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The AaFoxA factor regulates female reproduction through chromatin remodeling in the mosquito vector Aedes aegypti

Yang Cheng[a,b], Xuesong Wang[c,d], Yike Ding[a], Houhong Zhang[a], Zhenyu Jia[d], and Alexander S. Raikhel[a,1]

Affiliations are included on p. 9.

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Female mosquitoes are vectors of many devastating human diseases because they require blood feeding to initiate reproduction. Thus, elucidation of molecular mechanisms managing female mosquito reproduction is essential. Although the regulation of gene expression during the mosquito gonadotrophic cycle has been studied in detail, how this process is controlled at the chromatin level remains unclear. Chromatin must be accessible for transcription factors (TFs) governing gene expression. A specialized class of TFs, called pioneer factors (PFs), binds and remodels closed chromatin, permitting other TFs to bind DNA and activate the gene expression. Here, we identified a homolog of the vertebrate PF FoxA in the mosquito *Aedes aegypti* and used the CRISPR-Cas9 system to generate mosquitoes deficient in *AaFoxA*. We found that ovary development was severely retarded in mutant females. Multiomics and molecular biology analyses have shown that AaFoxA increased histone acetylation and decreased methylation of H3K27 by controlling the chromatin accessibility of histone modification enzymes and chromatin remodelers. AaFoxA is bound to the loci of chromatin remodelers, changing their chromatin accessibility and modulating their temporal expression patterns. AaFoxA increased the accessibility of the ecdysone receptor (EcR) and E74 loci, indicating the important role of AaFoxA in the hormonal regulation of mosquito reproductive events. Further, the CUT&RUN and ATAC-seq analyses revealed that AaFoxA temporarily bound closed chromatin, making it differentially accessible during the mosquito gonadotrophic cycle. Hence, this study demonstrates that AaFoxA modulates chromatin dynamics throughout female mosquito reproduction.

pioneer factor | chromatin remodeling | histone modification | CRISPR-Cas9 | transcription

In the eukaryotic nucleus, genomic DNA is packaged into chromatin. For physiological processes to occur, DNA must be transcribed into RNA, which guides protein synthesis (1). However, the condensed structure of chromatin makes DNA inaccessible to transcription factors (TFs), limiting the formation of gene regulatory networks. To initiate transcription, this tightly packed heterochromatin must be opened. A specific class of TFs known as pioneer factors (PFs) can bind to heterochromatin, facilitating its opening. This process allows other TFs to attach to their respective gene regulatory sites and enhancers, ultimately activating transcription (2, 3).

PFs can remodel compacted chromatin and subsequently trigger transcriptional competency by maintaining chromatin accessibility (4). Various PFs achieve their pioneering functions through diverse molecular mechanisms. They possess distinct DNA-binding domains that allow them to scan motifs on the surface of a nucleosome, facilitating the engagement of other TFs with chromatin (5). Previous studies have shown that some PFs can evict histones, promoting chromatin accessibility. For example, Hepatocyte Nuclear Factor 3 (HNF3), a PF from the Forkhead box family, has a winged-helix DNA-binding domain structurally like histones H1 and H5. This similarity enables HNF3 to replace histones on nucleosomes, leading to chromatin opening (5, 6).

PFs can further target histone modifications to modulate the chromatin landscape. For instance, during the differentiation of macrophages and B cells, the PF PU.1 promotes the deposition of H3K4me1 (monomethylation of lysine 4 on histone 3) at enhancers, facilitating nucleosome repositioning and chromatin opening (7). The DNA-binding domain of OCT4, a key PF for maintaining pluripotency and reprogramming cells, interacts with the N-terminal tail of histone H3 and with H3K27ac (acetylation of lysine 27 on histone H3). This interaction enhances OCT4 binding to internal nucleosome sites (8).

Moreover, some PFs can initiate transcription by directly interacting with RNA polymerase II (Pol II) (9). For example, during the foregut development in *Caenorhabditis elegans*, the binding of PF PHA-4 to promoters recruits Pol II, leading to its accumulation near transcription start sites (TSSs) and triggering gene expression (10).

Significance

Understanding the molecular mechanisms that regulate female mosquito reproduction is crucial for managing vectors of harmful human diseases. We have a good understanding of gene expression regulation during the mosquito gonadotrophic cycle; the control at the chromatin level needs further clarification. Using multiomics approaches, we found that modification of the *AaFoxA* gene via CRISPR-Cas9 leads to reduced chromatin accessibility and reproductive disorders in *Aedes aegypti* mosquitoes. AaFoxA controls the temporal expression patterns of histone modification enzymes and chromatin remodelers by altering their genomic accessibility. The key hormonal regulator, Ecdysone Receptor (EcR), was significantly affected by AaFoxA. Thus, AaFoxA appears to act as a pioneer factor, temporally regulating chromatin dynamics during the reproduction of this important disease vector.

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[1]To whom correspondence may be addressed. Email: araikhel@ucr.edu.

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In *Drosophila melanogaster*, Fkh is a versatile factor in organogenesis, tissue regeneration, and longevity. Fkh regulates the expression of most salivary gland genes to maintain fate decisions and control the physiological activities of these glands (11). Cooperating with the TF Daughterless, Fkh prevents intestinal progenitors from precocious differentiation (12). *Drosophila* AKT and TOR have been shown to bind and phosphorylate Fkh, increasing nutrient uptake and a longer lifespan (13). FoxA is also involved in hormone-regulated processes. Human FoxA1 is a key determinant of estrogen receptor (ER) function; knockdown of FoxA1 blocks the association of ER with chromatin and estrogen-induced gene expression (14, 15).

Despite this progress, the mechanisms behind PFs' regulation of female reproduction in insects, especially vectors, have yet to be elucidated in detail. The *Aedes aegypti* mosquito is a significant vector insect involved in transmitting many human diseases, such as dengue fever, yellow fever, chikungunya fever, and Zika syndrome, threatening human health worldwide (16). Due to the lack of effective vaccines and increasing insecticide resistance, control of the mosquito population by inhibiting reproduction is considered a promising strategy for disease prevention (17–19). Previous research has revealed that 20-hydroxyecdysone (20E) plays a critical role in female reproduction (20). After blood feeding, 20E controls gene expression by the ecdysone receptor (EcR) and a suite of ecdysone-induced proteins (17, 21). Although the downstream signals of EcR are well studied, it remains unclear how this critical TF is regulated.

In this study, we used the CRISPR-Cas9 genomic editing method to demonstrate the essential role of AaFoxA in regulating female reproduction in *A. aegypti* mosquitoes. Our RNA-seq and ATAC-seq data revealed that AaFoxA influences the accessibility of histone modification genes and chromatin remodelers, thereby controlling their expression patterns. Additionally, using the CUT&RUN technique, we showed that AaFoxA binds to closed chromatin regions, enhancing its accessibility. This finding underscores the role of AaFoxA as a pivotal factor during the female mosquito reproductive cycle. Consequently, our study contributes to a deeper understanding of gene regulatory mechanisms.

Results

AaFoxA Is Highly Expressed at PBM24h in the Female Mosquito Fat Body. We searched for FoxA homologs in the *Ae. aegypti* mosquito genome and found two genes (AAEL003173 and AAEL003163) that contained the Forkhead domain. They were designated as *AaFoxA1* (AAEL003173) and *AaFoxA2* (AAEL003163). Because the expression level of *AaFoxA2* was extremely low, we focused on investigating *AaFoxA1* (thereafter *AaFoxA*). The amino acid sequence alignment showed that the Forkhead domain of AaFoxA presented a high level of similarity to those of *D. melanogaster, Mus musculus,* and *Homo sapiens* (*SI Appendix*, Fig. S1). We collected fat body tissue at different previtellogenic (PE) and postvitellogenic (PBM) time points of the gonadotrophic cycle. We determined the relative transcript level of *AaFoxA* using real-time quantitative PCR (RT-qPCR). Results showed that the *AaFoxA t*ranscript level exhibited a high peak at PBM24h, coinciding with the highest level of vitellogenesis (*SI Appendix*, Fig. S2*A*). Most reproductive genes activated by 20E are highly expressed at this time point. (22, 23). We selected PBM24h, PBM36h, and PBM60h, as well as PE72h as a control, for studies involving RNA-seq, ATAC-seq, and CUT&RUN analyses.

To measure its protein levels, we generated an antibody against AaFoxA. The full length of the *AaFoxA* open reading frame (ORF) was cloned into a protein expression vector (*SI Appendix*, Fig. S3*A*), and the recombinant protein was expressed and purified successfully in the *Escherichia coli* system (*SI Appendix*, Fig. S3 *B* and *C*). The polyclonal antibody was produced and used for further experiments. The specificity of mosquito AaFoxA antibody was confirmed by a western blotting assay (Fig. 1*C*). We also checked the protein levels of AaFoxA at four time points using a western blotting assay. The results showed that the AaFoxA protein had the highest level at PBM24h and was lower at PBM36h and PBM60h (*SI Appendix*, Fig. S2*B*), consistent with RT-qPCR data on the AaFoxA transcript level. To examine the chromatin accessibility of AaFoxA, we used the ATAC-seq approach. We demonstrated that AaFoxA exhibited temporal chromatin dynamics corresponding to the major reproductive events in reproducing female mosquitoes with maximum accessibility at PBM24h (*SI Appendix*, Fig. S2*C*).

The *AaFoxA* CRISPR-Cas9 Mutation Results in Female Reproduction Disorders. To elucidate *AaFoxA's* function during female mosquito reproduction, we attempted to generate its knockout (KO) using CRISPR-Cas9. We introduced four sgRNAs into the *AaFoxA* coding sequence to generate genomic disruption (*SI Appendix*, Fig. S4*A*). Three hundred fifty-eight eggs were injected, and 91 of them hatched. In the G0 family, 13 died at the larval stage, eight died at the pupal stage, seven during eclosion, six at the adult stage, and 57 survived (22 males and 35 females) (Fig. 1*A*). All surviving adults were used to perform the self-crossing and generate offsprings for further experiments. Only heterozygous mutant mosquitoes were alive, while KO homozygosity was lethal. PCR amplification using genomic DNA as a template indicated that AaFoxA mutated individuals had smaller bands in addition to a regular band that was the same as in the wild-type (WT); this indicates that *Cas9* partially disrupted *AaFoxA* in combination with certain sgRNAs (*SI Appendix*, Fig. S4*B*). We designated this modification as the *AaFOXA* CRISPR-Cas9 mutation.

Further DNA sequencing of the smaller bands confirmed that sequence deletions and insertions occurred within the *AaFoxA* ORF (*SI Appendix*, Fig. S4*C*). To verify the *AaFOXA* CRISPR-Cas9 mutation efficiency, AaFoxA levels at both mRNA and protein were measured using RT-qPCR and western blotting, respectively, at PE72h, PBM24h, PBM36h, and PBM60h. The relative AaFoxA mRNA levels in mutant female mosquitoes were down-regulated significantly at all time points (Fig. 1*B*). Similarly, the AaFoxA protein level was lower in mutant individuals (Fig. 1*C*). Our tests have demonstrated that we have generated an AaFoxA functionally deficient mosquito line, which we used for the clarification of AaFoxA function.

We examined the development of ovaries in WT and mutant females after blood feeding. We found that it was severely retarded in *AaFoxA* mutant individuals at three time points during the vitellogenic periods (Fig. 1*D*). The average follicle size in mutants decreased to 16%, 23%, and 15% at PBM24h, PBM36h, and PBM60h, respectively, relative to WT (Fig. 1*E*). Consistent with ovarian defects, the *AaFoxA* mutation led to a dramatic reduction in egg deposition (37 versus 127 eggs per female) (Fig. 1*F*).

Another tissue essential for female reproduction is the fat body, a functional analog of the vertebrate liver. We stained lipid droplets from WT and mutant fat body and found that *AaFoxA* mutation decreased lipid level in mosquitoes (*SI Appendix*, Fig. S5). During the vitellogenesis of female mosquitoes, the fat body produces the yolk protein precursor vitellogenin (Vg) under the control of the 20E gene regulatory cascade (20, 21). Considering that the 20E pathway plays a pivotal role in female reproduction, we examined the effects of AaFoxA on the 20E receptor EcR. The results showed that in *AaFoxA* mutant mosquitoes, the *EcR* mRNA dropped to about 12% at PBM24h (Fig. 2*A*). We also

checked the expression of *Vg*, the downstream target of the 20E pathway and found the dramatic reduction in these mosquitoes (Fig. 2*B*). Western blotting results indicated that the *AaFoxA* mutation led to a significant reduction of both EcR and Vg at the protein level (Fig. 2*C*).

Next, we assessed the effect of AaFoxA on the expression of genes encoding the 20E gene regulatory cascade and vitellogenin. In the WT mosquitoes, expression of all genes belonging to the above categories peaked at PBM24h. At the same time, these gene levels diminished significantly in the *AaFoxA* mutant (Fig. 2*D*). Thus, the limited Vg supply in *AaFoxA* mutant female mosquitoes could contribute to developmental defects in their ovaries.

AaFoxA Temporally Activates the Accessibility of the 20E Regulatory Genes. To investigate further how AaFoxA regulated the expression of EcR, we compared genomic accessibility at the PE and PBM phases. ATAC-seq data showed that the *AaFoxA* mutation did not change the accessibility of *EcR* at PE72h. Still, it led to a dramatic reduction of EcR accessibility at PBM24h (Fig. 3*A*). CUT&RUN data revealed the binding of AaFoxA on the EcR cis-regulatory element (CRE) at PBM24h but not at PE72h (Fig. 3*A*). ATAC-seq and CUT&RUN data of E74B, a 20E-inducible early gene, indicated that AaFoxA bound to its CRE at PBM24h but not at PE72h to increase the genomic accessibility (Fig. 3*B*). Then, we retrieved 2 kb TSS upstream

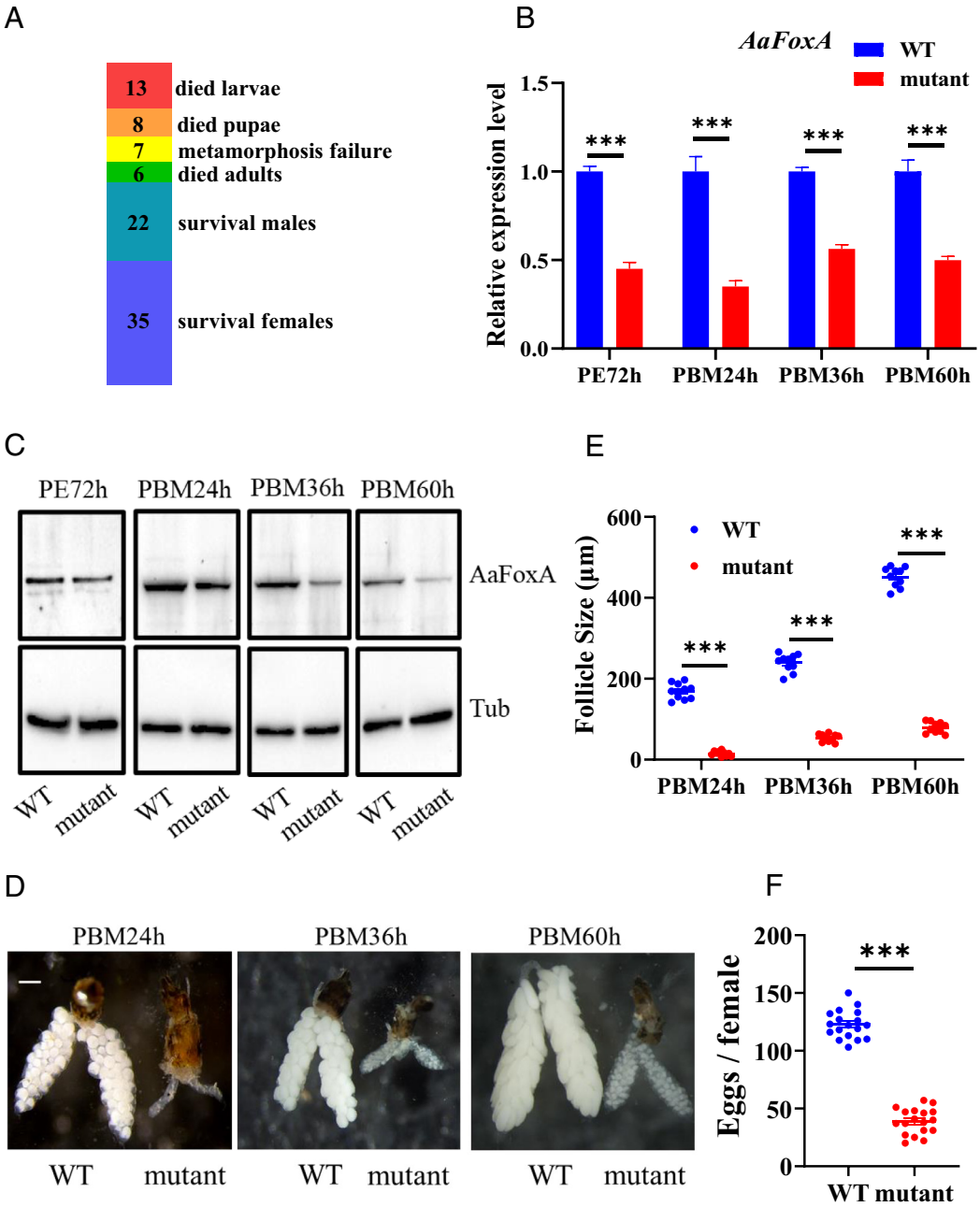

Fig. 1. Mutating *AaFoxA* by the CRISPR-Cas9 gene modification technique resulted in reproduction disorders in female mosquitoes. (*A*) Phenotype distribution of mutated individuals. The digits on each box indicated the number of individuals. Expression analysis of *AaFoxA* at different time points in wild-type (WT) and AaFoxA mutant female fat bodies at both mRNA (*B*) and protein (*C*) levels. *Rps7* and α-Tubulin were the internal controls for RT-qPCR and western blotting, respectively. (*D*) Phenotypes of ovaries in WT and mutant mosquitoes at different vitellogenic time points. (Scale bar, 1 mm.) (*E*) The follicle lengths of WT and mutant mosquitoes at different vitellogenic time points. (*F*) Egg deposition number of each WT or mutated mosquito. *B*, *E*, and *F* data were represented as mean ± SEM, ***$P < 0.001$.

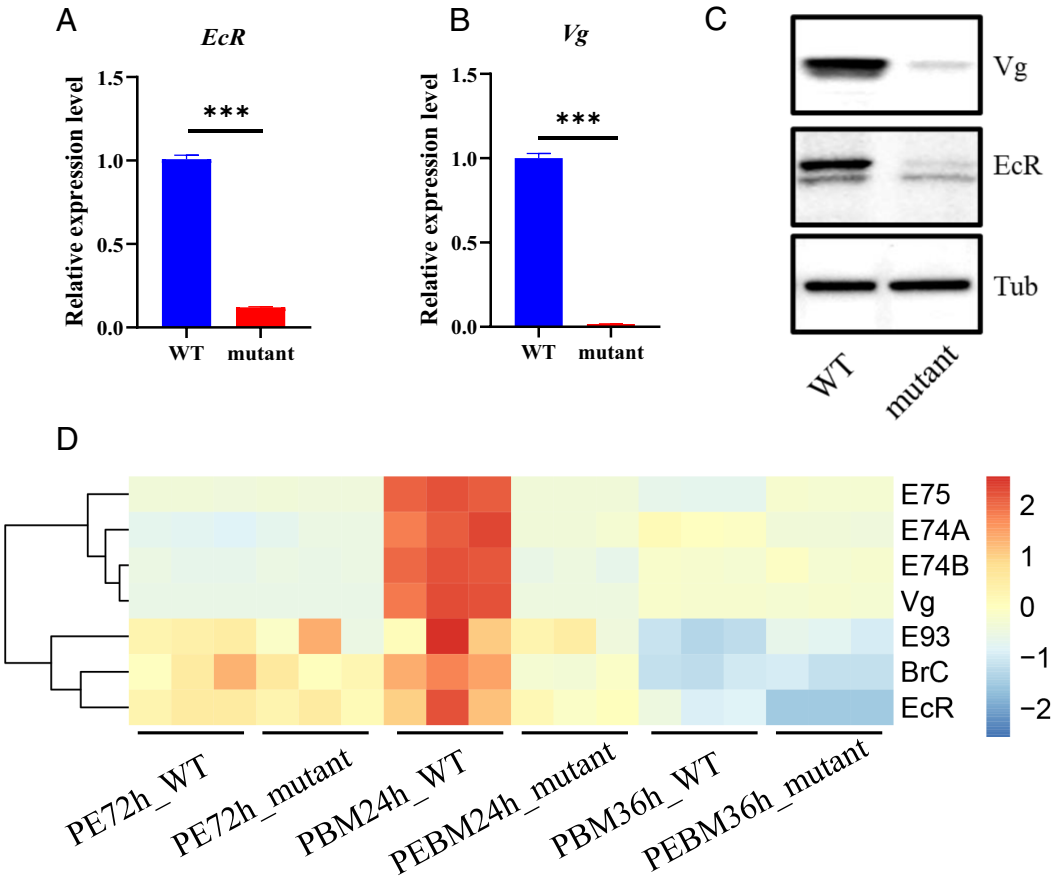

Fig. 2. The AaFoxA mutation inhibited 20E signaling in the female mosquito fat body. mRNA level of *EcR* (*A*) and *Vg* (*B*) in female fat body after the AaFoxA mutation at PBM24h. Data were shown as mean ± SEM, ***$P < 0.001$. (*C*) Protein levels of EcR and Vg in female fat body after the AaFoxA mutation at PBM24h. α-Tubulin was set as the internal control. (*D*) Hierarchical clustering analysis of 20E regulatory TFs and Vg in WT and mutated mosquitoes at different time points

sequences of EcR and E74B to predict the binding sites of AaFoxA using JASPAR online tool. The results showed 16 and 18 AaFoxA putative binding sites within these *EcR* and *E74B* TSS sequences, respectively (Fig. 3 *C* and *D*), suggesting the importance of AaFoxA binding sites for regulating these hormonal factors. The results demonstrated that AaFoxA activated the 20E signaling by controlling the genomic accessibility and expression of the critical genes in the ecdysone-regulatory pathway in a time-specific manner.

AaFoxA Regulates Chromatin Accessibility by Employing Histone Modifications. Previous reports on mammals have shown that FoxA functions as a PF (24, 25). Therefore, we examined the effect of the *AaFoxA* mutation on histone modifications during the vitellogenic period in *Ae. aegypti*. Western blotting results of the female fat body showed that in *AaFoxA* mutated mosquitoes at PBM24h, the level of H3K27 acetylation, which signifies transcriptional activation, was lower. At the same time, H3K27 trimethylation, a sign of gene repression, was higher (Fig. 4 *A* and *B*). That implied that AaFoxA functioned as a chromatin modifier, leading to gene transcription activation. To seek out the reasons why AaFoxA can contribute to histone modifications, we performed RNA-seq of fat body samples from WT and *AaFoxA* mutant mosquitoes at PE72h, PBM24h, PBM36h, and PBM60h. We selected the genes related to histone acetylation and methylation from the annotated RNA-seq libraries and generated a heatmap to show their expression profiles. We found that the expression levels of most transcription activation-related genes (including histone acetyltransferases and demethylases) were down-regulated in *AaFoxA* mutant mosquitoes. In contrast, most of the transcription repression-related genes (including histone methyltransferases and deacetylases) were up-regulated in these mosquitoes (Fig. 4*C*). This indicates that AaFoxA contributes to histone acetylation and methylation processes by modulating the expression of critical enzymes in histone modifications. To further confirm whether AaFoxA could regulate the expression of histone modification-related genes via chromatin accessibility, we selected two genes as examples of transcription activation and repression markers and checked their accessibility using ATAC-seq. The results showed that *KAT8* (histone lysine acetyltransferase 8, a transcription activation-related gene) became less accessible after the *AaFoxA* mutation at PBM24h, PBM36h, PBM60h, and *RMT1* (arginine methyltransferase-1, a transcription repression-related gene) was more accessible after the *AaFoxA* mutation at PE72h, PBM24h, PBM36h (Fig. 5). This indicated that AaFoxA could open or close gene loci to regulate their transcription temporally. We also performed the CUT&RUN assay to localize the binding sites of AaFoxA on chromatin. We found that AaFoxA was bound to the open region of *KAT8* at PBM24h and PBM36h, while it was bound to the open region of *RMT1* only at PE72h (Fig. 5). Thus, the CUT&RUN assay confirmed the temporal nature of AaFoxA binding.

Transcriptomic Analysis of the *AaFoxA* CRISPR-Cas9 Mutation Identified Genes Related to Chromatin Remodeling. To elucidate how AaFoxA regulated chromatin dynamics during the female mosquito gonadotrophic cycle, we performed RNA-seq of fat body samples from WT and *AaFoxA* CRISPR-Cas9 modified mosquitoes at PE72h, PBM24h, PBM36h, and PBM60h. The expression levels of all genes were calculated and normalized (Dataset S1). Notably,

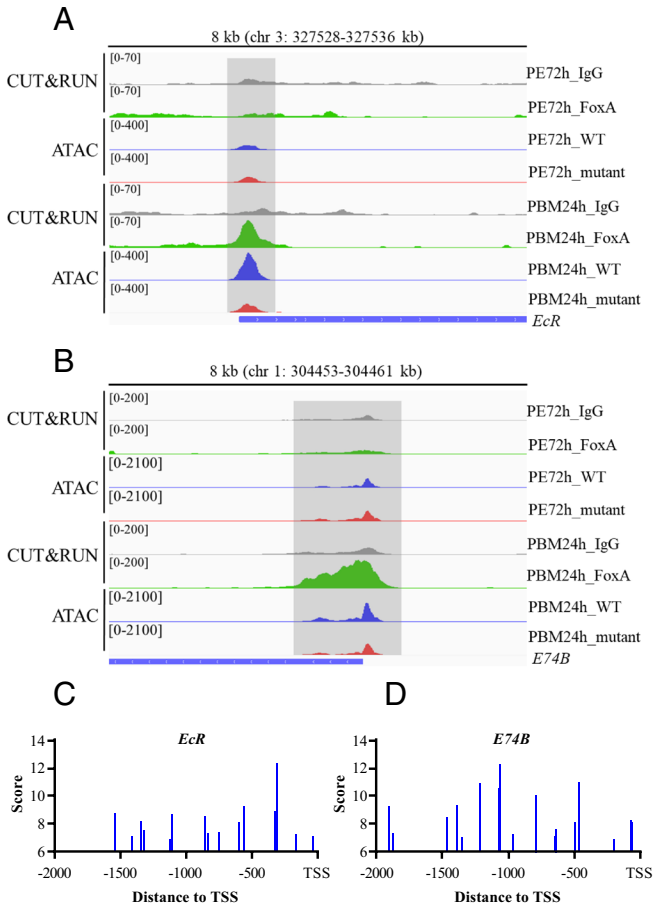

Fig. 3. AaFoxA bound to EcR and E74B CRE and increased their genomic accessibility. *AaFoxA* CUT&RUN and ATAC-seq signal tracks at *EcR* (*A*) and *E74B* (*B*) loci at PE72h and PBM24h. Differential chromatin accessibility or AaFoxA binding regions were highlighted with gray shading. AaFoxA binding motifs on *EcR* (*C*) and *E74B* (*D*) CREs.

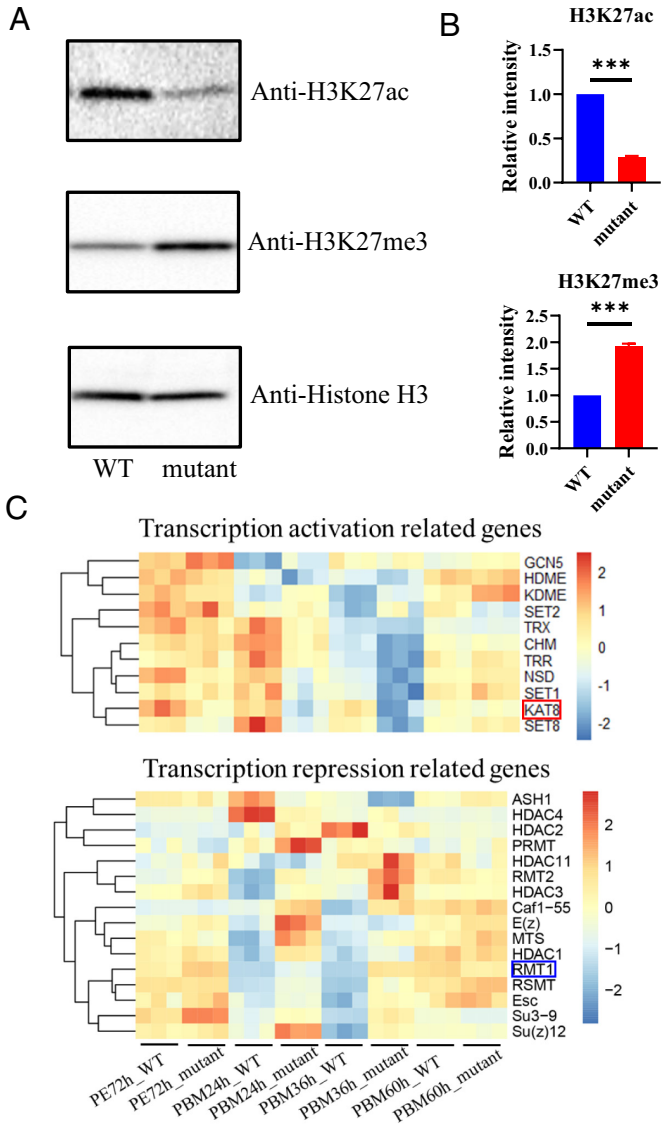

Fig. 4. The *AaFoxA* CRIPR-Cas9 mutation altered histone modifications in the fat body of *Ae. aegypti* female mosquitoes. (*A*) Protein levels of H3K27 acetylation (H3K27ac) and trimethylation (H3K27me3) after the *AaFoxA* mutation at PBM24h. Histone H3 was used as the loading control. (*B*) Bands in (*A*) were quantified using Image Lab software and normalized with Histone H3. Data were represented as mean ± SEM, ***$P < 0.001$. (*C*) Hierarchical clustering analysis of genes related to histone modifications in WT and at different time points of the reproductive cycle of *Ae. aegypti* female mosquitoes.

many gene cohorts were significantly affected by the AaFoxA mutation at PBM24h (1,857 up-regulated, 1,310 down-regulated) and at PBM36h (2,894 up-regulated, 2,100 down-regulated), while only a small number at PE72h (413 up-regulated, 583 down-regulated) and PBM60h (408 up-regulated, 651 down-regulated) (*SI Appendix*, Fig. S6). To perform a genome-wide examination of how the loss of AaFoxA impacts different categories of genes, we also analyzed RNA-seq data from four-time points comparing DEGs of WT vs. AaFoxA at PE72h, PBM24h, PBM36h, and PBM60h. Four thousand two hundred seventy-two genes differentially expressed in at least three of the four time points were plotted to the heatmap. The gene counts were normalized using DESeq2's size factors and scaled using z-scores for heatmap visualization. K-means clustering was then applied, resulting in distinct gene clusters. Cluster 1, containing 585 genes, was enriched for amide and peptide metabolic processes. Cluster 4, containing 417 genes, was enriched for amino acid metabolism and oxidation-reduction process. Cluster 6, comprising 692 genes, was associated with DNA metabolism and protein modification process (Fig. 6). We also performed KEGG enrichment of the DEGs at all four times. We found that most were enriched by metabolism-related pathways (*SI Appendix*, Fig. S7). These RNA-seq results have shown that AaFoxA plays an essential regulatory function in metabolism, which is critical for mosquito survival. Additionally, the regulation of 17 genes (7 up-regulated, 10 down-regulated) changed significantly at all four time points due to the *AaFoxA* mutation (*SI Appendix*, Fig. S8 *A* and *B*). Gene annotation revealed that two genes, out of these 17 genes, were involved in chromatin modifications: the mutation up-regulated gene encoding protein phosphatase 6 (PP6) and the mutation down-regulated gene encoding homeobox protein NK-2 (NK2) (Fig. 7*A*). Expression patterns of these two genes revealed that *PP6* was significantly up-regulated in the *AaFoxA* mutated mosquitoes. In contrast, *NK2* was down-regulated (*SI Appendix*, Fig. S9 *A* and *B*). These observations were consistent with the RNA-seq results. ATAC-seq data showed that *PP6* became more accessible, while *NK2* was less accessible after the *AaFoxA* mutation at PBM24h (Fig. 7 *B* and *C*). Notably, the CUT&RUN analysis demonstrated that AaFoxA binds to the upstream of target genes to open *NK2* but close *PP6* (Fig. 7 *B* and *C*).

To further verify the functions of *PP6* and *NK2* during mosquito reproduction, we performed the RNA interference (RNAi) knockdown of these two genes (*SI Appendix*, Fig. S9 *C* and *E*). Measurements of follicle size indicated that the RNAi knockdown of either *PP6* or *NK2* resulted in smaller ovaries and follicles at

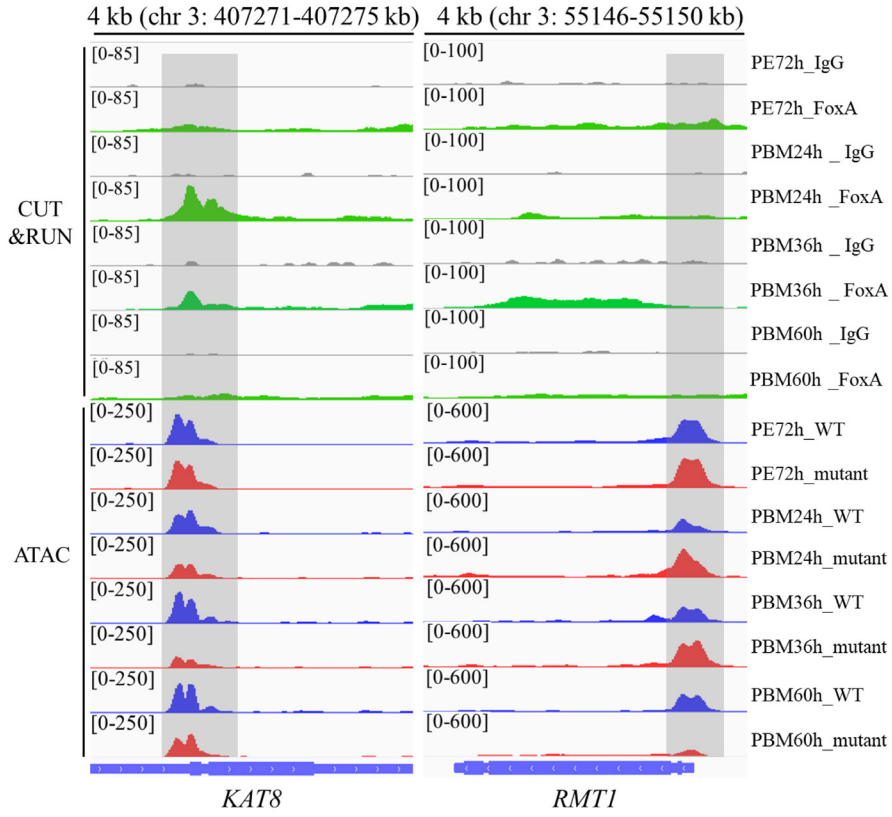

Fig. 5. Signal tracks of AaFoxA CUT&RUN and ATAC-seq at the *KAT8* and *RMT1* (two representatives boxed with red and blue in Fig. 4*C*) loci. Differential chromatin accessibility or AaFoxA binding regions were highlighted with gray shading.

PBM24h (*SI Appendix*, Fig. S9 *D* and *F*). Western blotting analysis demonstrated that PP6 up-regulated trimethylation and down-regulated acetylation of H3K27, while NK2 up-regulated acetylation without affecting trimethylation of H3K27 (Fig. 8 *A* and *B*). We also examined the changes of the histone-modifying enzymes (*KAT8* and *RMT1*) in *PP6* and *NK2* knockdown mosquitoes at PBM24h and found that *KAT8* was down-regulated. In contrast, *RMT1* did not change significantly with the knockdown of *NK2*. Neither *KAT8* nor *RMT1* did not show any significant differences following the *PP6* knockdown (*SI Appendix*, Fig. S9 *G* and *H*). That suggested that chromatin modifier NK2 could induce the expression of KAT8 to promote histone acetylation. Thus, our experiments suggest that AaFoxA remodels chromatin via control of chromatin modifiers such as PP6 and NK2.

AaFoxA Affects the Expression of TFs. We investigated the effect of AaFoxA on mosquito TFs at the genome-wide level. We gathered all the *Drosophila* TFs from the JASPAR database and obtained their *Ae. aegypti* homologs using protein BLAST analysis. In total, 253 TFs were attained from *Ae. aegypti* (*SI Appendix*, Table S1). Differentially expressed TFs were analyzed, and most of them were down-regulated in response to the *AaFoxA* mutation at PE72h (1 up-regulated and 19 down-regulated), PBM24h (8 up-regulated and 34 down-regulated), and PBM36h (21 up-regulated and 34 down-regulated); in contrast, at PBM60h, we observed nine up-regulated and seven down-regulated TFs (*SI Appendix*, Fig. S10*A*). These results infer that AaFoxA activates the expression of most TFs. The Venn diagram analysis showed that two TFs—AaFoxA itself and NK2—were down-regulated at all four time points (*SI Appendix*, Fig. S10*B*); this TF (NK2) could be induced by AaFoxA and participated in H3K27 acetylation (Fig. 8*A* and *SI Appendix*, Fig. S9*B*). To understand the AaFoxA function further, we analyzed whether AaFoxA is bound to the loci of TFs and modulated their genomic accessibility. The ATAC-seq and CUT & RUN data of some representative TFs showed that the *AaFoxA* mutation increased the accessibility of *Hey*, *Ftz-f1*, *Tj* and *Sug* at PE72h, PBM24h, PBM36h and PBM60, respectively, while decreasing the accessibility of *Dmrt99B*, *Cad*, *Blimp-1* and *Ro* at PE72h, PBM24h, PBM36h, and PBM60, respectively (Fig. 9 and *SI Appendix*, Fig. S11). AaFoxA bound to the closed region of *Hey* and *Dmrt99B* at the PE phase, PE72h. In contrast, it is attached to open regions of *Ftz-f1* and *Cad* at PBM24h (Fig. 9). These data imply that AaFoxA interacted with both open and closed gene chromatin regions to control the expression of TFs.

AaFoxA Is Involved in Remodeling Chromatin Accessibility. To understand the effect of AaFoxA on chromatin accessibility, we performed the correlation analysis of ATAC-seq peaks in all the WT and mutant samples. They were clustered into WT and mutant groups (*SI Appendix*, Fig. S12). Next, we counted the number of differentially enriched peaks between WT and mutant samples at each time point, and the results showed that down-regulated peaks were more abundant than up-regulated ones in mutant samples at both PE and PBM periods (Fig. 10*A*), indicating that AaFoxA l increases chromatin accessibility in female mosquitoes. Genomic distribution analysis showed that most of the peaks were located on introns, promoters, and intergenic regions (Fig. 10*B*), implying that AaFoxA remodels chromatin by changing the accessibility of regulatory sequences. We mapped the CUT&RUN peaks enriched by FoxA antibody to open or closed chromatin regions identified by ATAC-seq and found that AaFoxA tended to bind open chromatin at PE72h. In contrast, it bound to closed chromatin at PBM60h (Fig. 10*C*). That indicated that AaFoxA could bind to both open euchromatin and closed nucleosome-bound chromatin in a temporal- and content-dependent manner during the female reproductive cycle.

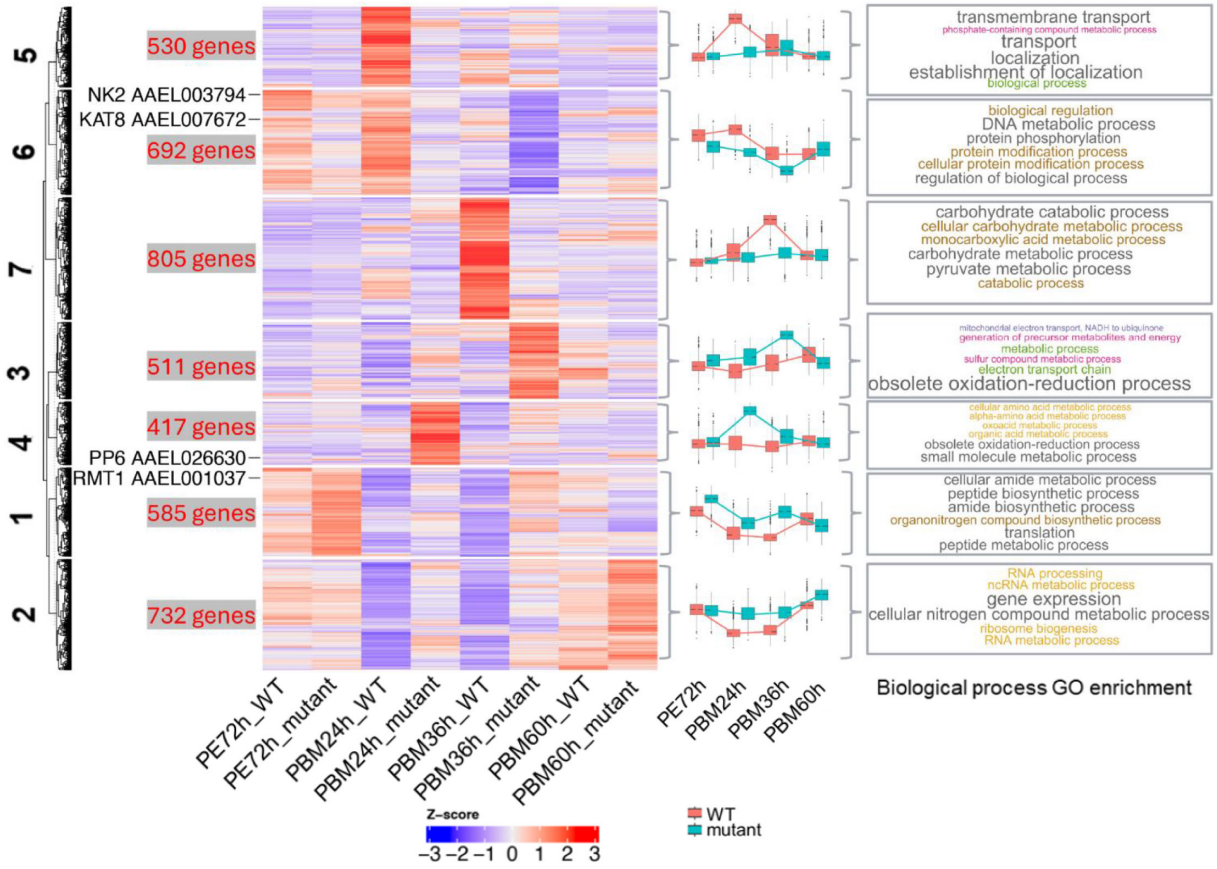

Fig. 6. Hierarchical clustering analysis of differentially expressed genes (DEGs) after the *AaFoxA* CRIPR-Cas9 mutation. DEGs were clustered into seven categories: heatmaps of all categories were shown on the left, expression patterns in the middle, and GO enrichments on the right. The four genes (KAT8, RMT1, PP6, and NK2) involved in this study were marked on heat maps.

Discussion

The PFs can bind and open the condensed chromatin, making it accessible for TFs for the genes located in the closed chromatin regions. Having accessible or open chromatin is the prerequisite for TF binding and transcription initiation, which is, in turn, essential for the differential expression of genes at the appropriate developmental stages. Our data have revealed that the AaFoxA motif shows the highest enrichment across most groups of accessible chromatin peaks during the PBM phase, indicating that AaFoxA plays an essential role during female reproduction. In this paper, we report that AaFoxA regulates genomic accessibility during the gonadotrophic cycle of female mosquitoes. AaFoxA controls histone modification–related genes and chromatin remodelers in the mosquito fat body. In addition, AaFoxA activates the 20E gene regulatory pathway by changing the genomic accessibility of EcR, the key TF in this pathway. Besides EcR, AaFoxA also modulates the expression of many other TFs, demonstrating the critical roles of AaFoxA in transcription regulation. Further CUT&RUN and ATAC-seq analyses have also revealed that AaFoxA binds to closed chromatin, proving its pioneering functions. Therefore, our investigations provide insights into how AaFoxA modifies the chromatin dynamics during the reproductive cycle of female mosquitoes.

A nucleosome, the basic structural unit of chromatin, consists of a segment of DNA wrapping around eight histone proteins. Previous studies have suggested that histone modifications can modulate transcription activities. Following methylation, the histone proteins H3K9, H3K27, and H3K20 function as transcription repressors. In contrast, the acetylation of H3K4, H3K9, and H3K27 leads these histone proteins to be transcription activators (26, 27). The AaFoxA mutation increased trimethylation and decreased acetylation of H3K27 at PBM24h (Fig. 4 *A* and *B*), implying that AaFoxA induced transcription by trimming methylation and adding acetylation to histone proteins.

Further RNA-seq analysis revealed that AaFoxA up-regulated the expression of histone acetyltransferase and demethylase genes and down-regulated the expression of histone methyltransferase and deacetylase genes (Fig. 4*C*). This suggests that the function of AaFoxA in histone modifications relied on controlling the expression of methylation and acetylation enzymes. KAT8 is a histone lysine acetyltransferase that catalyzes the acetyl coenzyme A-dependent lysine acetylation on histone and other proteins (28) and has essential roles in female mouse fertility (29). Our ATAC-seq data showed that the *AaFoxA* mutation decreased the genomic accessibility at the *KAT8* genomic region. The CUT&RUN analysis indicated the direct binding of AaFoxA to *KAT8* at PBM24h and PBM36h, to *RMT1* at PE72h (Fig. 5). RMT1, an arginine methyltransferase, critical for the establishment and maintenance of silent chromatin, inhibits gene transcription in *Saccharomyces cerevisiae* (30). Mutating of AaFoxA increased the genomic accessibility at the *RMT1* promoter regions (Fig. 5). These results indicate that AaFoxA stimulates gene transcription by increasing the accessibility of transcription activators and decreasing the accessibility of transcription repressors temporally.

Chromatin remodelers influence gene expression programs by controlling access to genomic DNA (31). We identified two chromatin remodelers (PP6 and NK2) from RNA-seq data and found that AaFoxA regulated them at both PE and vitellogenic stages of the female mosquito reproductive cycle (Fig. 7*A* and *SI Appendix*, Fig. S8 *A* and *B*). PP6 is a serine/threonine

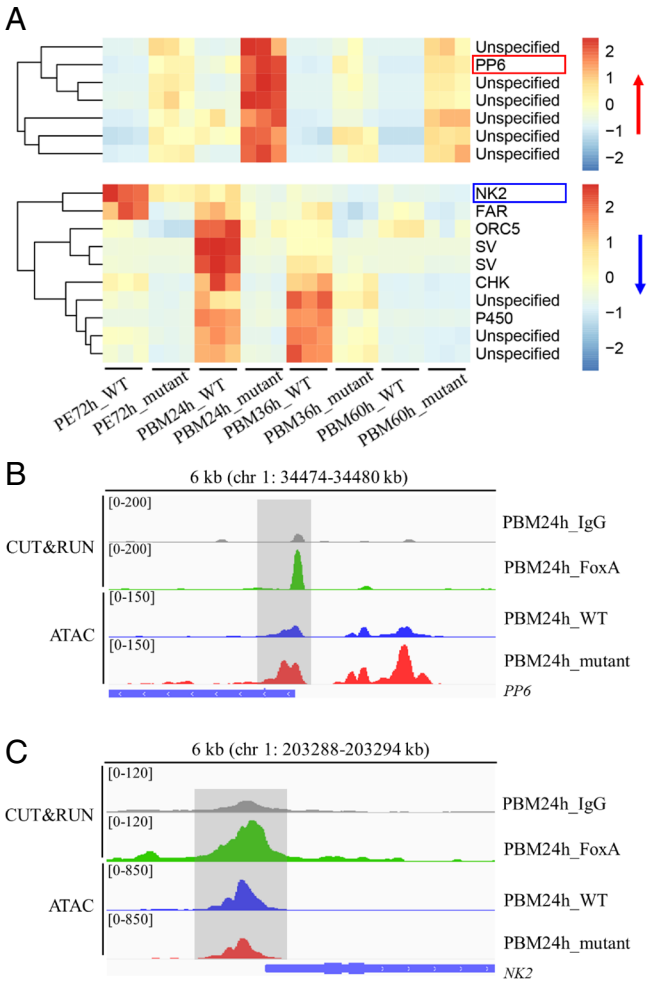

Fig. 7. Expression patterns of AaFoxA-regulated genes at different reproduction time points. (*A*) Hierarchical clustering analysis of the up-regulated and down-regulated genes at all four time points. Two genes related to chromatin modifications were boxed with red (PP6) and blue (NK2). AaFoxA CUT&RUN and ATAC-seq signal tracks at the *PP6* (*B*) and *NK2* (*C*) loci at PBM24h. Differential chromatin accessibility or AaFoxA binding regions were highlighted with gray shading.

phosphatase that plays critical roles in cell phase transitions, mitotic spindle formation, and chromosome segregation (32, 33). PP6 is also reported as the dephosphorylating factor in the activation of condensin I complex, which plays a vital role in chromatin folding, making it inaccessible (34, 35). Here, our multiomics data showed that AaFoxA inhibited the expression of *PP6* by decreasing its accessibility at the *PP6* CRE (Fig. 7 *A* and *B*). Furthermore, we demonstrated that PP6 increased trimethylation and decreased acetylation of H3K27 (Fig. 8 *A* and *B*), supporting its role in chromatin remodeling. NK2 is a homeodomain-containing TF that regulates the genes in developing ventral regions of the central nervous system in both *Drosophila* and mammals (36). NK2 interacts with HDAC1 (Histone Deacetylase 1) to control histone modification and the expression of downstream genes (37). We showed that NK2 changed the H3K27 acetylation level in female mosquitoes (Fig. 8 *A* and *B*). Moreover, AaFoxA bound to the *NK2* CRE and induced the transcription (Fig. 7*C*). Taken together, our data have demonstrated that AaFoxA increases chromatin accessibility by controlling the expression of chromatin remodelers.

Previous reports have proven that FoxA1 must associate hormone receptors (ER, androgen receptor, and glucocorticoid receptor) with chromatin and expression of downstream genes (14, 38,

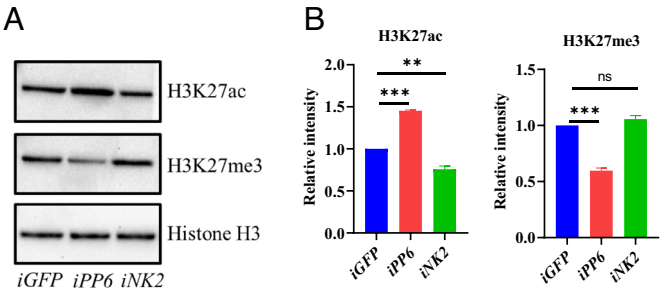

Fig. 8. Knockdown of *PP6* or *NK2* affected histone modifications. (*A*) Protein levels of H3K27 acetylation (H3K27ac) and trimethylation (H3K27me3) in fat body after *PP6* or *NK2* knockdown at PBM24h. Histone H3 was used as the loading control. (*B*) Bands in (*A*) were quantified using Image Lab software and normalized with Histone H3. Data were shown as mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$; ns, no significance.

39). 20E is the principal hormone that oversees insect development and reproduction via its nuclear receptor, EcR. Here, we demonstrated that AaFoxA was temporarily bound to the EcR CRE at PBM24h, opening the EcR locus to induce EcR expression (Fig. 3 *A* and *C*). E74 is an early 20E-responsive gene that activates Vg and plays a critical role in mosquito reproduction (40). In our study, we reveal that AaFoxA can mediate the genomic accessibility

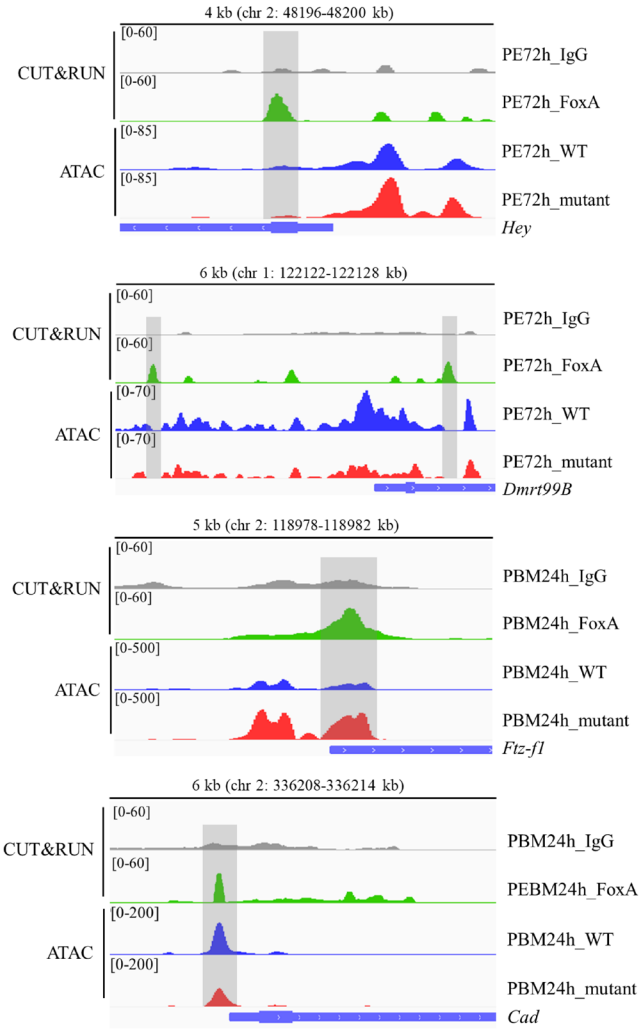

Fig. 9. The *AaFoxA* CRISPR-Cas9 mutation affected the expression patterns of TFs in female mosquitoes at PE72h and PBM24h. Signal tracks of AaFoxA CUT&RUN and ATAC-seq at specific TF gene loci. Differential chromatin accessibility or AaFoxA binding regions were highlighted with gray shading.

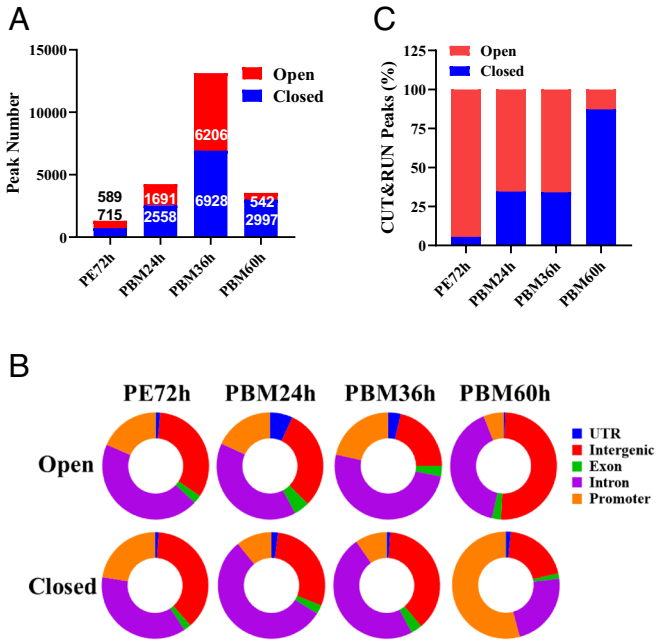

Fig. 10. AaFoxA remodeled chromatin accessibility at different reproduction time points. (*A*) Number of opening and closing peaks in *AaFoxA* mutant mosquitoes compared with WT. Digits marked on the columns indicated the peak numbers. (*B*) Genomic distribution of opening and closing peaks in *AaFoxA* mutant mosquitoes compared with WT. (*C*) Comparison of CUT&RUN peaks with open/closed chromatin at different time points.

of E74B (Fig. 3 *B* and *D*). This finding stresses the essential role of AaFoxA in governing the 20E hormonal network during the vitellogenic phase of mosquito reproduction.

In summary, our genome editing integrated with multiomics techniques demonstrated that AaFoxA binds to closed chromatin, suggesting its role as a PF. AaFoxA modulates genomic accessibility and controls the expression of both chromatin remodelers and TFs. These findings deepened our understanding of this factor's vital role in regulating chromatin dynamics and gene transcription during female mosquito reproduction.

Materials and Methods

Detailed *Materials and Methods* used in this study are described in *SI Appendix, Materials and Methods* (41–44). RNA isolation, RT-qPCR, expression and purification of recombinant AaFoxA, polyclonal antibody production, protein extraction, western blotting, CRISPR-Cas9-mediated AaFoxA mutation, identification of AaFoxA disruption, measurement of follicle size, dsRNA-mediated gene knockdown, RNA-seq, ATAC-seq, and CUT&RUN assay were performed. Primers used in this study are shown in *SI Appendix*, Table S2.

Data, Materials, and Software Availability. All the RNA-seq, ATAC-seq, and CUT&RUN data described in this manuscript are available under the BioProject accession number: PRJNA1219208 (45). All other data are included in the article and/or supporting information.

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Author affiliations: [a]Department of Entomology and Institute of Integrative Genome Biology, University of California, Riverside, CA 92521; [b]Department of Biotechnology, College of Bioscience and Biotechnology, Yangzhou University, Yangzhou 225009, China; [c]Interdepartmental Graduate Program of Genetics, Genomics and Bioinformatics, University of California, Riverside, CA 92521; and [d]Department of Botany and Plant Sciences, University of California, Riverside, CA 92521

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