Molybdenum cofactor (Moco) biosynthesis is linked to c-Jun N-terminal kinase (JNK) signaling in Drosophila through MoaE, a molybdopterin (MPT) synthase subunit that is also a component of the Ada Two A containing (ATAC) acetyltransferase complex. Here, we show that human MPT synthase and ATAC inhibited PKR, a double-stranded RNA-dependent protein kinase, to facilitate translation initiation of iron-responsive mRNA. MPT synthase and ATAC interacted with PKR and suppressed latent autophosphorylation of PKR and its downstream phosphorylation of JNK and eukaryotic initiation factor 2α (eIF2α). The suppression of eIF2α phosphorylation by MPT synthase and ATAC prevented sequestration of the guanine nucleotide exchange factor eIF2B, which recycles eIF2-GDP to eIF2-GTP, resulting in the promotion of translation initiation. Indeed, translation of the iron storage protein, ferritin, was reduced in the absence of MPT synthase or ATAC subunits. Thus, MPT synthase and ATAC regulate latent PKR signaling and link transcription and translation initiation.

Keywords: ATAC, Moco biosynthesis, MPT synthase, PKR

Introduction

The ATAC histone acetyltransferase complex consists of 13 subunits and serves as a transcriptional co-activator (Suganuma et al., 2008, 2010). Drosophila ATAC contains dMoaE, a subunit of MPT synthase that plays a role in JNK signaling and suppresses JNK phosphorylation in response to osmotic stress (Suganuma et al., 2010). Human ATAC contains homologous protein kinase (MAPK) upstream kinase-binding inhibitory protein (MBIP) instead of the human MoaE homolog MOCS2B (Wang et al., 2008). Through analysis of protein sequences, we have proposed that MBIP is evolutionarily derived from the ancestral MoaE protein (Suganuma et al., 2012).

Molybdenum-containing enzymes catalyze redox reactions using a pterin-molybdenum cofactor, Moco (Schwarz et al., 2009). Human Moco deficiencies lead to neurological abnormalities and death in early childhood (Schwarz et al., 2009). In humans, Moco biosynthesis begins with MOCS1A and MOCS1B, which catalyze the conversion of GTP into cyclic pyranopterin monophosphate (cPMP). cPMP is then converted to MPT by MPT synthase, which includes MOCS2A and MOCS2B. In MPT synthase, MOCS2B homodimerizes and forms a heterotetramer by binding two copies of MOCS2A. The heterodimerization of MOCS2A and MOCS2B is critical for their enzymatic activity as MPT synthase. Molybdenum and the pterin moiety form the redox reactive Moco, which is an essential component of enzymes such as sulfite oxidase and xanthine oxidase (Daniels et al., 2008; Schwarz et al., 2009).

The enzymes involved in Moco synthesis carry out a variety of enzymatic reactions. MOCS1A is a member of the radical S-adenosyl-L-methionine (SAM)-dependent methyltransferase (RSMT) superfamily and contains two iron sulfur [4Fe-4S] clusters that catalyze reductive cleavage of SAM (Hanzelmann et al., 2004). The reaction of MPT synthesis is thought to be ancestral to ubiquitin pathways where MOCS3 structurally and functionally resembles an E1 activating enzyme, and MOCS2A has an ubiquitin-fold and is believed to be the ancestor of ubiquitin-like proteins (Ubls) (Rudolph et al., 2001). In humans, MOCS3 activates not only MOCS2A but also ubiquitin-related modifier 1 (Urm1), which is involved in the tRNA thiolation (Chowdhury et al., 2012). In yeast, tRNA thiolation has been shown to alter translational capacity (Laxman et al., 2013). Further functions of Moco biosynthetic enzymes beyond Moco biosynthesis are largely unknown.

Here, we show novel functions of human MPT synthase and ATAC...
through interactions with PKR. PKR phosphorylates JNK and eIF2α in response to nutrient and inflammatory stress (Nakamura et al., 2010). GTP-bound eIF2α is assembled with a methionyl initiator tRNA (Met-tRNAi) to form a translation initiation ternary complex (TC), which promotes translation initiation (Hinnebusch, 2011). A number of stress-activated kinases phosphorylate eIF2α, resulting in sequestration of the nucleotide exchange factor eIF2B, which promotes conversion of eIF2-GDP to eIF2-GTP, and the reduction in TC concentration (Aitken and Lorsch, 2012; Hinnebusch, 2014). Furthermore, PKR has been shown to partially inhibit translation of the iron-responsive mRNA encoding ferritin through eIF2α phosphorylation (Daba et al., 2012). We found that MBIP directly interacts with MOCS2B and PKR, suppresses latent PKR autophosphorylation, and prevents its downstream phosphorylation of JNK and eIF2α. MPT synthase and ATAC prevent the sequestration of eIF2B by phosphorylated eIF2, favoring TC formation. Indeed, MPT synthase and ATAC were required for translation of ferritin mRNA. Thus, our data demonstrate that the translation initiation of iron-responsive mRNA is facilitated by the association of MPT synthase with ATAC and the subsequent inhibition of PKR.

**Results**

**Human MPT synthase associates with ATAC**

In *Drosophila*, the extended C-terminal region of dMoaE is homologous to mammalian MBIP and incorporates dMoaE into dATAC (Figure 1A). Moreover, the N-terminus domain of dMoaE, which is homologous to MOCS2B, inhibits JNK activation (Suganuma et al., 2010). Human MBIP is a component of ATAC, which has homology to MOCS2B in its N-terminus (MoaE-like moiety) but is a separate protein (Wang et al., 2008; Suganuma et al., 2012) (Figure 1A). Previously, we showed that only 2 of 7 residues, which are implicated in binding MPT and catalysis, are conserved in MoaE-like moiety of human MBIP, suggesting human MBIP unlikely to be catalytically active (Suganuma et al., 2012). Moreover, only 4 of 10 residues, which mediate the interaction of MoaE with MoaD, are conserved in MoaE-like moiety of human MBIP. Since the active MPT synthase MOCS2B homodimerizes and forms a heterotetramer by binding two copies of MOCS2A (Daniels et al., 2008), we asked whether MBIP interacted with MOCS2B or MOCS2A (human MoaD) (Schwarz and Mendel, 2006). As we expected, purified recombinant MOCS2A bound to MOCS2B (Figure 1B). Purified

![Figure 1](image-url)

**Figure 1** Human MPT synthase directly associates with ATAC. (A) The diagram illustrates the location of MoaE domain or MoaE-like moiety in *Drosophila* MoaE, human MOCS2B, and MBIP. Diagram of the MoaE—MoaD heterotetramer that was determined from the crystal structure of bacteria MoaE—MoaD (Daniels et al., 2008). (B–D) Purified recombinant human MOCS2B directly bound to recombinant human MOCS2A and MBIP, whereas association of recombinant MOCS2A with MBIP was not detected (Supplementary Figure S1). (E) Endogenously expressed MBIP and NC2β in the cytoplasm or nucleus of HEK293 cells were co-immunoprecipitated with MOCS2 or IgG (control). Precipitates were analyzed by western blots.
recombinant MBIP also bound to MOCS2B (Figure 1C and Supplementary Figure S1A–C), but did not interact with MOCS2A (Figure 1D). Since MBIP is a component of ATAC, we tested the interactions of MOCS2 with ATAC. In human HEK293 cells, we found that the endogenous MOCS2B interacted with MBIP and NC2B, which are subunits of human ATAC (Figure 1E). Thus, ATAC associates with MPT synthase through direct interaction of MBIP with MOCS2B.

**Human MPT synthase and ATAC interact with PKR**

We have previously shown that dMoaE inhibited JNK phosphorylation (Suganuma et al., 2010). This led us to investigate upstream kinases of JNK that might be suppressed by the homologous human MBIP protein (Figure 1A). Potential clues were discovered in the *Drosophila* ATAC purifications where peptides from the PKC-like family protein CG31974 were enriched and peptides from the DSRM family protein blinks were found (Supplementary Figure S2A–C). Both DSRM and PKC-like domains are found in mammalian PKR (Ehrenfeld and Hunt, 1971), which phosphorylates and activates JNK and phosphorylates and inhibits eIF2α (Ehrenfeld and Hunt, 1971; Garcia et al., 2006; Nakamura et al., 2010). We therefore examined whether human ATAC and MPT synthase interacted with PKR. Purified recombinant PKR directly bound to MOCS2B (Figure 2A). Purified recombinant MBIP also directly interacted with PKR (Figure 2B, lanes 3–5, and Supplementary Figure S2D). Interestingly, when PKR, MOCS2B, and MBIP were all present, MOCS2B facilitated the interaction of MBIP with PKR (Figure 2C, lane 4 vs. 5). Since autophosphorylation of PKR is required for its kinase activity, which is important for recognition of eIF2α (Romano et al., 1998; Dey et al., 2005), we then examined whether MBIP affected PKR phosphorylation. PKR phosphorylation was suppressed by MBIP in a dose-dependent manner (Figure 2B, lanes 3–5). MBIP also inhibited PKR activity in the presence of MOCS2B (Figure 2D, lanes 5–7). Thus, MOCS2B and MBIP associated with PKR and inhibited its autophosphorylation (Figure 2E). PKR is activated upon binding to dsRNA in respond to virus infection (Stark et al., 1998), and its dimerization promotes the autophosphorylation (Dey et al., 2005). However, PKR is thought to exist in cells in a latent monomeric state in which kinase activity is inhibited (Wu and Kaufman, 1997). The suppression of latent PKR autophosphorylation by MBIP and MOCS2B may be caused by preventing the dimerization of PKR, which is required for its kinase activity (Figure 2E).

**Human MPT synthase and ATAC inhibit PKR phosphorylation of JNK and eIF2α**

We further examined whether ATAC was involved in the PKR inhibitory activities of MBIP. We first examined the interactions of PKR with ATAC in HEK293 cells. PKR and MOCS2B associated with MBIP in both cytoplasmic and nuclear extracts (Figure 3A, lanes 3 and 6), while ATAC subunits (NC2B, ATAC2, and MBIP) also interacted with MBIP in nuclear extracts (Figure 3A, lane 6), suggesting nuclear and cytoplasmic functions of PKR. Proteins involved in ribosome biogenesis, mRNA stability, and chromatin organization were identified by mass spectrometry of nuclear PKR purifications (Blalock et al., 2014). Moreover, PKR overexpression inhibited ferritin translation (Daba et al., 2012). Thus, MPT synthase and ATAC might affect the translation initiation pathway through interactions with PKR. We therefore examined the effects of MPT synthase and ATAC on the latent PKR kinase activities by monitoring its downstream phosphorylation of eIF2α and JNK. We used CRISPR/Cas9 constructs in HEK293 cells to knockdown MOCS1 that encodes both MOCS1A and MOCS1B, MBIP, ATAC2/CSRP2BP, MOCS2 that encodes both MOCS2A and MOCS2B, and PKR, respectively (Lee et al., 2002; Reiss and Johnson, 2003). The knockdown efficiency for each target gene was confirmed by real-time quantitative RT-PCR (qRT-PCR) in cells from three biological repeats (Supplementary Figure S3A). The levels of phosphorylated PKR (p-PKR), eIF2α (p-eIF2α), and JNK (p-JNK) increased when MOCS1, MOCS2, and ATAC subunits MBIP and ATAC2 were knocked down, respectively (Figure 3B and Supplementary Figure S3B), indicating that Moco synthetic enzymes including MPT synthase and ATAC inhibited PKR activation and its downstream phosphorylation of eIF2α and JNK. Since phosphorylated eIF2α reduces TC concentration (Hinnebusch, 2011), our data suggest that Moco synthetic enzymes and ATAC inhibit eIF2α phosphorylation through inhibition of PKR autophosphorylation, an upstream event, and thus may favor translation initiation. These results also suggest that MPT synthase and ATAC participate in coordinating transcription and translational responses to cellular signaling (Figure 4C).

**MPT synthase and ATAC reduce binding of eIF2α to eIF2B**

Translation is initiated by the formation of the TC, which includes eIF2-GTP and methionyl-tRNA (Hinnebusch, 2011). For the next round of initiation, eIF2-GDP is recycled to eIF2-GTP by eIF2B, which functions as guanine nucleotide exchange factor (GEF) and GDP dissociation inhibitor (GDI) displacement factor (GDF) (Jennings et al., 2013). Stress kinases, including PKR, phosphorylate eIF2α resulting in a stable unproductive interaction with eIF2B (Aitken and Lorsch, 2012). Thus, eIF2α phosphorylation inhibits GEF activity of eIF2B by increasing the affinity of phosphorylated eIF2α for eIF2B, resulting in the reduction of TC concentration (Jennings et al., 2013). Hence, we monitored whether the phosphorylation of eIF2α by knockdown of MPT synthase or ATAC increased the interaction of eIF2α and eIF2B. We used siRNA in HEK293 cells to knockdown MOCS2, MBIP, and ATAC2/CSRP2BP, respectively, and confirmed the knockdown efficiency by real-time quantitative RT-PCR (qRT-PCR) and western blots for three biological repeats (Supplementary Figure S4A–C). To compare the eIF2α–eIF2B interaction among the control and knockdown cells, we immunoprecipitated eIF2α, and found that the expression levels of eIF2α are similar among these cells (Figure 4A, lanes 1–4). The association of eIF2B with eIF2α was increased in MPT synthase or ATAC knockdown cells (Figure 4A, lanes 6–8), suggesting that the increased phosphorylation of eIF2α by MPT synthase or ATAC knockdown increases its sequestration of eIF2B and reduces the TC concentration. Thus, MPT synthase and ATAC may promote the dissociation of eIF2B from eIF2α and TC formation through inhibition of PKR phosphorylation.
Cap-dependent translation of the iron-responsive mRNA encoding ferritin has been shown to be inhibited by PKR, which is partially overcome through an internal ribosomal entry site in the presence of iron (Daba et al., 2012). To understand the effects of MPT synthase and ATAC on translation via inhibition of PKR, we examined the translation of the iron-responsive mRNA for ferritin (Wallander et al., 2006). We metabolically labeled HEK293 cells with $^{35}$S-methionine and immunoprecipitated ferritin in cytoplasmic extracts of control and MOCS2 or ATAC knockdown cells. Ferritin-H was only detected in the immunoprecipitates of control cells, demonstrated by both staining for total proteins and detection of $^{35}$S-methionine incorporation (Figure 4B). However, ferritin transcription levels were not affected by MOCS2 or ATAC knockdown (Supplementary Figure S4D), indicating that only the translation of ferritin was favored by ATAC and MPT synthase (Figure 4C).

In summary, through inhibition of PKR autophosphorylation,
Figure 3 MPT synthase and ATAC inhibit PKR downstream phosphorylation of JNK and eIF2α. (A) Endogenously expressed PKR, MOCS2B, and ATAC in the cytoplasm and nucleus of HEK293 cells co-immunoprecipitated (IP) with MBIP or IgG (control). Precipitates were analyzed by western blots with the indicated antibodies. (B) Cytoplasmic extracts from MOCS2, MBIP, ATAC2, MOCS1, or PKR knockdown HEK293 cells using CRISPR/Cas9 constructs were analyzed by western blots. Band intensities of phosphorylated PKR (p-PKR), PKR, phosphorylated eIF2α (p-eIF2α), eIF2α, phosphorylated JNK (p-JNK), and JNK in western blots were quantified from three independent experiments (two more shown in Supplementary Figure S3B) and plotted as averaged ratios of each signal relative to the signal from control lane (bottom panels). The error bars show standard deviation.
ATAC and MPT synthase suppress the activation of c-Jun and subsequent stress-responsive transcription and suppress phosphorylation of eIF2α favoring translation (Figure 4C).

**Discussion**

PKR was identified as a trigger of cellular defense against viral infection (Marsollier et al., 2011). It has been shown that activation of PKR requires its autophosphorylation and is stimulated by double-strand RNA (dsRNA) during virus replication or cellular stresses (Garcia et al., 2006). PKR is thought to be present in a latent monomeric state (Wu and Kaufman, 1997; Nanduri et al., 2000). However, it was unknown how the activity of latent PKR is controlled in cells. In this study, we demonstrated that the latent PKR autophosphorylation is directly suppressed by ATAC. This may be by preventing the dimerization of PKR, which is required for its kinase activity, and may permit basal levels of translation initiation. Furthermore, we found that MOCS2B enhances the interactions of MBIP with PKR and promoted the MBIP inhibitory activity on PKR phosphorylation (Figure 2C). Overexpression of PKR or iron regulatory protein 1 (IRP1) has been suggested to suppress the translation of ferritin (Daba et al., 2012). In addition, excess iron can be stored in ferritin, and ferritin translation is suppressed by IRPs when iron levels are low (Wallander et al., 2006). Thus, the suppression of PKR by ATAC and MPT synthase regulates iron-responsive translation initiation, and PKR may respond to iron homeostasis directly or indirectly as a cellular signal through the interaction with ATAC and MPT synthase. Enzymes involved in Moco biosynthesis may function in diverse regulatory pathways (Schwarz et al., 2009). Moco is an essential component of at least 50 proteins, and a number of enzymes including MPT synthase are involved in Moco biosynthesis. It will be interesting to study whether other enzymes in the Moco biosynthesis pathway interface with translation regulation.

**Materials and methods**

**Expression and purification of recombinant proteins**

Sf21 cells were cultured and infected with recombinant baculoviruses encoding the human MOCS2B tagged with FLAG, human
MOCS2A with FLAG or HA, human MBIP with HA, or PKR with FLAG or HA, as previously described (Suganuma et al., 2010). Lysates were prepared and each tagged protein was individually purified using anti-FLAG M2 affinity gel or monoclonal anti-HA-agarose. The individually purified recombinant proteins were co-immunoprecipitated as previously described (Suganuma et al., 2010) and described in Supplementary Materials and methods.

Protein extraction, RNAi knockdown, CRISPR-Cas9 knockdown, [35S]-methionine labeling translation assay, protein purifications, and MudPIT analysis
Details are indicated in Supplementary Materials and methods.

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

Acknowledgements
Dr Chris Tachibana (Group Health Research Institute, Seattle, USA) provided scientific editing of the manuscript. We thank Workman Lab members and Stowers core facilities for support during this project.

Funding
This research was supported by private funding from the Stowers Institute for Medical Research to the Workman laboratory.

Conflict of interest: none declared.

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