Mediator MED23 Links Insulin Signaling to the Adipogenesis Transcription Cascade

Wei Wang,[1,4] Lu Huang,[1,4] Yan Huang,[1] Jing-wen Yin,[1] Arnold J. Berk,[2] Jeffrey M. Friedman,[3] and Gang Wang[1,*]
[1]State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China
[2]Molecular Biology Institute, University of California at Los Angeles, Los Angeles, CA 90095, USA
[3]Laboratory of Molecular Genetics, Rockefeller University, New York, NY 10021, USA
[4]These authors have contributed equally to this manuscript
*Correspondence: gwang22@sibs.ac.cn
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SUMMARY

Adipocyte differentiation is orchestrated by multiple signaling pathways and a temporally regulated transcriptional cascade. However, the mechanisms by which insulin signaling is linked to this cascade remain unclear. Here we show that the *Med23* subunit of the Mediator Complex and its interacting transcription factor *Elk1* are critical regulators of adipogenesis. *Med23*$^{-/-}$ embryonic fibroblast cells were refractory to hormone-induced adipogenesis. Knockdown of either *Med23* or *Elk1*, or overexpression of dominant-negative *Elk1*, inhibited adipogenesis. In the absence of either *Elk1* or *Med23*, *Krox20*, an immediate early gene stimulated by insulin during adipogenesis, was uninducible. Moreover, the adipogenic defect in *Med23*-deficient cells was rescued by ectopic expression of *Krox20* or one of its downstream factors, *C/EBPβ* or *PPARγ*. Mechanistically, the insulin-stimulated, *Med23*-deficient preinitiation complex failed to initiate robust transcription of *Krox20*. Collectively, our results suggest that *Med23* serves as a critical link transducing insulin signaling to the transcriptional cascade during adipocyte differentiation.

INTRODUCTION

Understanding the molecular basis underlying adipocyte differentiation is crucial for better management of obesity and its associated diseases, the prevalence of which have escalated worldwide in recent years (Rosen and Spiegelman, 2006). Regulation of adipogenesis involves signaling by multiple extracellular factors and a temporally regulated transcription cascade (Rosen and MacDougald, 2006; Rosen and Spiegelman, 2006). Although these multiple signaling pathways can either positively or negatively regulate adipogenesis (Farmer, 2006), insulin signaling plays a critical role in promoting lipid accumulation and energy storage. It has been previously demonstrated that insulin transiently activates MAPK signaling (Bost et al., 2005; Porras et al., 1992; Prusty et al., 2002) and the expression of several rapid response genes during the early phase of adipogenesis (Farmer, 2006). However, the mechanism by which insulin-MAPK signaling transmits information to the adipogenic transcription cascade still remains unclear (Rosen and MacDougald, 2006).

The Mediator Complex is a multiprotein complex that bridges gene-specific transcription factors and the basal transcription machinery. Different transcription factors, in response to environmental and developmental cues, physically interact with different Mediator subunits to control specific genes and biological processes (Malik and Roeder, 2005). The Mediator *Med23* (Sur2) subunit was originally identified as a genetic suppressor of a hyperactive *ras* phenotype in *C. elegans* (Singh and Han, 1995). In vivo and in vitro studies have demonstrated a specific interaction between mammalian *Med23* and the ternary complex factor *Elk1*, which is activated by MAPK signaling (Stevens et al., 2002; Wang et al., 2005). Gel filtration chromatography and immunoprecipitation (IP) revealed that *Med23*$^{-/-}$ cells contain a stable Mediator Complex that supports the transcription of most genes (see Figure S1 available online) (Stevens et al., 2002; Wang et al., 2005). Furthermore, microarray analyses revealed a small set of genes that are downregulated in *Med23*$^{-/-}$ cells (Stevens et al., 2002; Wang et al., 2005), suggesting that *Med23* may regulate specific cellular functions. Because the Ras-MAPK pathway has been shown to be involved in insulin signaling and adipogenesis, in this study we investigated the biological function of *Med23* and the possible involvement of *Med23* and *Elk1* in the regulation of insulin signaling and adipogenesis. We found that both *Med23* and its binding partner *Elk1* are regulators of adipogenesis. By controlling the expression of *Krox20* (*Egr2*), the first transcription factor induced during adipogenesis (Chen et al., 2005; Gonzalez, 2005), the MAPK-activated *Elk1-Med23* interaction functions as a critical link that connects insulin signaling to the adipogenic transcriptional cascade.

RESULTS

Med23 Is Required for Hormone-Induced Adipogenesis

The *Med23*$^{-/-}$ (KO) mice were embryonic lethal around 10.5 days of gestation (data not shown). Therefore, the embryonic fibroblasts (MEFs) were prepared from day 9.5 wild-type (WT) and KO mouse embryos, self-immortalized, and subjected to the standard hormone induction of adipogenesis. A three-hormone cocktail (insulin, 3-isobutyl-1-methylxanthine [IBMX],
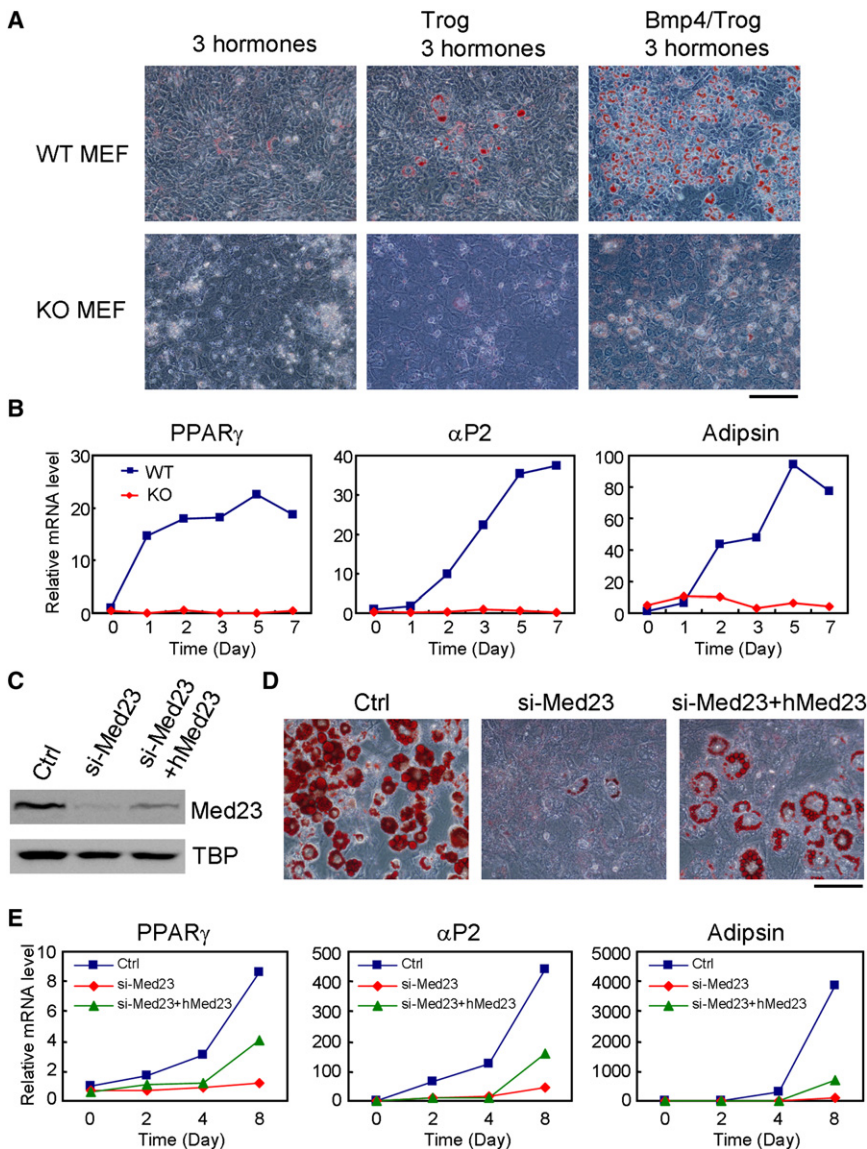
Figure 1. *Med23* Deficiency Results in Defects in Adipogenesis

(A) WT MEFs and KO MEFs were treated as indicated. At day 8 postinduction, the cells were stained for lipid droplets with ORO. Scale bar, 100 μm.

(B) WT MEFs and KO MEFs were treated with insulin, IBMX, and Dex plus troglitazone. At different time points posttreatment, total RNA samples were extracted and subjected to real-time PCR analysis of PPARγ, αP2, and adipsin. Expression was normalized to EF2 mRNA expression.

(C) 3T3-L1 cells were coinfected with retroviruses encoding either hMED23 or GFP and retroviruses encoding either a *Med23*-specific (si-*Med23*) or control siRNA and then selected for hygromycin and puromycin resistance. A western blot was used to confirm both the siRNA-mediated knockdown of endogenous *Med23* expression and the ectopic expression of hMED23. The anti-TBP blot was included as a control.

(D) Hormone-induced adipogenesis of si-Ctrl, si-*Med23*, and si-*Med23*+hMED23 3T3-L1 cells. At day 8 postinduction, cells were stained for lipid droplets with ORO. Scale bar, 100 μm.

(E) Real-time PCR analysis of the expression of PPARγ, αP2, and adipsin in 3T3-L1 cells at different time points postinduction. The expression was normalized to EF2 mRNA expression.

To further verify that *Med23* plays an essential role in adipogenesis, *Med23* expression was reduced in 3T3-L1 preadipocytes by RNAi. A stable *Med23* knockdown cell line was established by retrovirus-mediated siRNA delivery in 3T3-L1 cells. A control cell line expressing a negative control oligonucleotide was generated in a similar manner. Following selection with puromycin, the cells were cultured to confluence and treated with the hormone cocktail. The

and dexamethasone [Dex]) induced a small amount of lipid accumulation in WT MEFs, but not in KO MEFs (Figure 1A). Troglitazone, a diabetes drug that promotes glucose uptake and fat accumulation, efficiently potentiated hormone-induced adipogenesis in WT MEFs (over 20%), but failed to do so in KO MEFs (Figure 1A). As confirmed by real-time PCR, expression of the adipocyte markers αP2, adipsin, and PPARγ at different time points postinduction was increased in wild-type cells, but not in KO cells (Figure 1B). It has been previously shown that BMP4 promotes the differentiation of mesenchymal stem cells into adipocytes (Tang et al., 2004). Similarly, we observed that pretreatment of cells with BMP4, followed by the application of troglitazone and the hormone cocktail, was highly potent in converting WT MEFs into adipocytes (over 80%). However, this powerful treatment failed to induce adipogenesis in KO cells (Figure 1A), suggesting that *Med23*-deficient MEFs are refractory to hormone-induced adipogenesis.

MED23 protein level in the si-*Med23* cells was reduced by approximately 90% compared with that of the control cell line (Figure 1C). Oil Red O (ORO) staining demonstrated that si-*Med23* cells accumulated far fewer lipids than the control cells (Figure 1D), and real-time PCR confirmed the greatly attenuated expression of adipocyte markers in si-*Med23* cells during differentiation (Figure 1E). To exclude off-target effects of the siRNA, the human *Med23* gene, which is resistant to the siRNA specific for the mouse *Med23*, was reintroduced into the si-*Med23* cell line using retroviral transduction. Cells with the incorporated gene were selected with hygromycin and then induced to differentiate. Ectopic expression of human MED23 partially rescued the defective adipogenesis in the si-*Med23* 3T3-L1 cells, as assessed by ORO staining and the expression of αP2, PPARγ, and adipsin (Figures 1C, 1D, and 1E). Collectively, the experiments in both the MEFs and the 3T3-L1 cells demonstrate that *Med23* is essential for hormone-induced adipogenesis.
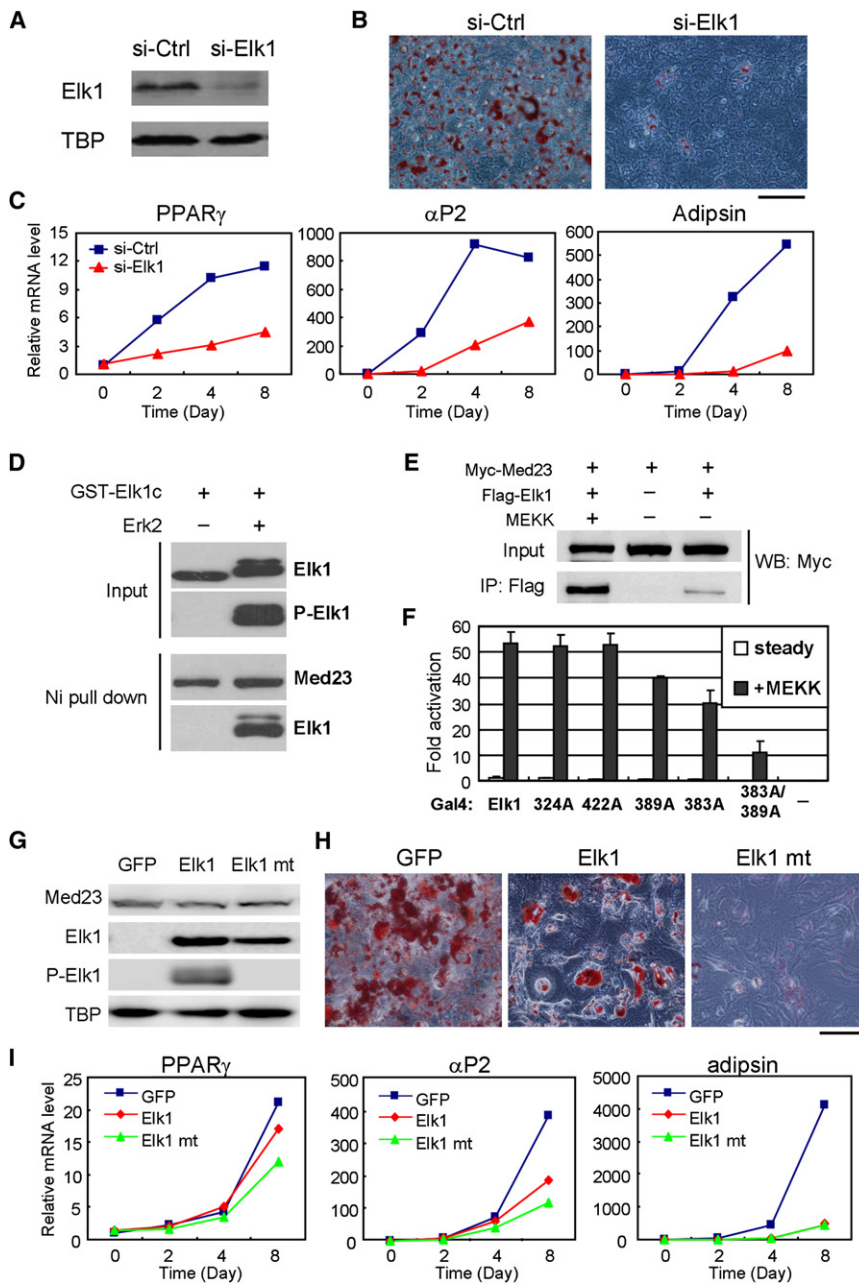
Figure 2. *Elk1* Regulates Adipogenesis

(A) Stable 3T3-L1 cell lines were generated by retroviral expression of an *Elk1*-specific siRNA (si-*Elk1*) or a control oligo (si-Ctrl). After puromycin selection, the cell lysates were analyzed by western blot.

(B) Inhibition of adipocyte differentiation by *Elk1* knockdown. si-*Elk1* and si-Ctrl cells were cultured to confluence, induced to differentiate, and stained with ORO at day 8 postinduction. Scale bar, 100 μm.

(C) Reduced expression of adipocyte markers following *Elk1* knockdown. Total mRNA was prepared from samples taken at the indicated time point postinduction and analyzed using real-time PCR. The expression was normalized to EF2 mRNA expression.

(D) Ni-NTA-immobilized His-FLAG-*Med23* was incubated at 4°C overnight with purified GST-ELK1 or *Erk2*-treated GST-ELK1. After being washed, the bound proteins were eluted by boiling and immunoblotted with the indicated antibodies.

(E) Co-IP of ELK1 with MED23. Plasmids encoding FLAG-*Elk1* and *Myc-Med23* were cotransfected into 293T cells with or without a Mekk expression plasmid. Whole-cell extracts were generated, immunoprecipitated with an anti-FLAG antibody, and analyzed by western blot using an anti-MYC antibody.

(F) 293T cells were transfected with a 5 × Gal-E1B-TATA-luciferase reporter construct and a plasmid encoding the Gal4-*Elk1* activation domain or Gal4-*Elk1* mutants, with or without a Mekk expression plasmid. The Firefly luciferase activity was normalized to Renilla luciferase activity. Error bars represent the standard deviation of three independent experiments.

(G) 3T3-L1 cell lines stably expressing wild-type *Elk1* (*Elk1*), the S383A/S389A mutated *Elk1* (*Elk1* mt), or GFP (GFP) were generated. After hygromycin selection, cell lysates were analyzed by western blot.

(H) Cells were cultured to confluence, induced to differentiate, and stained with ORO at day 8 postinduction. Scale bar, 100 μm.

(I) The expression of adipocyte markers was examined by real-time PCR. The expression is normalized to EF2 mRNA expression.

Elk1 Regulates Adipogenesis

Elk1 is a member of the ternary complex factor family. Upon phosphorylation by MAPK, ELK1 interacts with the Mediator Complex via the *Med23* subunit (Cantin et al., 2003; Stevens et al., 2002; Wang et al., 2005) and therefore is considered upstream of *Med23*. Although *Elk1* has been shown to be involved in proliferation and neuronal differentiation (Buchwalter et al., 2004), its role in adipogenesis has not yet been reported. We established a 3T3-L1 cell line stably expressing an *Elk1*-specific siRNA (Figure 2A) and observed attenuated hormone-induced adipogenesis, as detected by decreased ORO staining and adipocyte marker gene expression (Figures 2B and 2C). A similar observation was made in MEFs expressing the *Elk1*-specific siRNA (data not shown).

Next, we further characterized the ELK1 and MED23 interaction. The MED23 protein is highly hydrophobic, and soluble recombinant MED23 has been difficult to obtain. However, it is now possible to express and purify a soluble form of tagged MED23 protein from baculovirus-infected insect cells. In vitro, only the *Erk*-phosphorylated GST-ELK1 activation domain bound to the immobilized MED23 protein, whereas the nonphosphorylated GST-ELK1 did not (Figure 2D). In 293T cells, a weak interaction between ELK1 and MED23 was detected when *Myc-Elk1* and *Flag-Med23* expression plasmids were cotransfected. However, a much stronger interaction between ELK1 and MED23 was observed when a plasmid expressing active Mekk was also cotransfected (Figure 2E). These results suggest that phosphorylation of ELK1 facilitates its binding to MED23.

Given the importance of ELK1 phosphorylation, we mapped the ELK1 phosphorylation sites that are important for MAPK activation. Simultaneous mutation of two serine residues (S383 and S389) in the ELK1 activation domain to alanine significantly inhibited the activity of ELK1 in a luciferase assay (Figure 2F). We further analyzed the adipogenic phenotypes of 3T3-L1 cells overexpressing GFP, Elk, or *Elk1* mutant (Figure 2G). Overexpression of either *Elk1* or the S383A/S389A mutant (*Elk1*-mt) in 3T3-L1 cells slowed down cell growth and inhibited adipogenesis (Figures 2G and 2H). However, the generation of adipocytes from cells expressing *Elk1*-mt was more severely reduced (5%) compared with that from cells expressing wild-type *Elk1* (30%; Figures 2G and 2H). The morphological result was confirmed by the real-time PCR-based quantification of adipocyte markers (Figure 2I). Thus, the S383A/S389A mutant displayed characteristics of a dominant-negative mutant. Taken together, these results suggest that *Elk1* is an important regulator of adipogenesis.

Effect of *Med23* Deficiency on Insulin Signaling and Cell Cycle Progression

In an effort to understand the mechanism by which *Med23* regulates adipogenesis, we examined whether or not *Med23* deficiency affects insulin signaling and cell cycle regulation. Although *Med23* is an effector of growth signaling and is required for adipogenesis, siRNA knockdown of *Med23* did not alter insulin-activated signaling, as indicated by the equal expression and phosphorylation levels of AKT, ERK, and ELK1 in the si-Ctrl and si-*Med23* 3T3-L1 cells following insulin stimulation (Figure S2A, compare lanes 2 and 4). Interestingly, however, the basal phosphorylation level of ERK and AKT appeared to change, possibly due to crosstalk or feedback regulation (Figure S2A, compare lanes 1 and 3). Importantly, the *Med23* knockdown did not change the expression pattern of cell cycle regulators such as cyclin A, E, and D in a 24 hr time period after hormone induction (Figure S2B).

Postconfluent 3T3-L1 cells typically undergo one or two rounds of the cell cycle upon adipogenic hormone induction (mitotic clonal expansion), which is considered a prerequisite step for 3T3-L1 differentiation (Otto and Lane, 2005). It has been previously shown that MAPK signaling is required to initiate this mitotic clonal expansion during adipogenesis (Tang et al., 2003). However, knockdown of either *Elk1* or *Med23* in 3T3-L1 cells did not change cell doubling or cell cycle progression in response to the induction of adipogenesis by the three hormones (Figure S3). This suggests that *Elk1* and *Med23* most likely regulate adipogenesis by mechanisms other than an effect on insulin signaling, cell cycle progression, or mitotic clonal expansion.

Insulin-MAPK-*Elk1-Med23* Pathway Targets *Krox20* to Modulate the Adipogenic Transcription Cascade

In previous studies using microarrays and chromatin IP (ChIP) assays, it has been demonstrated that the *Elk1-Med23* interaction controls the expression of the early growth response genes, including *Krox20*, in response to serum growth factor (Wang et al., 2005). *Krox20* is highly expressed in adipose tissues (Soukas et al., 2001) and is the first transcription factor expressed upon insulin stimulation during adipocyte differentiation, although the mechanism of how insulin induces *Krox20* expres-

sion is not clear (Chen et al., 2005; Gonzalez, 2005). Therefore, to better understand how *Med23* controls adipogenesis and how insulin signaling affects *Krox20* expression, we investigated whether *Med23* also controls insulin-stimulated *Krox20* expression in the early phase of adipocyte differentiation. RNA samples were collected from confluent WT and KO MEFs at different time points after insulin stimulation and analyzed by real-time PCR. Consistent with the previous report (Chen et al., 2005), in WT cells the expression of *Krox20* was rapidly induced by insulin, reached a peak 30 min poststimulation, and decreased to the basal level 60 min poststimulation. However, this induction was abolished in KO cells (Figure 3A). *Krox20* seems to be specifically induced by insulin, because insulin treatment with or without the other two hormones (IBMX and Dex) did not alter the intensity of *Krox20* induction (data not shown). Consistent with the fact that *Elk1* regulates adipogenesis, insulin-stimulated expression of *Krox20* was also greatly reduced in cells following knockdown of *Elk1* (Figure S4) or overexpression of a dominant-negative *Elk1* mutant (data not shown). These data demonstrate that both *Elk1* and *Med23* control insulin-stimulated *Krox20* expression.

To determine the specific effect of the insulin-MAPK-*Elk1*-*Med23* pathway on *Krox20* expression, we treated the cells with various inhibitors of signal transduction during insulin induction (Figure 3B). Only a MEK1 inhibitor, U0126, selectively inhibited the insulin-mediated expression of *Krox20* in WT MEFs; inhibitors specific to other signaling pathways, including SB203580 (to p38), H-89 (to PKA), and LY294002 (to PI3K) did not inhibit insulin-stimulated *Krox20* expression. Interestingly, the inhibition of p38 further enhanced the insulin-induced expression of *Krox20*, possibly due to the crosstalk between p38 and *Erk* (Keeton et al., 2003). In KO cells, none of the inhibitors had an obvious effect on the low level of *Krox20* expression, because deletion of *Med23* already abolished the *Krox20* response to insulin (Figure 3B). These data suggest that insulin-induced expression of *Krox20* is MAPK dependent and that *Med23* is required for connecting insulin-MAPK signaling to *Krox20* gene activation.

It has been previously reported that following insulin-activated expression of *Krox20*, both C/EBPβ (Farmer, 2006) and KLF5 (Oishi et al., 2005) are expressed during the early phase of adipogenesis. Therefore, we tested whether C/EBPβ and KLF5 are also affected by *Med23* in WT and KO MEFs. To eliminate the mixed effects of multiple hormones, the cells were treated with insulin alone (without IBMX and Dex). The mRNA expression level of both C/EBPβ and KLF5 in WT MEFs was modestly increased after insulin treatment. Expression reached the peak level (2- to 3-fold induction) 2 hr poststimulation and then returned to the basal level 4 hr poststimulation (Figures 3C and 3D). In contrast, in KO MEFs the expression level of both C/EBPβ and KLF5 did not change significantly throughout the time course, and a reduced basal level of expression was observed (Figures 3C and 3D). This suggests that loss of *Med23* attenuates both the insulin-induced and basal level expression of C/EBPβ and KLF5. These results demonstrate that *Med23* not only controls the MAPK-dependent expression of an early immediate gene, *Krox20*, but also modulates downstream adipogenic transcription factors. Furthermore, because PPARγ expression was also abolished in KO cells (Figure 1B),
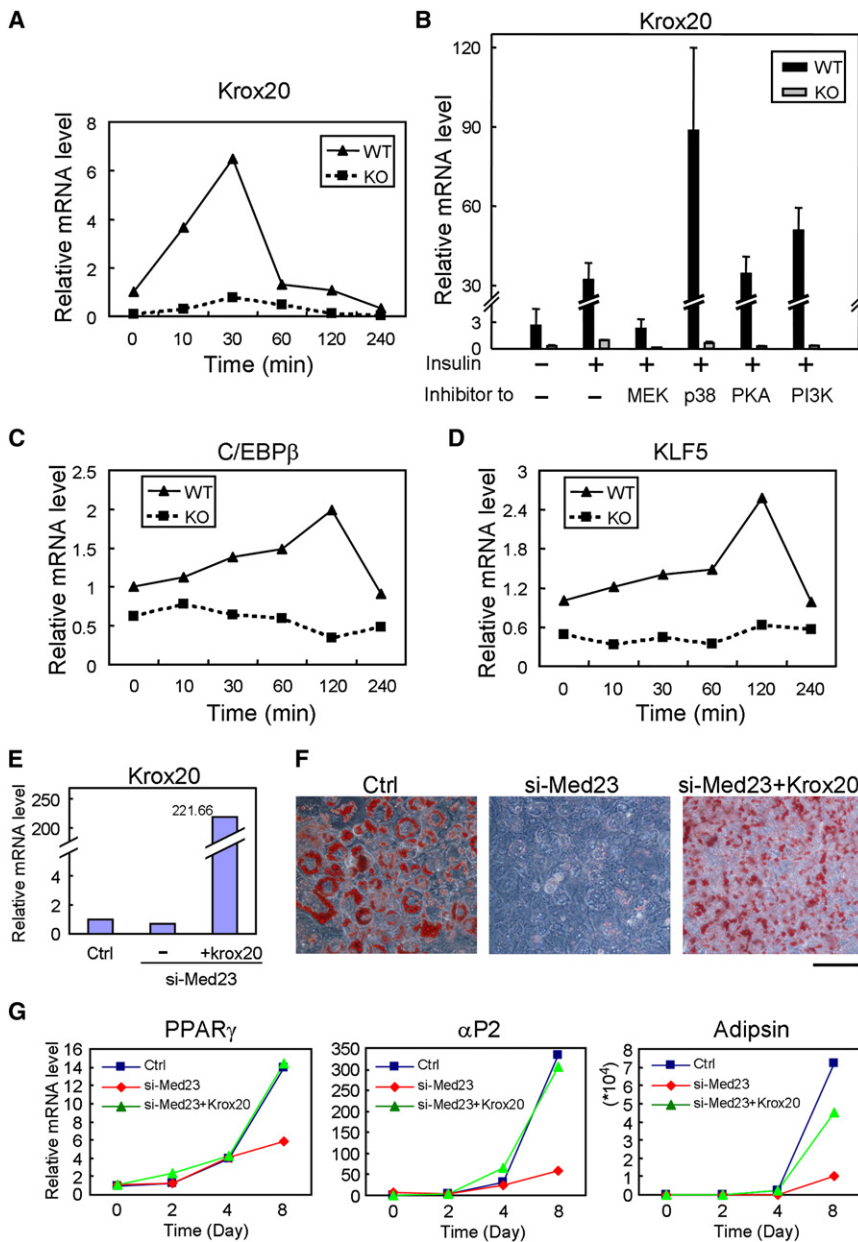
Figure 3. *Med23* Controls the Insulin-Stimulated *Krox20* Expression

(A) *Krox20* expression at the indicated time points following insulin induction.

(B) Insulin-stimulated expression of *Krox20* is selectively blocked by a MAPK inhibitor. Inhibitors of various signaling pathways were added to the culture medium 30 min before insulin application. Total RNA samples were harvested 30 min after insulin addition. Error bars represent the standard deviation of three independent experiments.

(C and D) Real-time PCR analysis of the expression of C/EBPβ and KLF5 at the indicated time points following insulin induction (without IBMX/Dex). The expression is normalized to EF2 mRNA expression.

(E) si-*Med23* 3T3-L1 cells were infected with a retrovirus encoding the *Krox20* cDNA and selected with hygromycin. Real-time PCR was used to quantify the *Krox20* expression level of the indicated cell lines. The expression is normalized to EF2 mRNA.

(F) Hormone-induced adipogenesis of si-Ctrl, si-*Med23*, and si-*Med23+Krox20* 3T3-L1 cells. At day 8 postinduction, cells were stained for lipid droplets with ORO. Scale bar, 100 μm.

(G) Real-time PCR analysis of the expression level of adipocyte markers in the indicated 3T3-L1 cell lines at different time points postinduction. The expression is normalized to EF2 mRNA expression.

Med23 appears to be required for the entire adipogenic transcriptional program.

Ectopic Expression of *Krox20* or Its Downstream Factors Rescues the Adipogenic Defect in *Med23*-Deficient Cells

Our data suggest that *Med23* acts upstream of *Krox20* and the adjunct adipogenic transcription cascade. Therefore, we expect that overexpression of *Krox20* should reverse the defect in adipogenesis resulting from *Med23* deficiency. To test this, si-*Med23* 3T3-L1 cells were infected with a retrovirus encoding *Krox20* and the hygromycin-resistance gene for selection (Figure 3E). As shown in Figures 3F and 3G, overexpression of *Krox20* rescued the differentiation defect of si-*Med23* 3T3-L1 cells, as indicated by ORO staining and real-time PCR analysis

of adipocyte marker gene expression. Additionally, forced expression of *Krox20* in the KO MEFs also partially restored hormone-induced adipogenesis (data not shown).

Because *Elk1* and *Med23* may regulate the expression of other genes besides *Krox20*, it is possible that other *Med23*-regulated genes are also involved in adipogenesis. Therefore, a gene profiling experiment was performed in the si-Ctrl and si-*Med23* 3T3-L1 cells, and a small number of genes (34) were identified that are both *Med23* regulated and insulin responsive (Table S1). *Krox20* was the most insulin-responsive (11.9-fold induction) of the 34 genes identified. Although we cannot rule out the possibility that one of the other genes may also regulate adipogenesis, the facts that *Krox20* overexpression rescues adipogenesis in *Med23*-deficient cells and that *Krox20* knockdown blocks adipogenesis in both 3T3-L1 and NIH 3T3 cells (Chen et al., 2005) suggest that *Krox20* is an essential factor connecting insulin signaling to adipogenesis. We also noticed that another transcription factor, *Egr1*, which is a closely related family member of *Krox20* that is also targeted by *Med23* (Wang et al., 2005), is significantly induced by insulin (Table S1). However, in contrast to the effect of *Krox20*, ectopic expression of *Egr1* failed to restore hormone-induced adipogenesis in the si-*Med23* 3T3-L1 cells (Figure S5), which further underscores the specific role of *Krox20* in the adipocyte lineage.
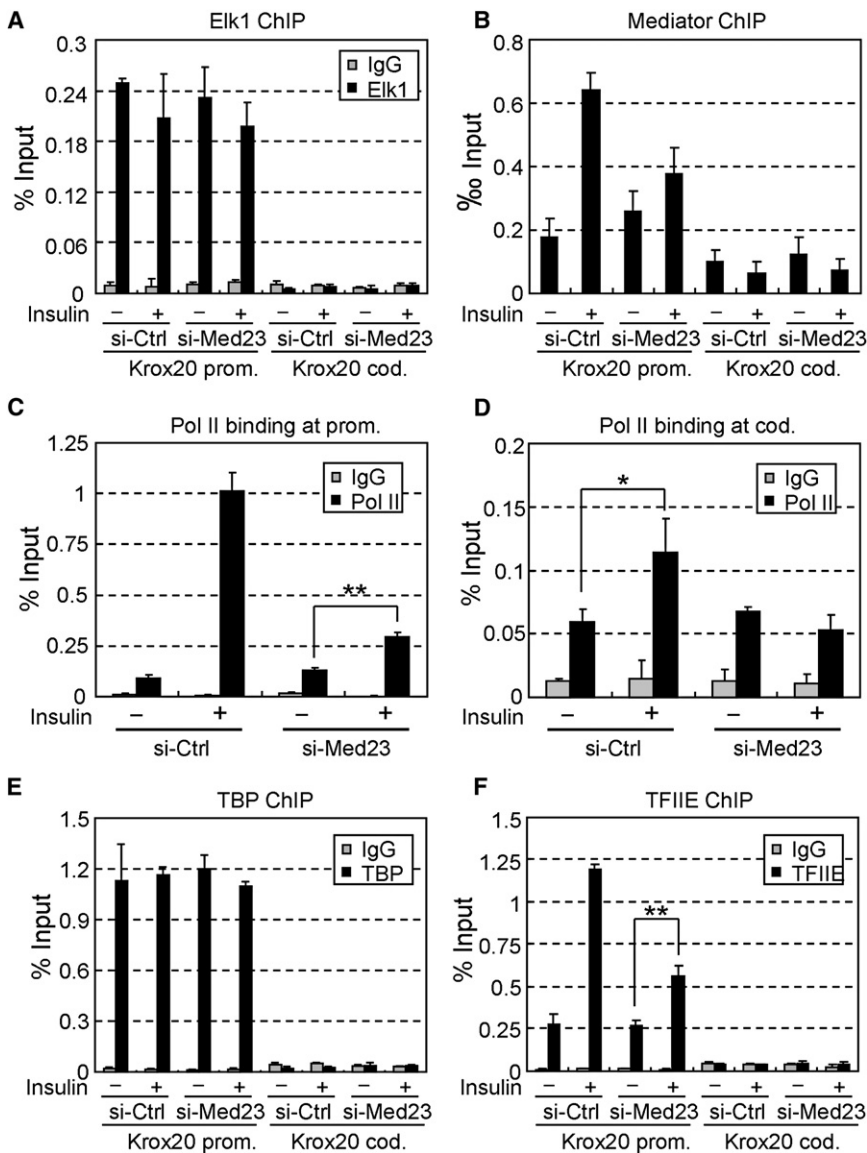
Figure 4. Effect of Insulin on the Binding of ELK1, Mediator, and Pol II to the Krox20 Promoter

3T3-L1 cells were grown to confluence and then treated with insulin at a final concentration of 5 μg/ml for 30 min. ChIP experiments were performed using antibodies against *Elk1* (A), the Mediator Complex (*Med1* and *Med17*) (B), Pol II (C and D), TBP (E), TFIIE (F), and IgG as a control. The precipitated DNA was analyzed by real-time PCR with primers targeting the *Krox20* promoter region (*Krox20p*) and coding region (*Krox20c*). The primer sequences are provided in Table S2. The relative binding level of each factor was calculated by normalization to the input DNA. The mean of three separate experiments is shown, and the standard deviation is indicated. Student's t test, *p < 0.05, **p < 0.01.

measure the binding of ELK1, the Mediator Complex, Pol II, and general transcription factors to the *Krox20* locus in response to insulin in confluent si-Ctrl, si-*Elk1*, and si-*Med23* 3T3-L1 cells. Both ELK1 and TBP constitutively bind to the *Krox20* promoter, but not to the coding region, at an equivalent level regardless of *Med23* status and insulin application (Figures 4A and 4E). This most likely indicates that they function as the platform for the formation of the preinitiation complex (PIC). The knockdown of *Elk1* reduced ELK1 binding at the *Krox20* promoter by 50% (Figure S7A), which led to a 50% decrease in both the basal and the insulin-stimulated levels of the Mediator Complex binding at the *Krox20* promoter (Figure S7B). Consistent with the in vivo and in vitro data demonstrating that phosphorylated ELK1 interacts with MED23 (Figures 2D, 2E, and 2F), the addition of insulin enhanced the co-occupancy of ELK1 and Mediator at the *Krox20* promoter (6-fold increase), as detected in a sequential ChIP (re-ChIP) experiment (Figure S7C). These results suggest that recruitment of the Mediator Complex at the *Krox20* promoter is dependent on ELK1 and insulin stimulation. Consistently, insulin stimulation increased the binding of the Mediator Complex, Pol II, and TFIIE at the *Krox20* promoter (3- to 6-fold increase) in si-Ctrl 3T3-L1 cells (Figures 4B, 4C, and 4F). The level of Pol II binding at the *Krox20* promoter in the presence of insulin and *Med23* is comparable to that at the promoter of a constitutively expressed gene, EF2, but is significantly above that at the promoter of an inactive gene, IL12b (Figure S8). Interestingly, in the si-*Med23* cells, there seems to be a *Med23*-independent enhancement of Pol II and TFIIE binding to the *Krox20* promoter (approximately 1-fold increase) upon insulin stimulation (Figures 4C and 4F), and Mediator Complex binding is also slightly increased (Figure 4B). These enhancements are probably not due to the residual amount of *Med23*, because similar

To further verify the pivotal role of *Med23* in regulating the adipogenic transcription cascade, we tested whether factors downstream of *Krox20* (C/EBPβ and PPARγ) are able to reverse the adipogenic defect in *Med23*-deficient cells. To address this question, KO MEF cell lines stably expressing C/EBPβ or PPARγ were generated. A GFP-expressed cell line was also generated as a control. These cells were cultured to confluence and then subjected to hormone-induced differentiation. The ectopic expression of C/EBPβ or PPARγ in KO cells robustly promoted adipogenesis (>90%) and adipogenic marker gene expression (Figure S6). These results further support that *Krox20*, C/EBPβ, and PPARγ are downstream of *Med23*, because ectopic expression of any one of them is able to bypass the need for *Med23* in adipogenesis.

Med23 Is Required for the Productive Transcription of Krox20 in Response to Insulin Stimulation

To determine how *Med23* controls insulin-MAPK-activated *Krox20* transcription, ChIP experiments were performed to

increases in Mediator Complex and Pol II binding were also observed in the insulin-stimulated KO MEFs (Figures S9B and S9C). However, only in the WT cells, and not in the *Med23*-deficient cells, Pol II at the *Krox20* promoter was able to initiate transcription efficiently, as indicated by the enhanced Pol II binding to the *Krox20* gene coding region in the presence of insulin (Figure 4D and S9D) and the greatly enhanced *Krox20* mRNA level (Figure 3A). Taken together, these data suggest that insulin stimulation and *Med23* are both required for significant recruitment of Pol II and active transcription from the *Krox20* promoter. Upon insulin stimulation, the *Elk1-Med23* interaction promotes a productive PIC that leads to robust transcription, whereas the *Med23*-deficient PIC fails to do so.

DISCUSSION

This report demonstrates that the Mediator Complex subunit *Med23* and *Elk1* are pivotal regulators of adipogenesis. By controlling insulin-MAPK-stimulated expression of *Krox20*, *Med23* connects the insulin signaling pathway to the adipogenic transcription cascade. Our study reveals a function of the *Elk1-Med23-Krox20* axis in regulating adipocyte formation, adding *Elk1* and *Med23* to the growing list of key early regulators of adipogenesis, such as *Krox20* (Chen et al., 2005), *Klf5* (Oishi et al., 2005), *Klf4* (Birsoy et al., 2008), and CREB (Reusch et al., 2000; Zhang et al., 2004). Collectively, these studies suggest the existence of an early transcriptional factor network that initiates the adipogenesis program, and *Elk1/Med23* may represent the earliest point of regulation.

The effect of *Med23* in adipogenesis is highly specific, because *Med23* deficiency does not prevent the differentiation of embryonic stem cells into many other cell types of the three germ layers during embryoid body and teratoma formation (data not shown). Furthermore, knockdown of *Med23* in C2C12 myoblasts did not inhibit induced skeletal myogenesis (Figure S10), emphasizing the specific role for *Med23* in adipogenesis. Because the deletion of *Med23* is embryonic lethal, the in vivo function of *Med23* during adipogenesis awaits future study using a conditional knockout approach.

Growing evidence has demonstrated that the Mediator Complex functions as a control center in directing diverse biological processes. For example, another Mediator subunit, *Med1*, controls adipogenesis by binding to PPARγ, a transcription factor that is critical for the terminal stage of adipocyte differentiation (Ge et al., 2002). Other Mediator subunits are required for different biological functions, such as *Med12* for regulating neuronal development (Wang et al., 2006) or *Med15* for controlling TGFβ signaling during development (Kato et al., 2002) and SREBP-controlled cholesterol and fatty acid homeostasis (Yang et al., 2006). Modulation of the various interactions between different activators and Mediator subunits may provide novel strategies to manipulate these diverse biological functions. Importantly, the *Elk1-Med23* interaction may represent a potential therapeutic target for obesity and its associated diseases.

EXPERIMENTAL PROCEDURES

Cell Culture and Adipocyte Differentiation

Differentiation of MEFs and 3T3-L1 into adipocytes was described previously (Chen et al., 2005; Ge et al., 2002). For more detail, including methods for insulin stimulation and signaling inhibition, see Supplemental Experimental Procedures.

Retrovirus Plasmids and Infection

Establishing stable cell lines to knock down or overexpress a gene of interest were based on the manufacturer's recommendation (Clontech). For more detail, see Supplemental Experimental Procedures.

Real-Time PCR and Western Blot Analysis

Methods for Real-time PCR and western blot analysis were described previously (Wang et al., 2005). See Supplemental Experimental Procedures for more detail.

Recombinant Protein Production, In Vitro Binding, and Co-IP

See Supplemental Experimental Procedures.

Site-Specific Mutagenesis and Luciferase Assay

Multiple serine residues within the *Elk1* activation domain of Gal4-*Elk1* (307–428) were mutated by site-directed mutagenesis to alanine residues using the KOD Hot Start DNA Polymerase kit (TOYOBO). Cotransfection and luciferase assay are described in the Supplemental Experimental Procedures.

ChIP

ChIP assays were performed as described previously (Wang et al., 2005); however, the immunoprecipitated DNA was quantified using real-time PCR. The primers for analyzing the ChIP DNA are provided in Table S2. More detail is offered in the Supplemental Experimental Procedures.

SUPPLEMENTAL DATA

Supplemental Data include Supplemental Experimental Procedures, ten figures, and two tables and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00168-3.

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