The subunit assembly state of the Mediator complex is nutrient-regulated and is dysregulated in a genetic model of insulin resistance and obesity

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The Mediator complex plays a critical role in the regulation of transcription by linking transcription factors to RNA polymerase II. By examining mouse livers, we have found that in the fasted state, the Mediator complex exists primarily as an approximately 1.2-MDa complex, consistent with the size of the large Mediator complex, whereas following feeding, it converts to an approximately 600-kDa complex, consistent with the size of the core Mediator complex. This dynamic change is due to the dissociation and degradation of the kinase module that includes the MED13, MED12, cyclin-dependent kinase 8 (CDK8), and cyclin C (CCNC) subunits. The dissociation and degradation of the kinase module are dependent upon nutrient activation of mTORC1 that is necessary for the induction of lipogenic gene expression because pharmacological or genetic inhibition of mTORC1 in the fed state restores the kinase module. The degradation but not dissociation of the kinase module depends upon the E3 ligase, SCF<sup>FBW7</sup>. In addition, genetically insulin-resistant and obese db/db mice in the fasted state displayed elevated lipogenic gene expression and loss of the kinase module that was reversed following mTORC1 inhibition. These data demonstrate that the assembly state of the Mediator complex undergoes physiologic regulation during normal cycles of fasting and feeding in the mouse liver. Furthermore, the assembly state of the Mediator complex is dysregulated in states of obesity and insulin resistance.

Gene transcription in eukaryotes is orchestrated through complex multistep processes that include chromatin reorganization coupled with transcription initiation, elongation, and termination, with the initiation being the most studied regulatory step in this process (1, 2). In the case of protein-encoding mRNA, the RNA polymerase II (Pol II)<sup>3</sup> and the general transcription factors, such as TFIIA, TFIID, TFIIE, TFIIF, and TFIIH, constitute the basal transcription machinery (3, 4). The regulation of gene activation or repression engages specific sets of DNA-binding transcription factors. However, it is generally believed that the majority of transcription factors in eukaryotic cells do not directly interact with Pol II but instead engage a series of transcription cofactors, particularly the Mediator complex that interacts with multiple transcription factors and Pol II to integrate the transcriptional signals to the basal transcriptional machinery (5–7).

Biochemical isolation studies of the Mediator complex from cultured cancer cells have suggested the presence of at least two forms: the small or core Mediator complex composed of ~26 subunits with a size of ~600 kDa and the large Mediator complex that also contains the kinase module subunits (MED13, MED12, CDK8, and CCNC) but lacks the MED26 subunit with a size of ~1.2 MDa (8–11). Although some reports have suggested that the presence of multiple forms of the Mediator complex may be generated during the biochemical purification or due to the use of different cell lines (12, 13), other studies have reported that the small Mediator complex functions to activate transcription initiation, whereas the large Mediator complex is inactive or acts as a transcription repressor (14). In addition, the large Mediator complex has been linked to activation of transcription elongation (15), and the kinase module was found to function in a context-specific manner to either repress or activate transcription, depending on cell context, transcription factors, and/or target gene promoters (16–18).

Recently, we reported that CDK8 and CCNC play an important role in the control of lipogenic gene expression through a nutrient-stimulated down-regulation in the liver (19). However, it is unclear whether the subunit assembly state of the whole Mediator complex is regulated under physiological or pathophysiological states.

In this study, we now demonstrate that the subunit assembly state of the Mediator complex in mouse liver undergoes dynamic normal physiologic regulation through a mTORC1-dependent down-regulation of the entire kinase module to generate the small Mediator complex. In addition, the degradation of the kinase module, but not its dissociation from the large Mediator complex, depends upon the E3 ligase SCF<sup>FBW7</sup>.
Results

The kinase module is dissociated from the large Mediator complex and degraded upon nutrient signals

We have previously shown that the degradation of two subunits in the kinase module, CDK8, and its binding partner, CCNC, is triggered by nutrient signals in the liver (19). To determine whether other subunits of the Mediator complex are also regulated by nutrient availability, we compared the protein levels of additional subunits of the Mediator complex in the livers between mice that were fasted overnight and those that were fed for 4 h following the overnight fast. As observed previously, equal amounts of liver nuclear extracts (normalized for the nuclear TATA-binding protein (TBP)) displayed reduced levels of CDK8 and CCNC in the fed state (Fig. 1A). Similarly, there was also a reduction in MED13 and MED12 proteins with little effect on the levels of the Mediator subunit MED1. Immunoprecipitation with an antibody against MED1, which is present in both the small and large Mediator complexes (8–11), demonstrated a reduction in all four kinase module subunits that are associated with MED1 in the fed state (Fig. 1A), suggesting a shift from the large Mediator complex to the small Mediator complex upon feeding. To confirm the specificity of kinase module subunit antibodies, here we show the immunoblots of liver-specific Ccnc knockout mice that display the expected decrease in CDK8 and CCNC protein without significant effect on MED13 or MED12 protein levels (Fig. S1A). In contrast, liver-specific knockout of Med13 knockout results in decreased protein levels of all four kinase module proteins MED13, MED12, CCNC, and CDK8 (Fig. S1B).

The reduction in the protein levels of the kinase module subunits occurred without any significant change in the amounts of mRNAs encoding for the Mediator subunits (Fig. S2). To further confirm this apparent change in the Mediator subunit assembly state, we examined the size distribution of the Mediator complex by glycerol gradient fractionation. Based on the molecular size standards, the small Mediator complex (~600 kDa) should be in fractions 12–15, whereas the large Mediator complex (~1.2 MDa) should be in fractions 16–19 (Fig. 1B). In fasted state, it was apparent that all of the subunits were in the large Mediator form, as both the core subunit (MED25) and kinase module subunits were enriched in the later fractions that match with the size of the large Mediator complex (~1.2 MDa) (Fig. 1B). In contrast, the enrichment of all subunits was shifted to the left with the major distribution located in fractions 12–15 in the fed livers to the size close to the small Mediator complex or the kinase module alone (Fig. 1B). It is also important to note that the glycerol gradient fractionation analyses do not reflect the relative amounts of proteins present between fasting and feeding, but only the relative sizes of protein complexes that they are associated with, as greater amounts of nuclear extracts from fed livers were used, and the gel exposures were not identical in order to observe the less abundant kinase module subunits.

The nutrient-sensitive mTORC1 signaling pathway is responsible for the degradation and dissociation of the kinase module

To explore the role of mTORC1 signaling in the regulation of protein levels of the kinase module subunits, we first took a pharmacological approach by using the mTORC1-specific inhibitor rapamycin. Mice were fasted, fed, or treated with rapamycin before induction of the fed state. As observed by others, rapamycin effectively blocked the feeding-induced activation of mTORC1 kinase activity, as demonstrated by the inhibition of S6K1 and S6 phosphorylation (Fig. 2A). In addition, rapamycin blocked the induction of lipogenic gene expression in the fed state but has no significant effect on gluconeo-
genic gene expression levels (Fig. S3, A and B). In concert, the reduction in the kinase module subunits MED12, MED13, CDK8, and CCNC in the fed state was prevented when the mice were pretreated with rapamycin (Fig. 2A). To determine whether mTORC1 inhibition also prevents the feeding-induced formation of the small Mediator complex, we analyzed the liver nuclear extracts by glycerol gradient fractionation. Consistent with the data in Fig. 1, in the fasted state, the Mediator complex primarily resolved as the large Mediator complex (centered around ~1.2 MDa), whereas in the fed state, it shifted to a smaller size consistent with the small Mediator complex (Fig. 2B). However, feeding of the rapamycin-treated mice prevented the formation of the small Mediator complex, as the Mediator complex mostly fractionated in the position of the large Mediator complex (Fig. 2B). The specificity of rapamycin for mTORC1 was verified by examining the phosphorylation of AKT, in which both Ser-473 and Thr-450 are thought to be mTORC2 target sites (20, 21). As expected, the phosphorylation of Thr-450 was unaffected by rapamycin, and both Ser-473 and Thr-308 were found to be further increased in the fed state following rapamycin pretreatment (Fig. 2C). These data confirm the specificity of rapamycin for mTORC1 and are also consistent with mTORC1 activation acting in a feedback pathway to reduce AKT activation (22).

To further examine the role of mTORC1 in the regulation of the Mediator complex, we generated hepatocyte-specific Raptor knockout mice via injecting AAV8-Tbg-Cre in the tail vein of Raptor<sup>fl/fl</sup> mice. RAPTOR is a critical component of mTORC1, and knockout of Raptor completely abolishes the mTORC1 pathway but not mTORC2 (23, 24). These liver-specific Raptor knockout mice displayed a marked reduction of the S6K1 phosphorylation consistent with reduced mTORC1 activation along with prevention of the loss of the kinase module proteins in the fed state (Fig. 3A). Although Raptor deficiency did not affect the suppression of gluconeogenic genes in the fed state (Fig. 3B), there was a substantial reduction of lipogenic gene expression (Fig. 3C). In parallel, Raptor deficiency also blocked the conversion of the large Mediator complex to the small Mediator complex in the fed state (Fig. 3D).

The effect of loss of function of mTORC1 activity by rapamycin and Raptor deficiency was further confirmed by analyses of mTORC1 gain of function using liver-specific Tsc1 knockout mice, in which the mTORC1 pathway is constitutively active. The constitutive activation of mTORC1 was confirmed by the increased levels of S6K1 phosphorylation in both the fasted and fed state in the Tsc1 knockout liver hepatocytes. As observed previously, control mice in the fed state had reduced MED13, MED12, CDK8, and CCNC protein levels compared with those in the fasted state (Fig. S4). In contrast, following Tsc1 deletion, the levels of these proteins were relatively low in the fasted state and refractory to any further change following feeding (Fig. S4).
Degradation and dissociation of the kinase module subunits are increased in insulin-resistant and obese mice

Previously, we reported that the livers of the insulin-resistant and obese db/db mice display elevated mTORC1 activation in the fasted state, and this was confirmed in Fig. 4A. Consistent with this fasted state activation of mTORC1, the Mediator kinase module subunits were reduced compared with the fasted control mice. Importantly, treatment with rapamycin to suppress mTORC1 activation resulted in a concomitant rescue of the kinase module proteins (Fig. 4A), suggesting that mTORC1 is the major driver for the loss of the kinase module. As expected, rapamycin treatment of the db/db mice also suppressed the abnormal elevation of lipogenic gene expression in the fasted state (Fig. 4B) with little effect on the key gluconeogenic gene Pck1 (Fig. 4C) but with a small reduction of G6pc mRNA by ~50%. In any case, glyceral gradient fractionation demonstrated a large shift to smaller sizes of the Mediator complex in the fasted db/db mice compared with control mice, and the complex was mostly reversed back to the large Mediator complex following rapamycin treatment (Fig. 4D).

Dissociation of the kinase module from the large Mediator complex is not dependent on protein degradation

Next, we examined whether blocking the degradation of the kinase module is sufficient to prevent the feeding-induced formation of the small Mediator complex. In vitro studies have suggested that the E3 ubiquitin ligase component FBW7 can induce the degradation of MED13 (25), which links the kinase module to the core Mediator complex (14, 26, 27). We therefore tested whether FBW7 is necessary for the degradation and/or dissociation of the kinase module in vivo using acute hepatocyte-specific Fbw7 knockout mice generated by tail vein injection of AAV–Tdg-Cre into Fbw7fl/fl mice. Control mice in the fed state displayed the typical decrease in the Mediator kinase module subunits that was prevented in the hepatocyte-specific Fbw7-deficient mice (Fig. 5A). Consistent with functioning downstream of mTORC1, Fbw7 deficiency had no significant effect on the activation of mTORC1 kinase activity in the fed state (Fig. 5B). Interestingly, despite the restoration of the kinase module proteins in the Fbw7 knockout livers, the Mediator complex is still converted to the small Mediator and the kinase module complexes following feeding (Fig. 5D), indicating that stabilizing the kinase module subunits is not sufficient to block the feeding-induced dissociation of this module from the large Mediator complex. In parallel, both the control and Fbw7 knockout livers displayed elevated gluconeogenic genes in the fasted state that was suppressed in the fed state, whereas the lipogenic genes were increased in the fed state and suppressed in the fasted state (Fig. 5, C and D). Although the relative changes in Pck1 and Fasn were not significantly different between control and Fbw7 knockout livers, we have consistently observed that the fasting-induced expression of G6pc was reduced, whereas the feeding-induced expression of Acly, Acaca, and Scd1 were enhanced in the liver-specific Fbw7 knockout mice.

Figure 3. Inhibition of mTORC1 pathway protects kinase module from degradation and dissociation. Twelve-week-old Raptorfl/fl mice were tail vein–injected with either AAV8-Tdg-eGFP (Control) or AAV8-Tdg-Cre (Raptor knockout) and fed ad libitum for 10 days. The control and Raptor knockout mice were subjected to the fasting and feeding protocol described under “Experimental procedures” (n = 6 per group). A, Western blot analysis of the Mediator subunits and cytosolic kinases from two independent isolated liver nuclear extracts are shown. B and C, real-time quantitative RT-PCR (qRT-PCR) of genes involved in gluconeogenesis (Pck1 and G6pc) and lipogenesis (Fasn, Scd1, Acaca, and Acly) in livers of fasted, fed, or liver-specific Raptor knockout fed mice. p values were obtained by Student’s t test. *, p < 0.05 versus fasted liver; **, p < 0.05 versus all others. Values are the mean ± S.E. (error bars). D, shift of various subunits to the size that correlates with different complex sizes shown on gels. Samples were obtained by glycerol gradient column separation with ultracentrifugation. Fractions 2–6 represent free subunits, fractions 8–13 represent small complexes, and fractions 14–18 represent large complexes according to size standards.
The Mediator complex is a multisubunit protein complex that directly binds to multiple DNA sequence-specific transcription factors (28–31). The Mediator complex simultaneously engages Pol II and thereby integrates and conveys gene-specific regulation for transcription initiation and/or...
The SCFFBW7 E3 ligase ubiquitinates target proteins that are phosphorylated at a conserved phosphodegron motif, \( \Phi X \Phi \Phi \Phi (T/S) \) (25). In human embryonic kidney HEK293 cells, co-expression of MED13 with the SCFFBW7 E3 ligase was shown to induce the degradation of the MED13 protein, and mutation of Ser-323 or Ser-326 reduced FBW7-mediated degradation (25). In agreement with these cell culture results, using liver-specific \( Fbw7 \) knockout mice, we also found that the degradation of the kinase module depends on SCFFBW7. However, despite the stabilization of the kinase module, the Mediator complex is still converted to the small Mediator complex in the fed state. These data indicate that FBW7 is necessary for the degradation of the kinase module but is independent of the mTORC1-dependent signals required for the dissociation of the kinase module from the large Mediator complex. The SCFFBW7 phosphodegron motif is present in a number of important regulatory proteins, including SREBP-1c, and is required for the termination of lipogenic gene expression by mediating the degradation of nuclear SREBP-1c protein (35). This also accounts for the small increase in both basal and nutrient-stimulated lipogenic gene expression in the liver-specific \( Fbw7 \) knockout mice.

Although we have yet to identify the specific kinase(s)/phosphatase(s) regulating the phosphorylation of the MED13 phosphodegron motif, it is unlikely that either of the identified mammalian orthologs of the yeast kinases is responsible. In the fed state, the mammalian ortholog of SNF1, the AMP-dependent protein kinase, is inactive in mouse liver. Although MAP kinases can be activated in the fed state, the MED13 phosphodegron motif is not a consensus sequence for the mammalian orthologs of MAP kinase (34). It is also less likely that mTORC1 is the direct kinase responsible as mTORC1 is primarily thought to be active at the lysosome (36), and evidence for nuclear localization has yet to be compelling. Alternatively, certain subunits in the small Mediator complex may also be post-translationally modified upon feeding, resulting in conformational changes of the Mediator complex so that the kinase module may be dissociated from the large Mediator complex. Future work will identify these molecular details. Nevertheless, our study shows for the first time that the assembly state of the Mediator complex is dynamically regulated at least in the liver under physiological and pathophysiological conditions, and the mTORC1 signaling is the major upstream regulator of the Mediator complex in vivo.

**Experimental procedures**

**Animals**

All animal experiments followed the guidelines that were approved and in agreement with the Albert Einstein College of Medicine Institutional Animal Care and Use Committee (2016-0901). \( C57B6/\), \( Raptor^{\text{fl/fl}}/\), \( Fbw7^{\text{fl/fl}}/\), \( Tsc1^{\text{fl/fl}}/\), and \( db/db \) mice in \( C57BLKS/J \) background were obtained from the Jackson Laboratory. \( Ccn2^{\text{fl/fl}}/\) mice were obtained as described previously.
(37), and Med13
tm mice were kindly provided by Dr. Eric Olson. AA8-Tbg-Cre (Vector Biolabs) was introduced by intravenous injection at 5 × 1011 genome copies/mouse to generate the respective liver-specific knockout mice. For rapamycin studies, 1 mg/kg body weight of rapamycin (LC Laboratories) was injected every 6 h over a 48-h period. Mice were housed in groups and in a facility equipped with a 12-h light/dark cycle with free access to food and water and were fed with a normal chow diet unless noted otherwise.

Mice were provided standard laboratory mouse chow (LabDiet catalog no. 5053) that contains 25% protein, 13% fat, and 62% carbohydrates. For fasting and feeding experiments, mice were trained for 3 days before the experiment by removing food at 5 p.m. and feeding at 9 a.m. the next day (16-h fast overnight and 8-h feeding daytime). On day 4, the fasting group of mice was sacrificed in the morning at 11 a.m. (18-h fast), whereas the 9 a.m. fed group was also provided drinking water that contained 15% sucrose (w/v). The fed mice were sacrificed at 1 p.m., equal to 4 h of feeding.

**Antibodies and immunoblotting**

Antibodies against the following proteins were used in this study: MED13 (Fisher PA5-35929 (1:1000) or Bethyl A301-278A (1:1000)), MED12 (Abcam ab70842 (1:1000), CDK8 (Abcam ab54561 (1:1000) or Bethyl A302-501A-M (1:1000)), CCNC (Bethyl A301-989A (1:1000)), MED1 (Bethyl A300-793A (1:1000)), MED25 (Fisher PA5-43616 (1:1000)), MED15 (Bethyl A302-422A (1:1000)), nucleolin (Cell Signaling 14574S (1:1000)), tubulin (Cell Signaling 2144S (1:1000)), phospho-p70 S6 kinase (Cell Signaling 9234S (1:1000)), p70 S6 kinase (Cell Signaling 2708T (1:1000)), phospho-AKT Ser-473 (Cell Signaling 4060 (1:1000)), AKT Ser-473 (Cell Signaling 9272 (1:1000)), and RAPTOR (Cell Signaling 4056P (1:1000)), S6 ribosomal protein (Cell Signaling 2708S (1:1000)), phospho-S6 ribosomal protein (Cell Signaling 4858P (1:1000)), S6 ribosomal protein (Cell Signaling 2317S (1:1000)), TBP (Cell Signaling 8515S (1:1000)), phospho-AKT Ser-473 (Cell Signaling 4060 (1:1000)), Thr-308 (Cell Signaling 4058 (1:1000)), Thr-450 (Cell Signaling 12178 (1:1000)), total AKT (Cell Signaling 9272 (1:1000)), and RAPTOR (Cell Signaling 2280 (1:1000)). Total liver tissue lysates were prepared using the Pierce IP lysis buffer (87787, Thermo Fisher Scientific) followed by silver staining and compared with the fractions from the lysate samples.

**Nuclear extraction and glycerol gradient fractionation**

Fresh mouse liver nuclear extracts were prepared as described previously with some modifications (38). Mouse liver nuclear were incubated in extraction buffer A containing 20 mM Tris-HCl, pH 8.0, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20% glycerol, 12% sucrose, 1 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, and 1 mg/liter aprotinin. The nuclear extracts were then dialyzed against 500 volumes of buffer D (20 mM Tris-HCl, pH 8.0, 100 mM KCl, 1.5 mM MgCl2, 0.1 mM EDTA, 20% glycerol, 1 mM DTT, 2.5 mM phenylmethylsulfonyl fluoride, and 1 mM benzamidine). After centrifugation at 20,000 × g for 20 min at 4 °C, the resulting supernatants were used for immunoprecipitation. Ten microliters of anti-MED1 antibody was bound to 20 μl of protein A/G beads (Pharmacia) for 30 min at room temperature and incubated with 200 μl of liver nuclear extracts for 3 h at 4 °C. The beads were washed five times with 1 ml of buffer containing 0.1% Nonidet P-40. Interacting proteins were eluted with buffer containing 0.3% Sarkosyl.

**Statistical analysis**

Data are presented as the mean ± S.E. unless otherwise noted and compared between two groups using Student’s t test. A two-sided p < 0.05 was considered statistically significant.

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