performed as described previously (46). The quantitation of enriched genomic DNA relative to inputs was performed in duplicate or triplicate using SYBR green master mix (Applied Biosystems). Primer sequences are listed in Table S3.

Generation of tissue-specific PAGR1 KO mice. The Myf5-Cre mice with a mixed C57BL/6J and 129 Sv background (no. 007893) were obtained from The Jackson Laboratory. *Pagr1*$^{lox/lox}$ mice with congenic C57BL/6J background were crossed with Myf5-Cre mice. The resulting heterozygous mice (*Pagr1*$^{lox/+}$; *Cre*) in F1 were then crossed with *Pagr1*$^{lox/lox}$ mice to generate tissue-specific PAGR1 KO mice (*Pagr1*$^{lox/lox}$; *Myf5-Cre*). *Pagr1*$^{lox/lox}$ mice were used as the control. Genotyping of *Myf5-Cre* was done by PCR using 3 primers: 5′-CGTAGACGCCTGAAGAAGGTCAACCA-3′, 5′-CACATTAGAAAACCTGCCAACACC-3′, and 5′-ACGAAGTTATTAGGTCCCTCGAC-3′. PCR amplified the wild-type (603 bp) and *Myf5-Cre* (400 bp) allele. All mouse work was approved by the Animal Care and Use Committee of NIDDK, NIH.

Histology and IHC. E18.5 embryos were removed by cesarean section and fixed in 4% paraformaldehyde overnight at 4°C. Embryos were further dehydrated, embedded in paraffin, and sectioned at 7 to 10 μm with a microtome. Mouse inguinal adipose tissues were fixed in 10% formalin, embedded in paraffin, and sectioned at 5 μm with a microtome. H&E staining and immunohistochemistry on paraffin sections were done as described previously (47). The primary antibodies used for IHC were a 1:20 dilution of antimyosin (MF20; Developmental Studies Hybridoma Bank) and a 1:400 dilution of anti-UCP1 (ab10983; Abcam). Fluorescent secondary antibodies used were Alexa Fluor 488–goat anti-mouse IgG2b and Alexa Fluor 555–goat anti-rabbit IgG (Molecular Probes).

Data availability. Microarray data have been deposited in the NCBI GEO database (accession number GSE20157).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

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J.-E.L., Y.-W.C., and K.G. designed the study and analyzed the data. J.-E.L., Y.-W.C., and C.D. performed the experiments. J.-E.L. and K.G. wrote the manuscript.

We have no conflict of interest to declare.

REFERENCES

1. Cristancho AG, Lazar MA. 2011. Forming functional fat: a growing understanding of adipocyte differentiation. Nat Rev Mol Cell Biol 12:722–734. https://doi.org/10.1038/nrm3198.
2. Rosen ED, MacDougald OA. 2006. Adipocyte differentiation from the inside out. Nat Rev Mol Cell Biol 7:885–896. https://doi.org/10.1038/nrm2066.
3. Lefterova MI, Haakonsson AK, Lazar MA, Mandrup S. 2014. PPARgamma and the global map of adipogenesis and beyond. Trends Endocrinol Metab 25:293–302. https://doi.org/10.1016/j.tem.2014.04.001.
4. Rosen ED, Hsu C-H, Wang X, Sakai S, Freeman MW, Gonzalez FJ, Spiegelman BM. 2002. C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. Genes Dev 16:22–26. https://doi.org/10.1101/gad.948702.
5. Tontonoz P, Hu E, Spiegelman BM. 1994. Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. Cell 79:1147–1156. https://doi.org/10.1016/0092-8674(94)90006-x.
6. Freytag SO, Paielli DL, Gilbert JD. 1994. Ectopic expression of the CCAAT/enhancer-binding protein alpha promotes the adipogenic program in a variety of mouse fibroblastic cells. Genes Dev 8:1654–1663. https://doi.org/10.1101/gad.8.14.1654.
7. Morrison RF, Farmer SR. 1999. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem 274:17088–17097. https://doi.org/10.1074/jbc.274.24.17088.
8. Yeh WC, Cao Z, Classon M, McKnight SL. 1995. Cascade regulation of terminal adipocyte differentiation by three members of the C/EBP family of leucine zipper proteins. Genes Dev 9:168–181. https://doi.org/10.1101/gad.9.2.168.
9. Lane MD, Tang Q-Q, Jiang M-S. 1999. Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. Biochem Biophys Res Commun 266:677–683. https://doi.org/10.1006/bbrc.1999.1885.
10. Tanaka T, Yoshida N, Kishimoto T, Akira S. 1997. Defective adipocyte differentiation in mice lacking the C/EBP[beta] and/or C/EBP[delta] gene. EMBO J 16:7432–7443. https://doi.org/10.1093/emboj/16.24.7432.
11. Cao Z, Umek RM, McKnight SL. 1991. Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. Genes Dev 5:1538–1552. https://doi.org/10.1101/gad.5.9.1538.
12. Lee JE, Schmidt H, Lai B, Ge K. 2019. Transcriptional and epigenomic regulation of adipogenesis. Mol Cell Biol 39:e00601-18. https://doi.org/10.1128/MCB.00601-18.
13. Reusch JEB, Colton LA, Klemm DJ. 2000. CREB activation induces adipogenesis in 3T3-L1 cells. Mol Cell Biol 20:1008–1020. https://doi.org/10.1128/mcb.20.3.1008-1020.2000.
14. Gonzalez GA, Montminy MR. 1989. Cyclic AMP stimulates somatostatin gene transcription by phosphorylation of CREB at serine 133. Cell 59:675–680. https://doi.org/10.1016/0092-8674(89)90013-5.
15. Zhang JW, Klemm DJ, Vinson C, Lane MD. 2004. Role of CREB in transcriptional regulation of CCAAT/enhancer-binding protein beta gene during adipogenesis. J Biol Chem 279:4471–4478. https://doi.org/10.1074/jbc.M311327200.
16. Steger DJ, Grant GR, Schupp M, Tomaru T, Lefterova MI, Schug J, Manduchi E, Stoeckert CJ, Lazar MA. 2010. Propagation of adipogenic signals through an epigenomic transition state. Genes Dev 24:1035–1044. https://doi.org/10.1101/gad.1907110.
17. Siersbæk R, Nielsen R, John S, Sung M-H, Baek S, Loft A, Hager GL, Mandrup S. 2011. Extensive chromatin remodelling and establishment of transcription factor "hotspots" during early adipogenesis. EMBO J 30:1459–1472. https://doi.org/10.1038/emboj.2011.65.
18. Park YK, Ge K. 2017. Glucocorticoid receptor accelerates, but is dispensable for, adipogenesis. Mol Cell Biol 37:e00260-16. https://doi.org/10.1128/MCB.00260-16.
19. Cho YW, Hong S, Jin Q, Wang L, Lee JE, Gavrilova O, Ge K. 2009. Histone methylation regulator PTIP is required for PPARgamma and C/EBPalpha expression and adipogenesis. Cell Metab 10:27–39. https://doi.org/10.1016/j.cmet.2009.05.010.
20. Lee J-E, Wang C, Xu S, Cho Y-W, Wang L, Feng X, Baldridge A, Sartorelli V, Zhuang L, Peng W, Ge K. 2013. H3K4 mono- and di-methyltransferase MLL4 is required for enhancer activation during cell differentiation. Elife 2:e01503. https://doi.org/10.7554/eLife.01503.
21. Cho Y-W, Hong T, Hong S, Guo H, Yu H, Kim D, Guszczynski T, Dressler GR, Copeland TD, Kalkum M, Ge K. 2007. PTIP associates with MLL3- and MLL4-containing histone H3 lysine 4 methyltransferase complex. J Biol Chem 282:20395–20406. https://doi.org/10.1074/jbc.M701574200.
22. Gong Z, Cho Y-W, Kim J-E, Ge K, Chen J. 2009. Accumulation of Pax2 transactivation domain interaction protein (PTIP) at sites of DNA breaks via RNF8-dependent pathway is required for cell survival after DNA damage. J Biol Chem 284:7284–7293. https://doi.org/10.1074/jbc.M809158200.
23. Munoz IM, Rouse J. 2009. Control of histone methylation and genome stability by PTIP. EMBO Rep 10:239–245. https://doi.org/10.1038/embor.2009.21.
24. Kumar A, Lualdi M, Loncarek J, Cho Y-W, Lee J-E, Ge K, Kuehn MR. 2014.

Loss of function of mouse Pax-interacting protein 1-associated glutamate rich protein 1a (Pagr1a) leads to reduced Bmp2 expression and defects in chorion and amnion development. Dev Dyn 243:937–947. https://doi.org/10.1002/dvdy.24125.
25. Farmer SR. 2006. Transcriptional control of adipocyte formation. Cell Metab 4:263–273. https://doi.org/10.1016/j.cmet.2006.07.001.
26. Tallquist MD, Weismann KE, Hellstrom M, Soriano P. 2000. Early myotome specification regulates PDGFA expression and axial skeleton development. Development 127:5059–5070.
27. Seale P, Bjork B, Yang W, Kajimura S, Chin S, Kuang S, Scime A, Devarakonda S, Conroe HM, Erdjument-Bromage H, Tempst P, Rudnicki MA, Beier DR, Spiegelman BM. 2008. PRDM16 controls a brown fat/skeletal muscle switch. Nature 454:961–967. https://doi.org/10.1038/nature07182.
28. Park YK, Wang L, Giampietro A, Lai B, Lee JE, Ge K. 2017. Distinct roles of transcription factors KLF4, Krox20, and peroxisome proliferator-activated receptor gamma in adipogenesis. Mol Cell Biol 37:e00554-16. https://doi.org/10.1128/MCB.00554-16.
29. Gupta RK, Arany Z, Seale P, Mepani RJ, Ye L, Conroe HM, Roby YA, Kulaga H, Reed RR, Spiegelman BM. 2010. Transcriptional control of preadipocyte determination by Zfp423. Nature 464:619–623. https://doi.org/10.1038/nature08816.
30. Cristancho AG, Schupp M, Lefterova MI, Cao S, Cohen DM, Chen CS, Steger DJ, Lazar MA. 2011. Repressor transcription factor 7-like 1 promotes adipogenic competency in precursor cells. Proc Natl Acad Sci U S A 108:16271–16276. https://doi.org/10.1073/pnas.1109409108.
31. Raghav SK, Waszak SM, Krier I, Gubelmann C, Isakova A, Mikkelsen TS, Deplancke B. 2012. Integrative genomics identifies the corepressor SMRT as a gatekeeper of adipogenesis through the transcription factors C/EBPbeta and KAISO. Mol Cell 46:335–350. https://doi.org/10.1016/j.molcel.2012.03.017.
32. Cohen DM, Won KJ, Nguyen N, Lazar MA, Chen CS, Steger DJ. 2015. ATF4 licenses C/EBPbeta activity in human mesenchymal stem cells primed for adipogenesis. Elife 4:e06821. https://doi.org/10.7554/eLife.06821.
33. Hirning U, Schmid P, Schulz WA, Kozak LP, Hameister H. 1989. In developing brown adipose tissue c-myc protooncogene expression is restricted to early differentiation stages. Cell Differ Dev 27:243–248. https://doi.org/10.1016/0922-3371(89)90704-1.
34. Gensch N, Borchardt T, Schneider A, Riethmacher D, Braun T. 2008. Different autonomous myogenic cell populations revealed by ablation of Myf5-expressing cells during mouse embryogenesis. Development 135:1597–1604. https://doi.org/10.1242/dev.019331.
35. Tang Q-Q, Otto TC, Lane MD. 2004. Commitment of C3H10T1/2 pluripotent stem cells to the adipocyte lineage. Proc Natl Acad Sci U S A 101:9607–9611. https://doi.org/10.1073/pnas.0403100101.
36. Winnier G, Blessing M, Labosky PA, Hogan BL. 1995. Bone morphogenetic protein-4 is required for mesoderm formation and patterning in the mouse. Genes Dev 9:2105–2116. https://doi.org/10.1101/gad.9.17.2105.
37. Segre JA, Bauer C, Fuchs E. 1999. Klf4 is a transcription factor required for establishing the barrier function of the skin. Nat Genet 22:356–360. https://doi.org/10.1038/11926.
38. Swiatek PJ, Gridley T. 1993. Perinatal lethality and defects in hindbrain development in mice homozygous for a targeted mutation of the zinc finger gene Krox20. Genes Dev 7:2071–2084. https://doi.org/10.1101/gad.7.11.2071.
39. Deng CX, Xu X. 2004. Generation and analysis of Brca1 conditional knockout mice. Methods Mol Biol 280:185–200. https://doi.org/10.1385/1-59259-788-2:185.
40. Xu X, Li C, Garrett-Beal L, Larson D, Wynshaw-Boris A, Deng C-X. 2001. Direct removal in the mouse of a floxed neo gene from a three-loxp conditional knockout allele by two novel approaches. Genesis 30:1–6. https://doi.org/10.1002/gene.1025.
41. Ge K, Cho YW, Guo H, Hong TB, Guermah M, Ito M, Yu H, Kalkum M, Roeder RG. 2008. Alternative mechanisms by which mediator subunit MED1/TRAP220 regulates peroxisome proliferator-activated receptor gamma-stimulated adipogenesis and target gene expression. Mol Cell Biol 28:1081–1091. https://doi.org/10.1128/MCB.00967-07.
42. Jin Q, Yu L-R, Wang L, Zhang Z, Kasper LH, Lee J-E, Wang C, Brindle PK, Dent SYR, Ge K. 2011. Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation. EMBO J 30:249–262. https://doi.org/10.1038/emboj.2010.318.
43. Todaro GJ, Green H. 1963. Quantitative studies of the growth of mouse embryo cells in culture and their development into established lines. J Cell Biol 17:299–313. https://doi.org/10.1083/jcb.17.2.299.
44. Ahn S, Olive M, Aggarwal S, Krylov D, Ginty DD, Vinson C. 1998. A dominant-negative inhibitor of CREB reveals that it is a general mediator of stimulus-dependent transcription of c-fos. Mol Cell Biol 18:967–977. https://doi.org/10.1128/mcb.18.2.967.
45. Prifti E, Zucker JD, Clement K, Henegar C. 2008. FunNet: an integrative tool for exploring transcriptional interactions. Bioinformatics 24:2636–2638. https://doi.org/10.1093/bioinformatics/btn492.
46. Wang L, Jin Q, Lee JE, Su IH, Ge K. 2010. Histone H3K27 methyltransferase Ezh2 represses Wnt genes to facilitate adipogenesis. Proc Natl Acad Sci U S A 107:7317–7322. https://doi.org/10.1073/pnas.1000031107.
47. Feng X, Krebs LT, Gridley T. 2010. Patent ductus arteriosus in mice with smooth muscle-specific Jag1 deletion. Development 137:4191–4199. https://doi.org/10.1242/dev.052043.