# The landscape of DNA methylation associated with the transcriptomic network in laying hens and broilers get insight into embryonic muscle development in chicken

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## Abstract

As DNA methylation is one of the key epigenetic mechanisms involved in embryonic development, elucidating its relationship with non-coding RNA and genes was essential for understanding early development of life. In this study, we performed single-base-resolution bisulfite sequencing together with RNA-seq to explore the genetic basis of embryonic muscle development in chicken. Comparison of methylome profiles between broilers and layers revealed that lower methylation in broilers may contribute to the muscle development. Differential methylated region analysis between two chicken lines showed that the majority of DMRs were hypo-DMRs for broilers. Differential methylated genes were significantly enriched in muscle development related terms at E13 and E19. Furthermore, we identified a lncRNA named MYH1-AS that potentially regulates muscle development by constructing the network of the lncRNA. These findings depicted an integrative landscape of late period of embryonic myogenesis in chicken and give rise to a better understanding of epigenetic and transcriptional regulation in the skeletal muscle development. In addition, our study provided a reliable epigenetic resource for further muscle studies.

## Introduction

Epigenetics including DNA methylation, histone modification,

42 non-coding RNAs and chromatin remodeling fascinate researchers in
43 recent year because of their essential roles in various biological
44 processes[1,2]. The functions of epigenetics have been reported in many
45 aspects such as in human diseases[3], oogenesis and spermatogenesis[4] as
46 well as in adipose and muscle development[5-7]. DNA methylation is one of
47 the epigenetic mechanisms that has been reported to exert considerable
48 influence within regulation of the gene expression without changing the
49 DNA methylation[8]. Its role in muscle development has been illustrated in
50 human[9], pig[5,6], rabbit[10], bovine[11] and chicken as well[12].

51 Embryonic stage is crucial for mammal's muscle development as the
52 number of muscle fiber keeps stable after birth. Therefore, it is interesting
53 to study the embryonic muscle development from DNA methylation
54 aspect. DNA methylation functioning in embryonic muscle development
55 has been widely reported. For instance, ELVIRA CARRIO at el[13] built the
56 methylome of myogenic stem cell and proved the importance of DNA
57 methylation-mediated regulation of the cell-identity Myf5 super-enhancer
58 during muscle-stem cell differentiation. Besides, lncRNAs were also
59 proved to be important in regulation of muscle development for example,
60 linc-MD1 interact with miR-133 and miR-135 to regulate the expression
61 of transcription factors MAML1 and MEF2C that activate the
62 muscle-specific gene expression[7]. Recently, the methylation and lncRNA
63 regulatory relationship has drawn extensive attentions of researchers. A

database of methylation and lncRNA regulatory relationship has been built for human diseases studies[14]. In human, DNA methylation and lncRNA regulatory relationship were widely reported to be involve in tumorigenesis[15-17] whereas this regulatory relationship about muscle development is limited. The role of methylation in embryonic muscle development still remains unclear, although studies have been done in related field, such as Zhang at el[5] reported the regulatory relationship of lincRNA and DNA methylation functions in muscle development in pig. Yang at el[6] revealed that DNA methylation potentially affects gene expression in skeletal muscle to influence the propensity for obesity and body size.

The chicken is an ideal model for studying the embryogenesis and early muscle development because the accessibility of egg. Several genome-wide methylation studies have been reported in chicken. Basically, the relationship between DNA methylation level of promoter and expression level of genes were identified[18-20]. However, its role in chicken's embryonic muscle development has not been fully understood although global methylation landscape of muscle development was described in chicken using juvenile and later laying-period hens[12]. The ROSS 308 is one of the broilers bred and raised specifically for meat production whereas the Lohmann pink hen is a kind of layer bred and raised specifically for laying edible eggs. As they have

extremely different muscle accumulation and similar genetic background, they are good contrast model for muscle study. Here we used the whole genome bisulfite sequencing to produce the methylomes of 12 ROSS 308 and 12 Lohmann pink hen. In order to explore the effect of methylation and lncRNAs relationship on muscle development, we sequenced the whole transcriptome of these 24 samples by RNA-seq simultaneously for the multi-Omics integrative analyses.

## Results

### Overview of DNA Methylation

For genomic methylation data among 24 samples, the average sequence depth is about 30.3X. Approximately 3.4 billion reads were generated by the Illumina HiSeq in total and an average of 71.99% clean reads were mapped to the Gallus gallus genome (version 5.0) (Supplementary Table S1). The coverage analysis revealed that approximately 82% of the Gallus gallus genome were covered by reads at least one-fold, whereas nearly 78% of genome were covered more than 5-fold and 75% of genome were covered more than 10-fold (Supplementary Table S2). Those result indicated a reliable sequencing outcome.

The methylation level of each developmental stages was displayed in Fig 1a, revealing the layers and broilers have a similar global

methylation profile. The mCpGs in three different contexts showed similar proportion among 4 developmental stages (Fig 1b). Next, the methylation level distribution of mCpGs were analyzed at 4 developmental stages. Generally, mCpGs showed a high methylation level in mCG context whereas showed a low methylation level in mCHG and mCHH contexts (Fig 1c and Supplementary Fig 1a). Then we measured the methylation level of different regions of gene and compared those in different stages and populations. Interestingly, we found that broilers showed statistically lower methylation level at all stages in mCG context than layers (Fig 1d). Besides, CpG islands (CGIs) were identified and the numbers of CGIs at different regions were counted (Supplementary Fig 1b). we observed more CGIs located in promoter regions of gene in broilers than layers, which indicates methylation in CGIs may involve in muscle development as CGIs located at promoters regions are important for controlling gene expression[21].

Furthermore, the methylation level of lncRNAs assembled in RNA-seq of this experiment was also analyzed in similar way and compared with those of genes. Generally, broilers still showed lower methylation level in various types of lncRNA in mCG and mCHH contexts compared to laying hens whereas similar methylation level was observed among different types of lncRNA (Fig 2b and Supplementary Fig 2 c-d). Genes and lncRNAs had similar global methylation level and

both showed significant difference between two populations (Fig 2a and Supplementary Fig 2a-b). Those results suggest that faster muscle development of broilers may due to the lower methylation level in late embryonic stage compared with layer. Besides, the genomic distribution pattern of DNA methylation around genes and lncRNAs were analyzed. The upstream (2kb), first exon, first intron, internal exon, internal intron, last exon and downstream (2kb) of genes and lncRNAs across the genome were divided as different features and their methylation levels were measured through 20 bins, respectively. In general, the 5' upstream and 3'downstream regions were lower methylated than gene body regions. Besides, we also compared methylation level of features of gene with features of lncRNA (Fig 2c-d). It resulted that lncRNAs have relatively higher methylation level around TSS compared with genes ($P<0.001$). In addition, methylation levels of different types of repeat region were also analyzed across the genome. Beside the significant differences between two populations, short interspersed nuclear elements (SINE) particularly showed lower methylation level across 4 stages in mCG context (Fig 3 and Supplementary Fig 3).

**Identification of differential methylation regions and genes.**

To explore the potential causes of divergences in muscle development between broilers and layers, the differential methylation loci

(DMLs) were identified in DSS package. Then DMRs were identified for E10, E13, E16 and E19 respectively based on DMLs. The DMRs were subsequently annotated to the genome and the distribution of the DMRs in whole genome were analyzed (Fig 4a and Supplementary Table S4-S7). Generally, the majority of DMRs located in intronic regions whereas a small part of DMRs distributed in promoters of gene (Fig 4a). The proportion analysis revealed that broilers had more hypomethylated regions across the genome in four developmental stages, indicating that low methylation in muscle development-related genes may account for broiler's fast muscle development (Fig 4b).

Subsequently, the differential methylation genes (DMGs) were defined as genes overlapped with at least one DMR in its body region. The Gene Ontology (GO) enrichment analyses were performed to investigate potential biological functions of the DMGs. Generally, DMGs in four developmental stages were most significantly enriched in terms related to nervous system. However, many muscle-related terms were also found in the lists especially at DMGs of E13 and E19 such as muscle organ development (47 genes; Q-value < 0.001), myotube cell development (12 genes; Q-value < 0.005), positive regulation of muscle organ development (17genes; Q-value < 0.001), and muscle cell differentiation (51 genes; Q-value < 0.003) etc. (Fig 4c, Supplementary Table S8-S11). Because DMRs were not unanimous among different

developmental stages, we merged the genomic position of DMRs of 24 samples to form common DMRs and re-calculated the methylation level for each common DMR. The clustering analysis was performed using the common DMRs and displayed through heatmap. Different developmental stages were shown to cluster together which is indicative of the high quality of sampling and DMR calling in this experiment (Fig 5a). Moreover, the result of PCA was coincided with the clustering analysis (Fig 5b).

**Integrative analyses of DNA methylation and transcriptome**

To further explore whether methylation influences the gene and lncRNA expression in chicken, RNA-seq were used to measure the expression of genes and assembled novel lncRNAs. We identified 20656 lncRNAs in total (Fig 6a). Most of them are lincRNAs (63.6%) (Fig 6b). Heatmap of 24 samples and PCA suggested developmental stages accounted for most variances (Fig 6c). We divided genes and lncRNAs into 5 groups respectively based on their expression level (highest, medium high, medium low and lowest) using a quantile way. Then we measured their methylation level in different groups of genes and lncRNAs, respectively. Generally, broilers and layers were shown to have similar methylation levels and negative correlation was observed in genes in both populations as the highest expression level group showed lowest

methylation level around TSS whereas the lowest expression level group showed the highest methylation level (Fig 6d, e). Interestingly, this negative correlation trend between expression and methylation was observed in downstream region of lncRNAs but not around TSS (Fig 6f, g). Moreover, the lncRNAs are usually higher methylated around TSS compared to genes (Fig 6d-g).

Next, the differential expression genes (DEGs) and lncRNA (DELs) calling were performed for subsequent analysis. Meanwhile, the cis-target and trans-target of lncRNAs were predicted, respectively. The DMRs were assigned to lncRNAs generated from RNA-seq in this study (Supplementary Table S12-S15) and the differential methylation lncRNA (DM lncRNA) were defined as DEL overlapped with DMR. The result showed that 55 DM lncRNAs were identified (13,16,11,15 in 4 stages, respectively) (Supplementary Table S16). Subsequently, we looked for DM lncRNA that was potential to regulate muscle development. In particular, the expression of a lncRNA (we named it MYH1-AS, Fig 7a) was highly correlated with methylation level of the DMR assigned to it (Spearman, Cor=-0.7513, p<10^-4, Fig 7c). The expression of MYH1-AS was detected to dramatically increase in broilers compared to laying hens at E16 and E19 (Fig 7b). As the lncRNA was predicted by lncTar to target several genes in MYH1 chicken-specific isoforms like MYH1A, MYH1G, MYH1E etc., the expression correlations between the lncRNA

and its targets were calculated to search for its most likely target. Among its targets, MYH1E showed the highest correlation with MYH1-AS (Fig 7d), indicative of potential target of MYH1-AS. To further explore the role of MYH1-AS in muscle development, the gene-lncRNA networks were constructed based on their mRNA expression connectivity using WGCNA and the subnetwork of MYH1-AS was extracted from the whole network. It revealed that MYH1-AS had a high correlation with some muscle-related genes in this subnetwork (Fig 7e). Moreover, the relationship between connectivity and correlation was visualized in Fig 7f. Interestingly, genes highly negatively correlated with MYH1-AS did not show high connectivity with it. All the gene showing the high connectivity with MYH1-AS were also highly positively correlated with the lncRNA (Fig 7f). Then a total of 168 genes with top 50% both high connectivity and correlation with MYH1-AS were selected to perform GO enrichment analysis in order to confirm the role of MYH1-AS in muscle (Fig 7g-j and Supplementary S17). The result showed that the majority of terms enriched by those genes were muscle related.

## Discussion

The chicken provides a unique model to study embryology research of animal because of the accessibility of egg. As one of the most important energy sources for human diet, the muscle development of

chicken is a significant commercial feature worthy for studies. In this study, the broilers and laying hens were used to explore the muscle development of chicken in late embryonic period as they are artificially selected for different commercial use thereby are divergent in muscle development. Because of the crucial role of methylation in embryogenesis[13], we performed whole genome bisulfite sequencing (WGBS) and RNA-seq for to systematically explore the prenatal landscapes of chicken muscle development. Previous methylome studies have been done using prenatal chicken or born chicken muscle[12,22,23], however, those studies fails to display a comprehensive landscape of embryonic stages. We focused on more systematical study range from E10 to E19 between two chicken lines and aimed to elucidate the detain of embryonic muscle development.

The methylation level and proportion of different methylation contexts (mCG, mCHG, mCHH) of each developmental stage (Fig 1 a-d) indicated the layers and broilers have a similar global methylation profile. Additionally, the methylation level of different types of mCgG were measured (Fig 1 e-g). Those results are coincided with previous studies in chicken muscle[18]. The distribution proportion of mCpG in genome was different from the study of Zhang at el[23] as the mCpG in repeat region accounts for less genomic proportion in our study, probably because they used born chicken whereas the we performed the experiment on prenatal

chicken. However, more studies were required to explore the detain.

We next comprehensively compared the methylation level of genes and lncRNAs among different developmental stages and chicken lines (Fig 2a). Generally, laying hens showed a significantly higher methylation level than broilers in mCG context in both genes and lncRNAs, which may be responsible for their divergences in muscle development. Furthermore, different types of lncRNA (sense, intronic, antisense and lincRNA) were globally compared at methylation level and there were no significant differences among different types of lncRNAs, although layers and broilers still revealed significant variances (Fig 2b). Then genomic methylation around genes and lncRNAs were measured across the genome and the transcription start sites (TSS) were detected to be low methylated in genes (Fig 2c). The broilers and layers showed the similar trends around the transcription start site (TSS) which is coincident with the patterns of previous studies in chicken[12,18], as well as in bovine muscle tissue[11] and pig[24]. However, TSS of lncRNAs were usually higher methylated compare to genes (Fig 2c-d), which is able to explain why mRNA expression of lncRNAs are usually lower than genes ($P < 10^{-8}$) because methylation in promoter region usually affects gene expression[25]. In addition, the methylation level of different types of TEs (SINE, LINE, LTR, DNA and Satellite) were also measured in genome (Fig 3) and layers were found higher methylated than broilers in TEs regions.

Transposable elements are usually inactivated in animals but TEs were reported to have a present-day function in early development of human and other mammals to provide cis-regulatory elements that co-ordinate the expression of groups of genes[26]. As epigenetic regulation is important for activity of TEs[27], the difference showed in the two chicken lines may also account for the divergence in development.

The clustering heatmap and principle component analysis (PCA) were performed using common DMRs among 4 developmental stages. The expected classifications were observed in both analyses and indicated the reliable outcomes of sequencing and DMR calling (Fig 5a-b). Moreover, we found that DMRs between two chicken lines mainly distributed in intron regions and intergenic regions (Fig 4a), whose result is coincide with previous study in chicken[12], indicative of its important role in development regulation. However, as methylation in gene body region affects gene expression in several sophisticated ways[21], further studies on how methylation of the intron regions influences gene expression are required to elucidate the complicated epigenetic mechanism underlying development in chickens. Furthermore, the proportion of hyper and hypo methylated regions were analyzed and the majority of DMRs were detected to be hypomethylated regions in broilers, indicating that low methylation may be responsible for fast muscle development. This result was coincided with former result in this study

(Fig 4b, Fig 2a-b). Subsequently, genes with overlapped with DMR at different times were regarded as DMGs and used for GO enrichment analysis, respectively. We found that DMGs at E13 and E19 were significantly enriched in muscle related terms, suggesting that methylation play an important role in embryonic stage muscle development. Additionally, DMGs among 4 stages were both significantly enriched in nerve development related terms, which may relate to the impact of domestication and artificial breeding. Integrative analysis was conducted to study the association between methylation level and mRNA expression. We noticed that mRNA and methylation level around TSS were negative correlated in genes which was widely proved but not lncRNAs, indicating that DNA methylation regulates lncRNA expression in a more complex way.

To explore which lncRNA may potentially influence muscle development, the DM lncRNAs were identified and the correlation between DM lncRNA and DMR assigned to it were measured. In particular, we noticed that MYH1-AS showed high correlation with its target MYH1E and the DMR located in its intron region. Further WGCNA analysis revealed that some muscle related genes were highly correlated with MYH1-AS in its subnetwork (Fig 7e). For example, MYLK2, a muscle-specific gene, expresses skMLCK specifically in skeletal muscles[28,29]. ABLIM1 was reported to be related to muscle

weakness and atrophy[30]. Increased PDK4 expression may be required for the stable modification of the regulatory characteristics of PDK observed in slow-twitch muscle in response to high-fat feeding[31] and some other genes in the network such as MyoZ1, MYPN, ZBTB16 etc. were also revealed to be muscle or meat quality related genes[32-35]. Therefore, it is reasonable that MYH1-AS functions in muscle development. Notably, as we noticed that high correlation did not exactly mean high connectivity either (Fig 7f), we also performed GO enrichment analysis using 168 genes which had top 50% both high connectivity [36] and correlation values with MYH1-AS in its network as input. It resulted in GO terms of which the majority were muscle related terms (Fig 7f-g), strongly indicative of the MYH1-AS functioning in muscle development. Therefore, it is reasonable to assume that MYH1-AS was regulated by DNA methylation and participated muscle development during embryonic stage.

Our experiment revealed a comprehensive landscape of DNA methylome and transcriptome during embryonic developmental stage. Besides, we also found one lncRNA named MYH1-AS may potentially play a part in muscle development in chicken and provided evidence for this conclusion. Moreover, we provided a resource for further investigating the genetic regulation of methylation and gene expression in embryonic chicken. However, more studies are needed to elucidate the detailed mechanism how DNA methylation impacts lncRNA expression

and how the lncRNA regulates myogenesis.

## Materials and Methods

### Sample collection

The fertilized eggs of Rose and WhiteLoghorn were incubated in the same condition. The breast muscle and blood were collected at E10, E13, E16, E19. After sex determination, only samples identified as male were kept for next experiment. A total of 24 embryonic chicken were used in the study to form eight groups: E10, E13, E16, E19 for Rose and WhiteLoghorn, respectively. Each group included 3 individuals as biological replicates.

### DNA and RNA extraction

Genomic DNA was extracted using an animal genomic DNA kit (Tiangen, China) following the manufacturer's instructions. The DNA integrity and concentration were measured by agarose gel electrophoresis and NanoDrop spectrophotometer, respectively. Total RNA was isolated using TRIzol (TAKARA, Dalian, China) 110 reagent according to the manufacturers' instruction. RNA was reverse 111 transcribed by TAKARA PrimeScriptTM RT reagent kit (TAKARA) 112 according to the manufacturers' instruction.

## Library construction and sequencing

Bisulfite sequencing libraries were prepared using the TruSeq Nano DNA LT kit (Illumina, San Diego, CA, USA). The genomic DNAs were then fragmented into 100–300 bp by sonication (Covaris, USA) and purified using a MiniElute PCR Purification Kit (QIAGEN, Silicon Valley Redwood City, CA, USA). The fragmented DNAs were end repaired and a single 'A' nucleotide was appended to the 3′ end of each fragment. After ligating the DNAs to the sequencing adapters, the genomic fragments were bisulfite converted via a Methylation-Gold kit (ZYMO, Murphy Ave. Irvine, CA, USA). The converted DNA fragments were PCR amplified and sequenced as paired-end reads using the Illunima HiSeq xten platform by the Biomarker Technologies company (Beijing, China).

## Data alignment and process

The raw data in the FastQ format generated by the Illumina HiSeq were pre-processed by removing reads containing adapters, N (unknown bases) > 10%, and those which over 50% of the sequence exhibited low quality value (Qphred score ≤ 10). During the process, we also calculated the Q20, Q30, CG content for each sample data. The reads remained after this procedure were clean reads and used for subsequent analysis. The methylation data were aligned to reference genome Gallus gallus 5.0 by

Bismark software[37]. Meanwhile, the number of aligned clean reads in unique position of reference genome were calculated as unique mapped reads number. The proportion of the number of aligned reads in the total number of reads was calculated as the mapping rate. Subsequently, the methylation level of single base was then calculated by the ratio of the number of methylated reads to the sum of total reads covered the locus. Finally, we used a binominal distribution teat approach to determine whether a locus was regarded as methylated locus with the criteria: coverage depth > 4 and FDR<0.05[37].

The transcriptional libraries were sequenced on an Illumina HiSeq xten platform at the Biomarker Technologies Company (Beijing, China). The obtained transcriptome data were filtered by removing sequences containing adaptors, low-quality reads (Q-value < 20), and reads containing more than 10% of unknown nucleotides (N) and were aligned to reference genome Gallus gallus 5.0 by HISAT2[38] then the transcript assembly and FPKM calculation were performed using the StringTie[39]. Transcripts mapped to the coding genes of reference were used to subsequent differential expression gene calling.

**lncRNA identification**

In order to identify the potential lncRNA, the assembled transcripts generated from the StringTie were submitted to CPC[40], CNCI[41], CPAT[42]

416 and pfam[43] software with defeat parameters to predict the potential
417 lncRNAs. Only transcripts predicted as lncRNA shared among four tools
418 were regarded as candidate lncRNA. Then the cis-target gene of lncRNA
419 were defined as neighbor gene in 100 kb genomic distance from the
420 lncRNA and were identified using in-house script. The trans-target
421 prediction of lncRNAs was performed by LncTar software[44].
422

423 **DMLs and DMRs calling**

424 The differential methylation locus (DMLs) and differential
425 methylation regions (DMRs) between broilers and layers at each
426 comparison were detected separately using Dispersion Shrinkage for
427 Sequencing Data (DSS) package in R[45-48]. The differential methylation
428 regions (DMRs) were then calculated in with default parameters.
429 Subsequently, DMRs were annotated using ChIPseeker package in R[49].

430 Gene overlapped with at least one DMR is defined as differential
431 methylation gene (DMG). Common DMRs among 4 developmental
432 stages were identified by merging all positions of DMRs in 24 samples
433 and re-calculating the methylation level for each merged DMR position
434 with an average approach using mCpG data.
435

436 **DEGs and DELs calling**

437 The differential expression genes (DEGs) calling and the differential

expression lncRNA (DEL) calling between two populations at each time point were performed separately using the DEseq[50]. The results were filtering with the criteria: (1) fold change >2 (2) FDR<0.5. The transcripts satisfied both standards were regarded as DEGs or DELs.

**Validation for RNA-seq by quantitative Real-time RCP(Q-PCR)**

Total RNA was purified and reversely transcribed into cDNA using PrimerScriptR RT reagent Kit with gDNA Eraser (Takara Biotechnology (Dalian) Co., Ltd) following the specification. Quantities of mRNA were then measured with qRT-PCR using a CFX96TM real-time PCR detection system (Bio-Rad, USA). The qRT-PCR assays were then performed with a volume of 20 μL containing 10 μL SYBR Green Mixture, 7 μL deionized water, 1 μL template of cDNA, 1 μL of each primer and with following thermal conditions: 95 °C for 5 min, 45 cycles of 95 °C for 10 sec, 60 °C for 10 sec, 72 °C for 10 sec. Primer sequences used for qRT-PCR assays are displayed in Supplementary Table Last. β-catin gene was used as internal control. Each qPCR assay was carried out in triplicate. The relative gene expression was calculated by using the $2^{-\Delta\Delta Ct}$ method[51].

**Functional enrichment analysis and WGCNA analysis**

Gene ontology enrichment analyses were conducted for DMGs at

460 E10, E13, E16, E19 comparisons respectively to explore their potential
461 roles in muscle development. These analyses were performed by
462 clusterProfiler package implemented in R[52]. A hypergeometric test was
463 applied to map DMGs to terms in the GO database to search for
464 significantly enriched terms in DMGs compared to the genome
465 background.

466 The WGCNA analysis was performed using WGCNA package
467 implemented in R[36]. We used all the differential expression lncRNAs and
468 all the genes as input. Then, variable coefficient was used to filter
469 transcripts with low expression change. The variable coefficient was
470 calculated as follow: $C_v = \sigma/\mu$. The $\sigma$ is the standard deviation and $\mu$
471 represents the mean value of expression of input transcripts. Only
472 transcripts with ranked top 30% high $C_v$ value were used for WGCNA
473 analysis. After the entire network was constructed, only genes with
474 connectivity more than 0.15 were selected for subsequent subnetwork
475 analysis.

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**Fig 1**. Genome-wide profiles of DNA methylation among different sample groups. **(a)** Genomic methylation level in either layers or broilers at E10, E13, E16, E19, respectively. Methylation level were range from 0 to 1. **(b-d)** Proportion of mCpG in different genomic features at different developmental stages in mCG, mCHG and mCHH contexts, respectively. **(e-g)** Methylation level of CpGs was equally divided into 10 intervals and the percentage of each interval were measured using E10 as example.

**Fig 2**. Comparatively measurement of methylation level of genes and lncRNA. **(a)** Comparison of methylation level of genes or lncRNAs between layers and broilers in three different contexts. **(b)** Measurement of methylation level of different types of lncRNAs. * $P$ <0.05, ** $P$ <0.01 for comparison between two chicken lines. The red star means the methylation level of layers is significantly higher than broilers whereas the green star represents an opposite result. **(c-d)** Genomic methylation around genes and lncRNAs were measured across the genome, respectively. Transcripts were separated into seven regions (upstream, first exon, first intron, inner exon, inner intron, last exon and downstream) and each region was equally divided into 20 bins for visualization.

**Fig 3**. Methylation level of different types of TEs using E19 as an example. **(a)** Comparatively measurement of methylation of SINE, LINE,

504 LTR, DNA, Satellite regions between two chicken lines in mCG context.
505 **(b)** Methylation of different types of TEs for upstream, body and
506 downstream regions in three different contexts using 20 bins across the
507 whole genome.
508
509 **Fig 4**. Analyses of DMRs at 4 developmental stages. DMR calling
510 were performed in mCG, mCHG and mCHH, respectively. **(a)** Numbers
511 of DMRs in different genomic features (promoter, exon, intron, intergenic,
512 and UTR regions). **(b)** Relative proportion of hyper DMRs to hypo
513 DMRs in different CpG contexts. **(c)** The results of Gene Ontology (GO)
514 analysis for genes with overlapped with DMR. Only part of the terms was
515 selected for display. The red color means GO-BP terms, the blue color
516 means GO-CC terms whereas green color represents GO-MF terms. The
517 number in bracket means number of genes enriched in a specific term.
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519 **Fig 5**. Heatmap clustering analysis and PCA analysis. **(a)** Heatmap
520 clustering using merged common DMRs among 24 samples (see
521 Materials and Methods). **(b)** The result of PCA analysis using common
522 DMRs among 24 samples. Only the first component and the second
523 component were visualized.
524
525 **Fig 6**. LncRNAs idenditication and correlation analysis between

526 methylome and transcriptome. **(a)** Number of different types of lncRNAs
527 in all developmental stages. **(b)** Venn diagram of lncRNAs identified
528 through different software. **(c)** Hierarchical cluster analysis of lncRNAs
529 using their expression level. Replicates were merged together in the
530 analysis. **(d-g)** The genes and lncRNAs were divided into five groups
531 based on their expression levels, respectively. Then the methylation level
532 around TSS and TES of each group were measured using 20 bins across
533 the whole genome for layers and broilers.
534
535 **Fig 7**. Comprehensive analysis of lncRNA MYH1-AS. **(a)**
536 visualization of the transcript of MYH1-AS and DMR overlapped it. **(b)**
537 Expression level of MYH1-AS in layers and broilers at different
538 developmental stages. **(c)** Correlation between methylation of DMR and
539 expression of MYHA-AS using Spearman method. **(d)** Correlation
540 between expression of MYH1-AS and expression of its potential target
541 MYH1E. **(e)** The whole gene-lncRNA network and subnetwork including
542 MYH1-AS extracted from the entire network. (f) Relationship between
543 correlation and connectivity of gene and MYH1-AS. The red points
544 represent genes with both high connectivity and correlation with
545 MYH1-A and were selected for subsequent GO analysis. **(g)** Comparison
546 of connectivity value between genes selected (red points) and all genes
547 with in the subnetwork (background). * $P$ <0.05, ** $P$ <0.01 for

548 comparison between selected genes and background. **(h-j)** Results of GO
549 analysis for genes selected.
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Fig 1a

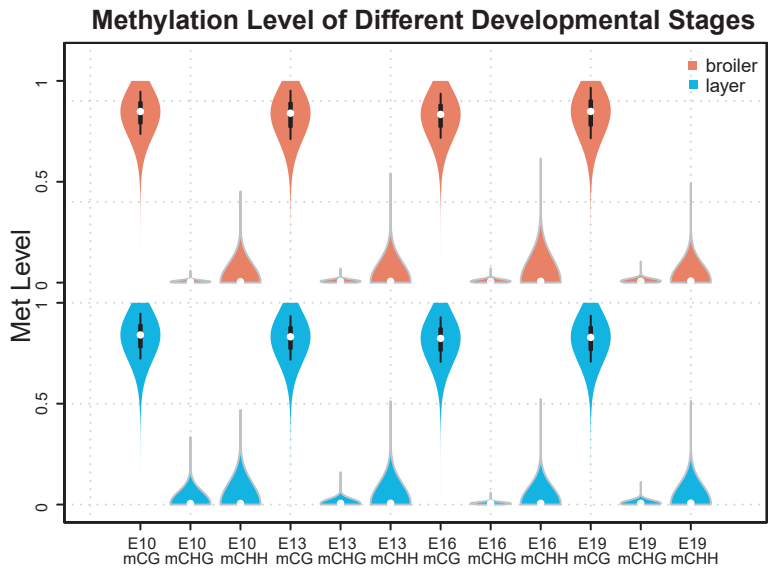



Fig 1b

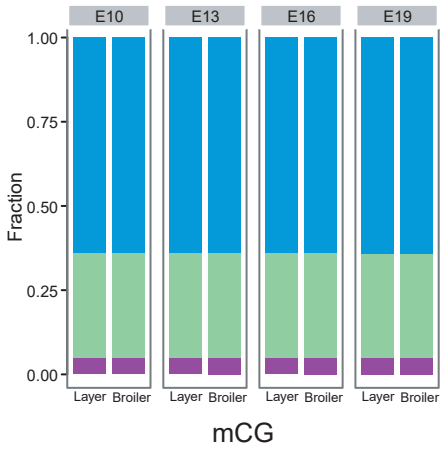



Fig 1c

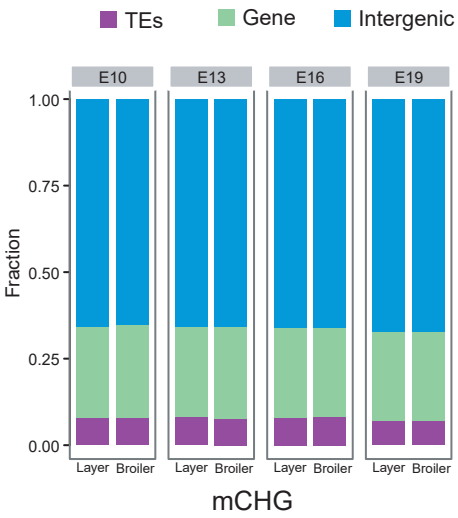



Fig 1d

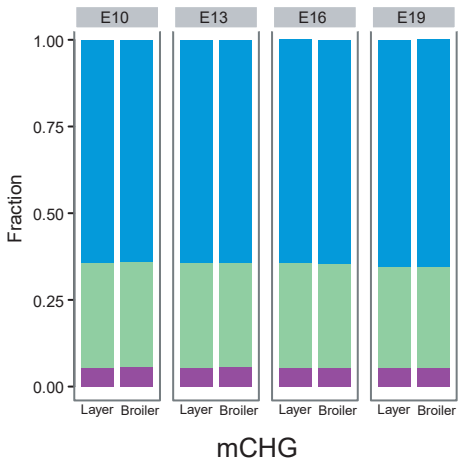
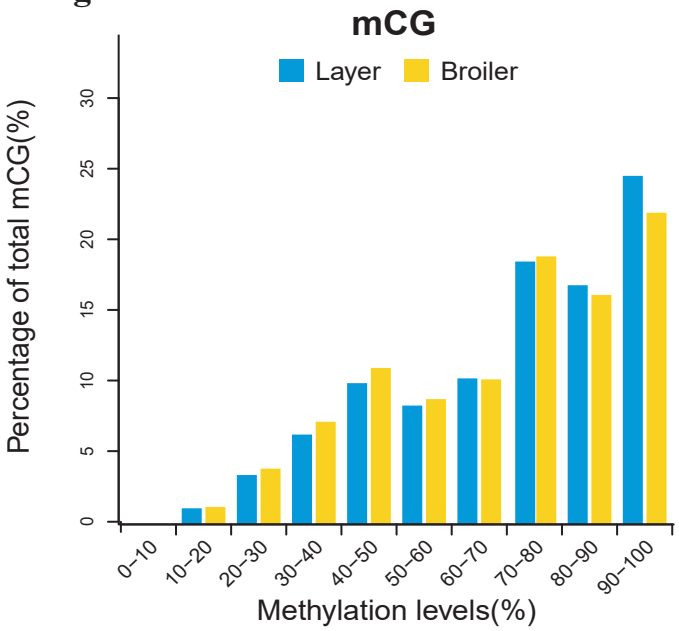



Fig 1e

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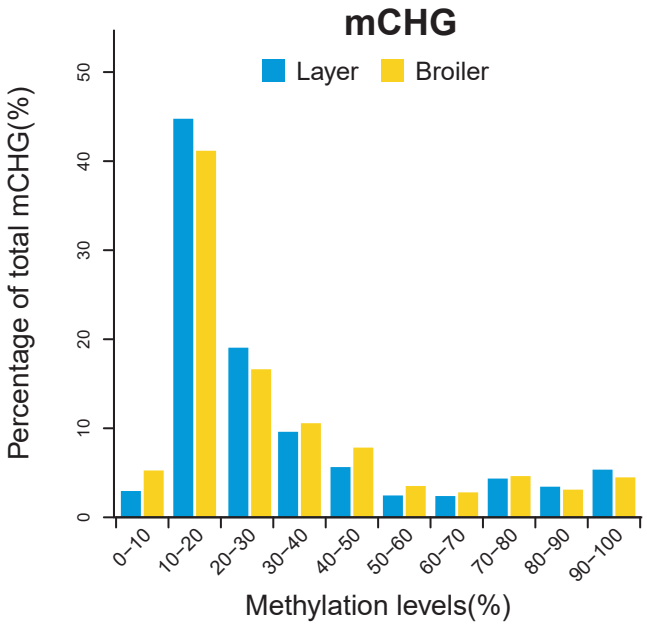



Fig 1g

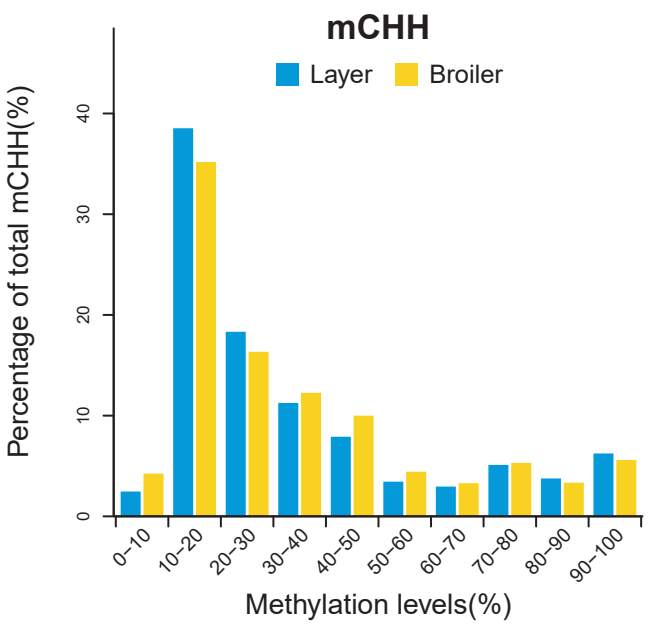


Fig 2a

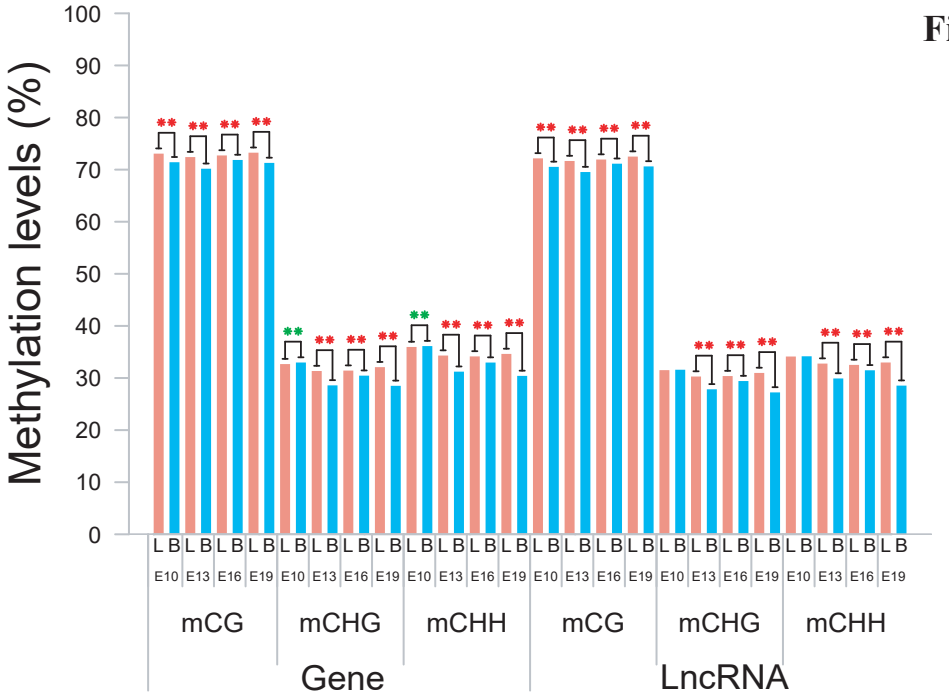



Fig 2b

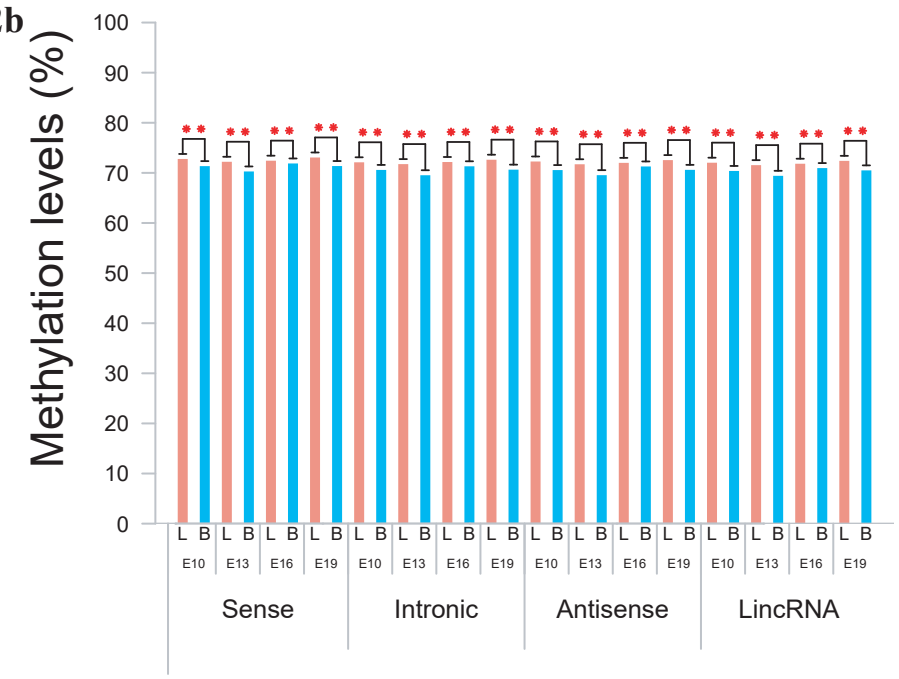



Fig 2c

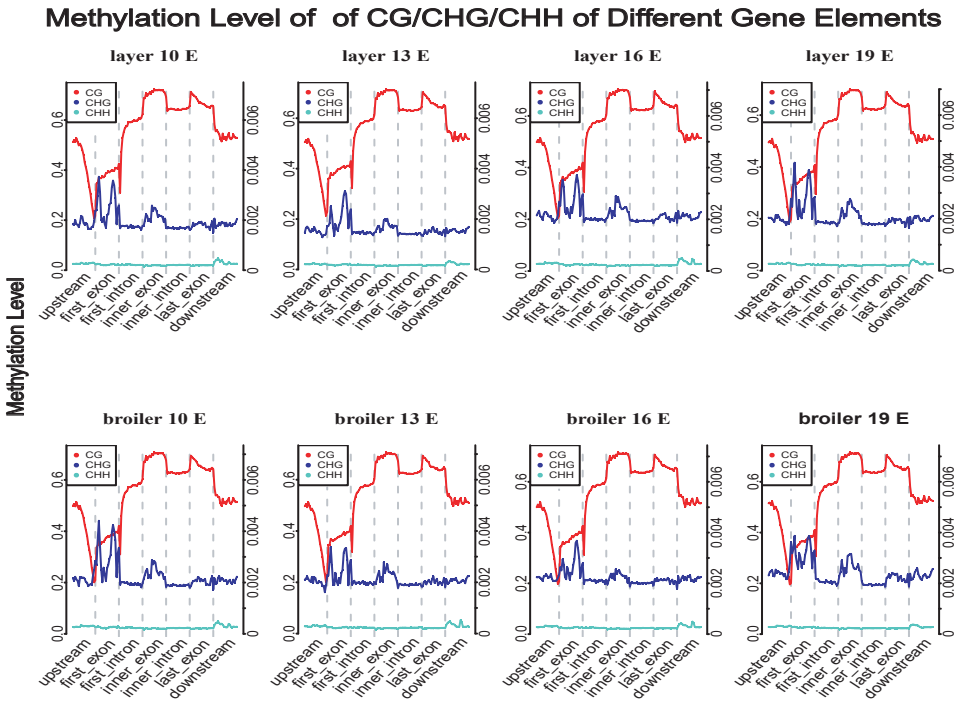



Fig 2d

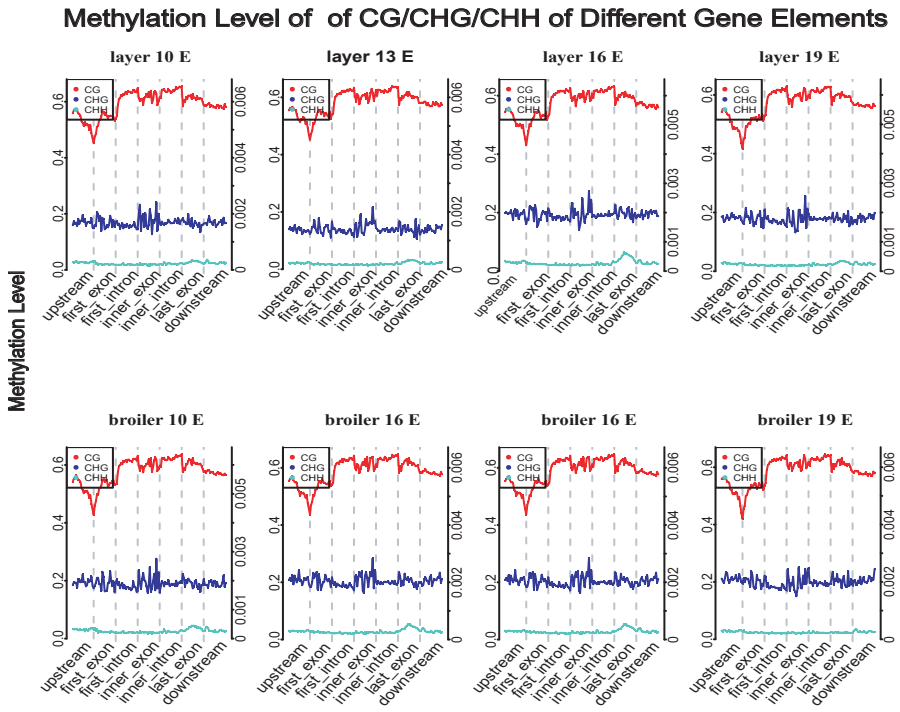


**Fig 3a**

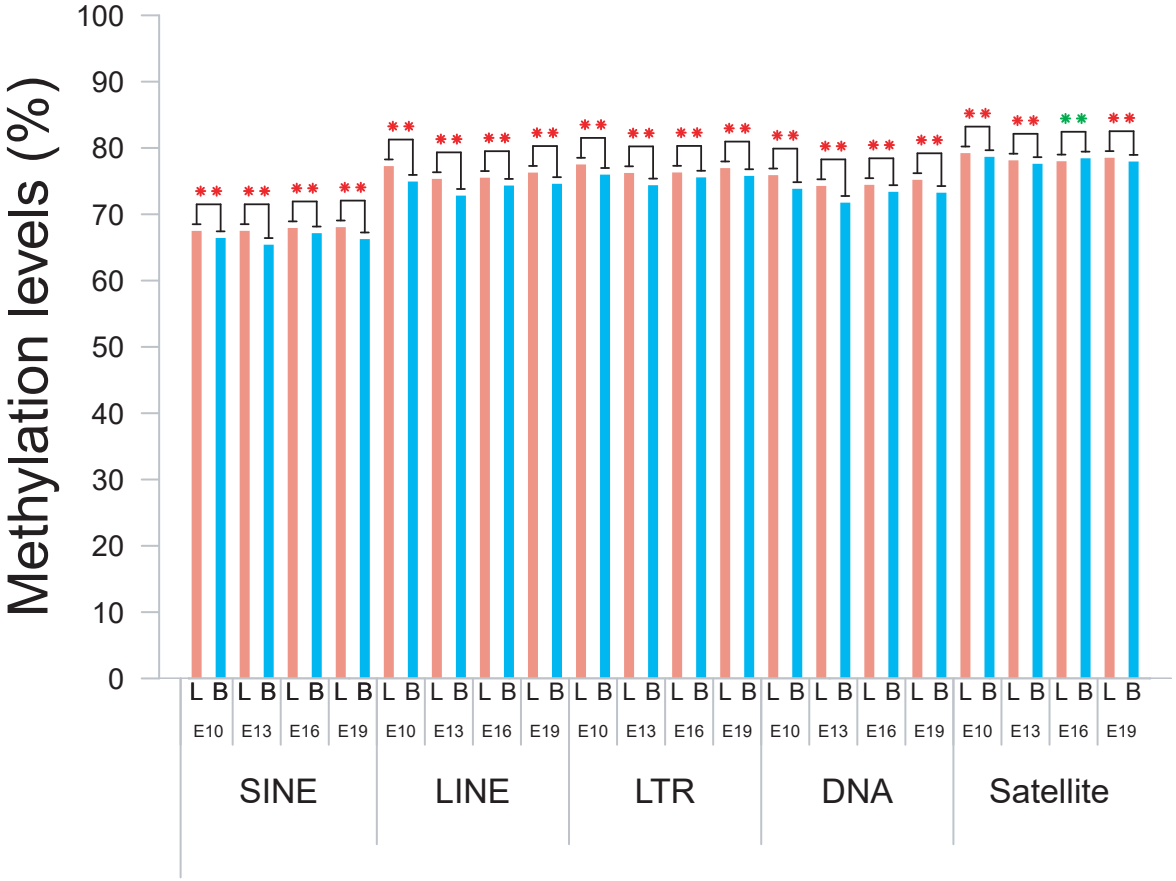

Methylation levels (%)
100
90
80
70
60
50
40
30
20
10
0
L B L B L B L B
E10 E13 E16 E19
SINE
LINE
LTR
DNA
Satellite

**Fig 3b**

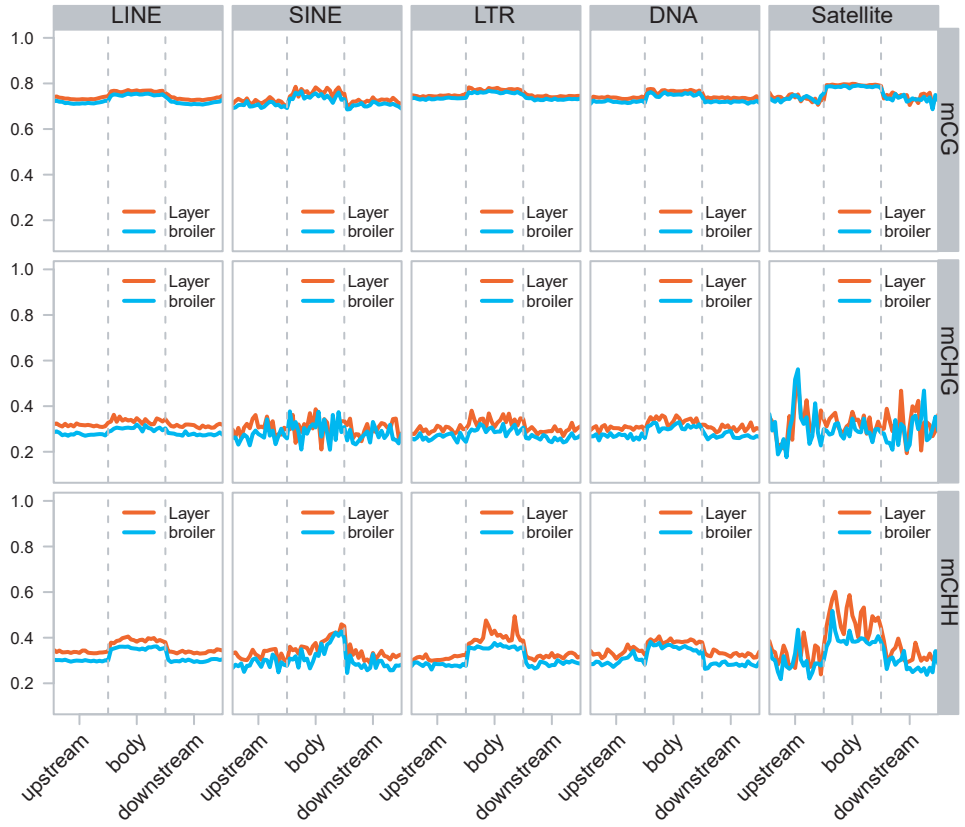

LINE
SINE
LTR
DNA
Satellite
mCG
mCHG
mCHH
1.0
0.8
0.6
0.4
0.2
Layer
broiler
upstream
body
downstream

Fig 4a

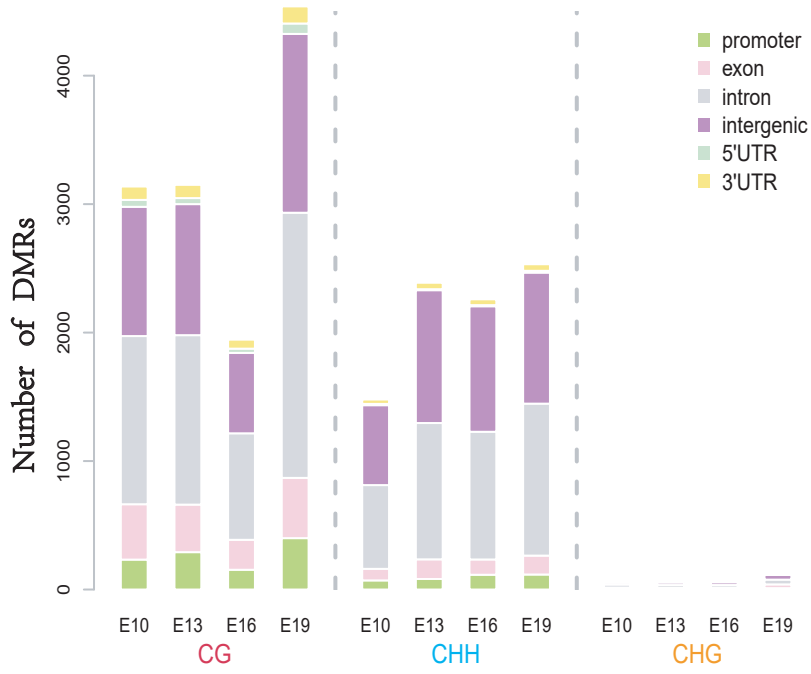



Fig 4b

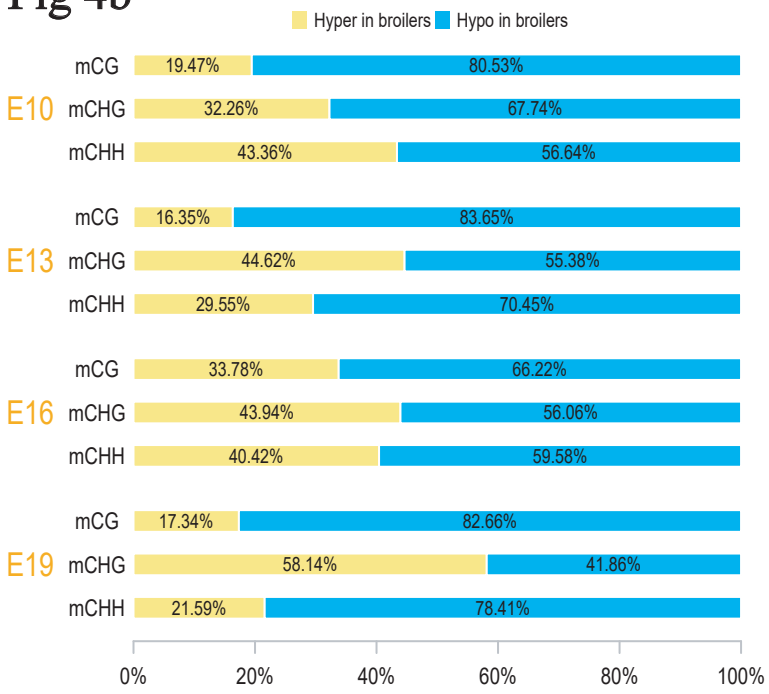



Fig 4c

## E10

**CC**

microtubule(38)

extracellular(45)

**BP**

positive regulation of nervous system(59)

neuromuscular process controlling balance(11)

positive regulation of neuron differentiation(43)

**MF**

signaling adaptor(15)

molecular adaptor activity(25)

## E13

**CC**

cell-cell junction(40)

extracellular matrix(45)

**BP**

muscle organ development(47)

neuromuscular junction development(11)

positive regulation of muscle organ development(17)

**MF**

protein binding, bridging(21)

molecular adaptor activity(22)

## E16

**CC**

axon part(30)

voltage-gated calcium channel complex(7)

**BP**

embryonic organ morphogenesis(30)

striated muscle tissue development(28)

developmental growth involved in morphogenesis(25)

**MF**

ion channel binding(15)

transcription coactivator activity(27)

## E19

**CC**

myofibril(27)

sarcolemma(22)

actin cytoskeleton(65)

**BP**

muscle cell development(30)

myotube cell development(12)

muscle cell differentiation(51)

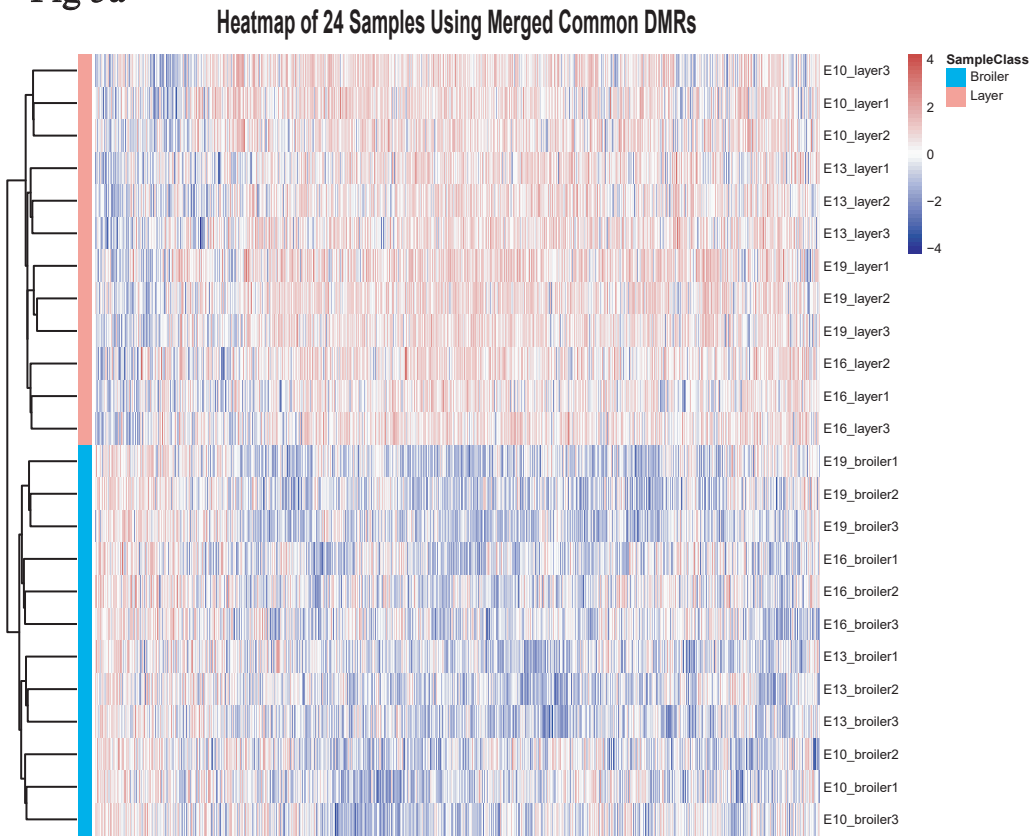
skeletal muscle organ development(32)

**MF**

actin binding(57)

actin filament binding(31)

Fig 5a


Heatmap of 24 Samples Using Merged Common DMRs
E10_layer3
E10_layer1
E10_layer2
E13_layer1
E13_layer2
E13_layer3
E19_layer1
E19_layer2
E19_layer3
E16_layer2
E16_layer1
E16_layer3
E19_broiler1
E19_broiler2
E19_broiler3
E16_broiler1
E16_broiler2
E16_broiler3
E13_broiler1
E13_broiler2
E13_broiler3
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E10_broiler1
E10_broiler3
4
2
0
−2
−4
SampleClass
Broiler
Layer

Fig 5b

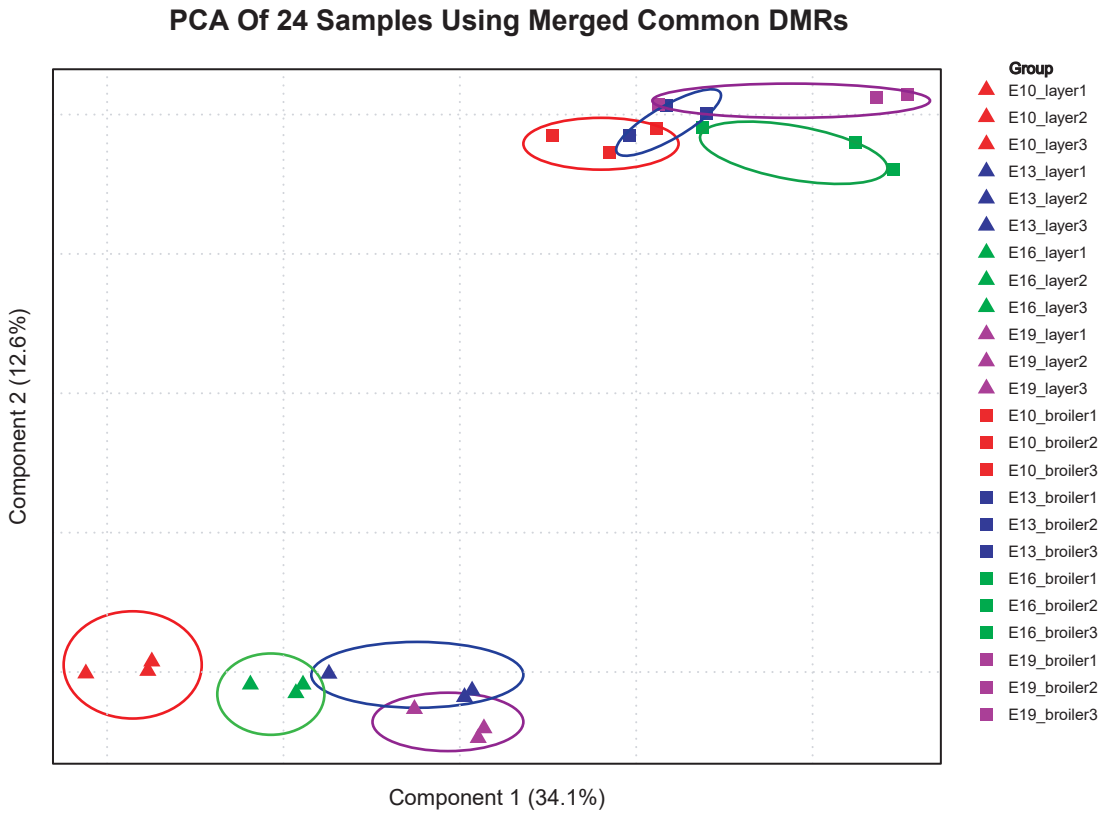

PCA Of 24 Samples Using Merged Common DMRs
Component 2 (12.6%)
Component 1 (34.1%)
Group
E10_layer1
E10_layer2
E10_layer3
E13_layer1
E13_layer2
E13_layer3
E16_layer1
E16_layer2
E16_layer3
E19_layer1
E19_layer2
E19_layer3
E10_broiler1
E10_broiler2
E10_broiler3
E13_broiler1
E13_broiler2
E13_broiler3
E16_broiler1
E16_broiler2
E16_broiler3
E19_broiler1
E19_broiler2
E19_broiler3

Fig 6a

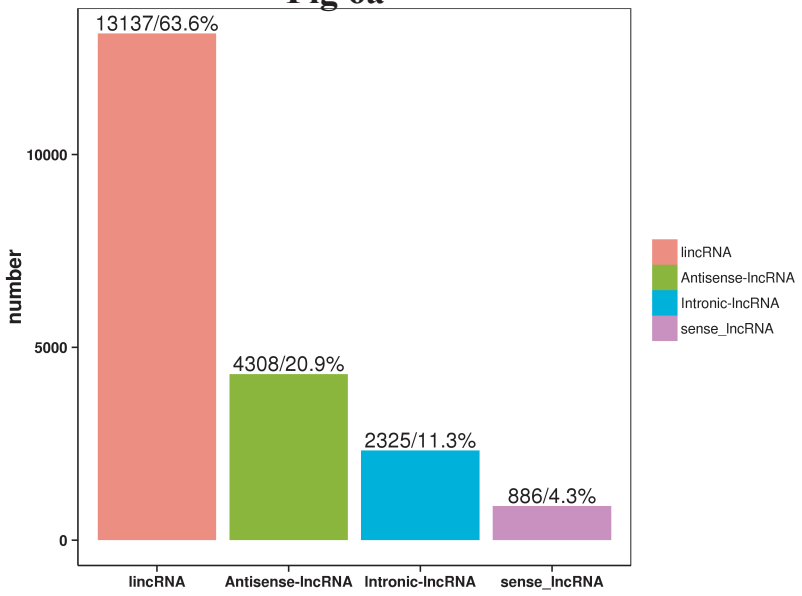



Fig 6b

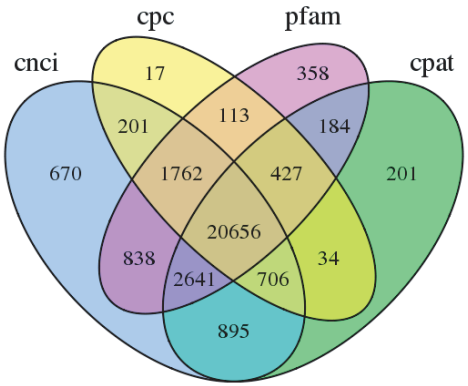



Fig 6c

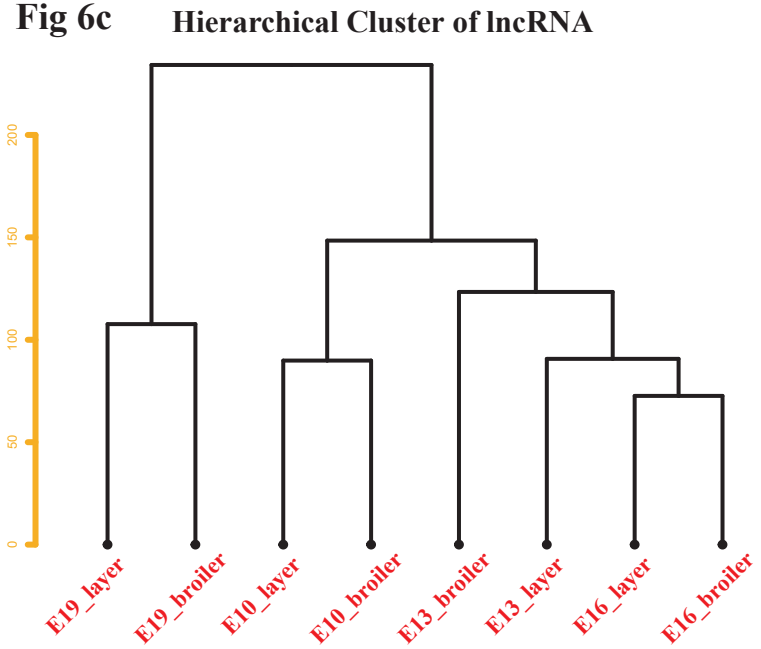



Fig 6d

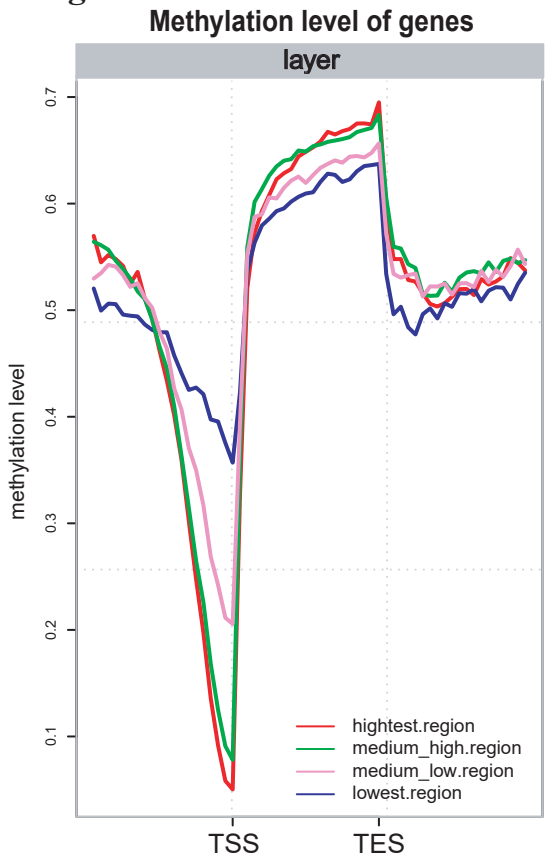



Fig 6e

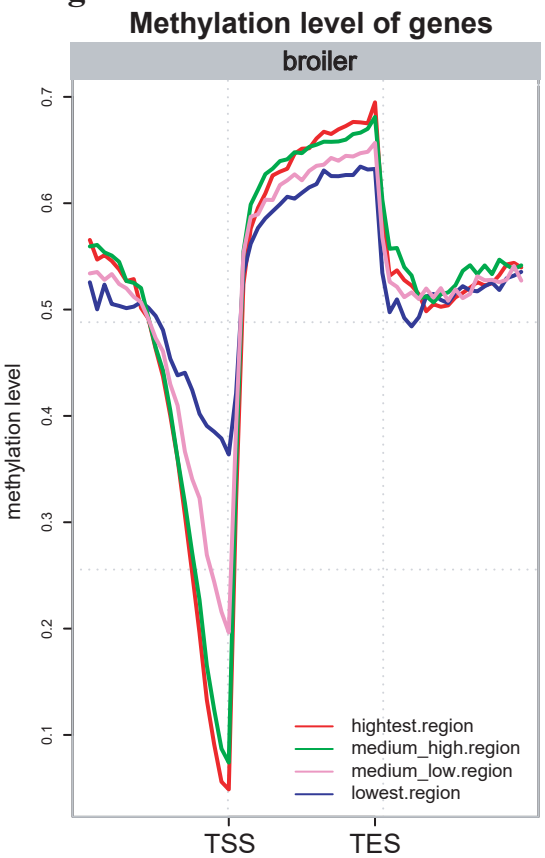



Fig 6f

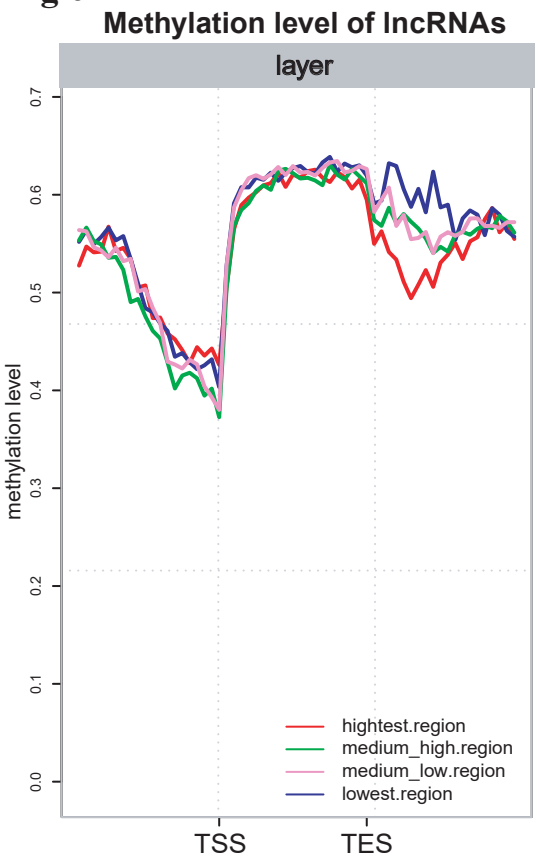
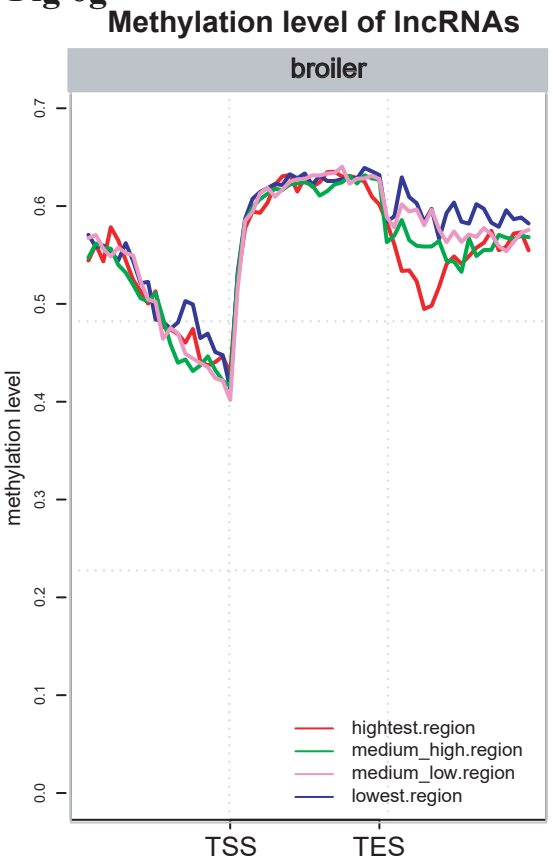



Fig 6g



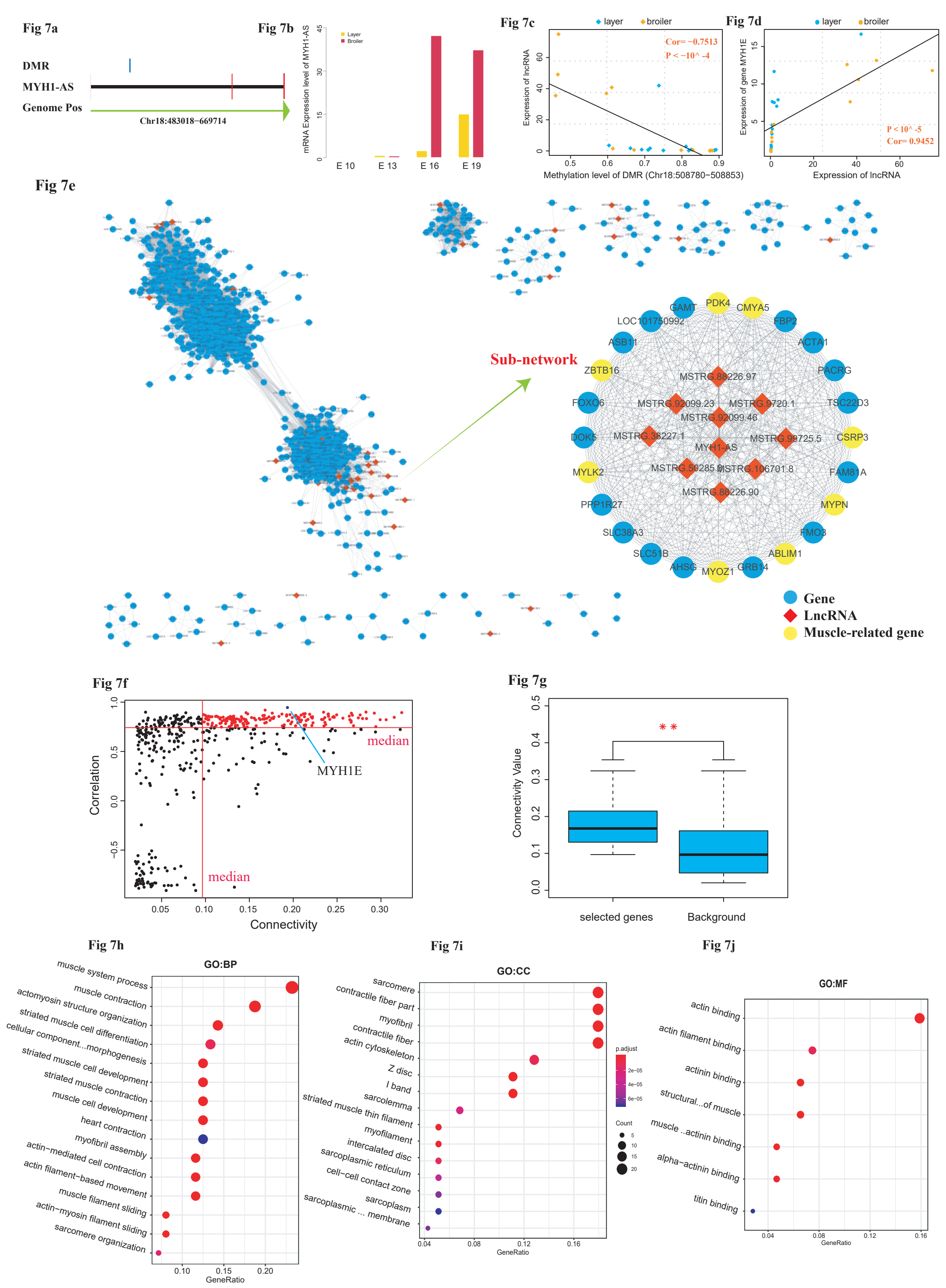


Fig 7a
DMR
MYH1-AS
Genome Pos
Chr18:483018−669714
Fig 7b
mRNA Expression level of MYH1-AS
Layer
Broiler
E 10
E 13
E 16
E 19
Fig 7c
layer
broiler
Cor= −0.7513
P < −10^ -4
Expression of lncRNA
Methylation level of DMR (Chr18:508780−508853)
Fig 7d
layer
broiler
P < 10^ -5
Cor= 0.9452
Expression of gene MYH1E
Expression of lncRNA
Fig 7e
Sub-network
GAMT
PDK4
CMYA5
LOC101750992
FBP2
ASB11
ACTA1
ZBTB16
PACRG
MSTRG.88226.97
FOXO6
MSTRG.92099.23
MSTRG.9720.1
TSC22D3
MSTRG.92099.46
DOK5
MSTRG.38227.1
MSTRG.99725.5
CSRP3
MYH1-AS
MYLK2
MSTRG.50285.8
MSTRG.106701.8
FAM81A
MSTRG.88226.90
PPP1R27
MYPN
SLC38A3
FMO3
SLC51B
ABLIM1
AHSG
MYOZ1
GRB14
Gene
LncRNA
Muscle-related gene
Fig 7f
Correlation
median
MYH1E
median
Connectivity
Fig 7g
Connectivity Value
selected genes
Background
Fig 7h
GO:BP
muscle system process
muscle contraction
actomyosin structure organization
striated muscle cell differentiation
cellular component...morphogenesis
striated muscle cell development
striated muscle contraction
muscle cell development
heart contraction
myofibril assembly
actin−mediated cell contraction
actin filament−based movement
muscle filament sliding
actin−myosin filament sliding
sarcomere organization
GeneRatio
Fig 7i
GO:CC
sarcomere
contractile fiber part
myofibril
contractile fiber
actin cytoskeleton
Z disc
I band
sarcolemma
striated muscle thin filament
myofilament
intercalated disc
sarcoplasmic reticulum
cell−cell contact zone
sarcoplasm
sarcoplasmic ... membrane
GeneRatio
p.adjust
Count
Fig 7j
GO:MF
actin binding
actin filament binding
actinin binding
structural...of muscle
muscle ..actinin binding
alpha−actinin binding
titin binding
GeneRatio