Heterochromatin remodeling by CDK12 contributes to learning in *Drosophila*

Lixia Pan[a], Wenbing Xie[b,1], Kai-Le Li[c], Zhihao Yang[a], Jiang Xu[a], Wenhao Zhang[d], Lu-Ping Liu[a], Xingjie Ren[a], Zhimin He[a], Junyu Wu[a], Jin Sun[a], Hui-Min Wei[c], Daliang Wang[a], Wei Xie[d], Wei Li[c], Jian-Quan Ni[a,2], and Fang-Lin Sun[c,a,2]

[a]School of Medicine, Tsinghua University, Beijing 100084, China; [b]Key Laboratory of Cell Proliferation and Differentiation of the Ministry of Education, School of Life Sciences, Peking University, Beijing 100871, China; [c]Research Center for Translational Medicine at East Hospital, School of Life Sciences and Technology, Tongji University, Shanghai 200120/200092, China; and [d]Tsinghua-Peking Center for Life Sciences, School of Life Sciences, Tsinghua University, Beijing 100084, China

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Dynamic regulation of chromatin structure is required to modulate the transcription of genes in eukaryotes. However, the factors that contribute to the plasticity of heterochromatin structure are elusive. Here, we report that cyclin-dependent kinase 12 (CDK12), a transcription elongation-associated RNA polymerase II (RNAPII) kinase, antagonizes heterochromatin enrichment in *Drosophila* chromosomes. Notably, loss of CDK12 induces the ectopic accumulation of heterochromatin protein 1 (HP1) on euchromatic arms, with a prominent enrichment on the X chromosome. Furthermore, ChIP and sequencing analysis reveals that the heterochromatin enrichment on the X chromosome mainly occurs within long genes involved in neuronal functions. Consequently, heterochromatin enrichment reduces the transcription of neuronal genes in the adult brain and results in a defect in *Drosophila* courtship learning. Taken together, these results define a previously unidentified role of CDK12 in controlling the epigenetic transition between euchromatin and heterochromatin and suggest a chromatin regulatory mechanism in neuronal behaviors.

epigenetic transition | HP1 | RNAPII C-terminal domain kinase | histone H3 on Lys9 methylation | neuronal function

Appropriate regulation of gene transcription is essential throughout the life of an organism. In eukaryotes, DNA and histone octamers are assembled into nucleosomes and further compacted into higher-order structures (1). Dynamic changes in the chromatin architecture affect gene transcription in many aspects of developmental and physiological processes, such as neural plasticity, memory formation, and cognition (2, 3). Eukaryotic genomes are composed of two basic forms, euchromatin and heterochromatin, which are originally characterized by their cytological chromatin packaging levels. Euchromatic regions are generally associated with a relatively open chromatin configuration and mainly contain transcriptionally active genes, whereas heterochromatin is highly compacted and less accessible to the transcriptional machinery (4). *Drosophila* heterochromatin is mainly localized at the pericentric and subtelomeric regions and enriched for methylation of histone H3 on Lys9 (H3K9me), which provides a docking site for heterochromatin protein 1 (HP1) (5, 6), a highly conserved protein involved in heterochromatin formation (7). Previous studies also show that HP1 and H3K9me associate with a subset of loci on euchromatic regions, and they presumably serve to fine tune the level of gene transcription (8–10). One significant question that comes up is how the chromatin structure is dynamically regulated to impact expression of genes in complex neuronal processes, such as learning and memory. Our earlier study on chromatin domain mapping of chromosome 4 suggests that heterochromatic domains may be regulated by the activities of RNA polymerase II (RNAPII) complexes, which form a "barrier" to prevent heterochromatin spreading and transcriptional gene silencing (11). However, the mechanism underlying such a mode of regulation and the consequence on reprogrammed transcription remain to be investigated.

Results

Cyclin-Dependent Kinase 12 Counteracts Heterochromatin Enrichment on Euchromatic Regions. To understand how heterochromatin domains are regulated on chromosomes, we intended to screen for factors that affect HP1 distribution in *Drosophila* polytene chromosomes. Because kinases play important roles in modulating the cellular localization of their substrates and kinase JIL-1 has been reported to regulate HP1 binding on chromosomes (12), we performed a pilot transgenic RNAi screen focusing on kinases. The "visible" and consistent cytological localization of HP1 at the chromocenter on giant polytene chromosomes from the third-instar larvae allows for evaluation of the switch between the heterochromatic and euchromatic domains through immunofluorescence assays. We screened transgenic RNAi lines against protein kinases in *Drosophila* using a salivary gland-specific (SG) GAL4 (*SG-GAL4*). Surprisingly, we found substantial accumulation of HP1 in euchromatic regions in a cyclin-dependent kinase 12 (CDK12) RNAi line (TH00344.N) (Fig. 1*A*). In addition, similar HP1 binding patterns were observed in male and female flies after CDK12 knockdown (Fig. S1*A*), suggesting that this

Significance

Eukaryotic genomes are compacted into chromosomes, in which heterochromatin is generally considered to be distinct from euchromatin in chromosomal packaging levels and locations. In *Drosophila*, heterochromatin is mainly found in pericentric and telomeric regions. In this study, we show that heterochromatin landscapes that interspersed in euchromatic arms are counteracted by CDK12, a major RNA polymerase II C-terminal domain kinase. After the depletion of CDK12, heterochromatin enrichment can be observed on euchromatic arms, especially on the X chromosome, which leads to transcriptional attenuation in targeted genes and defects in neuronal functions. Our findings provide insights into the regulation of heterochromatin domain in the natural chromosomal context and suggest a chromatin regulatory role of CDK12 in neuronal functions.

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The authors declare no conflict of interest.

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[1]Present address: The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, The Johns Hopkins University School of Medicine, Baltimore, MD 21287.

[2]To whom correspondence may be addressed. Email: nijq@tsinghua.edu.cn or flsun@mail.tsinghua.edu.cn.

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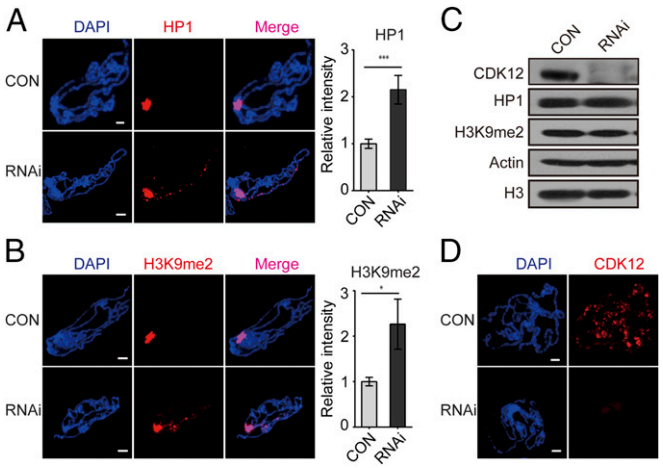

Fig. 1. Depletion of CDK12 triggers heterochromatin enrichment on euchromatic arms. (*A* and *B*) HP1 and H3K9me2 spread to euchromatic arms after CDK12 knockdown. Polytene chromosomes were squashed and stained with (*A*) anti-HP1 and (*B*) anti-H3K9me2. The relative fluorescence intensities of HP1 and H3K9me2 on the euchromatic arms based on raw data are shown in *Right*. Error bars indicate SD ($n = 3$). $*P < 0.05$; $***P < 0.001$. (*C*) Western blot results showing that CDK12 depletion has no obvious effect on the level of HP1 or H3K9me2. Whole-tissue extracts were prepared from third-instar larvae. Antibodies specific for CDK12, HP1, and H3K9me2 were used. Antibodies against Actin and histone H3 were used as loading controls. Three independent replicates were performed. (*D*) The efficiency of CDK12 depletion was validated by immunostaining. Polytene chromosomes were labeled with anti-CDK12 (red). CON, control. (Scale bar: 10 μm.)

phenomenon is sex-independent. We next studied whether the localization of H3K9me2 was also changed along with the distribution of HP1 after CDK12 knockdown, because these two heterochromatin components are often coregulated, and both are essential for heterochromatin formation (7). Indeed, H3K9me2 also accumulated on euchromatic arms after depletion of CDK12 (Fig. 1*B* and Fig. S1 *B* and *C*), with a pattern indistinguishable from that of HP1 (Fig. S1*D*), which suggests that CDK12 counteracts heterochromatin enrichment on euchromatic arms. To examine whether CDK12 depletion affects the overall levels of HP1 and H3K9me2, we performed Western blot analysis. There was no obvious change (Fig. 1*C*), indicating that heterochromatin enrichment on euchromatic arms is not caused by increased levels of HP1 and H3K9me2.

The CDK12 RNAi efficiency was validated by quantitative RT-PCR (qRT-PCR), Western blot analysis, and immunostaining. Our results showed that both the transcription level and the protein level of CDK12 were greatly reduced in third-instar larvae driven by *actin-GAL4* (Fig. 1*C* and Fig. S1*F*). By immunostaining with a CDK12-specific antibody, we found abundant CDK12 signals in control chromosomes as previously reported. However, after CDK12 knockdown, these signals were efficiently eliminated in the polytene chromosomes (Fig. 1*D*). Furthermore, we tested two other independent CDK12 RNAi lines and found a similar HP1 binding pattern to that observed with the original RNAi line (TH00344.N) (Fig. S1*G*). In addition, we found that depletion of cyclin K (CycK), a known functional partner of CDK12 (13–15), induced heterochromatin enrichment on the chromosomes (Fig. S2 *A* and *B*), supporting that CDK12 is involved in counteracting heterochromatin enrichment. Recently, CDK12 has been identified as an RNAPII C-terminal domain (CTD) kinase that catalyzes the bulk of Ser2 phosphorylation (Ser2P) in *Drosophila* (13). Immunostaining of polytene chromosomes showed a dramatic decrease of Ser2P after CDK12 knockdown (Fig. S2*C*). Consistently, clonal assays in the salivary gland and fat body also showed reduced Ser2P levels in CDK12 knockdown cells compared with those in the neighboring control cells (Fig. S2 *D* and *E*). Moreover, we observed heterochromatin enrichment in fat body cells as indicated by H3K9me2 on CDK12 depletion (Fig. S2 *F* and *G*), suggesting that this phenomenon is preserved between different cell types. Taken together, these results show that CDK12 is a previously unidentified regulator for counteracting heterochromatin enrichment on euchromatic arms in *Drosophila*.

Heterochromatin Enrichment Triggered by Loss of CDK12 Primarily Occurs on the X Chromosome. Consistent with previous reports that the X chromosome is a preferential target for heterochromatin enrichment (12, 16), the accumulation of HP1 induced by CDK12 depletion was prominent on the X chromosome marked by acetylation of histone H4 on Lys16 in males (Fig. 2*A*). However, the loss of CDK12 driven by *SG-GAL4* remarkably reduced the size of the salivary gland, increasing the difficulty in precisely mapping the distribution of HP1 on the X chromosome. Thus, we introduced *tub-GAL80^{ts}*, a temperature-sensitive inhibitor of GAL4, for controlling GAL4 activity. At 25 °C, GAL80 activity is partially inhibited and allows a low level of GAL4/UAS activation, and the size of the salivary gland was found to be comparable with that in control. We found that HP1 exhibited a prominent distribution pattern on the X chromosome (Fig. 2*B* and Fig. S2*H*). A similar binding pattern was observed for H3K9me2 (Fig. S2*I*). We then mapped HP1 signals to multiple regions on the X chromosome and found that HP1 mainly associated with band regions rather than interbands (Table S1). Band regions are shown by the deep staining of DAPI for DNA and generally considered to be transcriptionally inactive. In summary, our observations reveal that the depletion of CDK12 induces ectopic HP1 accumulation in transcription-inactive regions on the X chromosome.

Heterochromatin Enrichment Predominantly Occurs in Long Genes After CDK12 Depletion. To further determine the target regions of HP1 accumulation on the X chromosome, we performed ChIP and sequencing (ChIP-seq) using tissues from control and CDK12-depleted larvae. Compared with those in control, we found that

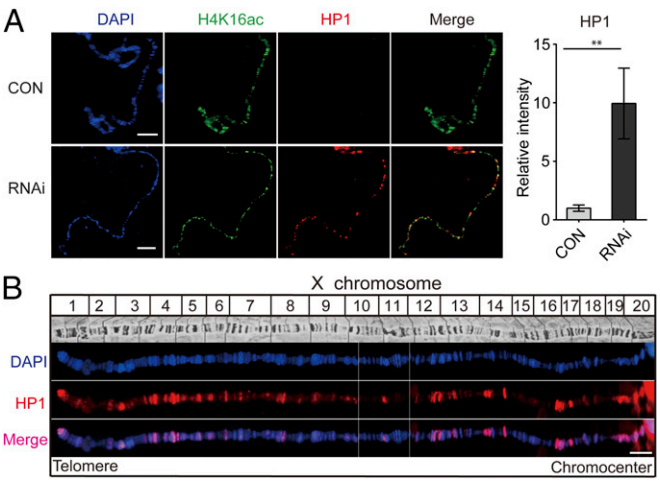

Fig. 2. Heterochromatin enrichment mainly occurs on the X chromosome after CDK12 knockdown. (*A*) HP1 spreads to the X chromosome in male flies after CDK12 depletion. Polytene chromosomes isolated from male CDK12-depleted and control larvae were stained with anti-HP1 (red), antiacetylation of histone H4 on Lys16 (anti-H4K16ac; green), and DAPI (blue). The relative fluorescence intensities of HP1 on the X chromosome based on raw data are shown. Error bars indicate SD ($n = 3$). $**P < 0.01$. (*B*) HP1 enrichment occurs in band regions on the X chromosome; *tub-GAL80^{ts}* was combined with the *SG-GAL4* to allow low levels of GAL4 activity at 25 °C. The presented chromosome is assembled from three immunostained X chromosomes. The photographic map of the X chromosome is reproduced from ref. 40, with permission from Elsevier. CON, control. (Scale bar: 10 μm.)

299 chromatin peaks showed an increase in HP1 binding, whereas only 29 peaks showed a decrease in HP1 binding after CDK12 depletion (Fig. 3*A* and Dataset S1). Similarly, a global increase in the binding of H3K9me2 was also evident, with 540 peaks exhibiting increased binding and only a few peaks showing decreased binding (Fig. 3*A* and Dataset S1), validating that the loss of CDK12 resulted in heterochromatic territories. In addition, among the euchromatic peaks with increased association of HP1, more than 30% of the peaks were located on the X chromosome (Fig. 3*B*), which is consistent with the preferential binding pattern of HP1 on the X chromosome as indicated by the cytological mapping after CDK12 knockdown. Additional analyses indicated that the enrichment of both HP1 and H3K9me2 occurred mainly in the gene bodies after CDK12 depletion, counting for ∼60% of the total binding detected (Fig. S3*A*). In accordance with previous reports that HP1 prefers to bind with long genes in euchromatic regions (for example, >10 kb) (17), we found that the increased association of HP1 was biased toward long genes with multiple exons after CDK12 knockdown (Fig. 3*C* and Fig. S3*B*).

Furthermore, we examined the distribution patterns of heterochromatin enrichment at single-gene resolution by focusing on a group of 10 X-chromosomal genes with highly increased HP1 and H3K9me2 bindings after loss of CDK12. Consistent with a previous report (9), low levels of HP1 and H3K9me2 bindings exist in transcribed regions in 10 chosen genes in control. Interestingly, after CDK12 depletion, abundant levels of HP1 and H3K9me2 bind the gene bodies of these loci (Fig. 3 *D*–*F* and Fig. S3*C*). The increased levels of HP1 and H3K9me2 in the X-chromosomal genes were also confirmed by quantitative PCR with another independent ChIP preparation (Fig. 3 *G* and *H*). Because we noticed that these X-chromosomal genes were initially covered with low levels of HP1 and H3K9me2, we continued to analyze whether this is a general phenomenon in the whole genome. We found that, in the average profile, the regions with increased heterochromatin enrichment are originally associated with low levels of HP1 and H3K9me2 in the control (Fig. S4*A*). Moreover, to test whether CDK12 initially associates with these regions, we performed ChIP-seq analysis. The results suggest a certain level of CDK12 association in these regions (Fig. S4*B*). Taken together, our ChIP-seq analysis supports that heterochromatin enrichment preferentially occurs on the X chromosome after CDK12 knockdown and that the increased association of HP1 predominantly locates in long genes, which were initially covered with a low level of HP1.

Heterochromatin Enrichment by CDK12 Depletion Impairs Neuronal Gene Activation. To further characterize the developmental functions of the targeted genes with heterochromatin accumulation after CDK12 knockdown, we selected the genes with increased HP1 binding on the X chromosome and analyzed their expression patterns in various wild type (WT) tissues using the public FlyAtlas database (18). Interestingly, about one-half of the affected X-chromosomal genes on CDK12 knockdown are preferentially expressed in the brain and ganglion of WT flies (Fig. 4*A* and Table S2), especially during the adult stage. A similar expression pattern was observed for genes with increased H3K9me2 binding (Fig. S5*A* and Table S2), indicating a major role of CDK12 in antagonizing heterochromatin enrichment on

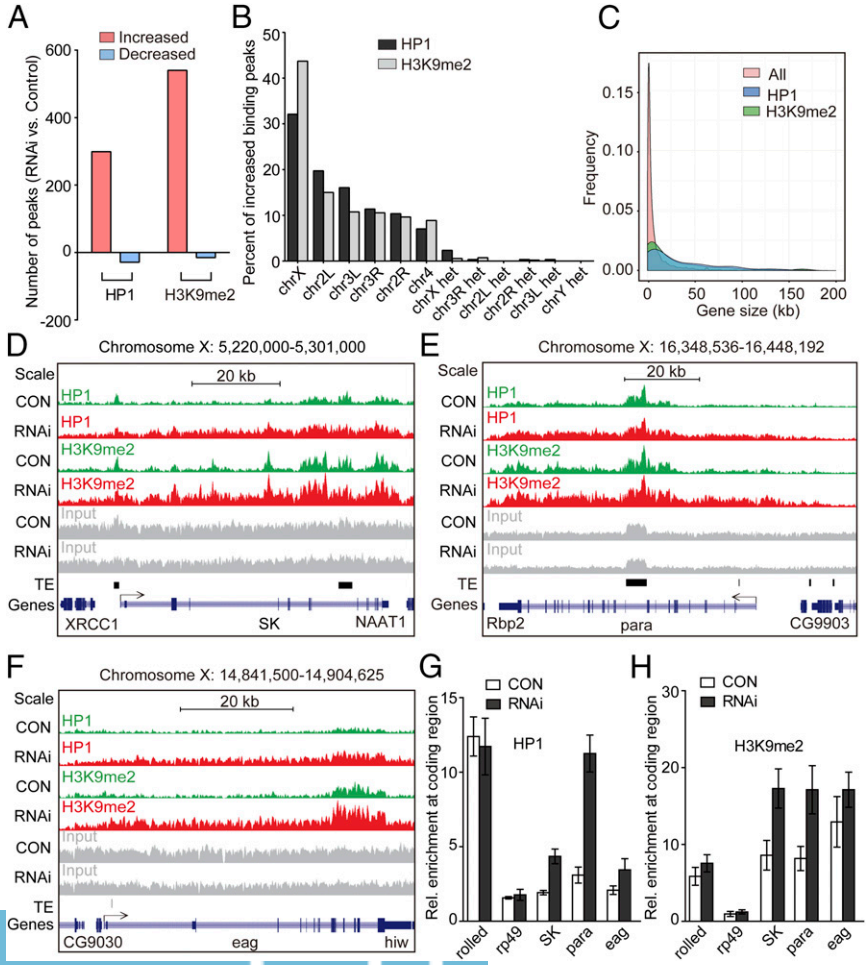

Fig. 3. ChIP-seq analysis of heterochromatin enrichment after CDK12 knockdown. (*A*) The binding of HP1 and H3K9me2 is increased genome-wide after CDK12 depletion. The numbers of increased (red) and decreased (blue) binding peaks of H3K9me2 or HP1 are indicated. (*B*) HP1 and H3K9me2 preferentially associate with the X chromosome. The percentages of increased binding peaks of HP1 (black) or H3K9me2 (gray) on different chromosomes and heterochromatic domains are indicated. (*C*) HP1 and H3K9me2 preferentially associate with long genes. The comparisons of all *Drosophila* genes and genes with increased HP1 and H3K9me2 bindings with regard to the gene size are shown. The data for the gene length were obtained from the University of California at Santa Cruz Genome Browser. (*D*–*F*) The distribution of HP1 and H3K9me2 at three long genes on the X chromosome, (*D*) *SK*, (*E*) *para*, and (*F*) *eag*, in control (green) and CDK12 depletion (red) larvae. ChIP-seq input (gray), transposable elements (TEs; black), and the reference genes (blue) are illustrated. All annotated TE insertions on the X chromosome were obtained from FlyBase. The direction of gene transcription is indicated by arrows. The height range for all peak panels in *D* and *F* is 10–215, and the height range for all peak panels in *E* is 10–410. (*G* and *H*) ChIP assay and quantitative PCR analysis validate the increased binding of HP1 and H3K9me2 at gene bodies on the X chromosome after CDK12 depletion. The bars show enrichment of HP1 or H3K9me2 normalized to input and the internal control gene *GS*, which shows no binding of HP1 and H3K9me2 in the ChIP-seq analysis; *rolled* serves as a positive control, whereas *rp49* serves as a negative control. The primers were designed based on sequences within the transcribed regions of these genes. Error bars indicate SD ($n = 3$). CON, control.

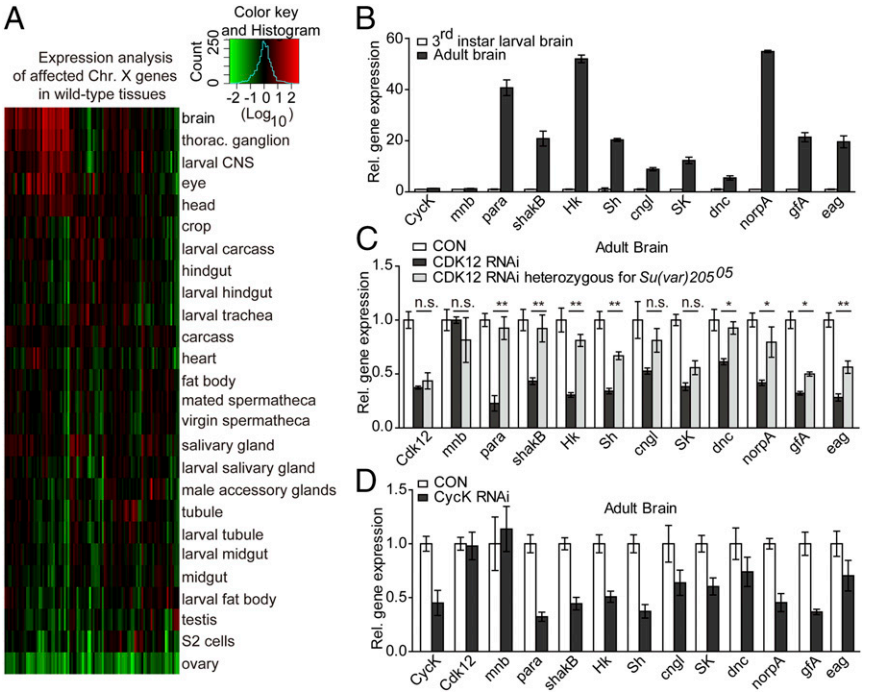

Fig. 4. Heterochromatin enrichment on CDK12 knockdown impairs neuronal gene activation. (*A*) Heat map shows that the affected X-chromosome genes are highly expressed in the brain of WT *Drosophila*; 52 X-chromosome genes with increased HP1 binding after CDK12 depletion are selected. Expression data were obtained from the FlyAtlas database (18). The relative expression levels were determined using a $\log_{10}$ scale and normalized to the whole-fly genome-wide average. (*B*) The neuronal genes are highly activated in the adult brain. The relative expression levels of neuronal genes in the third-instar larval brain and the adult brain in WT were monitored by qRT-PCR and normalized to *rp49* mRNA; *mnb* showed no association with HP1 or H3K9me2 in our ChIP-seq analysis and served as a negative control. Error bars indicate SD ($n = 3$). (*C* and *D*) The depletion of CDK12 reduced the transcription of neuronal genes, whereas introducing *Su(var)205*^{05} restored the expression level. The relative expression levels of neuronal genes in the adult brain after (*C*) CDK12 depletion or CDK12 depletion heterozygous for *Su(var)205*^{05} or (*D*) CycK depletion were monitored by qRT-PCR as described in *B*. Significance was analyzed by ordinary one-way ANOVA in GraphPad Prism. Error bars indicate SD ($n = 3$). CON, control; n.s., not significant. $^{*}P < 0.05$; $^{**}P < 0.01$.

neuronal genes. Based on neuronal functions, these genes are mainly divided into three categories. The first group of genes encodes for voltage-gated and mechanosensitive ion channels, including *Sh*, *Hk*, *eag*, and *para*. The second group of genes encodes for ligand-gated channels/receptors, including *SK*, *Cngl*, and *gfA*. The third group of genes includes *norpA*, a phospholipase C, and *dnc*, a cAMP phosphodiesterase that is expressed in *Drosophila* mushroom body (MB) neuropil and regulates learning acquisition in flies (19).

To detect whether the heterochromatinization on the X chromosome after CDK12 depletion could result in transcription downregulation, we performed qRT-PCR analysis with brain tissues on 10 selected neuronal genes. By using a panneuronal driver *elav-GAL4* to conduct neuron-specific knockdown of CDK12, we found no obvious change in the transcription of these genes in the larval brain after CDK12 depletion (Fig. S5*B*), which may be because of the relatively low expression levels of these genes in the larval brain compared with those in the adult brain as indicated by gene expression analysis using the public FlyAtlas database (Fig. 4*A*). These neuronal genes may be developmentally regulated, because insects, like *Drosophila*, go through metamorphosis in which the nervous system is remarkably remodeled from larva stage to adult stage (20). Therefore, we compared expression levels of 10 genes between third-instar larvae and 1-d adult in the brain. qRT-PCR results showed that expression levels of these genes were 3- to 60-fold higher in the adult brain compared with those in the larval brain (Fig. 4*B*).

Next, we wondered whether the depletion of CDK12 in the adult brain would cause neuronal defects. Notably, we found that flies failed to eclose from puparia after CDK12 knockdown driven by *elav-GAL4* at 25 °C (Fig. S5*C*) and that these flies exhibited a severely paralyzed phenotype if dissected out of puparia (Fig. S5*D*). A similar phenotype was also observed after CycK knockdown (Fig. S5 *C* and *D*). When the culture temperature was shifted to 18 °C, ∼50% of the flies were able to eclose from puparia, but all of them still displayed a paralyzed phenotype (Fig. S5 *E* and *F*). This paralyzed phenotype may be induced by the decreased expression of *para*, because a previous report showed that *para* mutants exhibit a paralyzed phenotype (21).

We next assessed the transcription levels of these neuronal genes in the brains of 1-d adults. The qRT-PCR results showed two- to threefold reduction in their transcription after CDK12 knockdown compared with those in control (Fig. 4*C*). Consistently, the depletion of CycK also resulted in a decrease in the transcription of the tested genes in the adult brain (Fig. 4*D*). To further address whether CDK12 affects the expression of these neuronal genes through modulating heterochromatin, we introduced HP1 loss-of-function allele *Su(var)205*^{05} into CDK12 transgenic RNAi stock. The qRT-PCR results showed that the expression levels of eight neuronal genes were significantly rescued (Fig. 4*C*). Moreover, we found that the paralyzed phenotype was clearly suppressed in CDK12 knockdown flies that are heterozygous for *Su(var)205*^{05} (Movies S1–S3). In addition, to detect whether CDK12 affects alternative splicing, we focused on the *Sh* gene, which is processed into multiple RNA transcripts (Fig. S5*G*). The qRT-PCR results showed that the different isoforms of *Sh* were uniformly decreased in the adult brain (Fig. S5*H*), suggesting that CDK12 is not associated with the alternative splicing of the *Sh* gene. Overall, these results indicate that the heterochromatinization on the X chromosome after CDK12 depletion impairs the transcriptional activation of neuronal genes during the adult stage.

Heterochromatin Enrichment on CDK12 Depletion Affects *Drosophila* Courtship Learning. The decreased expression of neuronal genes in the adult brain prompted us to test whether the loss of CDK12 or CycK would have any effect on neuronal behaviors. Because the potassium channel genes *Sh*, *Hk*, *eag*, and *SK* and the cAMP phosphodiesterase *dnc* are all known to be involved in *Drosophila* learning and memory (19, 22–25), we performed a courtship conditioning test using an *OK107-GAL4* driver, which is mainly active in the MBs (putative learning and memory centers in the *Drosophila* brain). Unsuccessful courtship reduces the subsequent courtship behavior of male flies toward virgin females, and this courtship conditioning behavior is known as one of the major learning and memory paradigms in *Drosophila*. Our results showed that knockdown of CDK12 in the MBs induced a severe courtship learning defect (Fig. 5 *A* and *B* and Movies S4 and S5). Furthermore, we tested two other independent CDK12 RNAi

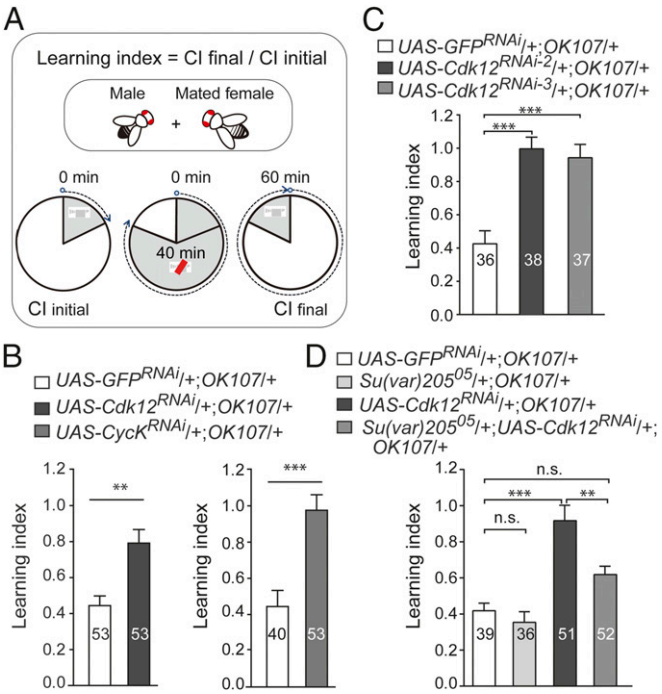

Fig. 5. Heterochromatin enrichment upon CDK12 depletion affects *Drosophila* courtship learning. (*A*) The schematics illustrating procedures used to analyze *Drosophila* courtship learning. The learning index was determined as the ratio of the courtship level during the final 10 min of training [courtship index final (CI_{final})] to that of the initial 10 min [courtship index initial ($CI_{initial}$)]. If the learning index is ≥1, it indicates that there is no learning. (*B*) Courtship learning indexes were measured in (*Left*) CDK12-depleted and (*Right*) CycK-depleted flies. Significance was estimated using Student's *t* test. (*C* and *D*) Heterozygous *Su(var)205*^{05} mutation suppresses the learning defects induced by CDK12 knockdown. Courtship learning indexes were measured in (*C*) two independent CDK12-depleted flies and (*D*) CDK12 RNAi flies heterozygous for *Su(var)205*^{05}. Significance was analyzed by ordinary one-way ANOVA in GraphPad Prism. The genotypes of tested flies are listed. Error bars indicate SEM. n.s., not significant. $^{**}P < 0.01$; $^{***}P < 0.001$.

lines and found a consistent courtship learning defect (Fig. 5*C*). We then tested the courtship memory behaviors of the control and the CDK12 RNAi flies at 2 h, 1 d, and 6 d after a 5-h training period based on the memory curves of *Canton-S* flies (Fig. S6*A*) and found no obvious change in memory indexes between the control and CDK12-depleted lines at these different time points (Fig. S6 *B* and *C*). Consistently, we also observed that the depletion of CycK resulted in similar defects in courtship learning but not in courtship memory (Fig. 5*B*, Fig. S6*D*, and Movie S6). To further address whether CDK12 affects courtship learning through modulating heterochromatin status, we performed a learning test on CDK12 knockdown flies that were also heterozygous for *Su(var)205*^{05} (Fig. 5*D* and Movie S7). We found that the learning index of these flies had decreased significantly compared with that of CDK12 knockdown flies, which shows that heterozygous HP1 mutation suppresses the learning defect induced by CDK12 knockdown in the MB. Overall, our results suggest that CDK12 regulates *Drosophila* courtship learning by antagonizing heterochromatin enrichment on neuronal genes.

Discussion

In *Drosophila*, heterochromatin is featured by strong enrichment of "silencing marks" HP1 and H3K9me2 (9). In this study, we have shown that the depletion of CDK12 induces heterochromatin accumulation within euchromatic arms, particularly on the X chromosome. Previous reports show that overexpression of Su(var)3–7 induces ectopic heterochromatin formation on the euchromatic arms, with preferential enrichment of HP1 and H3K9me2 in the male X chromosome (16). This pattern is associated with the dosage compensation complex, and it is distinct from CDK12 depletion-triggered heterochromatin enrichment. In addition, mutation of the histone H3S10 kinase JIL-1 results in heterochromatin spreading to the euchromatic arms, especially on the X chromosome in both male and female flies (12). However, there was no obvious change in the level of H3S10 phosphorylation (H3S10P) after CDK12 knockdown (Fig. S1*E*). Furthermore, we noticed that the overall levels of HP1 and H3K9me2 remain unchanged after CDK12 knockdown (Fig. 1*C*), which resembles what has been observed in the *JIL* null mutant (12). A similar pattern was reported in the *spindle-E* mutant, where HP1 was highly enriched on the entire chromosome, whereas the overall level of HP1 was not affected (26). There are two possibilities of why the HP1 level remained unchanged while an enrichment on the euchromatic regions was observed. The first possibility is that HP1 may be relocated in the nucleus. HP1 is also found in the nucleoplasmic fraction (27), and its association with chromatin is dynamic (28). After CDK12 depletion, there may be more HP1 becoming associated with chromatin from the nucleoplasm. A second possibility is that HP1 is redistributed on the chromosome. In certain heterochromatin regions, HP1 is highly enriched and may be saturated. The current research methods may not be sensitive enough to detect the slight decrease in HP1 levels in the heterochromatin regions.

Intriguingly, the X chromosome is shown to be a preferential target for heterochromatin enrichment in this study and previous reports. This phenomenon may be partly explained by modENCODE data in *Drosophila*, which show that the X chromosome contains large "facultative heterochromatin" domains with moderate levels of H3K9me2/me3 (9). These domains, thus, may serve as seed regions for ectopic heterochromatinization. Indeed, our ChIP-seq analysis on the X chromosome showed that genes with heterochromatin enrichment were initially covered with low levels of HP1 and H3K9me2 without CDK12 depletion.

RNAPII CTD serves as a scaffold for orchestrating chromatin modifiers and transcription apparatus during eukaryotic gene expression (29). Here, we provide the first evidence, to our knowledge, that an RNAPII CTD kinase functions as a heterochromatin antagonizer on euchromatic arms in *Drosophila*. Previous reports show that Ctk1, the yeast ortholog of CDK12, directed RNAPII CTD Ser2P to provide a docking site for the H3K36me3 methyltransferase (30), whereas HP1 promotes H3K36me3 demethylation through interaction with dKDM4A in *Drosophila* (31), which indicates a potential counteractive interplay between HP1 and the transcription elongation machinery. Moreover, our earlier study on chromatin domain mapping of chromosome 4 suggests that the transcriptional activity of the RNAPII complexes prevents heterochromatin spreading by forming a barrier (11). Because CTD Ser2 is the only known substrate for CDK12 (13), we speculate that CTD Ser2P catalyzed by CDK12 provides a platform for counteracting heterochromatin enrichment (Fig. S6*E*). Nevertheless, emerging evidence has shown that CDK12 contains the arginine/serine domain and is involved in RNA processing (32, 33). In addition, CDK12 has been implicated in mRNA splicing (15, 34, 35). However, from our data, we cannot exclude the possibility that the arginine/serine domain of CDK12 also contributes to heterochromatin remodeling. Additional research is needed to better understand the mechanism of heterochromatin enrichment on CDK12 depletion.

The mechanism of epigenetic regulation on neuronal and behavioral plasticity remains an intriguing issue in neuroscience. So far, ~30 chromatin regulators have been implicated in mental retardation and psychiatric disorders (36). Moreover, the pathology of Alzheimer's disease is linked to alterations in heterochromatin status (37). In this study, we provide evidence that heterochromatin

enrichment induced by CDK12 knockdown hinders the transcriptional activation of neuronal genes, leading to a defect in courtship learning (Fig. S6*E*). Previous reports show that large facultative heterochromatin domains are prominent in the *Drosophila* BG3 neuronal cell line (9), and our data show that heterochromatin enrichment mainly occurs on neuronal genes, especially on long genes encoding ion channel subunits. A partial explanation for these observations is that the facultative heterochromatin domain acts in a dynamic equilibrium to fine tune the transcription elongation rate of RNAPII to ensure RNA processing on long neuronal genes with multiple exons (38). However, after heterochromatin accumulates, the transcription efficiency is greatly impeded, especially for genes that need to be expressed at high levels for specific neuronal processes. Therefore, after CDK12 depletion, those neuronal genes that are robustly up-regulated in the adult brain are the ones affected the most in our study. Because evidence shows that CDK12 affects the expression of long genes in mammals (14), it will be interesting to determine whether heterochromatin enrichment on long genes triggered by CDK12 depletion is conserved in vertebrates and affects important neuronal processes, such as learning or memory.

Materials and Methods

For total larval chromatin preparation, ∼3,500 third-instar larvae were collected. Larvae extracts were treated as described previously with some modifications (39). The details for fly strains, antibodies, immunostaining, ChIP-seq analysis, qRT-PCR, Western blot analysis, and courtship learning tests are described in *SI Materials and Methods*.

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