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ORIGINAL ARTICLE

Genome-wide *Kdm4* histone demethylase transcriptional regulation in *Drosophila*

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Abstract

The histone lysine demethylase 4 (Kdm4/Jmjd2/Jhdm3) family is highly conserved across species and reverses di- and trimethylation of histone H3 lysine 9 (H3K9) and lysine 36 (H3K36) at the N-terminal tail of the core histone H3 in various metazoan species including *Drosophila*, *C.elegans*, zebrafish, mice and humans. Previous studies have shown that the Kdm4 family plays a wide variety of important biological roles in different species, including development, oncogenesis and longevity by regulating transcription, DNA damage response and apoptosis. Only two functional *Kdm4* family members have been identified in *Drosophila*, compared to five in mammals, thus providing a simple model system. *Drosophila Kdm4* loss-of-function mutants do not survive past the early 2nd instar larvae stage and display a molting defect phenotype associated with deregulated ecdysone hormone receptor signaling. To further characterize and identify additional targets of Kdm4, we employed a genome-wide approach to investigate transcriptome alterations in *Kdm4* mutants versus wild-type during early development. We found evidence of increased deregulated transcripts, presumably associated with a progressive accumulation of H3K9 and H3K36 methylation through development. Gene ontology analyses found significant enrichment of terms related to the ecdysteroid hormone signaling pathway important in development, as expected, and additionally previously unidentified potential targets that warrant further investigation. Since *Kdm4* is highly conserved across species, our results may be applicable more widely to other organisms and our genome-wide dataset may serve as a useful resource for further studies.

Keywords Kdm4 · Histone methylation · *Drosophila* · Development · Epigenetics · Histone

Introduction

The histone lysine demethylase 4 (Kdm4/Jmjd2/Jhdm3) family of histone demethylases is highly conserved across species and demonstrated to be a crucial regulator of various cellular processes. It reverses di- and tri-methylation of histone H3 lysine 9 (H3K9me2,3) and lysine 36 (H3K36me2,3) at the N-terminal tail of the core histone H3 in various metazoan species including *Drosophila*, *C.elegans*, zebrafish,

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mice and humans, although in *Arabidopsis*, its substrate was found to be histone H3 lysine 27 (H3K27) (Hillringhaus et al. 2011; Klose et al. 2006; Lu et al. 2011; Whetstine et al. 2006; Zhang et al. 2012). Various studies in different organisms have shown that Kdm4 family members play key roles in oncogenesis and development by its enzymatic activity towards H3K9me2,3 and H3K36me2,3. Furthermore, a recent study in *Drosophila* showed that it also catalyzes the demethylation of H3K56me3 in heterochromatin (Colmenares et al. 2017).

The Kdm4 family of demethylases was initially characterized in the context of tumorigenesis. It was found that *Kdm4C* is amplified in esophageal squamous cell carcinoma (Katoh and Katoh 2004) and *Kdm4A* overexpression was detected in various cancer cells (Gray et al. 2005). The first in vivo characterization of the biological role of *Kdm4* was in *C.elegans*, in which its depletion in the germline resulted in increased DNA damage and apoptosis, and further studies also demonstrated its role in DNA replication by targeting H3K9me2,3 and modulating heterochromatin protein 1γ (HP1γ) recruitment (Black et al. 2010, 2012; Whetstine et al. 2006). Multiple studies have linked the oncogenic potential of the Kdm4 family to its role as a co-activator of nuclear hormone receptor-mediated transcription. It was found that KDM4 interacts with the androgen receptor (AR) to mediate target gene activation by promoting the removal of the transcriptionally repressive H3K9me2,3 mark in prostate cancer cells and additionally playing a role in AR turnover (Coffey et al. 2013; Gaughan et al. 2013; Shin and Janknecht 2007; Wissmann et al. 2007). Similarly, studies in breast cancer cells found that KDM4 family members interact with the estrogen receptor (ER) and act as co-activators of target genes by removing the repressive H3K9me2,3 mark at target promoters and enhancers (Gaughan et al. 2013; Young and Hendzel 2013). Another study has described KDM4's oncogenic role in acute myeloid leukemia, where it transcriptionally activates *interleukin 3 receptor α* (*Il3ra*) and promotes survival (Agger et al. 2016). Furthermore, overexpression of various KDM4 family members resulted in defective DNA mismatch repair and genomic instability, thus suggesting another mechanism by which they contribute to tumorigenesis (Awwad and Ayoub, 2015).

Ample studies have also found that the Kdm4 family of demethylases plays key roles in stem cell differentiation and development. In murine embryonic stem cells, Kdm4C regulates self-renewal by removing H3K9me3 at the promoter of key stem cell regulator, *Nanog* to prevent the recruitment of transcriptionally repressive proteins, Heterochromatin Protein 1α (HP1α) and KRAB domain of KOX1 (KAP1) (Loh et al. 2007). Similarly, a different study demonstrated that conditional knockdown of *Kdm4a* and *Kdm4c* results in impaired embryonic stem cell self-renewal both in vivo and in vitro (Pedersen et al. 2016). In human mesenchymal stem cells, KDM4B regulates the transcriptional activation of *Distal-less* (*DLX*) to inhibit adipogenesis by H3K9me3 removal (Ye et al. 2012). Consistent with its ability to regulate stem cells, it also plays significant roles in organismal development. A study found that the sole rice *Kdm4* gene regulates the floral organ development phenotype by demethylation of H3K9me3 at the promoters of relevant key developmental genes (Sun and Zhou 2008). Furthermore, inhibiting Kdm4A during chick embryogenesis results in downregulation of various neural crest specification genes and increased H3K9me3 enrichment at the promoter of *Sox10*, a key regulator in neural crest regulation (Strobl-Mazzulla et al. 2010).

In *Drosophila*, two *Kdm4* family members have been identified and described to be functional H3K9me2,3 and H3K36me2,3 demethylases (Lloret-Llinares et al. 2008). Additional studies found that the interaction of Kdm4A with HP1a stimulates its activity towards H3K36 demethylation in vitro and that its overexpression in vivo results in male lethality with a concomitant decrease in bulk H3K36 methylation (Crona et al. 2013; Lin et al. 2008). Furthermore, *Drosophila* Kdm4A regulates lifespan and male-specific sex determination by transcriptional regulation of specific genes (Lorbeck et al. 2010). It has also been demonstrated that H3K9me2,3 demethylation by *Drosophila* Kdm4B controls the recruitment of factors involved in UV-induced DNA damage response (Palomera-Sanchez et al. 2010). A recent study described that following X-ray irradiation, Kdm4A mediates heterochromatic double-stranded DNA relocation by H3K56me3 demethylation and that Kdm4A loss-of-function, in combination with other double-stranded repair pathway mutation, impacts organismal survival (Colmenares et al. 2017). Kdm4A appears to additionally have a demethylase activity-independent role in heterochromatin organization (Colmenares et al. 2017).

Similarly to other organisms, *Drosophila* Kdm4 also appears to be important for development, as the loss-of-function of both *Kdm4A* and *Kdm4B* results in lethality in the early 2nd instar larval stage, with a molting defect phenotype associated with deregulation of the Ecdysone nuclear hormone receptor signaling cascade (Tsurumi et al. 2013). Given the important role of the Kdm4 family, in this study, we aimed to further elucidate additional potential transcriptional targets of *Kdm4A* and *Kdm4B* double mutants at different stages of development leading up to the time of lethality. The therapeutic potential of Kdm4 inhibitors for the treatment of various cancers (Chin and Han 2015; Duan et al. 2015; Kim et al. 2014; Lohse et al. 2011; Ye et al. 2015) and as anti-viral agents (Liang et al. 2013; Rai et al. 2010) has been demonstrated, further highlighting the relevance and importance of identifying biological mechanisms pertinent to the Kdm4 family of histone demethylases. Taking advantage of the highly conserved *Drosophila*

system where there are only two redundant *Kdm4* family members, compared to mammals where there are five, we sought to identify transcriptional targets at various stages of development comprehensively by employing a genome-wide approach. The dataset generated from this study could potentially also serve as an additional resource for further studies.

Materials and methods

Fly stocks/genetics and RNA sample preparation

All crosses were carried out at 25 °C on standard cornmeal/agar medium. Fly stocks of $Kdm4A^{KG04636}$, $Kdm4B^{EY10737}$ and *Sco/CyO-GFP* lines were obtained from the Bloomington *Drosophila* Stock Center (Bloomington, IN). For creating the double mutant line, the *Kdm4A* and *Kdm4B* lines were recombined with *CyO-GFP* as the balancer chromosome. We outcrossed the mutants to W^{1118} to minimize background differences. W^{1118} larvae collected at comparable stages were used as the control. Eggs were laid on an apple agar plate with yeast paste and early 1st, late 1st and early 2nd instar larvae were synchronized by egg laying time and morphology. Homozygous larvae were selected based on the lack of the GFP marker. The larvae were washed twice with deionized water and total RNA was prepared using the RNeasy Plus Mini kit (Qiagen) according to the manufacturer's manual. RNA quality was assessed using the Agilent 2100 Bioanalyzer and the RNA 6000 Nano kit (Agilent Technologies Inc., Palo Alto, CA, USA).

Microarray analyses

To prepare microarray samples from the RNA isolated, 200 ng of total RNA was used to prepare biotin-labeled RNA using Ambion MessageAmp Premier RNA Amplification Kit (Applied Biosystems, Forster City, CA, USA). Briefly, the first strand of cDNA was synthesized using ArrayScript reverse transcriptase and an oligo(dT) primer bearing a T7 promoter. Then, DNA polymerase I was used (in the presence of *E. coli* RNase H and DNA ligase) to convert single-stranded cDNA into double-stranded DNA (dsDNA), which was then used as a template for in vitro transcription in a reaction containing biotin-labeled UTP and T7 RNA Polymerase to generate biotin-labeled antisense RNA (aRNA). Twenty µg of labeled aRNA was fragmented and 15 µg of the fragmented aRNA was hybridized to Affymetrix Drosophila Genome 2.0 Array Chips according to the manufacturer's manual (Affymetrix, Santa Clara, CA, USA). Array Chips were stained with streptavidin–phycoerythrin, followed by an antibody solution (anti-streptavidin) and a second streptavidin–phycoerythrin solution, performed by a GeneChip Fluidics Station 450. The Array Chips were scanned with the Affymetrix GeneChip Scanner 3000.

R version 3.1.3 was used for the subsequent analyses. GCRMA was used for the numerical conversion to expression intensity (Wu et al. 2004), using the R packages, Affy and EMA (Gautier et al. 2004; Gentleman et al. 2004; Servant et al. 2010). The end result yielded normalized log_2 expression intensity of each of the probe sets for each sample (Supplementary Material 1). Control probe sets were filtered, as well as those where the wild-type and respective mutant intensities all had log_2 intensity below the 2.5 threshold as determined appropriate by the histogram plot in EMA. After these filtering steps, the total number of probe sets included for subsequent analyses was 10,724. Pearson–Ward metrics were used for hierarchical clustering and heatmap plot of the top 2000 differentially expressed probe sets.

Functional assessment of down- and upregulated genes

The log_2 fold change of the *Kdm4* mutant over wild-type samples was determined. Further analyses of assessing stage-specific differences of mutants were conducted using a 2-fold change cutoff (Supplementary Material 3), as this cutoff corresponds to the top 15th percentile upregulated and downregulated log_2 fold change of all probes, which were found to be 1.01 and − 1.09, respectively. As expected, the probe set for *Kdm4A* (1635774_at) showed more than the cutoff of 2-fold downregulation in the *Kdm4* mutant samples at all stages investigated. The probe set for *Kdm4B* (1629788_at) is labeled as also targeting CG17724 that shares overlapping genomic regions and, therefore, not expected to provide a reliable quantification of *Kdm4B*.

Database for Annotation, Visualization and Integrated Discovery (DAVID), version 6.8 was used for functional annotation and assessing the top 20 Fold Enrichment of Gene Ontology terms of putative H3K9me3 and H3K36me3-regulated gene probe sets (Dennis et al. 2003). Classification of different analyses groups into "ecdysone-related" vs. "non ecdysone-related" loci was determined by GO terms related to the ecdysteroid hormone pathway, chitin-based processes, molting, instar larval/pupal development and metamorphosis, or by previously published results that found evidence of regulation by ecdysone signaling by a genome-wide approach using mutants or 20-hydroxyecdysone (20E) hormone responsiveness (Bechstead et al. 2005; Davis and Li 2013; Gauhar et al. 2009; Gonsalves et al. 2011). To test whether the "ecdysone-related" probe sets were significantly enriched for the analyses groups given the background pool of all the 10,724 probe sets used for analyses (Supplementary Material 2), a hypergeometric test was performed to calculate the *p* value.

Analysis of modENCODE H3K9me3 and H3K36me3 enrichment sites

In the modEncode database (modEncode Consortium et al. 2010), H3K9me3 and H3K36 Chromatin Immunoprecipitation-sequencing (ChIP-seq) data are available for Oregon-R whole organisms at developmental stage 14–16 h after egg laying (AEL) and during the 3rd instar larval stage (sample numbers 4939, 4950, 4952 and 4941). First, we determined enrichment loci that exist in the early embryonic stage that did not show enrichment in the larval stage, as putative Kdm4-demethylated sites through development. We therefore, analyzed these developmentally removed H3K9me3 and H3K36me3 sites and assessed overlaps with loci of differentially regulated transcripts in the early late 2nd instar transcriptome dataset, when we expect these differences to be most relevant.

RT-qPCR and ChIP-qPCR validation

Early 2nd instar *Kdm4* double mutant or W^{1118} control larvae were collected as described in "Fly stocks/genetics and RNA sample preparation" section. For RT-qPCR, total RNA was isolated using RNeasy Plus Mini kit (Qiagen), as described in "Fly stocks/genetics and RNA sample preparation" section. The SuperScript III First-Strand Synthesis System (Thermo Fisher) was used to generate cDNA, according to the manufacturer's manual, and subjected to Sybr Green qPCR. Expression values are shown as normalized values relative to *rp49*.

Primer sequences are as follows:

rp49 Forward: TCCTACCAGCTTCAAGATGAC.
rp49 Reverse: CACGTTGTGCACCAGGAACT.
spok Forward: CACTCGCTGCATAGTGGTAAA.
spok Reverse: CCGCCAAAGAGCTTGTGATA.
Eip71CD Forward: GGTGCTGGAAATCGACTATGA.
Eip71CD Reverse: CCTCATCGTGGTACAGAATCAA.
ImpE2 Forward: GGCGCTAGTGAACACATCTT.
ImpE2 Reverse: GAGTACTCTGGCTTGGCTAATG.
Eip78C Forward: CACCCAAGATGACCAGCTTAT.
Eip78C Reverse: CCATCGTCCAGTGTCAATGT.
scu Forward: TCGGTCGTCTGGATCTGACT.
scu Reverse: AACGTGCCCACGGTATTGAT.
forked Forward: CTTCTTTTTGCCCCGAAGGC.
forked Reverse: GAGTACTCTACGCGACACCG.
Cpr12A Forward: GATGGAACCGCTCGCTATGA.
Cpr12A Reverse: AAGACGGTGATGTAACGCCC.

For ChIP-qPCR, samples were crushed in 200ul of 1X PBS with a pestle. Formaldehyde was added to a final concentration of 1% and samples were incubated for 15 min in room temperature, then glycine was added to a final concentration of 0.125 mM and incubated for 5 min at room temperature. The sample was centrifuged at 4000*g* and washed 3 times with PBS-T (1X PBS, pH 7.6 with 0.3% Triton-X). Lysis Buffer (50 mM HEPES–KOH, 140 mM NaCl, 1 mM EDTA, pH 8.0, 1% Triton-X, 0.1% Sodium Deoxycholate, 5 mM PMSF, 1X PIC) was prepared and 200 µl was added to each sample. The cells were sonicated 8 times with 15 s pulses and with 1 min rest in between pulses at 4 °C, with a Branson S-450 Sonifier using a setting of 40% and output of 5. The samples were centrifuged at 13,000*g* for 2 min at 4 °C to remove cell debris. 1% input control was removed and the remaining chromatin lysate was incubated overnight at 4 °C in 4 µg of anti-H3K9me3 (Upstate 07-442) or IgG control. 20 µl of pre-blocked Agarose-A beads was added to each sample and incubated overnight at 4 °C. The beads were washed three times with Wash Solution (0.1% SDS, 1% Triton-X, 2 mM EDTA, 150 mM NaCl, 20 mM Tris–Cl pH 8.0), then in Final Wash Solution (0.1% SDS, 1% Triton-X, 2 mM EDTA, 500 mM NaCl, 20 mM Tris–Cl, pH 8.0) for 2 h at 4 °C. The beads were then centrifuged and the wash solution was removed. Then, the beads were incubated in 100 µl Elution Buffer 1% SDS, 100 mM NaHCO3) at 65 °C overnight to de-crosslink, followed by centrifugation and the QIAquick PCR Purification Kit (Qiagen) was used to purify the DNA from the supernatant. The purified DNA was then subjected to Sybr Green qPCR. Relative enrichment of H3K9me3 of target genes was calculated and normalized to *actin5C* proximal promoter region enrichment.

Primer sequences are as follows:

spok promoter Forward: GCAGACAGATGGATACGG TTAG.
spok promoter Reverse: CAGCCTTAGTAAATAGTT CTCAACATAC.
Eip71CD promoter Forward: AATCGGGAGAGGGAG AAAGA.
Eip71CD promoter Reverse: TTTCTACGCGAATGT GGAGAG.
ImpE2 promoter Forward: TCGAGTCAACAAGGAATG AGAG.
ImpE2 promoter Reverse: ACCAACTGTGCAGCGATT A.
Eip78C promoter Forward: CTTGTGTGGCTGCTGTTA TTG.
Eip78C promoter Reverse: CGAGTACTGGAGGCTCTA TCT.
scu promoter Forward: TTGCCTGCTCGAGGTAATTT.
scu promoter Reverse: GGGCTCCTATCATTGGCT TAG.
forked promoter Forward: CTGCGTGGTAGAGTATTC ACAG.
forked promoter Reverse: AGCCCGAAATTATCCCAA AGA.

Cpr12A promoter Forward: AGTTAGCTGGCTTATTGC TAGG.
Cpr12A promoter Reverse: TATCCGAAAGGGTGACTG AGA.
actin5C proximal promoter Forward: ATTCAACACACC AGCGCTCTCCTT.
actin5C proximal promoter Reverse: ACCGCACGGTTT GAAAGGAATGAC.

Results

Kdm4 double mutants show progressively increased number of differentially regulated genes

We conducted hierarchical clustering with the top 2000 differentially expressed probe sets and found that in the early 1st instar larval stage, the wild-type and *Kdm4* double mutants clustered together. However, later in development, in the late 1st and early 2nd stages, wild-type and *Kdm4* double mutants separated out according to the genotype, suggesting that transcriptome pattern differences between the two genotypes become more apparent in the later stages of development (Fig. 1a). When comparing the number of probe sets showing 2-fold down- and upregulation compared to the wild-type during their respective larval stages, we found that the number of downregulated probe sets increased progressively and drastically from 482 in the early 1st, to 767 in the late 1st, and to 1685 in the early 2nd stage (Fig. 1b). The number of upregulated probe sets was similar between the early and late 1st instar larval stages (417 versus 413, respectively), but increased significantly in the early 2nd larval stage (1516) (Fig. 1b). For the early 1st down- and upregulated probe sets, there were more overlaps with the early 2nd instar (294 among downregulated and 165 among upregulated probe sets) compared to the late 1st instar (167 among downregulated and 83 among upregulated probe sets). This may be due to the cyclic nature of developmental signaling, in which there are common processes that are turned on in the earlier and later stages of each instar larval stage. However, in general, the most overlaps were found between the late 1st and early 2nd instar stages (502 among downregulated and 277 among upregulated probe sets). There were 128 downregulated probe sets that were shared between the three stages, whereas for the upregulated probe sets, there were only 63.

Since the ecdysteroid pathway-related developmental phenotype is most discernable, we focused on validating the select panel of genes relevant to this process (*spok, Eip71CD, ImpE2, Eip78C, scu, forked, Cpr12A*) that showed downregulation in the early 2nd instar larvae in the microarray results. We verified transcript levels by RT-qPCR (Fig. 1c) and, indeed, found more than 2-fold downregulation of all these genes in the early 2nd instar *Kdm4* mutants compared to the wild-type larvae, whereas in the early 1st instar stage, none was significantly more downregulated than the 2-fold threshold. We also assessed the promoter (within 1 kb upstream region) H3K9me3 enrichment of these genes by ChIP-qPCR and detected a concomitant increase in the repressive H3K9me3 mark in the early 2nd instar larvae (Fig. 1d).

Transcriptome alternations in *Kdm4* double mutants were detected for biological pathways relevant to development and other previously unlinked processes

The genome-wide approach found altered transcript levels of potential Kdm4 targets that have not previously been identified that may warrant further investigation. One such potential target is transposable elements, which were mostly found among the upregulated set, as compared to the downregulated set at each stage. In the early 1st instar stage, 2 among 482 downregulated probe sets mapped to transposable elements, compared to 5 among 417 in the upregulated set (Fisher's exact test $p=0.26$) (Supplementary Material 3). Significant differences could be detected in the late 1st instar stage, where no transposable elements were found among the 767 downregulated probe sets, while 4 among 413 were found in the upregulated set (Fisher's exact test $p=0.015$), and further difference was seen in the early 2nd instar stage where none of the 1685 downregulated probe sets mapped to transposable elements compared to 9 among the 1516 upregulated probe sets (Fisher's exact test $p=0.0012$).

Furthermore, we conducted Gene Ontology (GO) enrichment analyses and found that among significantly altered Biological Processes terms, the most prevalent terms for all the developmental stages investigated were those relevant to ecdysteroid signaling-mediated molting behavior, as expected from previously published phenotypes (Tsurumi et al. 2013). In the early 1st instar larval stage, GO biological processes terms relevant to the hormone biosynthesis pathway, molting behavior and cuticle development appeared among the top fold enrichment and significantly altered terms (Fig. 2a; Supplementary Material 4). Similar terms related to these pathways were also found to be significant in the late 1st instar larval stage among differentially regulated probe sets (Fig. 2b; Supplementary Material 4). Comparable with the findings in early and late 1st instar larvae, in early 2nd instar larvae, significant terms also included those relevant to the ecdysteroid hormone pathway, molting behavior and cuticle development (Fig. 2c; Supplementary Material 4). GO terms pertinent to development apart from ecdysteroid signaling-mediated molting behavior included those related to wing imaginal disc development in early 1st instar larvae, morphogenesis, cell polarity and various imaginal

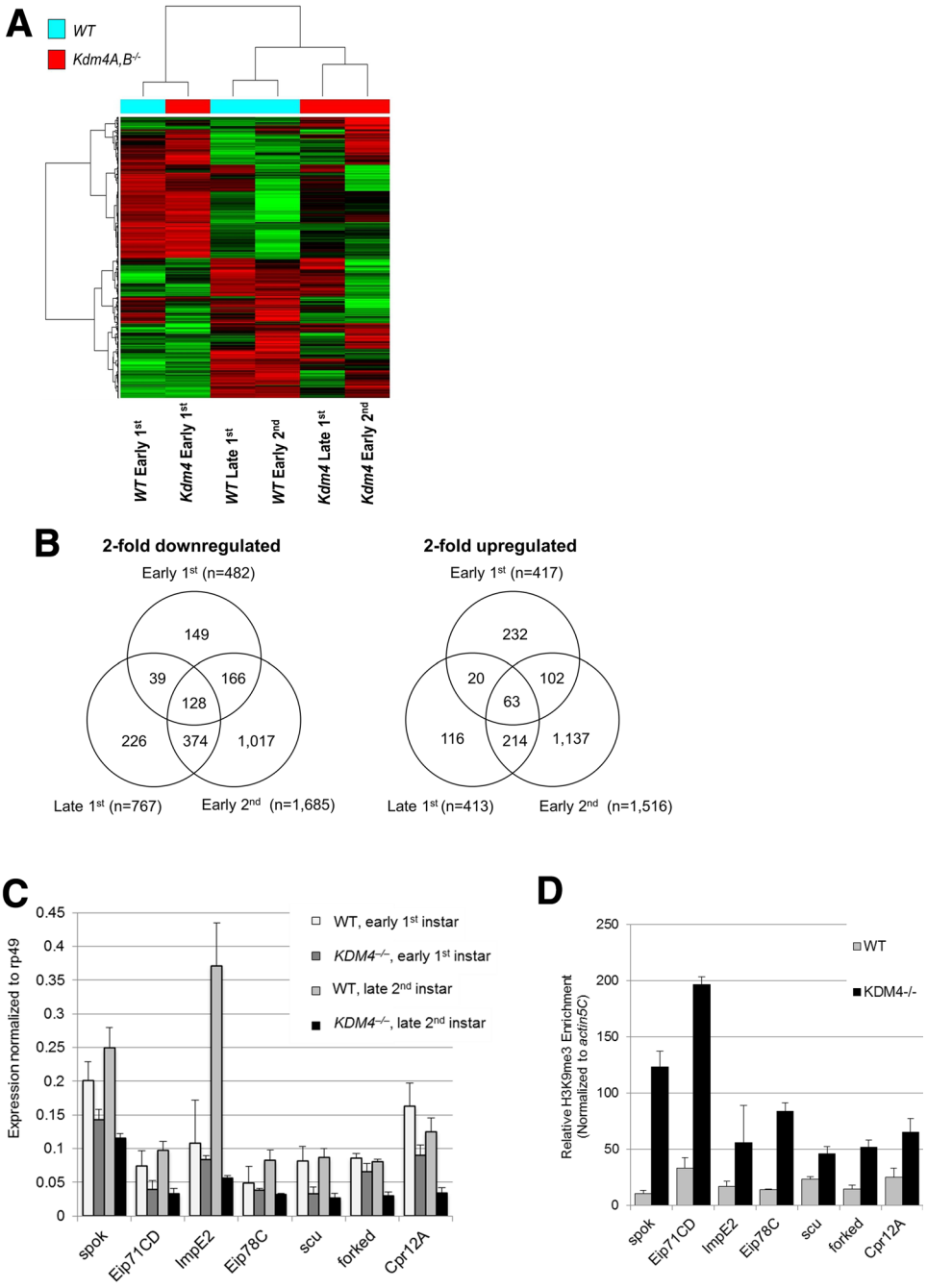

Fig. 1 *Kdm4* double mutants appear to have progressive accumulation of differentially regulated genes **a** Hierarchical clustering was performed using the top 2000 expressed probe sets among wild-type and *Kdm4A,B* double mutant larval samples at early 1st, late 1st and early 2nd instar larval stages to assess overall changes in gene expression. **b** The number of probe sets with above 2-fold change down- and upregulation compared to the wild-type was quantified and overlaps among the different stages were quantified. **c** RT-qPCR was performed to validate a subset of genes (*spok, Eip71CD, ImpE2, Eip78C, scu, forked, Cpr12A*) and found to be downregulated at least 2-fold in *Kdm4A,B* mutants compared to the wild-type during the 2nd instar larval stage, but not in the early 1st instar in the microarray. Expression values normalized to *rp49* are plotted. **d** ChIP-qPCR of the downregulated gene subset was performed to assess H3K9me3 in the early 2nd instar larval stage and relative H3K9me3 enrichment normalized to *actin5C* proximal promoter is shown

discs in late 1st instar larvae, and in addition to these terms, dendrite morphogenesis and eye photoreceptor development were also detected in early 2nd instar (Fig. 2a–c; Supplementary Material 4). In the early 2nd instar larval stage, "determination of adult lifespan" appeared among significantly altered GO terms (Supplementary Material 4), which is also consistent with a previous publication that reported that *Kdm4A* mutants have a shortened lifespan phenotype (Lorbeck et al. 2010).

Various GO terms relevant to DNA replication and the cell cycle were found in both early 1st and 2nd instar downregulated sets and additionally, terms relevant to mitosis were also significantly enriched in the early 2nd instar larvae. Among early 2nd instar larvae upregulated probe sets, GO terms relevant to male germ-line maintenance, telomere maintenance, double-stranded DNA repair, apoptosis and transcription also appeared as significant terms, which have all been implicated in mechanisms related to H3K9me2,3 and H3K36me2,3-dependent transcriptional/post-transcriptional regulation and heterochromatin organization (Fig. 2a–c; Supplementary Material 4).

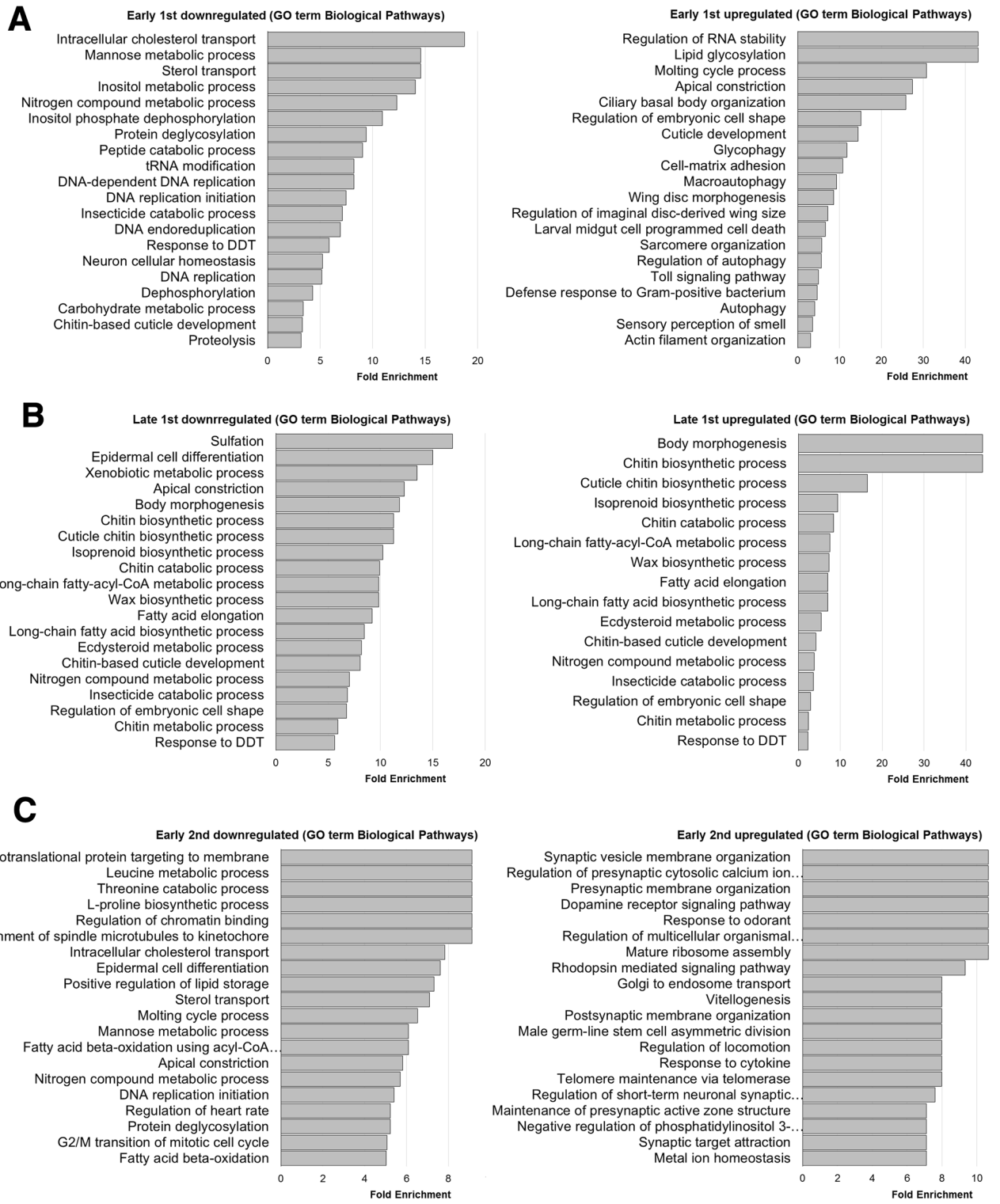

Fig. 2 Gene ontology analyses found significant enrichment in biological processes terms relevant to ecdysone signaling and other developmental processes and signaling cascades in *Kdm4* double mutants. GO enrichment analyses were performed using probe sets with at least 2-fold down- and upregulation in the **a** early 1st, **b** late 1st and **c** early 2nd instar larval stages, **d** common across the three stages, and biological process terms with top 20-fold change are shown in descending order

It may be interesting to note that terms relevant to immunity were found uniquely among upregulated genes in each of the developmental stages investigated, but not in the downregulated set. The molecular mechanism involving H3K9me2,3 in transcriptional silencing would suggest that the upregulated genes may be indirect effects. Upregulated gene sets were enriched with various GO terms relevant to the innate immune response and the Toll signaling pathway in all the three stages. GO terms pertaining to neuronal and sensory behavior, and odorant and light response were mostly found in the upregulated set in all the three developmental stages (Fig. 2a–c; Supplementary Material 4). There is also indication that signaling cascades other than ecdysteroid signaling may be impacted. GO terms identified among the early 2nd instar differentially regulated set include those for Toll and peptidoglycan recognition pathways, tyrosine receptor kinase (RTK), epidermal growth factor receptor (EGFR), phosphatidylinositol 3-kinase (PIP3K) and G-protein coupled receptor signaling pathways (Fig. 2c; Supplementary Material 4). GO terms relevant to metabolism were found frequently in both down- and upregulated sets in each of the developmental stages investigated (Fig. 2a–c; Supplementary Material 4). Further studies validating these GO term analyses would be beneficial.

We classified commonly down- and upregulated probe sets shared between the three developmental stages into "ecdysone-related" vs. "non ecdysone-related" loci by GO terms and previously published genome-wide studies (Bechstead et al. 2005; Davis and Li 2013; Gauhar et al. 2009; Gonsalves et al. 2011). When we classified the entire pool of probe sets used for the analyses (i.e., background set), we found 38.2% (4094 out of 10,724 total) to be "ecdysone-related" (Fig. 3a; Supplementary Material 2). Among the downregulated set, 61.7% (79 probe sets out of 128) were classified to be "ecdysone-related" and among the upregulated set, 52.4% (33 probe sets out of 63) were found (Fig. 3b; Supplementary Material 3). The enrichment of "ecdysone-related" probe sets was statistically significant for both groups ($p < 0.001$ and $p = 0.015$, respectively).

modENCODE ChIP-seq H3K9me3 and H3K36me3 developmentally regulated sites overlapped with various up- and downregulated genes

Taking advantage of the publicly available modENCODE database (modEncode Consortium et al. 2010) in which ChIP-seq data for H3K9me3 and H3K36me3 are available for Oregon-R early embryonic and 3rd instar larval whole animal samples, we postulated that genomic regions showing enrichment in early development that is later removed in the 3rd instar larval stage likely reflect loci that are demethylated by Kdm4 in the 2nd instar larval stage. Thus, failure of *Kdm4A,B* mutants to remove these marks may be one molecular mechanism that underlies the lethality phenotype. To further narrow down candidate direct targets of H3K9me3 separately from H3K36me3, we assessed overlaps between these developmentally demethylated H3K9me3 and H3K36me3 regions with corresponding altered transcripts. We evaluated downregulated probe sets for putative H3K9me3 targets and both down- and upregulated probe sets for putative H3K36me3 targets (Supplementary Material 5).

Various genes relevant to the EcR pathway, molting behavior, or cuticle formation were found among downregulated genes potentially regulated by Kdm4's H3K9me3 activity, including several *Ecdysone-inducible*

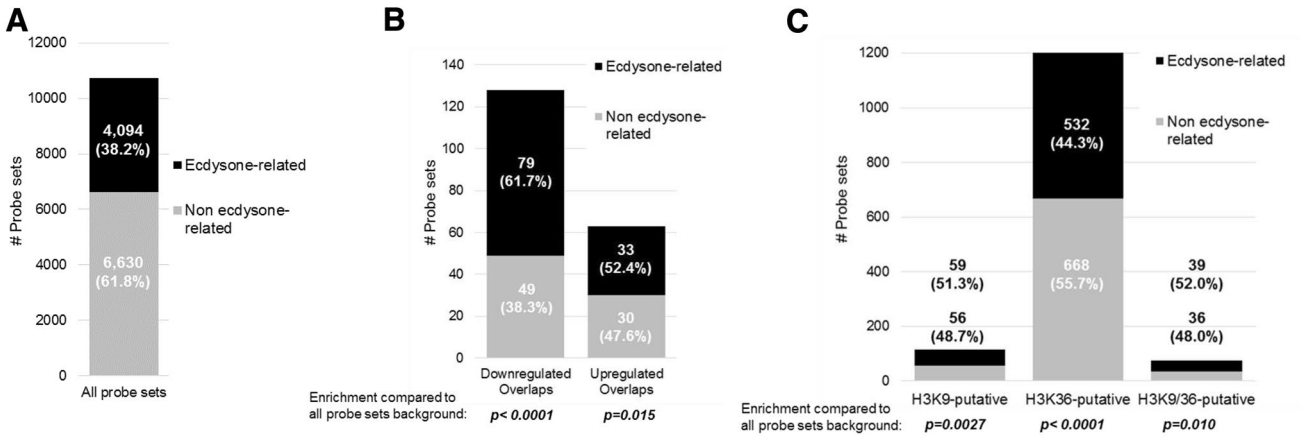

Fig. 3 Commonly altered transcripts and putative H3K9/K36me3 developmentally altered target regions are enriched with "ecdysone-related" genes. We classified **a** the background pool of all probe sets prior to analyses, **b** those commonly altered in the early 1st, late 1st and early 2nd instar larval stages, and **c** those representing potential H3K9me3 and/or H3K36me3 2nd instar stage target regions into "ecdysone-related" vs. "non ecdysone-related" genes. Statistical significance of enrichment of "ecdysone-related" genes was calculated using the hypergeometric test

(*Eip*), cuticle and ecdysone hormone biosynthesis genes (Table 1). For putative H3K9me3-regulated genes that were downregulated in the *Kdm4A,B* mutants, we also found several genes that perform critical cellular functions and required for cell viability, or for which loss-of-function mutation on their own leads to developmental lethality. Thus, they may also contribute to the nonviable *Kdm4A,B* phenotype (Supplementary Material 6; Apger et al. 2010; Cosgrove et al. 2012; Hernández et al. 2004; Jaspers et al. 2014; LaLonde et al. 2006; Larschan et al. 2007; Leshko-Lindsay and Corces 1997; Maynard et al. 2010; Ocorr et al. 2007; Ono et al. 2006; Schittenhelm et al. 2009; Schwed et al. 2001; Shtorch et al. 1995; Technau and Roth 2008; Torroja et al. 1998; Wang et al. 2003; Zhang and Ward 2010; Zhu et al. 2008). Since H3K36me3 is known to regulate post-transcriptional alternative splicing events, we considered both down- and upregulation of different isoforms as putative direct targets (Table 2). Noteworthy examples include multiple *Ecdysone-inducible* (*Eip*) and cuticle formation genes, and other highly relevant components, such as the ecdysone hormone biosynthesis enzyme, *shadow* (*sad*), *Ecdysis triggering hormone* (*ETH*) and *Hormone receptor-like in 38* (*Hr38*). We also assessed whether "ecdysone-related" probe sets were significantly enriched among the group representing putative H3K9me3, H3K36me3 and H3K9/K36me3 common targets. For the putative H3K9me3 target group, the proportion of "ecdysone-related" sites was found to be 51.3% (59 out of 115, $p = 0.0027$); among putative H3K36me3 targets, this proportion was 44.3% (532 out of 1200, $p < 0.0001$) and among putative H3K9/K36me3 common targets, it was 52.0% (39 out of 75, $p = 0.010$) (Fig. 3c; Supplementary Material 5).

Discussion

This study investigated the genome-wide transcriptional profile of mutants of the *Drosophila Kdm4* family of H3K9me2,3 and H3K36me2,3 dual demethylases. Considering the highly conserved function of Kdm4, as well as its substrates H3K9me2,3 and H3K36me2,3, the findings of this study may also be applicable to furthering the understanding of their in vivo functions in other organisms. Since there are only two members of the *Kdm4* family, *A* and *B* in *Drosophila* and the loss-of-function of both genes were found to have a drastic phenotype of lethality with molting defect associated with deregulated ecdysteroid hormone signaling cascade (Tsurumi et al. 2013), our findings are expected to be biologically relevant. Our study may provide insights into the regulatory role of Kdm4 in transcriptional activation by H3K9me2,3 demethylase activity and in post-transcriptional alternative splicing by H3K36me2,3 demethylase activity that may contribute to cellular events leading to the observed phenotypic outcomes (Fig. 4).

Drosophila Kdm4 has a role as a transcriptional co-activator for the ecdysteroid hormone receptor, analogously to the mammalian system involving KDM4 members as co-activators of the Androgen Receptor and Estrogen Receptor nuclear hormone signaling, thus further promoting studies using the simple *Drosophila* system (Gaughan et al. 2013; Shin and Janknecht 2007; Wissmann et al. 2007). *Kdm4A,B* double mutants are viable until the early 2nd instar larval stage, despite the ecdysone hormonal response being normally important also during the molting process to the 1st instar stage. It is well established that during *Drosophila* early development, maternal products are abundant and play essential roles. It may be likely

Table 1 Ecdysteroid pathway and molting behavior-related early 2nd instar downregulated putative H3K9me3 target transcripts

Probe set	Gene	Transcript
Downregulated		
1638505_at	*tan (t)*	CG12120-RA
1626830_at	*Larval cuticle protein 1 (Lcp1)*	CG11650-RA
1625276_a_at *Ecdysone-induced protein 28/29kD (Eip71CD)*	CG7266-RC	
1632561_at	*Laccase 2 (laccase2)*	CG30437-RC
1626695_at	*CG9518*	CG9518-RA
1625621_s_at *forked (f)*	CG5424-RB	
1634433_at	*Scully (scu)*	CG7113-RA
1622932_s_at *singed (sn)*	CG32858-RC	
1638601_at	*Spookier (spok)*	CG40123-RA
1634928_at	*Ecdysone-inducible gene E2 (ImpE2)*	CG1934-RA
1623060_at	*Cuticular protein 12A (Cpr12A)*	CG15757-RA
1640770_a_at *Ecdysone-induced protein 78C (Eip78C)*	CG18023-RA	
1638227_at	*Uninflatable (uif)*	CG9138-RA

Table 2 Ecdysteroid pathway and molting behavior-related early 2nd instar downregulated and upregulated putative H3K36me3 target transcripts

Probe set	Gene	Transcript
Downregulated		
1623060_at	*Cuticular protein 12A (Cpr12A)*	CG15757-RA
1623494_at	*Ecdysone-inducible gene E3 (ImpE3)*	CG2723-RA
1623668_at	*Shavenoid (sha)*	CG13209-RA
1624121_at	*Knickkopf (knk)*	CG6217-RA
1625470_s_at	*CG1520 (WASp)*	CG1520-RA
1625621_s_at	*Forked (f)*	CG5424-RB
1626235_at	*Miniature (m)*	CG9369-RA
1626485_at	*Shadow (sad)*	CG14728-RA
1626841_s_at	*Crinkled (ck)*	CG7595-RA
1626984_at	*Glucose dehydrogenase (Gld)*	CG1152-RA
1627051_at	*Larval cuticle protein 9 (Lcp9)*	CG16914-RA
1627571_at	*Cyclic-AMP response element binding protein A (CrebA)*	CG7450-RA
1628067_s_at	*Dusky-like (dyl)*	CG15013-RB
1628301_at	*Doublesex cognate 73A (dsx-c73A)*	CG32159-RB
1628930_at	*CG14485 (swi2)*	CG14485-RA
1628938_at	*Sec24CD ortholog (Sec24CD)*	CG10882-RA
1631375_a_at	*Ecdysone-inducible gene E1 (ImpE1)*	CG32356-RB
1631774_at	*Cuticular protein 66Cb (Cpr66Cb)*	CG7076-RA
1632378_at	*No mechanoreceptor potential A (nompA)*	CG13207-RA
1632561_at	*Laccase 2 (laccase2)*	CG30437-RC
1633919_s_at	*Lethal (3) malignant blood neoplasm (l(3)mbn)*	CG12755-RA
1634766_at	*Serrate (Ser)*	CG6127-RA
1637182_at	*CG9503*	CG9503-RA
1638610_at	*Neyo (neo)*	CG7802-RA
1638663_at	*Sec61 alpha subunit (Sec61alpha)*	CG9539-RA
1639979_at	*CG8927*	CG8927-RA
1640770_a_at	*Ecdysone-induced protein 78C (Eip78C)*	CG18023-RA
Upregulated		
1623084_at	*Shaggy (sgg)*	CG2621-RA
1623160_at	*Matrix metalloproteinase 1 (Mmp1)*	CG4859-RA
1623164_a_at	*Ecdysone-induced protein 75B (Eip75B)*	CG8127-RB
1628927_at	*CG17914 gene product from transcript CG17914-RB (yellow-b)*	CG17914-RA
1629747_at	*Cuticular protein 49Ag (Cpr49Ag)*	CG8511-RA
1630916_at	*MAP kinase kinase 4 (Mkk4)*	CG9738-RA
1631481_a_at	*Kruppel homolog 1 (Kr-h1)*	CG18783-RA
1631765_at	*Puckered (puc)*	CG7850-RA
1634405_s_at	*p21-activated kinase (Pak)*	CG10295-RC
1634573_a_at	*Grainy head (grh)*	CG5058-RC
1635128_a_at	*Absent, small, or homeotic discs 2 (ash2)*	CG6677-RA
1635331_at	*Cuticular protein 49Af (Cpr49Af)*	CG8510-RA
1635998_at	*Cuticular protein 67B (Cpr67B)*	CG3672-RA
1636202_s_at	*Ecdysone-inducible gene L2 (ImpL2)*	CG15009-RA
1636630_s_at	*Garnysstan (gny)*	CG5091-RA
1637421_at	*Chitinase 2 (Cht2)*	CG2054-RA
1639278_at	*Ecdysis triggering hormone (ETH)*	CG18105-RA
1639366_at	*Hormone receptor-like in 38 (Hr38)*	CG1864-RC
1639823_at	*Ebony (e)*	CG3331-RA

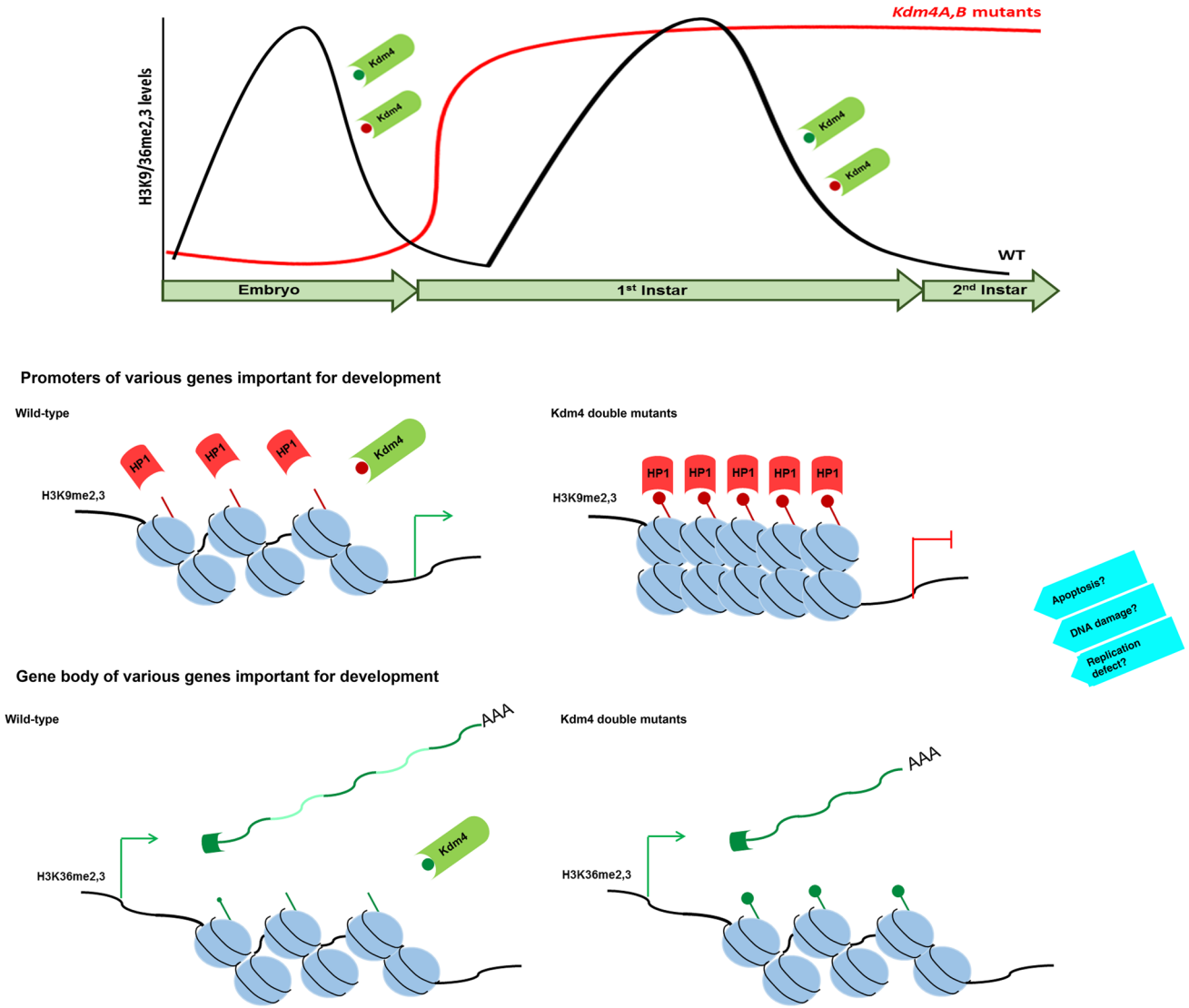

Fig. 4 Model of the transcriptional basis of lethality in *Kdm4* double mutants. In wild-type animals, Kdm4A and/or Kdm4B remove H3K9me2,3 at developmental gene loci to regulate transcriptional activation, whereas in the *Kdm4A,B* double mutants, H3K9me2,3 hypermethylation of direct target genes results in gene silencing by HP1a recruitment. In the gene body of target genes, in wild-type animals, Kdm4 removes H3K36me2,3 at appropriate exons to ensure correct splice site choice; however, *Kdm4A,B* double mutants are hypermethylated, resulting in improper and/or over-splicing events that result in mRNA without all the correct exons. Considering cellular events other than transcriptional control and alternative splicing regulated by Kdm4 that have been reported previously, it is also likely that additional mechanisms including apoptosis, DNA damage and defects in replication may also contribute to lethality

that maternal effects allow the *Kdm4* double mutants to undergo the first wave of hormone signaling, whereas in the later 2nd instar stage when maternal products become less abundant or no longer present, they are unable to complete the second wave of hormone signaling, contributing to lethality. Our results that found increased altered probe sets with each successive developmental stage may suggest that *Kdm4A,B* double mutants progressively accumulate H3K9me2,3 and H3K36me2,3 that eventually lead to transcriptional deregulation of a large number of genes, ultimately resulting in lethality. Most overlaps in deregulated probe sets were shared between the late 1st and early 2nd instar stages.

GO terms pertinent to the ecdysteroid hormone signaling and molting behavior were among those that appeared most frequently in our differential gene analysis. We also detected significant enrichment in probe sets that are relevant to this process among deregulated probe sets shared across the developmental stages. These observations are expected, given the specific phenotype associated with *Kdm4A,B* mutants. Our analyses also found various GO terms related to DNA damage, apoptosis and DNA replication, consistent

with observed *Kdm4* loss-of-function in both *C.elegans* and *Drosophila,* where previous studies have linked these phenotypes to deregulation of H3K9me2,3 and subsequent effect on HP1α/γ recruitment (Black et al. 2010, 2012; Palomera-Sanchez et al. 2010; Whetstine et al. 2006). Previous studies have also shown the role of H3K36 methylation in double-stranded break DNA repair (Jha and Strahl 2014). Considering the established role of H3K9me2,3 in heterochromatin, it is also feasible that *Kdm4A,B* mutants are susceptible to genomic instability, thus showing transcriptional changes in genes related to DNA damage. Our study also found a GO term relevant to adult lifespan, consistent with the observation that *Drosophila Kdm4A* mutant males have reduced lifespan (Lorbeck et al. 2010). *Drosophila Kdm4A* loss-of-function, in combination with loss-of-function of various double-stranded DNA repair component, also impacts organismal survival (Colmenares et al. 2017). HP1a also regulates lifespan (Larson et al. 2012) and thus our findings may indicate that altered H3K9me2,3 by *Kdm4* mutation may contribute to deregulation of genes related to lifespan. It was previously shown that HP1a and H3K9me2,3 are important for male germ-line stem cell maintenance in *Drosophila* (Xing and Li 2015) and analyses also found GO terms relevant in this process. Moreover, transposable elements were only found among the upregulated probe sets in the late 1st and early 2nd instar larval stages, and may reflect the dysregulation of heterochromatin maintenance as a result of *Kdm4* loss. Together with the likelihood that *Kdm4* mutants have altered DNA damage response, as suggested by our findings and shown in other studies, it may be one factor contributing to lethality. Future studies validating these findings will be advantageous.

The publicly available modEncode database provided the means to analyze H3K9me3 and H3K36me3 demethylated regions through the progression of the larval developmental program, where Kdm4 demethylase activity is likely to be responsible for removing these marks. When classifying probe sets commonly deregulated across developmental stages and putative H3K9me3 and H3K36me3-mediated direct targets into "ecdysone-related" versus "non ecdysone-related" genes in the 2nd instar stage, we found significant enrichment of "ecdysone-related" probe sets. The classifications were established by GO terms related to the ecdysteroid pathway and previously published genome-wide studies (Bechstead et al. 2005; Davis and Li 2013; Gauhar et al. 2009; Gonsalves et al. 2011). However, these studies investigated ecdysteroid pathway mutants during metamorphosis or treating cells with the 20E hormone and, therefore, additional validation studies are necessary. Furthermore, among this subset of downregulated and H3K9me3-relevant sites, we also detected various genes shown to have a developmentally nonviable phenotype involving other mechanisms, which may also be contributing to *Kdm4A,B* lethality. Taken these analyses together, our study suggests that ecdysone signaling may be a major molecular mechanism leading to lethality in *Kdm4* loss-of-function mutants, although other mechanisms are also likely to be involved. Thus, we propose that further studies will be beneficial for delineating these possibilities.

Recently, it was found that *Drosophila* Kdm4A has an additional histone residue target, H3K56me3, relevant to double-stranded DNA repair, as well as a demethylase-independent role (Colmenares et al. 2017). Mammalian KDM4B can function as a transcriptional co-activator of AR by catalyzing the removal of H3K9me3 at target gene promoters, meanwhile also enhancing AR protein stability by masking ubiquitination sites by protein–protein interactions, demonstrating its role independently of its catalytic activity (Coffey et al. 2013). It is possible that in *Drosophila,* there is also a similar Kdm4-EcR interplay that depends on both its catalytic role as a transcriptional co-activator and non-catalytic role of regulating protein stability. Conducting further meticulous genetic studies with catalytic-null versus whole gene knockouts and performing biochemical assays and protein–protein interaction studies would provide further important mechanistic understanding of catalytic versus non-catalytic roles of Kdm4.

It is also likely that additionally to its demethylase-independent function, non-histone catalytic targets of Kdm4 are also relevant. A study in human cell lines found that p53-KDM4A complex formation mediated by F-box22 (Fbxo22) promotes p53 degradation in a KDM4A demethylase activity-dependent manner, while ectopic expression of KDM4A catalytic mutant enhances p53's interaction with PHD Finger Protein 20 (PHF20) and leads to its stabilization in a demethylase-independent manner (Johmura et al. 2016). Studies have previously found *Drosophila Kdm4A* mutants to be susceptible to DNA damage due to its catalytic and non-catalytic functions (Palomera-Sanchez et al. 2010; Colmenares et al. 2017). Similarly, non-catalytic mechanisms may be relevant to our study on developmental lethality.

Various non-histone catalytic targets of JmjC-domain demethylases other than KDM4 have been found in mammals, including p53, DNA methyltransferase 1 (DNMT1), signal transducer and activator of transcription 3 (STAT3), myosin phosphatase target subunit 1 (MYPT1), and NFκB (reviewed in Zhang et al. 2012). G9a and EHMT1 H3K9me2 methyltransferases and the SETDB1 H3K9me3 methyltransferase add methyl marks on various signaling proteins such as p53, and epigenetic regulators including G9a itself and C/EBPβ (reviewed in Zhang et al. 2012). H3K36me2 methyltransferases, Nuclear Receptor Binding SET Domain Protein 1 (NSD1) and SET And MYND Domain Containing 2 (SMYD2) also have signaling protein targets including NFκB, p53 and Retinoblastoma protein (RB) (reviewed in Zhang et al. 2012). Taken together, it is feasible to postulate

that Kdm4 may also have an array of non-histone protein targets that may result in indirectly regulating transcriptional activity and post-transcriptional events, protein stability and protein–protein complex formation.

Our genome-wide approach uncovered previously unexplored putative *Kdm4* transcriptional targets that would be of interest to further validate. GO terms pertinent to neural and sensory behavior found in this study may represent a novel link between *Kdm4* and brain development. Various major signaling pathway cascades other than ecdysteroid signaling implicated from GO enrichment analyses include Toll, TOR, RTK, PGRP, EGFR, PIP3K and G-protein coupled receptor signaling pathways. Consistent with these signaling pathway terms, multiple GO terms related to immunity, cell growth and apoptosis were also suggested from our analyses. It is interesting to note that terms related to immunity were found among the upregulated set, suggesting a hyper-inflammatory state and that it may be due to indirect transcriptional de-regulation by *Kdm4* loss-of-function. Metabolism is also a previously unidentified *Kdm4* putative target, as suggested by our results. Since an established link exists between metabolism and heterochromatin (Bitterman et al. 2003; Meister et al. 2011), which Kdm4 regulates, it may indicate that *Kdm4A,B* mutants undergo altered metabolic states. Further investigation of these novel findings may aid in uncovering additional mechanisms relevant to Kdm4.

Since our aim was to elucidate putative Kdm4 targets in transcriptional/post-transcriptional events, we focused on H3K9me3 and H3K36me3 that previous literature suggests are most relevant. Taking into consideration the known molecular mechanisms of H3K9me2,3 and H3K36me2,3 in transcriptional and post-transcriptional processes, it is likely that heterochromatin-induced silencing by elevated levels of H3K9me2,3 would result in downregulation of direct target genes. As opposed to H3K9me2,3 which plays a role in transcriptional silencing, H3K36me2,3 regulates alternative splicing (Sorenson et al. 2016). Therefore, direct targets of Kdm4 with increased H3K36me2,3 may lead to altered expression of isoforms and reflected in both down- and upregulated transcripts. A direct approach to distinguishing between direct and indirect transcriptional/post-transcriptional targets genome-wide would be to conduct ChIP-seq analyses in the *Kdm4* mutants to identify regions with increased H3K9me2,3 levels and comparing to H3K36me2,3 enrichment. Alternatively, it would also be highly informative to conduct ChIP-seq of Kdm4A and Kdm4B pull-down, although limitations in available antibodies for wild-type protein pull-down, and artificial effects with transgenic overexpression of tagged Kdm4 present challenges. Furthermore, assessing Kdm4 non-histone targets or interacting proteins could provide additional insights into molecular mechanisms. Such further studies have immense potential to elucidate distinct H3K9 versus H3K36-specifc roles of Kdm4.

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Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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