Cis- and trans-regulation by histone H4 basic patch R17/R19 in metazoan development

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The histone H4 basic patch is critical for chromatin structure and regulation of the chromatin machinery. However, the biological roles of these positively charged residues and the mechanisms by which they regulate gene expression remain unclear. In this study, we used histone mutagenesis to investigate the physiological function and downstream regulatory genes of H4 residues R17 and R19 in Drosophila. We found all histone mutations including R17A/E/H and R19A/E/H (R17 and R19 of H4 are substituted by A, E and H respectively) result in a range of growth defects and abnormalities in chromosomal high-order structures, whereas R17E mutation is embryonic lethal. RNA-seq demonstrates that downregulated genes in both R17A and R19A show significant overlap and are enriched in development-related pathways. In addition, Western and cytological analyses showed that the R17A mutation resulted in a significant reduction in H4K16 acetylation and male offspring, implying that the R17 may be involved in male dosage compensation mechanisms. R19 mutation on the other hand strongly affect Gpp (Dot1 homologue in flies)-mediated H3K79 methylation, possibly through histone crosstalk. Together these results provide insights into the differential impacts of positive charges of H4 basic patch R17/R19 on regulation of gene transcription during developmental processes.

1. Introduction

Eukaryotic genomic DNA is packaged within the nucleus as a protein-rich complex known as chromatin. The nucleosome is the fundamental repeating unit of eukaryotic chromatin and consists of an octamer of histones with two copies of each of the histone proteins H2A, H2B, H3 and H4, around which approximately 146 base pairs of DNA are wrapped [1]. This structure not only permits compaction of the genome, but also serves as a regulatory mechanism for cellular processes such as transcription, replication and DNA repair [2–4]. Changes of chromatin, including the structure of DNA and its associated histone proteins, are key mechanisms by which transcription is regulated [5]. For example, histones are extensively decorated by covalent modifications that can affect chromatin compaction and thus influence the binding of transcription factors or recruitment of activators or inhibitors to promoters [2,6–8].

In addition to post-translational modifications (PTMs), which are major influencers of chromatin structures and functions, histones also contain basic and acidic regions, or ‘patches’, which govern nucleosome interactions and modulate their susceptibility to PTMs and their association with chromatin-binding proteins. The H4 basic patch, a positively charged patch in the H4 tail domain (residues 16-KRHR-19), contains multiple basic residues, i.e. arginine, histidine and lysine. This segment of basic residues plays an important role in regulating chromatin dynamics and multiple chromatin-modifying enzymes. For example, the H4 basic patch positively regulates the chromatin remodelers ISWI and
SNF2, which affects nucleosome sliding and positioning, and thereby allows regulatory proteins access to DNA [9–11]. In yeast, the entire H4 basic patch region is required for efficient Dot1-mediated histone H3K79 methylation and plays a key role in maintaining the balance of heterochromatin domains by interacting with the silencing protein Sir3 [12,13]. Recently, the H4 basic patch was shown to regulate SAGA-mediated H2B deubiquitination and histone acetylation [14]. Furthermore, full compaction of chromatin fibres and formation of higher-order chromatin structures require amino acids 14–19 of the H4 N-terminal tail [15]. Acetylation of H4K16 regulates higher-order chromatin organization [16–19] and is required for male X-chromosome dosage compensation, male viability and ovarian germ line stem cell maintenance [20]. However, it is poorly studied whether R17H18R19 affect chromatin structures or are involved in gene regulation in animals. Considering that H18 is a weakly positively charged residue, elucidating the biological roles of the strongly positively charged R17 and R19 is more meaningful for studying the chromatin regulation of the H4 basic patch.

In this study, we demonstrate the distinct functions of positive charges in the H4 basic patch during animal development using a combination of genetic animal models, biochemical analyses and transcriptome profiling. We show that the positive charge of R17 is essential for adult survival. By inducing charge-based mutations, we identified a potential cis-regulatory link between R17 and H4K16 acetylation, as demonstrated by the H4R17A mutation, which affects H4K16 acetylation and ultimately leads to a severe sex bias. We also identified a potential trans-regulatory pathway in which R19 is required for Dot1-mediated H3K79 methylation. Our charge-based mutation strategy provides new insights into the diverse roles of different positive charges in the H4 basic patch during animal development.

2. Material and methods

2.1. Fly strains and plasmids

H4K16A, H3K79A, ubi-GFP-FRT and HisD flies are available in our laboratory. Flies were grown at 25°C on standard food with 60% humidity and a 12 h light/dark cycle, or in vials placed in water baths at other indicated temperatures. Site-specific histone mutant transgenesis and fly genetics were performed as previously described [20].

The integration plasmid of attB 5xHis-GUs carrying the WT histone protein or H4R17A, H4R17H, H4R17E, H4R19A, H4R19H or H4R19E mutation was constructed using published procedures [20]. The human histone H4 CDNA sequence was used to generate wild-type constructions and H4 sequences with point mutations (R17A, R17H, R17E, R19A, R19H and R19E). The sequences were cloned into pCDH-CMV-MCS-Puro lentiviral vectors. To produce lentivirus, 293T cells were transfected with lentiviral vectors and helper plasmids (psPAX2 and pMD2.G). Supernatants containing lentivirus were collected, filtered, and concentrated at 72 h after transfection. Virus titre was determined before transduction.

2.2. Cell culture and generation of stable cell lines

Drosophila S2 cells were cultured in Schneider’s Drosophila Medium (Gibco) containing 10% fetal bovine serum (FBS, Gibco). 293T cells were cultured in Dulbecco’s modified Eagle medium (DMED, Gibco) with 10% FBS (Gibco). To generate cells stably expressing various histone mutants, cells were transduced with concentrated lentivirus (2 × 10^7 IFU). Transduced cells were grown under puromycin selection (2 μg ml⁻¹) for 48 h.

2.3. Antibodies

Primary antibodies against H3K4me3 (ab8580), H3K9me3 (ab8898), H3K27me3 (ab6002), H3K27me3 (Ab2621) and histone H3 (ab1791) were obtained from Abcam. Primary antibodies against histone H4 (07-108) were obtained from Millipore. Primary antibodies against H4K16ac (SC-8662-R) and LaminC (LC28.26) were obtained from Santa Cruz and DSHB, respectively.

2.4. Identification of lethal phases in histone mutants

Histone mutants were balanced over CyO-ActGFP to identify homozygous mutant embryos or larvae according to the absence of green fluorescent protein (GFP) expression. In total, 200 WT and 200 GFP-negative first instar larvae were collected on an apple-juice plate and tracked until adulthood. For each histone mutant, flies were counted at the second instar larval, third instar larval, pupal and adult stages. Experiments were performed in biological triplicate.

2.5. Developmental timing analyses

Histone mutants were balanced over CyO-ActGFP and allowed to lay eggs for 2 h on an apple-juice plate. The next day, homozygous mutant larvae lacking GFP expression were picked up in food vials. The number of animals that had pupariated was scored twice per day. Experiments were performed in biological triplicate.

2.6. Western blotting

Drosophila embryos or larvae were homogenized in phosphate-buffered saline (PBS). Protein loading buffer ( Takara, 9173) was added, and the mixture was heated for 5 min at 95°C. Protein samples were loaded onto a 15% SDS-PAGE gel for electrophoresis. After transfer to a PVDF membrane and blocking in 5% dried milk dissolved in tris-buffered saline containing 0.1% Triton X-100 (TBST), protein samples were incubated with primary antisera at 4°C overnight. After three washes with TBST for 10 min, membranes were incubated with secondary antisera from rabbit or mouse at room temperature for 1 h. After three washes with TBST for 10 min, and addition of enhanced chemiluminescence substrate, western blot signals were detected with a GE Amersham instrument.

2.7. DAPI staining of polytene chromosomes

Salivary glands of wandering third instar larvae were dissected in PBS, fixed in 45% acetic acid for 5 min, squashed and frozen in liquid nitrogen. Polytenes were stained with DAPI for DNA visualization. Micrographs were acquired using a Zeiss LSM880 inverted confocal microscope. Raw micrographs were processed with ImageJ.
2.8. Mosaic analysis and immunostaining

Eggs were deposited over 24 h at 25°C. Larvae were developed for 2 days and then heat shocked at 38°C for 1 h. L3 larva emerged 3 days after heat shock and were selected based on the lack of green balancer before dissection. Inverted larvae heads were fixed in PBS containing 4% paraformaldehyde (Sigma, 158127) for 20 min at room temperature. After washing with PBS, samples were permeabilized with PBS containing 0.3% Triton X-100 for 30 min at room temperature, blocked with PBS containing 1% bovine serum albumin and 0.03% Triton X-100 for 1 h and incubated with primary antibodies diluted in PBS containing 0.1% Triton X-100 overnight at 4°C. Samples were then incubated with secondary antibodies conjugated to Alexa fluorophores (Invitrogen) before DAPI staining. Thorough washes were performed between incubations. Imaginal discs were carefully removed and mounted with VECTASHIELD Mounting Medium (VECTOR, H-1200). Micrographs were acquired using a Leica SP8 inverted confocal microscope. Raw micrographs were processed with ImageJ.

2.9. RNA-sequencing and data analysis

Total RNA was separately isolated from embryos collected at 6–8 h and salivary glands of three instar larvae with TRIzol (Invitrogen, 15596-026). Sample quality was verified on an Agilent 2100 Bioanalyzer (Agilent). RNA-seq libraries were prepared using a NEBNext Ultra RNA Library Prep Kit for Illumina. RNA was sequenced on an Illumina NovaSeq 6000 system at Novogene. During the data-processing step, raw reads were filtered by removing adaptor sequences, reads shorter than 36bp, low-quality reads in which Phred Quality Scores of over 50% of bases is smaller than 20, and reads in which the percentage of unknown bases (N) was greater than 10%. The Q30 bases rate is about 93.5% after data pre-processing.

The cleaned data were aligned to the reference sequence using HISAT2 in default parameters. For alignment, a maximum of two mismatches were permitted. The *Drosophila melanogaster* genome and gene datasets were downloaded from FlyBase, which was used as a reference. To assess sequencing saturation, the number of genes identified was plotted against the number of cleaned reads to determine when no further genes could be detected by adding reads, which implied full saturation. To evaluate the quality of the RNA-seq dataset, the distribution of gene coverage in each sample was analysed. Gene expression levels were calculated using the Fragments Per Kilobase of transcript sequence per Million (FPKM) base pairs sequencing method. R package DESeq2 [21] was used to identify differentially expressed genes between two samples with biological replicates. In this approach, the \( \log_2(\text{FoldChange}) \geq 1 \) and FDR \( \leq 0.05 \) were used as the threshold of significance for differences in gene expression.

2.10. RT-qPCR

Total RNA was extracted with TRIzol following the manufacturer’s instructions and treated with DNase. In total, 1 mg RNA was reverse-transcribed using PrimeScript RT Master Mix (Takara, RR036A). Quantitative PCR analyses were performed with biological triplicates and technical duplicates.

2.11. GST-H4 binding assays

BL21 cells expressing GST (pGEX-4T-1) or GST-H4 tails (WT, H4R17A, H4R17E, H4R19A and H4R19E) were grown to mid-log phase, induced by treatment with 0.4 mM IPTG for 4 h at 25°C, and harvested. Cell pellets were lysed by sonication at 4°C in 200 µl lysis buffer (50 mM Tris-Cl at pH 8.0, 300 mM NaCl, 1 mM PMSF and 1 µg ml\(^{-1}\) each of leupeptin, aprotinin and pepstatin). Cell lysates were clarified by centrifugation at 14 000 rpm for 5 min at 4°C. The supernatants contained soluble protein. To isolate GST and GST-H4 tails, 10 µl of a 50% slurry of glutathione agarose (Sigma, G4510) in PBS was added to the soluble fraction and incubated for 1 h at 4°C. Thereafter, GST- and GST-H4 tail-bound agarose was pelleted, washed three times with lysis buffer for 5 min, and resuspended in 15 µl lysis buffer. Of this final slurry, 1.5 µl was analysed by SDS-PAGE to normalize GST and GST-H4 protein levels for binding reactions. *Drosophila* S2 cells were transfected with a plasmid bearing the Gpp-flag fusion, incubated for 3 days at 25°C and lysed by sonication at 4°C in 200 µl lysis buffer.

Binding reactions were performed by incubating 4 µl GST-H4 tail-bound glutathione agarose beads with 20 µl Gpp-flag lysates in a final reaction volume of 200 µl. The final buffer contained 50 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM PMSF and 1 µg ml\(^{-1}\) each of leupeptin, aprotinin and pepstatin. Ten microlitres of each reaction was removed as a control for Gpp input. Binding reactions were incubated with rotation for 2 h at 4°C. Beads were washed with a modified RIPA buffer (50 mM Tris at pH 7.4, 75 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1 mM PMSF and 1 µg ml\(^{-1}\) each of leupeptin, aprotinin, pepstatin) three times for 5 min. After washing, beads were resuspended in 12 µl of 4x SDS-PAGE sample buffer. Samples were loaded onto a 12% SDS-PAGE gel and probed for Gpp-flag using an anti-flag antibody (ABclonal, AE005).

3. Results

3.1. The positive charge of H4R17 is essential for development

Histone gene units (HisGU) reside in a tandem array in the *Drosophila* genome, and transgenic reintroduction of 20 copies of wild-type (WT) HisGU is sufficient to revert histone deficiency (HisD) to a WT phenotype [20]. To demonstrate the roles of the H4 basic patch in *Drosophila*, we used the previously described histone mutagenesis platform to induce charge-based mutations of R17 and R19 of H4 (R17 and R19 are substituted by neutrally charged alanine (A), positively charged histidine (H), and negatively charged glutamate (E), respectively), and obtained H4R17A, H4R17H, H4R17E, H4R19A, H4R19H and H4R19E mutants (figure 1a; electronic supplementary material, figure S1A, B) [20]. Each mutant had 20 identical amino acid substitutions in the diploid genome.
Molecular validation confirmed the genotypes of the mutant flies (electronic supplementary material, figure S1D). In adult progeny of experimental crosses obtained at 25°C, we did not obtain flies carrying the H4R17A or H4R17E mutation. However, approximately 30% of the first instar larvae of H4R17H...
survived to adulthood (figure 1b), indicating that the positive charge of R17 is essential for Drosophila development. Further examination revealed that a negatively charged substitution of R17 (H4R17E) resulted in embryonic lethality (figure 1b). Interestingly, the offspring of heterozygous parents comprised about 42% H4R19A, 51% H4R19H, and 9% H4R19E homozygous flies (figure 1b), suggesting that a positive charge at position 19 of H4 is, to some extent, dispensable for survival of Drosophila.

To further explore the biological function of R17/R19 in the H4 basic patch at the developmental level, we examined the weight and morphologies of third stage larvae at the same developmental period and quantified the developmental timing of H4R17 and H4R19 homozygous mutants. Comparison of age-matched larvae at 5.5 days after egg laying revealed that H4R17A mutants weighed less and had markedly smaller bodies than control larvae (20His-GUs) (figure 1c,d). Additionally, other mutants (H4R17H, H4R19A, H4R19H, and H4R19E) showed slight weight loss and smaller body sizes than control larvae. It took an average of 6, 7 and 8 days for 50% of control (20His-GUs), H4R17H homozygous mutant, and H4R17A homozygous mutant larvae to pupariate, respectively (figure 1e). These data indicate that the positive charge of R17 is more important than the positive charge of R19 for growth and development.

3.2. The positive charge of R17/R19 is essential for maintaining chromatin structure

According to the X-ray structure of nucleosomes [22], direct alterations of positive charges of histones can directly affect chromatin structures and thus influence gene transcription. To test whether loss of the positive charges of R17 and R19 affects chromatin structures, we cytologically analysed the polytene chromosome structure in salivary glands from H4R17A, H4R17H, H4R19A and WT flies. There were no detectable abnormalities in WT larvae (figure 2a). Notably, >90% of salivary glands (96/102) from third instar larvae with the H4R17A mutation contained structurally disorganized polytene chromosomes (figure 2a). Moreover, 40% (44/110) of H4R17H mutants had abnormal chromosome structures (figure 2a). In addition, we observed polytene chromosomal abnormalities in ~30% of H4R19A mutants. These results suggest that positive charges of the H4 basic patch, especially R17, are necessary for the maintenance of higher-order chromatin structures.

3.3. The positive charge of R17/R19 is essential for development-associated gene expression

To investigate the impact of genome-wide expression caused by disruption of the chromatin structure in H4R17A and H4R19A mutants, we performed RNA-seq by R17 and R19 affects chromatin structures, we cytologically analysed the polytene chromosome structure in salivary glands from H4R17A, H4R17H, H4R19A and WT flies. There were no detectable abnormalities in WT larvae (figure 2a). Notably, >90% of salivary glands (96/102) from third instar larvae with the H4R17A mutation contained structurally disorganized polytene chromosomes (figure 2a). Moreover, 40% (44/110) of H4R17H mutants had abnormal chromosome structures (figure 2a). In addition, we observed polytene chromosomal abnormalities in ~30% of H4R19A mutants. These results suggest that positive charges of the H4 basic patch, especially R17, are necessary for the maintenance of higher-order chromatin structures.

Transcriptome profiling revealed a partial co-regulatory link between R17 and R19 in the developmental pathway; therefore, we hypothesized that mutations that change the charges of these residues might affect the biological function of H4K16 due to neighbour steric hindrance effects [35]. H4K16 acetylation mediated by MOF–MSL complexes contributes to 2-fold transcriptional activation for X-chromosome dosage compensation [36]. To investigate whether the R17-R19 basic patch is involved in activation of X-chromosome genes in males, we first examined the male: female ratio of homozygous mutants. The H4R17A mutation was lethal in adults; therefore, we calculated the male: female ratio of third instar larvae. Interestingly, among randomly tested H4R17A mutant larvae, 17.5% (118/674) were male (figure 3a), yielding a male: female ratio of 1:5. Similarly, 12.9% of H4K16A mutant larvae were male (figure 3a).
However, the male: female ratio of R19 mutants (H4R19A, H4R19H and H4R19E) was approximately 1:1 (figure 3a). The male lethality observed with the H4R17A mutation is consistent with the 80% male adult lethal phenotype that we previously described with the H4K16A mutation [20]. To further investigate how mutations that change the charges of the basic patch affect male viability, we examined H4K16ac levels in third instar larvae with R17 (H4R17A and H4R17H) and R19 (H4R19A, H4R19H and H4R19E) mutations by western blot analysis. The H4K16ac level was approximately 75% lower in the H4R17A mutant than in the control (20His-GUs), whereas the compensatory substitution mutation to positively charged H (H4R17H) restored the H4K16ac level to 60% of that in the WT (figure 3b). However, the three charge mutations of R19 (H4R19A, H4R19H and H4R19E) did not significantly decrease the H4K16ac level (figure 3b). This finding suggests that maintenance of normal H4K16ac levels is dependent on the positive charge of R17 but not R19. This may help to explain why the positive charge of R17, but not R19, is important for male survival. To investigate crosstalk between R17 and H4K16ac, mosaic analyses of histone mutants were performed using FLP-FRT-mediated recombination [20] (electronic supplementary material, figure S3A). Homozygous HisD clones were detected when they are supplemented with 20X HisGUs of WT or mutated histones in larval tissues [20].

**Figure 2.** Positive charge of H4 R17/R19 is essential for maintaining chromatin structure and development associated gene expression. (a) Salivary glands from control (20His-GUs), H4R17A, H4R17H and H4R19A L3 larvae were squashed, and polytene spreads were stained with DAPI. The rate in the figures calculated as follow: the number of normal or disordered salivary gland samples / the number of total salivary gland samples observed. Scale bar = 20 μm. (b) Transcriptome comparisons of salivary glands between H4R17A mutants and 20His-GUs. |log2(FoldChange)| ≥ 1 and FDR ≤ 0.05 were used as the threshold of significance for differences in gene expression. (c) Transcriptome comparisons of salivary glands between H4R19A mutants and 20His-GUs. |log2(FoldChange)| ≥ 1 and FDR ≤ 0.05 were used as the threshold of significance for differences in gene expression. (d,e) Venn diagram showing overlap between the differentially expressed genes in H4R17A vs WT H4R17A–down and H4R19A–down. (f) Gene ontology categories enriched using genes significantly downregulated in H4R17A. (g) Gene ontology categories enriched using genes significantly downregulated in H4R19A. (h) Heatmap of differentially expressed genes that belong to metamorphosis GO term in panel f and g. Red denotes high expression values, and green denotes low expression values. Heatmap of the genes expression level (FPKM) using Z-score (ranged from −1 to 1) for normalized value.
We adopted this system to examine H4K16ac levels in WT, H4R17A, H4R17H and H4R19A flies. Consistent with the western blot results, the H4K16ac level was markedly decreased across the wing disc of H4R17A mutants but was weakly decreased in H4R17H mutants (figure 3c).

Staining of H4K16ac in the wing disc of H4R19A and WT flies was indistinguishable from that in neighbouring tissues (figure 3c).

To gain further insights into whether the H4R17A mutation affects male X-chromosome dosage compensation, we compared differentially expressed genes on the X-chromosome between H4R17A and H4K16A mutants. In H4K16A male flies, 16.5% (368) of X-linked genes were significantly downregulated compared with WT controls (figure 3d).

Transcriptome analysis revealed that the H4R17A mutation resulted in downregulation of 187 X-linked genes, 76%
(142) of which overlapped with genes downregulated in the H4K16A mutant (figure 3e). We confirmed the downregulation of eight known X-linked genes in H4K16A and H4R17A male flies relative to WT controls by RT-qPCR [37–44] (figure 3f). For example, expression of roxl (long non-coding RNA on the X-chromosome), which is required for male dosage compensation [45], was greater than 50% lower in H4R17A and H4K16A mutants than in the WT. To understand whether these downregulated X-linked genes presented in both H4K16A and H4R17A mutants are the direct targets of H4K16ac, we analysed the published anti-H4K16ac ChIP-seq data from salivary glands of male third instar larvae [36]. We found that 83% (307/368) of downregulated X-linked genes in H4K16A had the H4K16ac ChIP peaks and were therefore likely to be direct targeted genes regulated by H4K16ac (electronic supplementary material, figure S3B). 65% (121/180) of downregulated X-linked genes in H4R17A were enriched with H4K16ac peaks, while overlapping with downregulated genes in H4K16A (electronic supplementary material, figure S3C). Thus, the downregulation of these X-linked genes in H4R17A mutant is most likely due to the reduced modification of H4K16ac.

Furthermore, global transcriptome analysis demonstrated that 65% of genes upregulated in the H4R17A mutant were also upregulated in the H4K16A mutant (electronic supplementary material, figure S3D, E), while 70% of genes downregulated in the H4R17A mutant were also downregulated in the H4K16A mutant (electronic supplementary material, figure S3F). GO analyses suggested that dysregulated genes in the H4R17A and H4K16A mutants were enriched in very similar terms (figure 2f; electronic supplementary material, figure S2B, S3G, and S3H). For example, H4K16 and R17 co-regulated expression of genes involved in metamorphosis and ribonucleoprotein complex biogenesis (figure 2f; electronic supplementary material, figure S2B, S3G, and S3H). All these findings suggest that potent cis-regulatory crosstalk exists between H4K16 and R17.

3.5. R19 trans-regulates H3K79 methylation

Charge-based mutations of the basic patch broadly affect global gene expression according to transcriptome profiling. Therefore, we next investigated whether there is putative crosstalk between the H4 basic patch and other epigenetic signatures of gene silencing or activation. We examined the expression levels of several known important histone modifications (H3K4me3, H3K9me3, H3K27me3 and H3K79me3) in R17 and R19 mutants at the embryonic developmental stage (figure 4a,b). Western blot analysis of whole embryo extracts showed that when R17 was mutated to A or E (figure 4e), the levels of H3K4me3, H3K9me3 and H3K27me3 were unaffected compared with the WT (figure 4e). However, the level of H3K79me3 was slightly decreased in the H4R17A and H4R17E mutants (figure 4e). The levels of H3K4me3, H3K9me3 and H3K27me3 were not markedly decreased in the H4R19A and H4R19E mutants (figure 4b). However, mutation of R19 to A, H or E reduced the level of H3K79me3 by approximately 80% compared with the WT (figure 4b,d), indicating putative crosstalk between R19 and H3K79. To determine whether this crosstalk is conserved in other tissues, we examined H3K79me3 levels in the salivary glands of R19 mutants. The level of H3K79me3 was markedly lower in H4R19A and H4R19E mutants than in the WT, similar to the results obtained in embryos (electronic supplementary material, figure S5A).

The interaction between Dot1 and the H4 N-terminal tail is essential for H3K79 methylation [46]. Therefore, we examined the requirement of R19 for Gpp (Dot1 homologue in flies)-mediated generation of H3K79me3. We performed in vitro binding assays in which GST-H4 -34 was incubated with recombinant WT Gpp. Western blotting detected binding of Gpp to GST-H4 -34 (figure 4e,f). By contrast to the slight decrease in the interaction between Gpp and H4H1 -34 when R17 was mutated to A or E (figure 4e), mutation of R19 to A or E almost totally abolished binding of Gpp to the H4 N-terminal tail (figure 4f). All these data indicate that the interaction between Gpp and the H4 basic patch strongly depends on the arginine at position 19 of H4.

We further explored which genes are trans-regulated by crosstalk between R19 and H3K79 during early embryonic development. RNA-seq of H4R19A mutant embryos identified 567 dysregulated genes (5% FDR), of which 162 were downregulated and 405 were upregulated (figure 4g). In H3K79A mutants, we identified 416 dysregulated genes (5% FDR). Among these, 43% of downregulated genes (37/86) overlapped with downregulated genes in H4R19A mutants, while 66% of upregulated genes (219/330) overlapped with upregulated genes in H4R19A mutants (figure 4h,i; electronic supplementary material, figure S4B). GO analysis revealed that many co-upregulated genes were enriched in the ‘post-transcriptional regulation of gene expression’ cluster based on a comparison of both datasets (electronic supplementary material, figure S4C, D), including Smg, Qin, Ago3, Mael and Piwi [47–51] (figure 4j), which are involved in the piRNA pathway. To further delineate the possible role of trans-action between R19 and H3K79 in the piRNA pathway, we validated the expression of these co-regulated substrate genes and piRNAs by RT-qPCR. Consistently, these co-regulated genes and piRNAs were markedly upregulated in H3K79A and H4R19A mutants (figure 4k,l), suggesting that trans-histone crosstalk between R19 and H3K79 may play an important role in silencing of the PIWI-piRNA pathway.

4. Discussion

Although structural and biochemical studies suggest that the histone H4 basic patch regulates the binding and activity of chromatin-binding factors on nucleosome surfaces, few studies have directly elucidated its roles in biological functions and transcriptional regulation in metazoans. In this report, we utilized a previously published method to construct Drosophila mutant models bearing amino acid charge alterations in the histone H4 basic patch. We further elucidated the functional differences of the positively charged residues of this basic patch in animal development and their involvement in the regulation of gene transcription through cis and trans-mechanisms. Based on the growth and developmental phenotypic defects observed upon substitutions of R17 and R19 in the histone H4 basic patch with A, E or H, we concluded that the positive charge at position 17 plays a more critical role than the positive charge at position 19 during development. Interestingly, we identified cis-acting regulation between R17 and H4K16 acetylation as well as trans-acting regulation between R19 and H3K79 methylation. Furthermore, in human cultured cell lines, we investigated the finding that overexpression of
inputs were probed with flag antibodies to confirm equivalent amounts of grappa protein (Input). GST-histone constructs were Coomassie-stained to indicate the amount of GST histone fusion protein loaded per lane. (Figure 4. Histone H4 arginine 19 trans-regulates H3K79 methylation. (a,b) Western blots of whole-cell extracts using specific antibodies show the status of H3K4me3, H3K9me3, H3K27me3 and H4K16ac in embryos expressing wild-type histones or indicated mutation. Antibodies directed against histone H3, H4 and LaminC serve as loading controls. (c,d) Densitometry of western blot bands of H3K79me3 shown in panel a and b was quantified with Image J software. Values are means ± SEM of three biological replicates. (e,f) GST-H4 peptide fusion pull-downs were performed as described previously. Recombinant-purified grappa-flag was incubated in the presence of a GST control, or a GST-H4 tail peptide fusion encoding residues 1–34 of histone H4 (GST-H41–34) with indicated mutation. Bound grappa-flag was detected by flag antibodies (Bound). Reaction inputs were probed with flag antibodies to confirm equivalent amounts of grappa protein (Input). GST-histone constructs were Coomassie-stained to indicate the amount of GST histone fusion protein loaded per lane. (g) Transcriptome comparisons of embryos between H4R19A mutants and 20His-GUs. |log2(FoldChange)| ≥ 1 and FDR ≤ 0.05 were used as the threshold of significance for differences in gene expression. (h) Transcriptome comparisons of embryos between H3K79A mutants and 20His-GUs. |log2(FoldChange)| ≥ 1 and FDR ≤ 0.05 were used as the threshold of significance for differences in gene expression. (i) Venn diagram showing overlap between the differentially upregulated genes in H4R19A and H3K79A that belong to the GO terms (post-transcriptional regulation of gene expression). Red denotes high expression values, and green denotes low expression values. Heatmap of the genes expression level (FPKM) using Z-score (ranged from −1 to 1) for normalized value. (k,l) RT-qPCR verification of genes expression in H4R19A and H3K79A mutants. smg, qin, mael, AGO3 and piwi were chosen as the targets in panel k. 42AB piRNA cluster was chosen as the targets in panel l. Values are means ± SEM of three biological replicates (rp49 was the reference gene for normalization; RNA was extracted from embryos).
H4R17A mutated proteins remarkably reduces H4K16 acetylation levels and that overexpression of H4R19A also leads to a reduction in H3K79 methylation levels (electronic supplementary material, figure S5A, B). These results suggest that cis - and trans regulation between R17 and H4K16 acetylation and between R19 and H3K79 methylation in Drosophila may be conserved in other eukaryotic species. This strongly indicates that positive charges of the histone H4 basic patch affect downstream processes (e.g. transcription) through complex histone crosstalk, and contribute to regulation of chromatin structures during development.

Hierarchical packaging of eukaryotic chromatin plays a central role in transcriptional regulation and other DNA-related biological processes [52]. Although the cryo-electron microscopy structure of the nucleosome in vitro suggests that single mutations in the basic patch, such as H4K16A, H4R17A and H4R19A, do not affect 30 nm chromatin fibre folding [53], cytological examination of polytene chromosomes revealed disorganized structures in H4R17A and H4R19A mutant flies, particularly in the former. This may be due to several reasons. First, the H4 tail of nucleosomes interacts with acidic patch regions on the surface of adjacent nucleosomes to support compactation of the chromatin fibre [1,15,54]. When the positive charge of the H4 basic patch is altered, the internucleosomal interaction is weakened, possibly leading to chromatin decompaction. Second, the basic patch is required for DNA accessibility by chromatin-binding proteins such as ISWI, Snf2 and Chd1 [9–11,55–57]. Loss of positive charges in the H4 basic patch likely affects chromatin remodelling. Thus, we propose that positive charges in the H4 basic patch are essential for regulating higher-order chromatin structures, which is key for transcriptional regulation. Downregulated genes upon alteration of positive charges in the basic patch were ontologically enriched mainly in growth-, metamorphosis- and morphogenesis-related pathways, which further explains the developmental defects of H4R17A and H4R19 mutants. However, it is unclear why alterations of positive charges specifically affect genes involved in growth and development, and this merits future exploration.

Another question is whether important epigenetic modifications of R17 and R19 occur in vivo. Although in vitro biochemical experiments showed that R17 and R19 can be methylated [58], the presence of such modifications has not been examined in vivo. In this study, substitution of R17 with a positively charged H amino acid partially rescued the lethal phenotype observed with the A and E point mutations, suggesting that the positive charge itself, rather than modifications, may be essential for survival of multicellular organisms. However, these results do not exclude the possibility that R17 and R19 are modified in vivo, and the biological significance of such modifications requires further investigation.

4.1. Cis-crosstalk between R17 and H4K16

R17 carries a positive charge adjacent to H4K16 and can predictably influence H4K16ac based on charge steric hindrance. Although H4R17A mutation causes approximately 75% lower of H4K16ac level, RNA-seq data showed no significant differences in the expression of male-dosage related genes such as MOF, MSL1, MSL2, MSL3, and MLE between H4R17A and WT flies. This suggests that the point mutation of H4R17 did not result in decreased expression of components of the MOF-MSL complex. We reasoned that mutation of R17 might affect the binding or enzymatic activity of MOF, a histone acetyltransferase that specifically targets H4K16 [59]. MOF is abundantly localized on the X-chromosome in male Drosophila [60]; therefore, we examined its localization in H4R17A male Drosophila chromosomes. MOF protein still localized to the X-chromosome (data not shown), suggesting that charge-based mutation of R17 does not significantly affect binding of MOF to the X-chromosome. The positive charge of R17 may be required for the full catalytic function of MOF to acetylate H4K16 and form the activated conformation. In addition, the H4R17A mutation may induce the binding or activity of the H4K16ac deacetylase. Regardless, our results show that mutations of R17, but not R19, significantly decrease the H4K16ac level and downregulate many male X-linked genes (including the X dosage compensation gene roxl), which may explain why the male:female ratio was dysregulated in H4R17A mutants. However, we do not know whether downregulation of these X-linked genes in H4R17A male mutants is directly due to loss of the positive charge of R17, failure of H4R17A to facilitate MOF-mediated H4K16ac, or both. We propose that R17 plays an essential role in male X-chromosome dosage compensation, probably by acting synergistically with H4K16ac in cis.

4.2. Trans-crosstalk between the H4 basic patch and H3K79

In this study, R17 and R19 mutations variably affected H3K79 methylation by interfering with binding of the H3K79 methyltransferase Gfp [46] to the H4 N-terminal tail. Obviously, our results showed that R19 is more important than R17 for H3K79me3 during Drosophila embryonic development, probably because R19 residue plays a critical role in stabilizing the active-state of H3K79 catalyzed by Dot1. It is also worth mentioning that previous studies showed that H4K16ac and H4R17 are important for Dot1-mediated H3K79 methylation in yeast [12,62]. While in Drosophila, the H4R17A mutation resulted in a dramatic decrease in H4K16ac but less on H3K79me3. This is probably because the mode of Gfp binding to the K16 position in the H4 N-tail in Drosophila differs from that in yeast. Further resolution of the structural of Gfp in complex with nucleosomes will be helpful to address the above questions. Besides, ubiquitination of H2BK123 have been demonstrated to stimulate H3K79 methylation [62]. The above conclusions imply the existence of complex cross-talks between these different histone sites (electronic supplementary material, figure S5C).

In summary, our study shows how the basic patch of the nucleosome exerts its biological functions via a complex network through regulation of histone crosstalk in metazoans. The methods employed and insights provided in this study can be used to explore other regions of the nucleosome (e.g. the acidic patch). Further studies will help to fully understand how the basic patch of histone H4 regulates chromatin dynamics in different tissues and affects transcription of tissue-specific genes.

Data accessibility. Supplementary figures are available as electronic supplementary material. All data supporting the findings of the study...
are available in the submission. RNA-seq data are available under the accession number PRJNA514824.

The data are provided in electronic supplementary material [63].

Authors’ contributions. X.Z.: data curation, investigation, resources, validation, writing—original draft; X.W.: software, visualization; J.P.: investigation; A.S.: investigation; Y.G.: investigation; P.F.: investigation; G.G.: supervision, writing—review and editing.

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Conflict of interest declaration. The authors declare no competing interests.

References

1. Luger K, Mader AN, Richmond RK, Sargent DF, Richmond TJ. 1997 Crystal structure of the nucleosome core particle at 2.8 anstrom resolution. Nature 389, 251–260. (doi:10.1038/38444)

2. Kouzarides T. 2007 Chromatin modifications and their function. Cell 128, 693–705. (doi:10.1016/j.cell.2007.02.005)

3. Mersfelder EL, Parthun MR. 2006 The tale beyond silencing: identification of a new trans-histone nucleosomal epitope. Nucleic Acids Res. 34, 2653–2662. (doi:10.1093/nar/gkl338)

4. Ciccia A, Elledge SJ. 2010 The DNA damage response: making it safe to play with knives. Mol. Cell. 40, 179–204. (doi:10.1016/j.molcel.2010.09.019)

5. Swygart SG, Peterson CL. 2014 Chromatin dynamics: interplay between remodeling enzymes and histone modifications. Bio-Gene Rev. G. Mech. 1839, 728–736. (doi:10.1016/j.bbgrev.2014.02.013)

6. Rothbart SB, Strahl BD. 2014 Interpreting the language of histone and DNA modifications. Bio-Gene Rev. G. Mech. 1839, 627–643. (doi:10.1016/j.bbgrev.2013.03.001)

7. Strahl BD, Allis CD. 2000 The language of covalent histone modifications. Nature 403, 41–45. (doi:10.1038/47412)

8. Jayani RS, Ramanujam PL, Galande S. 2010 Studying histone modifications and their genomic functions by employing chromatin immunoprecipitation and immunoblotting. Method Cell Biol. 98, 35–56. (doi:10.1016/S0999-679x(10)80002-3)

9. Hamiche A, Kang JG, Dennis C, Xiao H, Wu C. 2001 Histone tails modulate nucleosome mobility and regulate ATP-dependent nucleosome sliding by NURF Proc. Natl Acad. Sci. USA 98, 14 316–14 321. (doi:10.1073/pnas.251421398)

10. Clapier CR, Nightingale KP, Becker PB. 2002 A critical epitope for substrate recognition by the nucleosome remodeling ATPase ISWI. Nucleic Acids Res. 30, 649–655. (doi:10.1093/nar/30.3.649)

11. Clapier CR, Cairns BR. 2012 Regulation of ISWI involves inhibitory modules antagonized by nucleosomal epitopes. Nature 492, 280. (doi:10.1038/nature11625)

12. Fingerman IM, Li HC, Briggs SD. 2007 A charge-based interaction between histone H4 and Dot1 is required for H3K79 methylation and telomere silencing: identification of a new trans-histone pathway. Gene Dev. 21, 2018–2029. (doi:10.1101/gad.1560607)

13. Alpar M, Utey RT, Lacoste N, Tan S, Briggs SD, Cote J. 2007 Interplay of chromatin modifiers on a short basic patch of histone H4 tail defines the boundary of telomeric heterochromatin. Mol. Cell. 28, 1002–1014. (doi:10.1016/j.molcel.2007.12.002)

14. Meriess Ha, Lerner AM, Chandrasekharan MB, Strahl BD. 2020 The histone H4 basic patch regulates SAGA-mediated H2B deubiquitination and histone acetylation. J. Biol. Chem. 295, 6561–6569. (doi:10.1074/jbc.RA120.013196)

15. Dorigo B, Schalch T, Bystricky K, Richmond TJ. 2003 Chromatin fiber folding: requirement for the histone H4 N-terminal tail. J. Mol. Biol. 327, 85–96. (doi:10.1016/s0022-2836(03)00025-1)

16. Shogren-Knaak M, Ishi H, Sun JM, Pazin MJ, Davies JR, Peterson CL. 2006 Histone H4-K16 acetylation controls chromatin structure and protein interactions. Science 311, 844–847. (doi:10.1126/science.1124000)

17. Robinson PJ, An W, Routh A, Martino F, Chapman L, Roedder RG, Rhodes D. 2008 30 nm chromatin fibre decompaction requires both H4-K16 acetylation and linker histone eviction. J. Mol. Biol. 381, 816–825. (doi:10.1016/j.jmb.2008.04.050)

18. Allahverdi A, Yang R, Koniev N, Fan Y, Davey CA, Liu CF, Nordenskiöld L. 2011 The effects of histone acetylation mimic, causes structural disorder of its N-terminal basic patch in the nucleosome. J. Mol. Biol. 411, 1680–1691. (doi:10.1039/nar/gkq900)

19. Zhou BR, Feng H, Ghirlando R, Kato H, Gruschus J, Bai Y. 2012 Histone H4 K16Q mutation, an acetylation mimic, causes structural disorder of its N-terminal basic patch in the nucleosome. J. Mol. Biol. 421, 30–37. (doi:10.1016/j.jmb.2012.04.032)

20. Zhang WM et al. 2019 Probing the function of metazoan histones with a systematic library of H3 and H4 mutants. Dev. Cell 48, 406. (doi:10.1016/j.devcel.2018.11.047)

21. Love MI, Huber W, Anders S. 2014 Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 15, 550. (doi:10.1186/s13059-014-0550-8)

22. Schalch T, Duda S, Sargent DF, Richmond TJ. 2005 X-ray structure of a tetranucleosome and its implications for the chromatin fibre. Nature 436, 138–141. (doi:10.1038/nature03686)

23. Yu GC, Wang LG, Han YY, He QY. 2012 clusterProfiler: an R package for comparing biological themes among gene clusters. Omics 16, 284–287. (doi:10.1089/omi.2011.0118)

24. Guay PS, Guild GM. 1991 The edysone-induced puffing cascade in Drosophila salivary-glands – a broad-complex early gene regulates intermolt and late gene transcription. Genetics 129, 169–175. (doi:10.1093/genetics/129.1.169)

25. Fletcher JC, Burts KC, Hogness DS, Thummel CS. 1995 The Drosophila E74 gene is required for metamorphosis and plays a role in the polytene chromosome puffing response to edysone. Development 121, 1455–1465. (doi:10.1242/dev.121.5.1455)

26. Stowers RS, Garza D, Rasde A, Hogness DS. 2000 The L63 gene is necessary for the edysone-induced 63ε late puff and encodes CDK proteins required for Drosophila development. Dev. Biol. 221, 23–40. (doi:10.1006/dbio.2000.9868)

27. Wright LG, Chen TH, Thummel CS, Guild GM. 1996 Molecular characterization of the 71E late puff in Drosophila melanogaster reveals a family of novel genes. J. Mol. Biol. 255, 387–400. (doi:10.1006/jmbi.1996.0032)

28. Ritter AR, Beckstead RB. 2010 Sox14 is required for transcriptional and developmental responses to 20-hydroxyecdysone at the onset of Drosophila metamorphosis. Dev. Biol. 239, 2685–2694. (doi:10.1016/j.ydbio.22407)

29. King-Jones K, Charles JP, Lam G, Thummel CS. 2005 The edysone-induced DHFR orphan nuclear receptor coordinates growth and maturation in Drosophila. Cell 121, 773–784. (doi:10.1016/j.cell.2005.03.030)

30. Zilian O, Frei E, Burke R, Brentrup D, Gutjahr T, Bryant PJ, Noll M. 1999 Double-time is identical to discs overgrown, which is required for cell survival, proliferation and growth arrest in Drosophila imaginal discs. Development 126, 5409–5420. (doi:10.1242/dev.126.23.5409)

31. Yang L et al. 2016 Minibrain and Wings apart control organ growth and tissue patterning through down-regulation of Cup1a Proc. Natl Acad. Sci. USA 113, 10 583–10 588. (doi:10.1073/pnas.1609471113)

32. Rintelen F, Stocker H, Thomas G, Hafen E. 2001 PDK1 regulates growth through at and S6K in
Drosophila. Proc. Natl Acad. Sci. USA 98, 15020–15025. (doi:10.1073/pnas.011318098)

33. Fusi B, Josten F, Feix M, Hoch M. 2004 Cell movements controlled by the Notch signalling cascade during foregut development in Drosophila. Development 131, 1587–1595. (doi:10.1242/dev.01057)

34. Lyukheva E, Taylor E, Michael M, Vehlow A, Tan SJ, Fletcher A, Krause M, Bennett D. 2008 Drosophila pico and its mammalian ortholog lamellipodin activate serum response factor and promote cell proliferation. Dev. Cell 15, 680–690. (doi:10.1016/j.devcel.2008.09.020)

35. Nurse NP, Yuan C. 2014 Cis and Trans Internucleosomal Interactions of H3 and H4 Tails in Tetranucleosomes. Biopolymers 103, 33–40. (doi:10.1002/bip.22560)

36. Conrad T, Cavalli FM, Hoitz H, Halladci E, Kind J, Ilk I, Vaquerizas JM, Luscombe NM, Akhtar A. 2012 The MGF chromobarrel domain controls genome-wide H4K16 acetylation and spreading of the MSL complex. Dev. Cell 22, 610–624. (doi:10.1016/j.devcel.2011.12.016)

37. Gelbart ME, Kuroda MI. 2009 Drosophila MSL complex activates the X chromosome of Drosophila melanogaster. Genetics 186, 1111–111285.

38. Meller VH, Wu KH, Roman G, Kuroda MI, Davis RL. 1997 roX1 RNA paints the X chromosome of male Drosophila and is regulated by the dosage compensation system. Cell 88, 445–457. (doi:10.1016/S0092-8674(00)81885-1)

39. Shanower GA, Muller M, Blanton JL, Honti V, Gyurkovics H, Schedl P. 2005 Characterization of the grappa gene, the S13059-020-02221-x)

40. Wang W, Han BW, Tipping C, Ge DT, Zhang Z, Weng Z, Theurkauf WE, Zamore PD. 2011 Heterotypic piRNA ping pong requires qin, a protein with both E3 ligase and tudor domains (vol 44, pg 572, 2011). Mol. Cell 44, 1005. (doi:10.1016/j.molcel.2011.12.002)

41. Pandey RR, Pillai RS. 2014 Primary piRNA biogenesis: caught up in a Maelstrom. Mol. Cell. 59, 819–830. (doi:10.1016/j.molcel.2015.08.007)

42. Wang C, Lin HF. 2021 Roles of piRNAs in transposon and pseudogene regulation of germine miRNAs and IncRNAs. Genome Biol. 22, ARTN 1200001. (doi:10.1186/s13059-020-02221-x)

43. Mukherjee P, Bhattacharjee S, Mandal DP. 2022 PWWP-interacting RNA (piRNA): a narrative review of its biogenesis, function, and emerging role in lung cancer. Asian Biomed. 16, 3–14. (doi:10.2478/abm-2022-0002)

44. Williams SK, Tyler JK. 2007 Transcriptional regulation by chromatin disassembly and reassembly. Curr. Opin. Genet. Dev. 17, 88–93. (doi:10.1016/j.gde.2007.02.001)

45. Song F, Chen P, Sun D, Wang M, Dong L, Liang D, Xu R-M, Zhu P, Li G. 2014 Cryo-EM study of the chromatin fiber reveals a double helix twisted by tetranucleosomal units. Science 344, 376–380. (doi:10.1126/science.1251413)

46. Dorigo B, Schalch T, Kolagana A, Duda S, Schroeder RR, Richmond TJ. 2004 Nucleosome arrays reveal the two-start organization of the chromatin fiber. Science 306, 1571–1573. (doi:10.1126/science.1103124)

47. Racki LR, Naber N, Pate E, Leonard JD, Cooke R, Narlikar GJ. 2014 The histone H4 tail regulates the conformation of the ATP-binding pocket in the SNF2h chromatin remodeling enzyme. J. Mol. Biol. 426, 2034–2044. (doi:10.1016/j.jmb.2014.02.021)

48. Liu XY, Li MJ, Xia X, Li XM, Chen ZC. 2017 Mechanism of chromatin remodelling revealed by the SmF2-nucleosome structure. Nature 544, 440. (doi:10.1038/nature22036)

49. Sundaramoorthy R, Hughes AL, El-Mkami H, Norman DG, Ferreira H, Owen-Hughes T. 2018 Structure of the chromatin remodelling enzyme Chd1 bound to a ubiquitylated nucleosome. Elife 7, ARTN e35720. (doi:10.7554/eLife.35720)

50. Feng Y et al. 2013 Mammalian protein arginine methyltransferase 7 (PRMT7) specifically targets RNR sites in lysine- and arginine-rich regions. J. Biol. Chem. 288, 37 010–37 025. (doi:10.1074/jbc.M113.525345)

51. Smith ER, Pannuti A, Gu W, Steunagel A, Cook RG, Allis CD, Lucchesi JC. 2000 The drosophila MSL complex acetylates histone H4 at lysine 16, a chromatin modification linked to dosage compensation. Mol. Cell. Biol. 20, 312–318. (doi:10.1128/MCB.20.1.312-318.2000)

52. Raja SJ et al. 2010 The nonspecific lethal complex is a transcriptional regulator in Drosophila. Mol. Cell. 38, 827–841. (doi:10.1016/j.molcel.2010.05.021)

53. Wodrow EJ, Hoffmann NA, Hicks CW, Wolberge C. 2019 Mechanism of cross-talk between H2B ubiquitination and H3 methylation by Dot1L. Cell 176, 1496. (doi:10.1016/j.cell.2019.02.002)

54. Valencia-Sanchez MI, De Ioannes P, Wang M, Truong DM, Lee R, Armachpe J, Boeke JD, Armarche K-J. 2021 Regulation of the Dot1 histone H3K79 methyltransferase by histone H4K16 acetylation. Science 371, eabc6663. (doi:10.1126/science.abc6663)

55. Zhang X, Wu X, Peng J, Sun A, Guo Y, Fu P, Gao G. 2022 Cis- and trans-regulation by histone H4 basic patch R17/R19 in metazaoan development. F12ghare. (doi:10.6084/m9.figshare.c.6277195)