The positive transcription elongation factor b (P-TEFb) is involved in physiological and pathological events including inflammation, cancer, AIDS, and cardiac hypertrophy. The balance between its active and inactive form is tightly controlled to ensure cellular integrity. We report that the transcriptional repressor CTIP2 is a major modulator of P-TEFb activity. CTIP2 copurifies and interacts with an inactive P-TEFb complex containing the 7SK snRNA and HEXIM1. CTIP2 associates directly with HEXIM1 and, via the loop 2 of the 7SK snRNA, with P-TEFb. In this nucleoprotein complex, CTIP2 significantly represses the Cdk9 kinase activity of P-TEFb. Accordingly, we show that CTIP2 inhibits large sets of P-TEFb- and 7SK snRNA-sensitive genes. In hearts of hypertrophic cardiomyopathic mice, CTIP2 controls P-TEFb-sensitive pathways involved in the establishment of this pathology. Overexpression of the β-myosin heavy chain protein contributes to the pathological cardiac wall thickening. The inactive P-TEFb complex associates with CTIP2 at the MYH7 gene promoter to repress its activity. Taken together, our results strongly suggest that CTIP2 controls P-TEFb function in physiological and pathological conditions.

Discovered in 1995 (1), P-TEFb (CyclinT1/Cdk9) is involved in physiological and pathological transcriptionally regulated events such as cell growth, differentiation, cancer, cardiac hypertrophy, and AIDS (for review, see refs. 2 and 3). It has been suggested to be required for transcription of most RNA polymerase II-dependent genes. However, a recent study suggests that a subset of cellular genes are distinctively sensitive to Cdk9 inhibition (4). P-TEFb is dynamically regulated by both positive and negative regulators. In contrast to Brd4, which is associated with the active form of P-TEFb (5, 6), the 7SK small nuclear RNA (7SK snRNA) and HEXIM1 inhibit Cdk9 activity in the inactive P-TEFb complex (7–10). P-TEFb elongation complexes are crucial for HIV-1 gene transactivation and viral replication. Recently, new P-TEFb complexes containing the HIV-1 Tat protein have been characterized (11, 12), providing evidence for the recruitment of an inactive Tat/P-TEFb complex to the HIV-1 promoter (13). However, defining the diverse nature and functions of the different P-TEFb complexes will require further investigations. The cellular protein CTIP2 (Bell1b) has been highlighted as a key transcription factor for thyroxine (14, 15) and neuron development (16), odontogenesis (17), cancer evolution (18), and HIV-1 gene silencing (19). Besides AIDS, hypertrophic cardiomyopathy is a well-described P-TEFb-dependent pathology (for review, see refs. 20 and 21).

Here, we report that CTIP2 represses P-TEFb function as part of an inactive P-TEFb complex. In hearts of hypertrophic cardiomyopathic mice, CTIP2 controls P-TEFb-sensitive pathways involved in the establishment of this pathology. Together with the inactive P-TEFb complex, CTIP2 associates with the β-myosin heavy chain promoter to repress its activity. Thereby, CTIP2 might contribute to the regulation of the size of heart sarcomeres in physiological or pathological conditions.

Results

CTIP2 Is Associated with the Inactive P-TEFb Complex. First, we investigated, whether or not CTIP2 associates with one of the previously described P-TEFb complexes. We performed coimmunoprecipitation experiments, revealing that CTIP2 coimmunoprecipitates with the CyclinT1 and Cdk9 components of the P-TEFb complex (Fig. 1A). To further define the CTIP2-containing complexes, we separated the previously described P-TEFb complexes by gel filtration chromatography (Fig. 1B). As shown in Fig. 1B, Cdk9 was detected in the low molecular weight (LMW) complex ("free" P-TEFb complex) and in the high molecular weight (HMW) complex that coeluted with HEXIM1 and the 7SK snRNA. Interestingly, we found CTIP2 in these latter fractions containing the HMW P-TEFb complex (Fig. 1B). These observations suggested that CTIP2 may be part of an inactive HEXIM1/7SK-including P-TEFb complex. To confirm this hypothesis, we performed additional coimmunoprecipitation experiments targeting the active and the inactive form of P-TEFb. We found that CTIP2 copurified with CyclinT1, Cdk9, HEXIM1, and 7SK but not with Brd4, making CTIP2 a previously undescribed component of the inactive P-TEFb complex. Confocal observations further confirmed the colocalization of CTIP2, P-TEFb, and HEXIM1 in the previously described CTIP2-induced nuclear structures (22) (Fig. S1). As suggested by the gel filtration elution profile, CTIP2 was not found in the active Brd4/P-TEFb complexes. The 7SK snRNA functions as a scaffold RNA facilitating the interaction between HEXIM1 and P-TEFb. To investigate how CTIP2 associates with the inactive P-TEFb complex, we performed coimmunoprecipitation experiments following RNase treatment.


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CTIP2 interacts with the 7SK snRNA. First, gel shift experiments were performed to define whether CTIP2 interacts with the 7SK snRNA in vitro. As shown in Fig. S2, increasing amounts of purified GST-CTIP2 protein shifted increasing amounts of 3P-labeled 7SK snRNA, indicating that CTIP2 interacts with the 7SK in vitro. As a control, GST-CTIP2 did not bind to TAR RNA in vitro. To define whether this interaction also exists in vivo, we performed immunoprecipitations of CTIP2-associated complexes and probed for the presence of 7SK RNA by RT-PCR. We found that the 7SK snRNA copurified with CTIP2, suggesting that this association exists in vivo (Fig. 24). Micro scale thermosesshion experiments confirmed this result and allowed us to precise the domain of 7SK involved in this interaction. CTIP2 associates with the Loop 2 domain of the 7SK snRNA, but not with a point-mutated Loop 2 (Fig. 2B and C). We next investigated which domain of CTIP2 is involved in the interaction with 7SK and P-TEFb. To do so, we performed immunoprecipititations experiments with truncated forms of CTIP2 (Fig. 2D). This mapping confirmed the need of the 7SK snRNA for the interaction with P-TEFb but not with HEXIM1 (Fig. 2D and Fig. S3). Indeed, a CTIP2 mutant lacking amino acids 355–813 was unable to associate with 7SK snRNA and P-TEFb, but still associated with HEXIM1 (Fig. 2D, column 3). Moreover, we identified the domain from 349 to 475 aa of CTIP2 as the potential interface for P-TEFb binding and thereby for the 7SK snRNA interaction (Fig. S4). Surprisingly, the deletion of the 717 first amino acids (out of 813) of CTIP2 was needed to release the interaction with HEXIM1 (Fig. 2D, column 1). Next, we tested whether or not CTIP2 regulates P-TEFb activity.

CTIP2 Inhibits Cdk9-Mediated Phosphorylation. To explain CTIP2-mediated repression of the P-TEFb function, we first hypothesized that CTIP2 may favor P-TEFb recruitment into the inactive complex. CyclinT1 immunoprecipitation experiments were performed to confirm this hypothesis. Surprisingly, CTIP2 overexpression did not favor recruitment of P-TEFb into the inactive 7SK snRNA-containing complex (Fig. 2E). Because the amount of P-TEFb in the inactive complex was not impacted by CTIP2, we next tested the inhibition of the P-TEFb kinase activity. We first quantified the Cdk9 kinase activity of the CyclinT1-immunoprecipitated complex in the presence or absence of CTIP2 overexpression. As shown in Fig. 2F, CTIP2 overexpression significantly inhibits Cdk9 activity (Fig. 2F and Fig. S5). To confirm that this repression also occurs in physiological conditions, we analyzed the global level of RNA Pol II serine 2 phosphorylation in CTIP2 knockdown cells. Accordingly, higher levels of RNA Pol II serine 2 phosphorylation were observed in CTIP2-depleted cells (Fig. 2G). These observations suggest that CTIP2 represses P-TEFb function by inhibiting Cdk9 activity.

CTIP2-Including Complexes Containing P-TEFb–and Chromatin-Modifying Enzymes Are Mutually Exclusive. We have reported that CTIP2 silences HIV-1 gene expression by the recruitment of HDAC1, HDAC2, and Suv39H1 to the viral promoter (19). To examine the existence of multiple CTIP2 complexes, we performed sequential immunoprecipitation experiments. For this purpose, CTIP2-associated complexes (IPI) were further immunoprecipitated with anti-HEXIM1 or anti-HDAC2 antibodies to discriminate between potential P-TEFb– and chromatin-modifying enzyme-containing complexes, respectively (IP2). As shown in Fig. 3, HDAC2 and Suv39H1 were excluded from the P-TEFb complex whereas neither Cdk9, CyclinT1, nor HEXIM1 was found in the complex containing
the chromatin-modifying enzymes. These data demonstrate that CTIP2 associates at least with two distinct nuclear complexes.

CTIP2 Regulates P-TEFb-Sensitive Genes. To validate the model emerging from our observations, establishing CTIP2 as a negative regulatory component of P-TEFb, we examined the genome-wide transcriptional consequences of a CTIP2 knockdown in microglial and HEK293 cells. Using DNA microarray analysis, genes whose expression was modulated by siRNA-mediated deletion of CTIP2 (Dataset S1) were compared with those regulated by the expression of a dominant negative mutant of Cdk9 (dnCdk9) (4). Hierarchical clustering of these genes revealed a significant anticorrelation between the two gene expression profiles (Fig. 4A). Clustering of highly coregulated genes (|ΔLog2 > 1 in both gene profiles) revealed that 86% of the Cdk9 sensitive genes are inversely regulated by the CTIP2 knockdown (Fig. 4B and Fig. S6). The comparison of the genes significantly (P < 0.05) regulated by CTIP2 overexpression, knockdown, and dnCdk9 expression in HEK293 cells confirmed the observations made in microglial cells (Figs. 4 C–E). The 25% of the genes whose expression was significantly (P < 0.05) altered following CTIP2 knockdown or overexpression were also sensitive to dnCdk9, and, among them, 76% were regulated consistently with a Cdk9-inhibitory activity of CTIP2. Note that the vast majority of these consistently regulated genes are repressed upon dnCdk9 expression, in concordance with the elongation-activating role of P-TEFb. Besides that, we identified 14% (microglial cells) and 24% (HEK293 cells) of the CTIP2 target genes to be divergently regulated upon dnCdk9 expression, pointing at a different mechanism, where CTIP2 can potentially also contribute to Cdk9 activation (Fig. 4D). Whether this mode of action is direct or indirect needs to be further investigated.

CTIP2 Regulates 7SK snRNA-Sensitive Genes. Because 7SK snRNA has been described as a key inhibitor of P-TEFb, we next compared the 7SK- and the CTIP2-dependent transcriptome (Fig. 4 F and G and Datasets S2–S5). About 48% of the genes were inversely affected by CTIP2 overexpression or 7SK knockdown. This observation is consistent with a P-TEFb–repressive role of CTIP2 and coincides with our model, in which both 7SK snRNA and CTIP2 contribute to the inactivation of Cdk9. Surprisingly, 52% of the genes were found to be similarly regulated following CTIP2 overexpression or 7SK knockdown, suggesting that CTIP2 regulates a subset of 7SK-sensitive genes by a still unknown, P-TEFb–independent mechanism (Fig. 4F).

CTIP2 Regulates Cdk9-Sensitive Genes Modulated During Cardiac Hypertrophy in Mouse. As a compound of the inactive P-TEFb complex, CTIP2 should contribute to the regulation of P-TEFb–related physiopathological events. To investigate the physiological relevance of our observations, we focused on one of the

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CTIP2 regulates P-TEFb

Role of NFAT in Cardiac Hypertrophy

Nitric Oxide Signaling in the Cardiovascular System

Cardiac Hypertrophy Signaling

Best-characterized P-TEFb-associated pathologies: hypertrophic cardiomyopathy (HCM). HCM was triggered by phenylephrine (PE) administration to adult mice for 15 d. PE is an α-adrenergic receptor agonist known to trigger cardiac hypertrophy in mice. Gene expression profiles from HCM mice were compared with those obtained upon Cdk9 overexpression and CTIP2 knockdown (Fig. 5). Cdk9 overexpression regulated half of the HCM-modulated genes, confirming the influence of P-TEFb on this specific pathological gene expression pattern (Fig. 5A and Dataset S6). We observed a significant correlation between the gene expression levels from both conditions (Fig. 5B). By comparing this cluster of genes with CTIP2-sensitive genes, we observed that 50% of the Cdk9-sensitive HCM genes were constantly regulated by CTIP2 knockdown (Fig. 5C and D and Dataset S7). Interestingly, key HCM pathways are enriched in the CTIP2/Cdk9 cluster of modulated genes (Fig. 5E). We found that the gene of the sarcomeric β-myosin heavy chain (MYH7) was regulated by CTIP2 consistently with a repression of Cdk9 function (Fig. 5F). Moreover, a network analysis of the HCM Cdk9- and CTIP2-sensitive genes highlights the major impact of CTIP2 on the regulation of cardiac hypertrophy (Fig. 5G). Indeed, key HCM pathways, such as the MAPK, the Ca²⁺/Calmodulin, the NF-κB/NFAT, and the PI3K/Akt pathways, are regulated by Cdk9 (Fig. 5G). We found endogenous CTIP2, CyclinT1, Cdk9, and HEXIM1 bound to the MYH7 promoter (Fig. 6). Moreover, overexpressed CTIP2 bound to the MYH7 proximal promoter but not to −1,000 bp and −2,000 bp upstream or +2,000 bp downstream of the transcription start site (Fig. 6B). Accordingly, with our model, the recruitment of Cdk9 (Fig. 6, column 3), CyclinT1 (Fig. 6, column 4), and HEXIM1 (Fig. 6, column 5) is favored by the presence of CTIP2. These observations argue for a CTIP2-mediated anchorage of an inactive P-TEFb complex at the MYH7 promoter and suggest a major influence of CTIP2 in the control of the size of heart sarcomeres. The proposed mechanistic model is presented in Fig. 7.

Discussion

The cellular factor CTIP2 is a key transcriptional repressor involved in development (14–17, 23), T lineage commitment (15, 24), cancer (18, 25), and HIV-1 gene silencing (19). In association with chromatin modifying enzymes, CTIP2 promotes the establishment of a local heterochromatin environment at target promoters such as the cellular p21 gene promoter (26) and the viral HIV-1 gene promoter (19, 22, 27). Interestingly, both genes have been reported to be highly sensitive to the P-TEFb elongation complex (28–30). To secure the cell integrity, P-TEFb is

Fig. 4. CTIP2 regulates P-TEFb-sensitive genes. (A) Genes modulated by the knockdown of CTIP2 in microglial cells were compared with the genes modulated by an inhibition of Cdk9 activity (expression of the dnCdk9 construct) (4). Shades of yellow and blue represent relative activation and repression, respectively. (B) Genes highly sensitive to both dnCdk9 expression and CTIP2 knockdown (modulation average: Log₂ > 1) were clustered and identified. (C) Transcriptome heatmap of genes significantly (P < 0.05) oppositely regulated by Cdk9 and CTIP2: comparison of the expression of dnCdk9 with CTIP2 overexpression and knockdown in HEK293 cells. (D) As in C, but for the statistically significantly coregulated genes. (E) Table showing the number of target genes differentially expressed in each of the comparisons from C and D. (F) Scatter plot of logQ values for the 112 genes significantly (P < 0.05) coregulated by CTIP2 overexpression (black arrow up) and 7SK knockdown (black arrow down). The logQ values of the same set of genes upon CTIP2 knockdown (gray arrow down) and 7SK overexpression (gray arrow up) are illustrated in gray. (G) Same as in F, but for the 105 genes antiregulated in these conditions.
Fig. 6. CTIP2 binds to the MYH7 gene promoter region. (A) Cells were subjected to chromatin immunoprecipitation experiments with the indicated antibodies. The associated promoter region of the MYH7 gene was quantified by Q-PCR. Enrichments are presented as % of input. (B) Cells transfected with the control FLAG-pcDNA3 or the FLAG-CTIP2 plasmids were subjected to chromatin immunoprecipitation experiments with the indicated antibodies 48 h posttransfection. The associated promoter, the −1,000 bp, the −2,000 bp, and the +2,000 bp regions of the MYH7 gene were quantified by Q-PCR. Fold enrichments are presented relative to the enrichments observed in the control condition. Results are representative of at least three independent experiments.

This subset of genes was identified as activated by CTIP2, but repressed by 7SK RNA. These genes are likely regulated by CTIP2 in a Cdk9-independent manner. D’Orso and Frankel showed the recruitment of the inactive P-TEFb complex to the Sp1 binding sites of the HIV-1 promoter (13). However, no mechanistic evidence was provided on the way of this recruitment. Because CTIP2 is anchored to the Sp1 region via an association with Sp1 and LSD1 proteins (27, 36), CTIP2 may constitute a platform for the recruitment of inactive P-TEFb to the HIV-1 promoter. Together with AIDS, hypertrophic cardiomyopathy is a well-described P-TEFb–dependent pathology (for review, see refs. 20 and 21). Upon HCM, the overexpression of the β-myosin heavy chain induces an increase of the size of the sarcomeres and a pathological thickening of the heart muscle. This final target gene expression is controlled by multiple pathways, all contributing to HCM (20). Our results suggest that CTIP2 contributes to the control of the MAPK, the Ca2+/Calmodulin, the NF-κB/NFAT, and the PI3K/Akt pathways. In addition, we show that CTIP2 inhibits the expression of the β-myosin heavy chain (MYH7) consistently with the repression of Cdk9 activity. Moreover, the CTIP2-mediated anchorage of an inactive P-TEFb complex at the MYH7 promoter further argues for the presence of the inactive P-TEFb complex at promoters of P-TEFb sensitive genes. In fact, a recent study by Ji et al. has demonstrated the assembly of 7SK RNA-containing complexes at gene promoters (37). Taken together, our results suggest that CTIP2 is a potent inhibitor of P-TEFb function in two of the best characterized P-TEFb sensitive pathologies: HCM and AIDS. As a part of the 7SK/HEXIM1/P-TEFb complex, CTIP2 controls P-TEFb–sensitive gene expression and thereby may constitute a pharmaceutical target for fighting P-TEFb–dependent pathologies.

Materials and Methods

Cell Culture. The human microglial cell line (provided by M. Tardieu, Paris, France) (38) and HEK293T cell lines were maintained in Dulbecco’s modified Eagle medium (DMEM) containing 10% (v/v) FCS and 100 U/ml penicillin/streptomycin.

Immunoprecipitation Assays and Antibodies. Two days posttransfection, immunoprecipitations were performed using the standard technique with M2 anti-FLAG (Sigma), anti-Hexim1 (Abcam), or anti-CyclinT1 (Santa Cruz) antibodies overnight at 4 °C with or without RNase or HMBA treatments. Finally, the immunoprecipitated complexes were processed for SDS/PAGE and Western blot analysis, real time quantitative PCR (qRT-PCR), or RT-PCR assays. Proteins were detected in Western blot analyses using antibodies directed against the FLAG epitope (M2 mouse monoclonal from Sigma), CyclinT1 and Cdk9 (Santa Cruz), and CTIP2, Brd4, and HEXIM1 antibodies (Abcam).

Gel Filtration Experiments. The 3 mg of microglial cell nuclear extracts were concentrated by using a Microcon YM-10 Centrifugal Filter Unit (3,000 Nominal Molecular Weight Limit) and separated by gel filtration on a
Superoxide 6 PC 3.2/30 column (Amersham Biosciences) as previously described (39). The 29 fractions collected were assessed by SDS/PAGE and Western blot analysis. Upon immunodetection, the presence of the 7SK was visualized by agarose gel electrophoresis.

Micro Scale Thermophoresis. Micro Scale Thermophoresis (MST) experiments were conducted with Fmoc-labeled 7SK L2 RNA or 7SK L2 m137 RNA and increasing concentrations of FLAG-CTIP2 using a Monolith NT.115 (Nano- temper Technologies) as described previously (40) (Laser-power 20%, Laser- on time 60s, LED-power 30%).

Transcriptome Analyses. Transcriptome analyses were performed using either Agilent Human whole-genome array (G2534A-60011), Applied Biosystems human arrays (Product nos. 4339628 and 4336875), or an Affymetrix Gen- eChip MOE 430 2.0 array according to the manufacturer’s instructions. Data quality was determined using a QC procedure (41). Data were normalized using NeuONORM with k = 0.02 (42–44). Subtraction profiling was performed as in refs. 31 and 32 using the CDS test (45).

Generation of Cardiac Hypertrophic Mice. Micropumps (Alzet 2002 Osmotic Pumps) were set up to deliver (R)-(-) Phenylephrine hydrochloride (Sigma P612-5G) at 80 mg kg⁻¹ day⁻¹ or vehicle buffer only (PBS, 0.002% ascorbic acid) for 15 d. The micropumps were implanted under the back skin of five C57BL/6N mice for each group under 2% Isoflurane (Abbott), 2% in combination of isoflurane induction followed by the increase of heart weight to body weight ratio in treated animals. Protocols are in accordance with the ethical recommendations of the University Pierre et Marie Curie, Paris 6 University.

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