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Mat1 Inhibits Peroxisome Proliferator-Activated Receptor γ-Mediated Adipocyte Differentiation▽†

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Mammalian Cdk7, cyclin H, and Mat1 form the kinase submodule of transcription factor IIH (TFIIH) and have been considered ubiquitously expressed elements of the transcriptional machinery. Here we found that Mat1 and Cdk7 levels are undetectable in adipose tissues in vivo and downregulated during adipogenesis, where activation of peroxisome proliferator-activated receptor γ (PPARγ) acts as a critical differentiation switch. Using both *Mat1*$^{-/-}$ mouse embryonic fibroblasts and Cdk7 knockdown approaches, we show that the Cdk7 complex is an inhibitor of adipogenesis and is required for inactivation of PPARγ through the phosphorylation of PPARγ-S112. The results demonstrate that the Cdk7 submodule of TFIIH acts as a physiological roadblock to adipogenesis by inhibiting PPARγ activity. The observation that components of TFIIH are absent from transcriptionally active adipose tissue prompts a reevaluation of the ubiquitous nature of basal transcription factors in mammalian tissues.

The Cdk7 kinase in complex with its cognate cyclin (cyclin H) and a RING domain-containing protein, Mat1, was first characterized as a *cdc2*-activating kinase (23, 29, 51), and subsequently identified as the kinase submodule of transcription factor IIH (TFIIH) (1, 29, 64, 66). Genetic analyses using temperature-sensitive or chemical genetic alleles of Cdk7 support the notion that Cdk7 is required for the activating phosphorylation of the T-loop of Cdc2 (45, 46, 75) and Cdk2 (45). However, genetic ablation of *Mat1* in mouse cells indicated that while *Mat1* is essential for embryonic development, it is not required for the viability of single cells (44, 63, 65) or for Cdc2 phosphorylation (65). As all studies indicate Mat1 and its orthologs in lower species function only as part of the Cdk7 kinase complex, and as deletion of murine *Mat1* leads to concomitant loss of Cdk7 (44, 65), the results suggest that Cdk7 kinase activity would not be critical for Cdc2 activation or cell viability in murine cells.

As part of TFIIH, the Cdk7 kinase submodule has been implicated in the phosphorylation of serine 5 of the carboxyl-terminal domain (CTD) heptapeptide repeat of the large subunit of RNA polymerase II (4, 28). RNA polymerase II CTD Ser 5 phosphorylation has been suggested to be required for appropriate mRNA processing and associated chromatin modifications (43, 54). While several studies especially with the budding yeast (*Saccharomyces cerevisiae*) ortholog Kin28 (27, 37, 73, 74) support the notion that Cdk7 within TFIIH is generally required for normal mRNA transcription, recent analyses in both budding (39) and fission (48) yeast suggest that Cdk7 kinase activity rather is involved in the regulation of specific transcriptional programs. Consistent with this notion, the ablation of Mat1 in murine myocardium resulted in a suppression of genes involved in energy metabolism (65) that is suggested to be mediated through suppression of the activity of the coactivator PGC-1.

The Cdk7 kinase complex has also been implicated in the regulation of specific transcription through its ability to phosphorylate a number of transcriptional regulators in vitro, including the nuclear hormone receptors retinoic acid receptors α and γ, estrogen receptor α, peroxisome proliferator-activated receptor α (PPARα), and PPARγ (9, 15, 20, 40, 59). The site Cdk7 phosphorylates on PPARγ2 in vitro is serine 112 (20), identified originally as an inhibitory mitogen-activated protein kinase site in the N-terminal activation domain (2, 12, 38). Phosphorylation of PPARγ-S112 inhibits PPARγ target gene activation by several mechanisms, including decreased ligand binding and impaired ability to recruit transcriptional coactivators (67).

PPARγ is a member of the nuclear hormone receptor family and is expressed preferentially in adipose tissue (10). Expression is low in preadipocytes and strongly induced during adipogenesis (14, 55). Adipogenesis is initiated in fibroblasts/preadipocytes through the activation of Krox-20, C/EBPß, and PPARγ transcription factors (17). In this transcriptional regulatory network, PPARγ has a central role, as its expression is necessary and sufficient for adipogenesis both in vitro and in vivo (62, 72). PPARγ activation is enhanced by ligand binding and agonists include high affinity synthetic ligands such as the antidiabetic thiazolidinediones (TZDs; troglitazone, pioglitazone) and endogenous ligands such as 15-deoxy-$\Delta^{12,14}$prostaglandin J_2 (49). In addition to ligand binding and phosphorylation, coactivators such as PGC-1 and SRC-1 further regulate PPARγ activity (56).

A putative link between PPARγ and Cdk7 in vivo was provided by the observation that mRNAs of PPARγ target genes were altered in adipose tissue of mice with mutations in the XPD subunit of TFIIH (20) modeling trichothiodystrophy and featuring fat hypoplasia. The altered XPD was proposed to specifically impact Cdk7 activity toward PPARγ-S112 in adipose tissue. This hypothesis is complicated by the variability of

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the alterations of PPARγ target mRNAs and PPARγ promoter occupancy in the analyzed samples (20).

The observations that the Cdk7 submodule of TFIIH may not be universally required for viability and potentially represents a specific transcriptional regulator prompted us to reevaluate the concept of the Cdk7 submodule as a constitutive ubiquitous kinase complex. Specifically, we were interested in identifying physiological circumstances where modulation of Cdk7 submodule activity would be used to achieve biological responses through regulation of the activity of specific transcription factors. The investigation was focused on adipose tissue based on the critical role of PPARγ in adipogenesis (62, 72), and the tentative link to Cdk7 (20), and reveals that the Cdk7 submodule acts as an inhibitor of PPARγ and as a physiological roadblock to adipogenesis.

MATERIALS AND METHODS

Immunostaining of murine tissue and cells in culture. All animal experiments were approved by the Committee for Animal Experiments of the District of Southern Finland and mice were housed according to their regulations. Mice were anesthetized, fixed by intracardiac perfusion with 1% paraformaldehyde, and tissue from neck was frozen in OCT medium (TissueTek). Seven-millimeter tissue sections were washed in phosphate-buffered saline (PBS), permeabilized using 0.3% PBS-Triton X (Fluka Biochemica), and blocked with PBS containing 5% donkey serum, 0.05% sodium azide, 0.2% bovine serum albumin, and 0.3% Triton X. Primary antibodies α-Mat1 (FL-309; Santa Cruz) and α-PPARγ (sc-7273; Santa Cruz) were incubated in blocking solution overnight and incubated with appropriate secondary antibodies as follows: anti-rabbit Alexa 594 and anti-goat Alexa 488 (Molecular Probes). Samples were mounted with Vectashield mounting medium (H-1200; Vector), and analyzed with a confocal microscope (Zeiss LSM 510; Carl Zeiss). Three-dimensional projections were digitally generated from confocal z stacks. Colocalization of signals was assessed from single confocal optical sections.

Immunostaining of cells was done as previously described (63). Immunolabeling was performed with the following antibodies: anti-Mat1 (FL-309; Santa Cruz), anti-PPARγ (sc-7273; Santa Cruz), anti-phosphorylated PPARγ-S112 (anti-P~PPARγ-S112) (PPAR-IF5; Euromedex), anti-C/EBPβ (sc-150; Santa Cruz), and antibromodeoxyuridine (Dako). Secondary antibodies included goat anti-mouse Alexa 488 (Molecular Probes) and goat anti-rabbit 549 (Molecular Probe). Stained coverslips were analyzed using Zeiss Axioplan 2 microscope and Axiovision software. For quantification of PPARγ-S112- and P~PPARγ-S112-positive cells in mouse embryonic fibroblasts (MEFs), 1,000 to 3,000 Hoechst-positive nuclei were scored.

Western blotting and kinase assays. Western blotting and kinase assays were as described previously (44, 50). Antibodies were as follows: anti-Mat1 (FL-309; Santa Cruz), anti-Cdk7 (sc-7344; Santa Cruz), anti-Cdk2 (sc-163; Santa Cruz), anti-phospho-Cdk2-T160 (2561; Cell Signaling), anti-phospho-Cdc2-T161 (9114; Cell Signaling), antiactin (AC-40; Sigma), anti-PPARγ (sc-7273; Santa Cruz), anti-P~PPARγ-S112 (PPAR-IF5; Euromedex), TAF10 (kind gift from L. Tora [52]). Secondary antibodies were as follows: anti-rabbit horseradish peroxidase (HRP), anti-mouse HRP, and anti-goat HRP (Chemicon International). For kinase assays, 100-μg portions of cell lysates were immunoprecipitated with anti-Cdk7 (50) and assayed for the ability to phosphorylate glutathione transferase (GST)-CTD or GST-Cdk2 (44). For rat fat lysates, adult rats were sacrificed with CO_2 and dislocation of the neck, and fat collected from various locations and snap-frozen. Proteins were isolated from tissue pieces by use of FastPrep FP120 (ThermoSavant) in ELB (150 mM NaCl, 50 mM HEPES, pH 7.4, and 5 mM EDTA with 10 mM β-glycerophosphate, 1 μg/ml leupeptin, 12.5 μg/ml aprotinin, 0.5 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol added before use) and centrifuged at 2,000 rpm for 5 min, followed by the collection of supernatants subsequently used in sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Adipocyte differentiation of MEFs and 3T3-L1 cells. Induction of adipogenesis was done as previously described (57). Briefly, 2-day postconfluent MEFs or 3T3-L1 preadipocytes (33) were treated with differentiation medium (Dulbecco's modified Eagle medium with 10% fetal calf serum supplemented with 10 μg/ml insulin, 1 μM dexamethasone, 0.25 mM 3-isobutyl-1-methylxanthine) for 2 days, then for 2 days in medium supplemented with insulin, and finally for 4 days in normal growth medium. For lipid staining, the cells were fixed at day 8 with 10% formalin for 30 min at 37°C and stained with Oil Red O. Troglitazone (Cayman Chemical Company) treatment was done at 10 μM for 4 to 24 h.

Establishment of *Mat1*$^{-/flox}$ primary and immortalized MEFs and deletion of *Mat1*. MEFs were isolated from embryonic day 12.5 *Mat1*$^{-/flox}$ (44) embryos and cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. To generate immortal *Mat1*$^{-/flox}$ MEFs, cells from passage 3 were infected with a retrovirus encoding residues 302 to 390 of p53 (42) and grown as pools following selection with hygromycin (Invitrogen) at 0.2 mg/ml. For the deletion of *Mat1*, MEFs were infected with adenoviruses encoding Cre recombinase (AdCre) (5) or green fluorescent protein (AdGFP) using a multiplicity of infection of 1,500 overnight at 37°C. Subsequent analyses of AdCre (*Mat1*$^{-/-}$)- and AdGFP (*Mat1*$^{-/flox}$)-infected cells were done 72 h later unless otherwise indicated.

Transfections and reporter gene assays. Transient transfections were performed in MEFs using Superfect transfection reagent (Qiagen) according to the manufacturer's instructions. For PPARγ activity assays, cells were transfected with PPARγ response element 3 ($PPRE_3$)-thymidine kinase (TK)-Luc (2) containing three copies of PPRE from acyl coenzyme A oxidase gene linked to the TK promoter ($PPRE_3$-TK-Luc) and PPARγ2 expression vector(s) (PPARγ2/pCMV-SPORT6 from MGC Gene Collection [7] or pSV-SPORT PPARγ and pSV-SPORT PPARγS112A [38], at 30 ng or 30 ng and 150 ng, respectively). For luciferase assays, cells were harvested 48 h after transfection. Relative luciferase activity was measured with a luciferase assay system (Promega). Values were normalized to *Renilla* luciferase values. Three technical replicates were performed from three separate experiments.

For short hairpin RNA (shRNA) knockdown experiments with U2OS cells, cells were transfected as follows: with shCdk7 (shRNA-K7/pENTR-H1; 3.5 μg) or with shControl (shControl/pENTR-H1; 3.5 μg) plus PPARγ2/pCMV (0.5 μg) plus pLL3,7-GFP (0.5 μg) by use of Fugene according to the manufacturer's instructions. Seventy-two hours after transfection, coverslips were fixed and stained as per the immunofluorescence protocol. Quantification of staining was performed with Image ProPlus 6.1 software, and 1,000 GFP-positive cells were counted using Zeiss Axioplan microscope and software. Sequences for shRNAs were as follows: for shCdk7, CCAATAGAGCTTATACAC; and for shControl, ATCCAAAAGCTGTCTTCGTTCTGCAG.

For small interfering RNA (siRNA) knockdown in MEFs, cells were transfected using siRNA targeting Cdk7 (siCdk7) or nonspecific siRNA (siControl) by use of Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Cdk7 siRNA oligonucleotides and nonspecific siRNA were synthesized by Dharmacon (Lafayette, CO) in a purified and annealed duplex form. The sequences are available upon request.

Retroviral transductions. Mat1 cDNA was cut from Mat1/pAHL with EcoRI and SalI and subcloned into the pBABEpuro vector. Amphotrophic retroviruses were produced in Phoenix 293T cells, and 1 ml of fresh viral medium/well (six-well plate) of MEFs plus 8 μg/ml Polybrene was used in spin transduction. Selection with puromycin (5 μg/μl) was started 48 h following transduction.

mRNA level assays using quantitative RT-PCR. RNA from MEFs was isolated following 72 h of AdCre (or control virus) infection using an RNEasy isolation kit (Qiagen) according to the manufacturer's protocol. double-stranded complementary DNA was amplified using power Sybr green PCR master mix (Applied Biosystems). Relative mRNA amounts from PPARγ, aP2, and adiponectin mRNAs were assayed by comparing PCR cycles to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) by use of a 7500 fast real-time PCR (RT-PCR) system software. Samples were normalized to the control genotype. Triplicate samples were run from three separate experiments. Primers used were as follows: for aP2, forward (5′-TCCTGTGCTGCAGCCTTTCTCA) and reverse (5′-CCAGGTTCCCACAAAGGCATCA); for GAPDH, forward (5′-AAGGTCGGAGTCAACGGATT) and reverse (5′-TTGATGACAAGCTTCCCGTT); for PPARγ2, forward (5′-TGACCCAGAGCATGGTGCCTTC) and reverse (5′TGTGGCATCCGCCCAAACC); and for adiponectin, forward (5′-GAAGATGACGTTACTACAAC) and reverse (5′-GCTTCTCCAGGCTCTCCTTT).

RESULTS

Cdk7 and Mat1 levels undetectable in WAT and downregulated during 3T3-L1 adipogenic differentiation. Studies to date have not supported the notion that the mammalian Cdk7 submodule would be regulated in cultured cells (8, 41, 69, 70) or in tissues (8, 21, 41, 44, 60, 63, 65). To assess the status of the Cdk7 submodule in adipose tissue, we initially used protocols set up during our previous studies (44, 63) to analyze Mat1

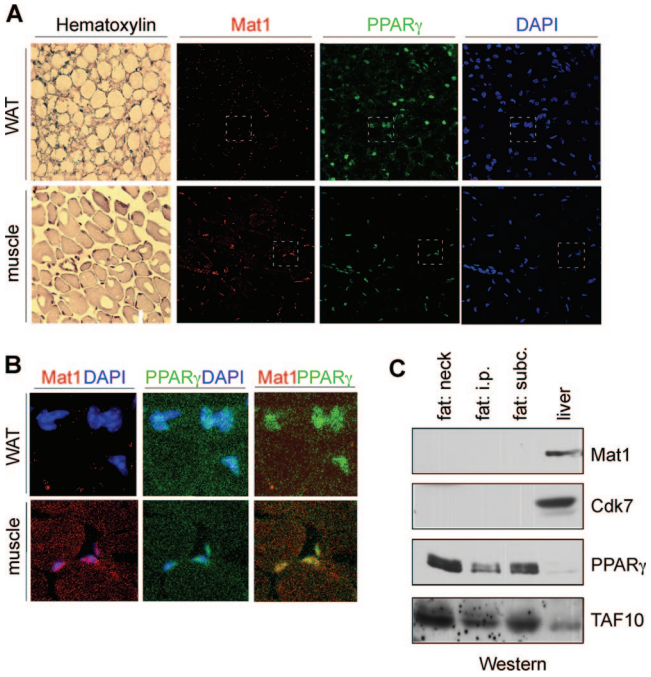

FIG. 1. Undetectable Mat1 and Cdk7 in terminally differentiated white adipocytes in vivo. (A) Micrographs from a single frozen section containing WAT and skeletal muscle from the neck of an adult mouse stained with hematoxylin and with Mat1, PPARγ, and DAPI; micrographs were made using confocal microscopy. Dashed lines indicate areas shown in detail in panel B. (B) To demonstrate the lack of specific nuclear Mat1 staining in WAT, higher magnifications of overlaid double stainings with indicated antibodies are shown, where the presence of Mat1 is visualized as pink and yellow (overlaid) color in muscle nuclei. (C) Western blotting of various fat depots (neck, intraperitoneal [i.p.], and subcutaneous [subc.]) showing adipocyte-specific downregulation of Mat1 and Cdk7. PPARγ is shown to identify fat tissue and TAF10 is shown for a nuclear loading control.

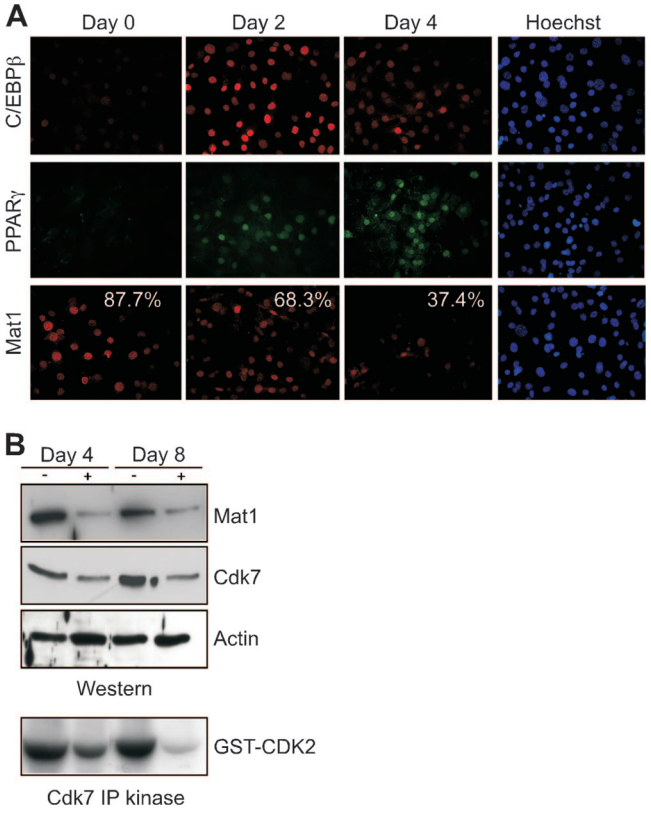

FIG. 2. Adipocyte differentiation involves the downregulation of Mat1 and Cdk7 levels. (A) Immunostaining of 3T3-L1 cells at days 0, 2, and 4 following induction of adipocyte differentiation with C/EBPβ, PPARγ, and Mat1 as indicated. Hoechst stain is shown for the day 4 samples. Fractions of Mat1-positive nuclei of Hoechst-positive nuclei are shown as percentages. (B) Western blotting analysis of Mat1, Cdk7, and actin levels on indicated days in control (−) or differentiation (+) medium. (Bottom) Kinase activity in Cdk7 immunoprecipitates (Cdk7 IP kinase) toward GST-Cdk2 from the same samples.

levels by confocal immunofluorescence from frozen murine white adipose tissue (WAT) sections (Fig. 1A and B). Unexpectedly, no Mat1 staining was detected in adipocyte nuclei, whereas PPARγ staining was robust (Fig. 1A and B, top). By contrast, Mat1 staining in striated muscle on the same section was readily detected and coincided with nuclear DAPI (4′,6′-diamidino-2-phenylindole) staining on the edges of myofibers (Fig. 1B, bottom), whereas PPARγ staining was lower than that in WAT.

To confirm the lack of Mat1 with another approach and to extend the analysis to Cdk7, levels of Mat1 and Cdk7 were analyzed by Western blotting from rat adipose tissue lysates isolated from neck, intraperitoneal, and subcutaneous fat depots. All WAT sources contained high levels of PPARγ as well as the TAF10 (52) TFIID subunit (Fig. 1C). Consistent with the immunofluorescence analysis, no Mat1 or Cdk7 could be detected in any of the fat samples, although they were readily detectable in control liver samples (Fig. 1C). The lack of detectable amounts of these two TFIIH subunits was highly unexpected, considering, e.g., the active mRNA transcription in adipocytes (32, 35). The results also establish adipocytes as the first cell type identified to lack detectable levels of these two TFIIH subunits.

To determine whether the lack of expression of Mat1 and Cdk7 noted for WAT would be recapitulated in the 3T3-L1 adipocyte differentiation model (33), Mat1 immunofluorescence staining was compared to what was seen for both PPARγ and C/EBPß during differentiation (Fig. 2A) As expected (13, 76), C/EBPß was transiently induced by day 2 and decreased by day 4. PPARγ was induced from day 0 to day 2 and remained high thereafter as noted before (14). Mat1 staining in turn was high in undifferentiated preadipocytes (87.7% Mat1-positive nuclei/Hoechst-positive nuclei) but decreased at day 2 (68.3% Mat1-positive nuclei/Hoechst-positive nuclei) and was almost undetectable at day 4 of differentiation (Fig. 2A). As with the adipose tissue, Western blotting analysis confirmed the decrease in Mat1 levels as well as in Cdk7 levels and activity during 3T3-L1 differentiation (Fig. 2B), indicating that the 3T3-L1 model was recapitulating events noted for WAT in vivo in this regard.

Activation of adipogenesis and adipogenic target genes in *Mat1*$^{-/-}$ MEF cultures. The dramatic loss of Mat1 and Cdk7 during adipocyte differentiation suggested that the presence of the Cdk7 kinase complex might be inhibitory for adipogenesis. To investigate this, we utilized MEFs with a floxed *Mat1* allele (63). When *Mat1* was depleted from immortalized *Mat1*$^{-/flox}$

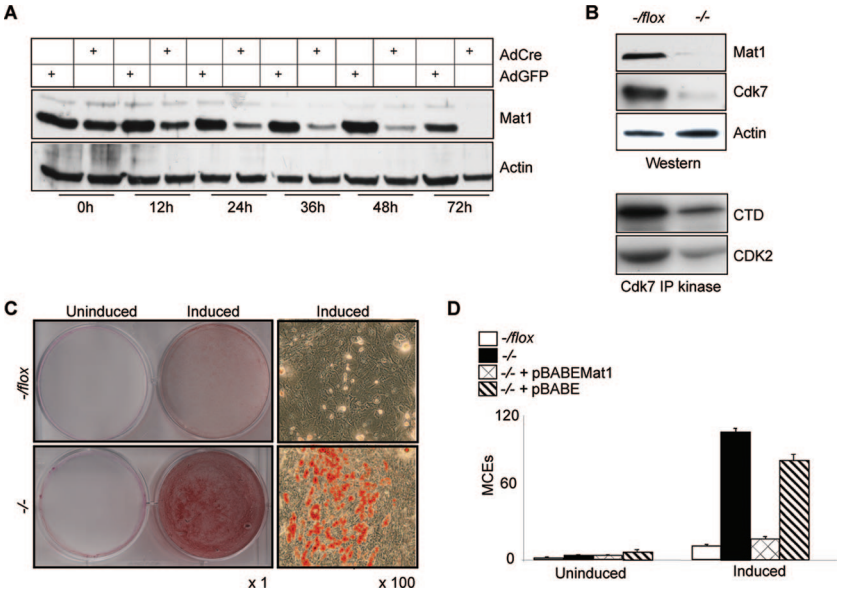

FIG. 3. *Mat1*$^{-/-}$ MEFs show increased capacity for adipocyte differentiation. (A) Western blotting of *Mat1*$^{-/flox}$ MEFs after the indicated number of hours following AdCre or AdGFP infection with indicated antibodies, demonstrating efficient depletion of Mat1 in AdCre-infected MEFs (*Mat1*$^{-/-}$) compared to AdGFP MEFs (*Mat1*$^{-/flox}$). (B) Mat1, Cdk7, and actin levels (Western) and kinase activity in Cdk7 immunoprecipitates (Cdk7 IP kinase) toward GST-CTD and GST-Cdk2 substrates in *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs. (C) Macroscopic (magnification, ×1) and microscopic (magnification, ×100) images of Oil Red O-stained of *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEF cultures following the indicated treatments. Stained areas represent triglyceride accumulation in MCEs in the induced *Mat1*$^{-/-}$ MEF culture. (D) Quantification of adipocyte differentiation as MCE number/10-cm plate following indicated treatments of *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs as well as *Mat1*$^{-/-}$ MEFs transduced with a control (pBABE) or Mat1-expressing (pBABE-Mat1) retrovirus, demonstrating rescue of the adipogenic phenotype. Error bars represent standard deviations from two independent experiments.

MEFs (Fig. 3A) with the use of AdCre, Mat1 protein was lost within 72 h following infection in contrast to control (AdGFP)-infected *Mat1*$^{-/flox}$ MEFs (Fig. 3A). The depletion of Mat1 resulted in a corresponding decrease in levels and activity of Cdk7 (Fig. 3B), demonstrating that Mat1 is required for a functional Cdk7 complex and supporting a role for Mat1 as a stabilizing factor of this complex, as suggested for sciatic nerves in vivo (44).

To study the effects of the depletion of the Cdk7 complex on adipogenesis, the abilities of *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs to undergo adipogenic differentiation in vitro were compared. In contrast to 3T3-L1 cells, immortalized MEFs generally differentiate poorly into adipocytes when grown in adipocyte differentiation medium, but occasional differentiating cells (0 to 2%) form mitotic clonal expansions (MCEs) and subsequently adipocytes (61). Consistent with this, only 7.33 ± 2.5 MCEs per 10-cm plate were detected with Oil Red O staining in control *Mat1*$^{-/flox}$ cultures. Strikingly, 115 ± 8 MCEs were found in *Mat1*$^{-/-}$ MEFs (Fig. 3C and D), demonstrating a 15-fold increase in adipogenesis. A similar increase in adipogenesis was observed for *Mat1*$^{-/-}$ primary MEFs (see Fig. S1 in the supplemental material). The average sizes (4 to 20 cells/MCE) and morphologies of MCEs were indistinguishable, suggesting that the lack of *Mat1* does not affect the proliferative response involved in MCE. This did not support the notion that Mat1 would be required for the activation of cell cycle cyclin-dependent kinases, and indeed, enhanced adipogenic properties in *Mat1*$^{-/-}$ MEFs were not associated with noticeable changes in MEF proliferation or Cdc2/Cdk2 T-loop phosphorylation acutely following *Mat1* deletion at 72 h (see Fig. S2A and S2B in the supplemental material). This suggests that another kinase is phosphorylating Cdk2 and Cdc2 in *Mat1*$^{-/-}$ MEFs, indicating these cells are a useful model to analyze cell cycle-independent functions of Mat1 and Cdk7.

The increased adipogenic response suggested that the loss of Mat1 may be required for cells to progress into adipogenesis and that Mat1 may therefore act as a block to this differentiation process. In order to confirm this, Mat1 was reintroduced using retroviral vectors after which MEFs were again induced for adipocyte differentiation. Indeed, the expression of Mat1 in MEFs resulted in a decrease in adipogenesis, demonstrating that Mat1 is able to inhibit adipocyte differentiation (Fig. 3D).

To determine whether the increased adipogenicity of *Mat1*$^{-/-}$ MEFs involved PPARγ activation, *Mat1*$^{-/-}$ and *Mat1*$^{-/flox}$ MEFs were transfected with a PPARγ-responsive reporter construct ($PPRE_3$-TK-Luc [2]) together with a PPARγ expression plasmid. Interestingly, *Mat1*$^{-/-}$ MEFs showed a sixfold increase in relative $PPRE_3$-TK-Luc reporter activity (Fig. 4A), suggesting that the loss of Mat1 relieves the inhibition of PPARγ-mediated transcription. The induction of PPARγ activity was directly attributable to Mat1 loss, as the reintroduction of Mat1 abolished induction in *Mat1*$^{-/-}$ MEFs (Fig. 4A), confirming that Mat1 acts as an inhibitor of PPARγ-mediated transcription.

The efficiency of *Mat1*$^{-/-}$ MEFs to differentiate into adipocytes without the expression of exogenous PPARγ (Fig. 3), and the increased activation of PPARγ in *Mat1*$^{-/-}$ MEFs (Fig. 4A), suggested a sensitization of the PPARγ pathway following Mat1 depletion. To investigate this possibility, *Mat1*$^{-/-}$ and *Mat1*$^{-/flox}$ MEFs were analyzed for the expression of adipo-

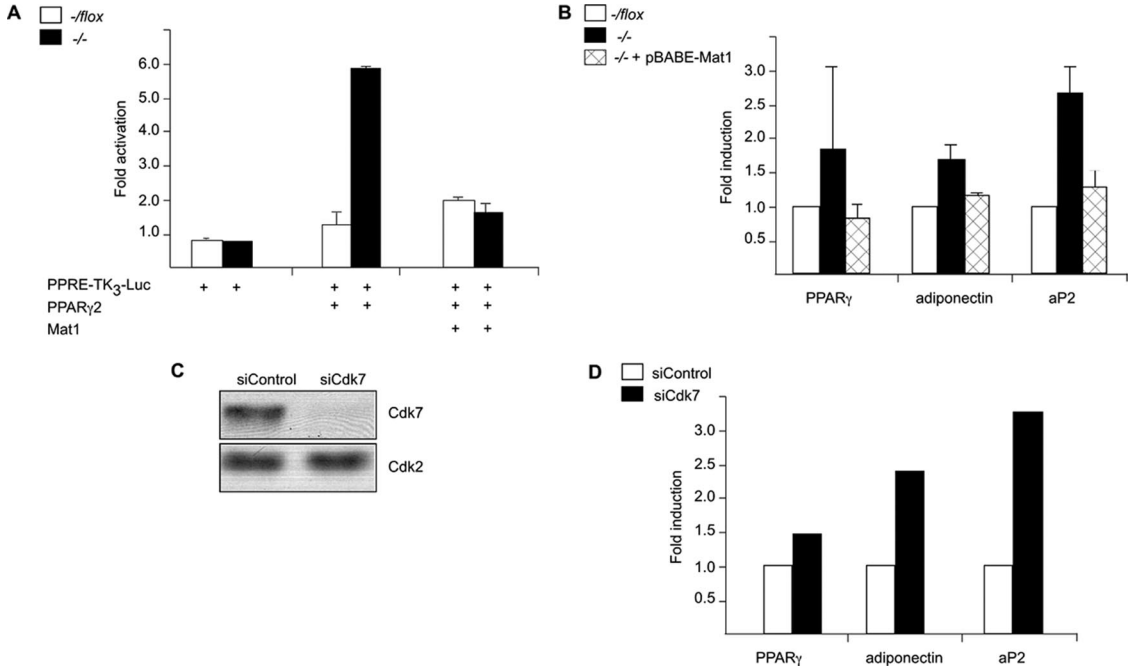

FIG. 4. Acquired PPARγ responsiveness and induction of adipogenic mRNAs in *Mat1*$^{-/-}$ and Cdk7 knockdown MEFs. (A) PPAR reporter (PPRE$_3$-TK-Luc) activity in *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs transfected as indicated with PPARγ2 and Mat1; sample values are compared to those for non-PPARγ2-transfected samples. (B) Relative mRNA levels of the adipogenic PPARγ (1.85-fold), adiponectin (1.70-fold), and aP2 (2.70-fold) genes in *Mat1*$^{-/-}$ MEFs compared to values for *Mat1*$^{-/flox}$ MEFs. Hatched bars show mRNA levels in *Mat1*$^{-/-}$ MEFs where Mat1 was reintroduced by retroviral transduction (pBABE-Mat1). Values for both panels A and B represent averages from three independent experiments, with standard deviations shown by error bars. (C) Western blotting of Cdk7 and Cdk2 (loading control) levels in MEFs transfected with control (siControl)- or Cdk7 (siCdk7)-targeting siRNAs as indicated, demonstrating efficient knockdown of Cdk7 in siCdk7-treated MEFs. (D) Relative mRNA levels of the indicated adipogenic PPARγ (1.47-fold), adiponectin (2.37-fold), and aP2 (3.23-fold) genes in MEFs transfected with siCdk7 compared to siControl-transfected MEFs from a representative experiment.

genic PPARγ target genes, which are normally expressed at very low levels in MEFs (31). Interestingly, quantitative RT-PCR analyses of mRNA levels of three well-characterized PPARγ target genes (the PPARγ, aP2, and adiponectin genes) demonstrated significant inductions in *Mat1*$^{-/-}$ MEFs, whereas no induction was noted for *Mat1*$^{-/flox}$ MEFs. (Fig. 4B) or for control (GAPDH) mRNA levels in either genotype. Treatment with troglitazone, a potent PPARγ agonist, caused a subtle further increase in these genes in *Mat1*$^{-/-}$ MEFs, which was not noticeable in *Mat1*$^{-/flox}$ MEFs (data not shown). Again, Mat1 reintroduction with retroviral vectors was able to restore the expression of these adipogenic genes to the baseline level seen for wild-type MEFs (Fig. 4B). While no Cdk7-independent functions of Mat1 have been identified, it was formally possible that the observed concomitant activations of PPARγ responses and Cdk7 loss were independent phenotypes of *Mat1*$^{-/-}$ MEFs. Therefore, to more directly establish the role of Cdk7 in this adipogenic response, Cdk7 levels were downregulated using siCdk7 (Fig. 4C). Quantitative RT-PCR analyses of the adipogenic genes demonstrated an induction in siCdk7-treated MEFs (Fig. 4D) similar to that seen for *Mat1*$^{-/-}$ MEFs, suggesting that the effects of Mat1 on adipogenesis are transmitted through the Cdk7 kinase. The results demonstrate that the loss of Mat1 and the subsequent Cdk7 activity leads to enhanced PPARγ activity and thus to increased adipocyte differentiation.

Mat1 and Cdk7 are required for the inhibitory phosphorylation of PPARγ at serine 112. The increased adipogenic capacity of *Mat1*$^{-/-}$ MEFs is shared by MEFs in which endogenous PPARγ is replaced with nonphosphorylatable PPARγ-S112A (57). As PPARγ-S112 can be phosphorylated by Cdk7 in vitro (20), it was of interest to analyze whether PPARγ-S112 phosphorylation was defective in *Mat1*$^{-/-}$ MEFs. For this, PPARγ2-transfected *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs were immunostained for total PPARγ and PPARγ phosphorylated on S112 (P∼PPARγ-S112). Whereas *Mat1*$^{-/flox}$ and *Mat1*$^{-/-}$ MEFs demonstrated comparable levels of total PPARγ (Fig. 5A), the levels of P∼PPARγ-S112 were dramatically lower in *Mat1*$^{-/-}$ MEFs (Fig. 5A), indicating that Mat1 is required for the inhibitory phosphorylation on PPARγ-S112.

To assess the role of Cdk7 directly in the phosphorylation of PPARγ-S112, Cdk7 levels were downregulated through introduction of a plasmid expressing shRNA targeting Cdk7 (shCdk7) in U2OS osteosarcoma cells together with a GFP-expressing reporter. As expected, GFP-positive transfected cells demonstrated efficient knockdown of endogenous Cdk7 by immunofluorescence analysis (green cells in Fig. 5B, left [shCdk7]): Cdk7 staining was detectable for only 3.4% of GFP-positive cells compared to 100% of GFP-negative cells. Similarly, Cdk7 staining was detected in all shControl-transfected GFP-positive cells (green cells in Fig. 5B, left [shControl]). Subsequently, S112 phosphorylation was compared in shCdk7-

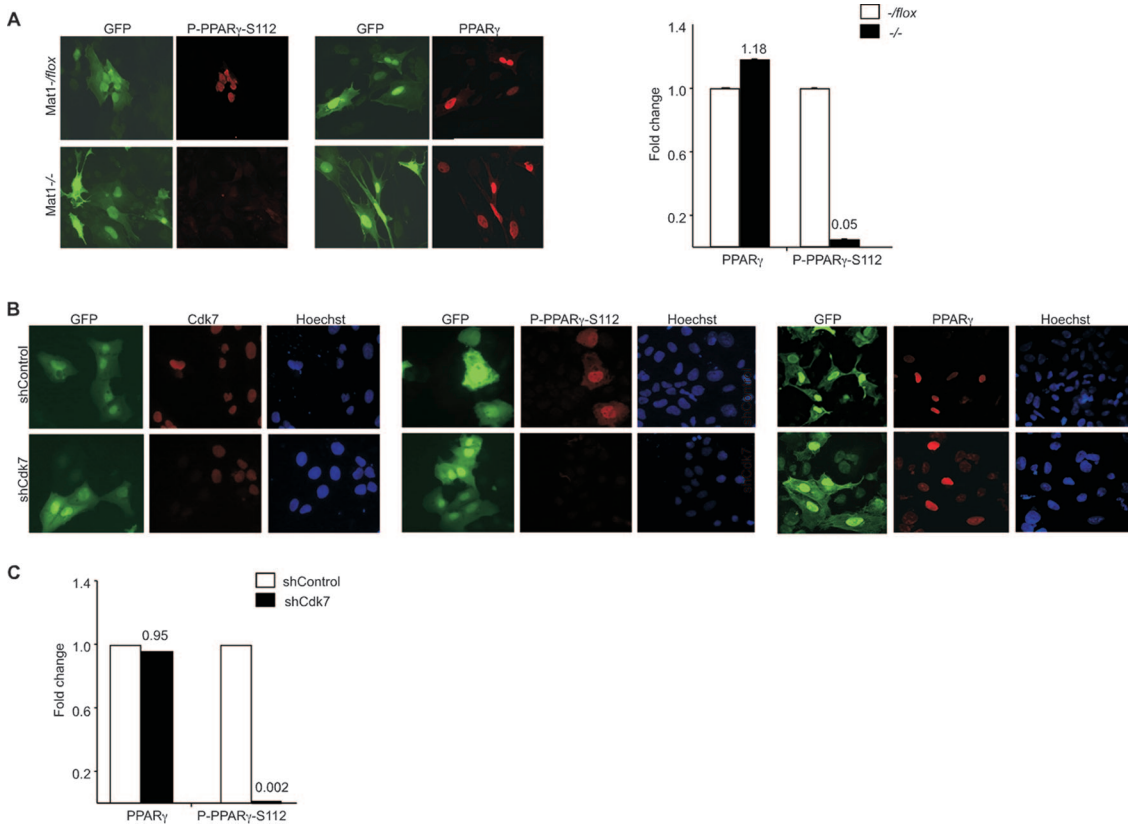

FIG. 5. Mat1 and Cdk7 are required for the inhibitory phosphorylation of PPARγ at serine 112. (A) Immunofluorescence staining and quantitation (bars) of total PPARγ and P~PPARγ-S112 for *Mat1*$^{-/-}$ MEFs and *Mat1*$^{-/flox}$ MEFs transfected with PPARγ2. Bars indicate changes for either total PPARγ- or P~PPARγ-S112-positive nuclei in *Mat1*$^{-/-}$ MEFs compared to *Mat1*$^{-/flox}$ MEFs. (B) GFP, Cdk7, P~PPARγ-S112, and total PPARγ immunofluorescence analysis of U2OS cells transfected with knockdown plasmids encoding a scrambled shRNA (shControl) or a Cdk7 shRNA (shCdk7) together with PPARγ2 and GFP expression plasmids. Nuclei were detected with Hoechst staining. Note the loss of signal for both endogenous Cdk7 signal (left) and P~PPARγ-S112 (middle) in shCDK7-transfected cells detected by GFP. (C) Quantitation of total PPARγ and P~PPARγ-S112 from the experiment described for panel B and analyzed as for panel A.

versus shControl-transfected cells also cotransfected with PPARγ (Fig. 5B, middle, [P~PPARγ-S112] and C) and GFP. Whereas PPARγ-S112 phosphorylation was readily detectable for 54% of shControl-transfected cells, only 0.22% P~PPARγ-S112-positive cells could be identified among shCdk7-transfected cells (Fig. 5B and C). The levels of total PPARγ were comparable in shControl- and shCdk7-transfected cells (Fig. 5B, right, and C). This result demonstrates the requirement for the Cdk7 kinase for the phosphorylation of PPARγ-S112 in cultured cells and provides evidence indicating that the activation of PPARγ and the increased adipogenic potential of *Mat1*$^{-/-}$ MEFs are due to attenuated Cdk7 kinase activity leading to the decreased phosphorylation of PPARγ-S112.

In order to directly assess the role of PPARγ-S112 phosphorylation in the acquired responsiveness to PPARγ in Cdk7 knockdown cells, MEFs were transfected with control and Cdk7 siRNAs together with the PPARγ reporter ($PPRE_3$-TK-Luc) and either wild-type PPARγ or a nonphosphorylatable mutant, PPARγ-S112A. As in *Mat1*$^{-/-}$ MEFs, Cdk7 knockdown resulted in a significant increase in PPARγ activity compared to what was seen for siControl-transfected MEFs (Fig.

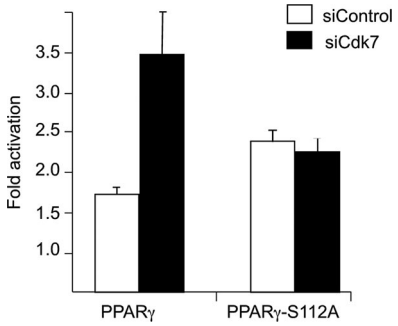

FIG. 6. Acquired responsiveness to PPARγ is dependent on PPARγ-S112 in Cdk7 knockdown MEFs. PPAR reporter ($PPRE_3$-TK-Luc) activity in MEFs transfected as indicated with control (siControl) or Cdk7 (siCdk7) siRNAs and either PPARγ or P~PPARγ-S112A, demonstrating that in contrast to wild-type PPARγ, the nonphospory-latable mutant displays similar activities in siCdk7 and siControl MEFs. Values represent averages from three technical replicates from two independent experiments, with standard deviations shown by error bars.

6). However, this difference was not noted when instead of wild-type PPARγ the nonphosphorylatable mutant PPARγ-S112A was used (Fig. 6), demonstrating that the acquired response in Cdk7-depleted cells is dependent on PPARγ-S112.

DISCUSSION

The results of this study implicate the Cdk7 submodule of TFIIH in inhibition of adipogenesis and provide evidence indicating that the mechanism of this inhibition involves Cdk7-mediated phosphorylation and inhibition of PPARγ. The results therefore contribute to the understanding of adipocyte biology and PPARγ regulation during adipogenesis and link regulation of the basal transcription machinery to differentiation in vivo.

The observation that *Mat1*$^{-/-}$ MEFs demonstrated increased activation of the PPARγ adipogenic pathway and that this activation was blocked by reexpression of Mat1 identified Mat1 as a negative regulator of PPARγ-mediated transcription. Regarding this, it is interesting to note the recent study on cardiac-specific ablation of Mat1, where Mat1 was proposed to function as an activator of the coactivator PGC-1 (65). While PGC-1 is involved in PPARγ-mediated transcription in some systems, it is not a plausible candidate to be involved in PPARγ regulation in *Mat1*$^{-/-}$ MEFs both because PPARγ-mediated transcription is activated—not repressed—in *Mat1*$^{-/-}$ MEFs and because neither PGC-1-mediated effects nor expression are detected in MEFs (65). A more direct role for Cdk7 in PPARγ regulation has been suggested by the observation that Cdk7 can phosphorylate PPARγ-S112 in vitro (20). This mechanism was more attractive, as phosphorylation of S112 represses PPARγ activity (2, 12, 38, 67), and MEFs from PPARγ-S112A mice demonstrate an increase in adipogenic potential similar to that seen for *Mat1*$^{-/-}$ MEFs (57). Consistent with this, results here from both Mat1-deficient fibroblasts and osteosarcoma cells following Cdk7 knockdown demonstrated that Mat1 and Cdk7 are required for PPARγ-S112 phosphorylation. A tentative link between Cdk7 and PPARγ has been provided previously for XPD mutant mice, which show deregulation of several PPARγ target genes (20). However, the results are complicated by the fact that in vivo the XPD mutant may deregulate Cdk7 and therefore PPARγ in a number of tissues, leading not only to primary changes but also to secondary responses in various tissues. The differences between these results and those present here may be due to, e.g., complexities of in vivo responses or to other proposed functions of XPD (16). The results presented here provide evidence that Cdk7 acts as an inhibitor of adipogenesis and that increased adipogenic potential following the loss of Mat1 and concomitantly Cdk7 activity are due to decreased PPARγ-S112 phosphorylation and the subsequent activation of the adipogenic PPARγ.

While the significance of PPARγ-S112 phosphorylation as a repressor of adipogenesis has been clearly demonstrated both in vitro (38, 67) and in vivo (57), it is not clear at what stage of adipogenesis it is important. The critical PPARγ inhibition through S112 phosphorylation is expected to be spatially and temporally restricted, as analysis of total cellular PPARγ by Western blotting has not demonstrated alterations in S112 phosphorylation during adipogenesis (58) or in adipose tissue (20). Inhibition of PPARγ activity by extracellular signal-regulated kinase 1/2-mediated PPARγ-S112 phosphorylation (2, 12, 38, 67) would be expected to be limited to the first hour of adipogenic differentiation based on lack of detectable extracellular signal-regulated kinase 1/2 activity thereafter (55). Inhibition of PPARγ activity by Cdk7-mediated PPARγ-S112 phosphorylation could occur either in preadipocytes (where PPARγ is expressed at low levels [55]) or more likely at early stages of adipogenesis during PPARγ induction prior to Cdk7 loss (Fig. 2). As a subunit of the basal TFIIH, the Cdk7 submodule is optimally poised to phosphorylate PPARγ on critical target gene promoters. For further characterization of the critical time and target genes of S112 phosphorylation-mediated inhibition of PPARγ activity, it would be useful to analyze PPARγ-S112~P distribution on PPREs of target genes during adipogenesis. The notion that PPARγ-S112 phosphorylation and subsequently PPARγ activity shows target gene specificity is supported by the phenotype of the PPARγ-S112A mouse (57), demonstrating similarities (increased insulin sensitivity) and differences (no weight gain) to what is seen for activation of PPARγ with TZD agonists. This specificity is also interesting from a therapeutic point of view, as it has been suggested that modulation of S112 phosphorylation might provide an improved alternative treatment modality to the currently widely used activation of PPARγ with TZDs (19, 57).

Investigation of adipocyte differentiation has been significantly strengthened by the widely used preadipocyte lines NIH 3T3-L1 and NIH 3T3-F442A (33, 34). More recently, important new tools have been provided through gene knockout MEFs, most of which result in reduced adipogenicity, often detected only after the overexpression of PPARγ (reviewed in reference 26). The identified negative regulators of adipogenesis whose deletion leads to enhanced adipogenicity similar to what is seen for Mat1 are GATA3 (71), p107/p130 (18), and E2F4 (24). Based on current understanding, the mechanisms involved appear to be less directly related to PPARγ transcriptional activation (reviewed in reference 26), and therefore *Mat1*$^{-/-}$ MEFs may provide a useful tool for the characterization of critical regulatory events during the transcriptional program of adipogenesis and cell lineage determination.

The implications of this study lie not only in characterizing mechanisms of adipocyte differentiation but additionally in providing insight into complexities of physiological roles of basal transcription factors. Based on several genetic models (47, 73–75) and expression studies (8, 11, 21, 41, 60, 69, 70), Cdk7 kinase activity has been considered as ubiquitous and essential. In this study, we found that both Mat1 and Cdk7 levels were downregulated in adipose tissue, reminiscent of what was seen for some TFIID subunits in other physiological settings (22, 25, 30). It will be of interest to characterize the mechanisms involved in the regulation of Mat1 and Cdk7 levels in more detail; based on observations of a C-terminally truncated Mat1 in ATRA-treated HL60 cells (36), it is plausible that the observed decrease in Mat1 is due to ubiquitin-mediated proteolysis leading to destabilization of Cdk7, consistent with results here and previously noted for sciatic nerves in vivo (44).

The loss of the Mat1 and Cdk7 subunits of TFIIH in adipose tissue suggests that TFIIH can function in tissue- and differentiation-specific forms. Recent studies of the *Drosophila* em-

bryo demonstrate that the Cdk7 kinase complex and core TFIIH are localized to different cellular compartments (16) and transcriptional loci during development (3). Cdk7 kinase activity has also been shown to be dispensable for the nucleotide excision repair function of TFIIH (6, 53, 68) as well as for the recently identified TFIIH coactivator function (21). The lack of detectable Mat1 or Cdk7 in adipocytes suggests that the apparently active mRNA transcription in adipocytes (32, 35) is independent of Mat1 and Cdk7 TFIIH subunits. From these recent findings on TFIIH and TFIID, it is beginning to emerge that differentiated cells may in fact contain very diverse compilations of the core transcription machinery.

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