

# Analysis of genome DNA methylation at inherited coat-color dilutions of Rex Rabbits

**Yang Chen**

Yangzhou University

**Shuaishuai Hu**

Yangzhou University

**Ming Liu**

Yangzhou University

**Bohao Zhao**

Yangzhou University

**Naisu Yang**

Yangzhou University

**Jiali Li**

Yangzhou University

**Qiuran Chen**

Yangzhou University

**Juan Zhou**

Yangzhou University

**Guolian Bao**

Zhejiang Academy of Agricultural Sciences

**Xinsheng Wu** (✉ [xswu@yzu.edu.cn](mailto:xswu@yzu.edu.cn))

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## Research article

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# Abstract

## Background

Dilution of color in rabbits is associated with many different genetic mechanisms that form different color groups. A number of previous studies have revealed potential regulatory mechanisms by which epigenetics regulate pigmentation. However, the genome-wide DNA methylation involved in animal coat-color dilution remains unknown.

## Results

We compared genome-wide DNA methylation profiles in Rex rabbit hair follicles in a Chinchilla group (Ch) and a diluted Chinchilla group (DCh) through whole-genome bisulfite sequencing (WGBS). Approximately 3.5% of the cytosine sites were methylated in both groups, of which the CG methylation type was in greatest abundance. In total, we identified 126,405 differentially methylated regions (DMRs) between the two groups, corresponding to 11,459 DMR-associated genes (DMGs). Gene ontology (GO) and KEGG pathway analysis revealed that these DMGs were principally involved in developmental pigmentation and Wnt signaling pathways. In addition, 2 DMRs were randomly selected to verify that the WGBS data were reliable using bisulfite treatment (BSP), and 7 DMGs were analyzed to establish the relationship between the level of DNA methylation and mRNA expression using qRT-PCR.

## Conclusion

These findings provide evidence that there is an association between inherited color dilution and DNA methylation alterations in hair follicles, greatly contributing to our understanding of the epigenetic regulation of rabbit pigmentation.

## Background

During the long-term domestication of the rabbit (*Oryctolagus cuniculus*), different coat colors and color patterns have been selected and bred, specific strains and breeds usually referred to by their color. The Chinchilla is among the more striking breeds, with characteristic coat patterning being displayed in interphase dark blue-gray. American Chinchilla rabbit colors include light, medium and dark shades. Light and medium Chinchilla rabbits exhibit a matte dilution relative to dark. In China, after long-term breeding, two genetically stable Chinchilla rabbits have been cultivated, one being the standard Chinchilla, the other a diluted Chinchilla (Fig. 1). The mechanism of hair color formation has not yet been explained.

rabbits.

Coat color dilution in animals is regulated by eumelanin and pheomelanin. Dilution of eumelanin in black and brown coat colors can produce blue and cream-brown, while dilution of pheomelanin in a yellow coat color can result in a cream-yellow coat. In addition to rabbits, there are several known dilution phenotypes that are identical and favored by breeding in different species, including cats[1], chicken[2], quails[3],

mice[4], foxes[5] and mink[6]. Dilution of coat color in dogs may be accompanied by alopecia[7, 8]. At present, dilution of color is associated with several different genetic mechanisms resulting in distinct color groups. The mechanisms exist in numerous species and varieties but are not consistent in the majority of cases.

It has been documented that modification by methylation is the cause of variations in mammalian coat color. Methyl donor nutrients can determine coat color and obesity in offspring based on levels of DNA methylation of Avy metastable epialleles[9]. When pregnant yellow agouti (Avy) mice were supplemented with a methyl donor component, the offspring's coat color shifted to gray, shown to result from methylation of the retroviral long terminal repeat (LTR) promoter[10]. Folic acid or the phytoestrogen genistein supplied by maternal nutrition can counteract DNA hypomethylation and transform coat color distribution[11]. The dilution of hair color in the somatic cell cloned pig is directly caused by methylation of the promoter region of the KIT gene[12]. Scrutiny of the studies described above suggests that modifications by methylation are important in genetic studies of animal hair color.

The present study aimed to identify the genome-wide DNA methylation patterns in Chinchilla and diluted Chinchilla rabbit hair follicles and thus the candidate genes responsible for coat color dilutions. This study provides the basis for speculation about the epigenetic mechanisms that lead to color dilution in the coats of Rex rabbits and provides a reference for the development of appropriate breeding programs.

## Results

### DNA methylation mapping, patterns and sequence preferences analysis

A total of  $105.40 \pm 2.26G$  and  $102.36 \pm 3.87G$  raw bases were generated from the Ch and DCh groups, respectively. After data filtering, more than 320 million clean reads were obtained in each group, which were detected in all chromosomal regions. The mapping rate ranged from 67.77–70.89%, suggesting that the data can be utilized in subsequent analysis (Table 1). In both groups, approximately 3.5% of all genomic C sites were methylated. Methylation was found in three sequence contexts in similar proportions in each group, namely CG, CHG and CHH (where H was A, C or T). The CG type was the most abundant form at 96.49%~96.79%. Overall genome-wide methylated cytosine levels of CHG and CHH were less than 3.60% in both of the two groups of rabbits (Fig. 2).

Table 1  
Read quality and genome coverage

Group	Sample name	Clean Base (Gb)	Clean Reads	Mapping rate (%)	Bisulfite Conversion Rate (%)	mC percent (%)
Ch	Ch1	95.79	343286864	68.43	99.25	3.98%
	Ch2	97.13	347831906	70.89	99.27	3.14%
	Ch3	92.26	330086043	70.76	99.25	3.55%
DCh	DCh1	90.44	323729478	67.77	99.16	3.64%
	DCh2	90.59	321049987	68.67	99.15	3.57%
	DCh3	97.12	347277157	69.18	99.24	3.7%
Clean Reads Trimmed, filtered sequencing data and subsequent bioinformatics analysis were based on clean reads						
Clean Bases (G) The number of bases included in clean reads						
Mapping rate (%) Percentage of the matched clean reads to total clean reads						
Bisulfite conversion rate (%) The ratio of conversion of C into T by bisulfite						
mC percent (%) Percentage of methylated cytosine in the whole genome as a percentage of all cytosines						

## DNA methylation levels in different genomic functional regions

To explore the role of methylation in transcriptional regulation, we analyzed DNA methylation levels in different genomic functional regions (e.g., promoter, exon, intron, repeat, etc., where the promoter region was 2 kb upstream of the transcription start site (TSS) in different sequence contexts, as shown in Fig. 3. Similar methylation levels were observed in each functional element for the mC in 6 samples of the CG type. DNA methylation levels were greatest in repeats, followed by exon, intron and CGI, the promoter region being the lowest. For CHG and CHH types, the CGI regions and promoter exhibited different methylation status. Methylation levels of the functional elements of the other genomes were consistent.

## Identification of differentially methylated regions (DMRs) between Ch and DCh

After annotation into genetic functional regions, a total of 126,405 DMRs were identified (Table S1). The distribution of lengths of the DMRs was calculated. The length of 71.12% of the DMRs was found to be less than or equal to 500 bp (Fig. 4A). The relationship between q-value and methylation level was visualized using a volcano map (Fig. 4B). The DMRs were aligned to different genomic elements, most in

the repeat region, followed by the intron and CGI shore (Fig. 4C). A total of 11,459 DMR-associated genes (DMGs) were identified, of which 9,410 were differentially hyper-methylated in the DCh rabbits and 2,049 in the Ch rabbits. Among them, 3,850 DMRs were detected that matched the promoter region, and these DMRs were annotated in the promoter region of 3,581 genes. More detailed DMR results are presented in Table S2.

## Functional enrichment analysis of DMGs

To explore function in these methylated regions based on coat color traits, gene ontology (GO) and Kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were conducted to annotate the 11,459 DMGs. GO analysis indicated that the DMGs were involved in biological processes important for the formation of coat color, including developmental pigmentation (GO:0048066), pigmentation (GO:0043473), positive regulation of MAPK cascade (GO:0043410), regulation of gene expression, epigenetic (GO:0040029), regulation of Wnt signaling pathway (GO:0030111), etc. KEGG analysis indicated that the DMGs were enriched in the PI3K-Akt signaling pathway, mTOR signaling pathway, Melanoma, MAPK signaling pathway, Melanogenesis, Wnt signaling pathway, Notch signaling pathway, etc. More detailed results of the GO and KEGG analyses are presented in Tables S3 and S4.

## Verification of DMGs by bisulfite treatment (BSP) and qRT-PCR

Two DMRs (1 hypo-DMR and 1 hyper-DMR) from the DCh group were randomly selected to verify the reliability of the WGBS data by BSP, namely DMR\_8\_8510614 (PKP2) and DMR\_14\_75103993 (USP13). The DCh group exhibited hypermethylation of PKP2, compared with the degree of methylation observed in the Ch group ( $P < 0.05$ ). Conversely, DCh exhibited hypo-methylation of USP13, compared with the extent of methylation in Ch ( $P < 0.05$ ). Methylation analysis of 2 DMRs was consistent with BSP and WGBS, indicating that the WGBS data in this study were reliable (Fig. 5).

To further validate the methylation status and expression of the DMGs, 7 that were annotated in the promoter region as identified from the results of WGBS were selected for quantification by qRT-PCR. As shown in Fig. 6, the mRNA expression of DCT, TCF7L1 and SZT2 were significantly lower and that of ARAF, GSTA4, EDA and WNT10A significantly higher in the DCh compared with the Ch group ( $P < 0.05$ ). The results were consistent with the known relationship between DNA methylation levels and mRNA gene expression.

## Discussion

DNA methylation plays an important role in genomic stability, gene activation, X-chromosome inactivation and other processes such as epigenetic regulatory mechanisms, that participate in numerous life processes, including pigmentation[9–12]. Based on an updated literature search, genomic regions targeted by the environment that escape epigenetic reprogramming could undergo epigenetic inheritance[13]. In the present study, we found that the hair color of Chinchilla rabbits was lighter when

directly exposed to sunlight. After long-term breeding, two genetically stable Chinchilla rabbits breeds were cultivated, one the standard Chinchilla and the other the diluted Chinchilla. In order to explore the role of epigenetics in hair color inheritance, we compared the genome-wide methylation patterns in the DCh and Ch rabbit hair follicles to identify DMRs related to the dilution of coat color. In both groups, the proportion and type of methylated cytosine site were similar to other species, such as humans, pigs, sheep and chicken[14–17]. This indicates that DNA methylation patterns of different species have similarities and are conserved.

We identified 126,405 DMRs and 11,459 genes related to these DMRs were obtained. In order to confirm the reliability of WGBS, we used a BSP method to detect the methylation levels of DMR\_8\_8510614 (PKP2) and DMR\_14\_75103993 (USP13), the results of which were consistent with the sequencing data. Generally, DNA methylation occurring within a promoter region or close to a transcription start site negatively regulates gene expression[18, 19]. Among the 126,405 DMRs found, only 3,966 were distributed within a promoter region, indicating that the majority of the DMRs are distributed within the gene body and intergenic regions. DNA methylation of the gene body is complex and a number of studies have demonstrated that methylation in this region can negatively regulate gene expression[20, 21]. However, a number of studies suggest that it does not inhibit, but promote, gene expression[22–24], even though some studies in plants have shown that DNA methylation in the gene body region may not regulate gene expression[25]. So the effect of methylation in the gene body and intergenic region remains controversial[26]. In this study, mRNA expression of the 7 DMGs annotated in the promoter region was analyzed by qRT-PCR. The results suggest that methylation in the promoter region negatively regulates gene transcription.

In the present study, DMGs involved in the regulation of pigmentation were identified by GO and KEGG enrichment analysis, including ASIP, MITF, RAB27A, MYO5A, MLPH, SLC36A1, SOX10, TYR, TYRP1, PMEL, WNT5B, USP13, etc. Of these, it is known that the complexes of RAB27A and MYO5A can affect the capture, transport and distribution of melanosomes and hence pigmentation[27]. The agouti and extension loci which encode ASIP and MC1R genes can affect mammalian pigmentation by regulating the relative quantity of 2 melanin types[28–30]. SLC36A1 helps regulate intracellular pH by participating in the maturation of melanin bodies, and its missense mutation in exon 2 is responsible for the champagne dilution in horses[31]. MITF regulates differentiation and development in melanocytes while its stability is regulated by the USP13 deubiquitinase[32, 33]. How the methylation modification of these DMGs is involved in coat color dilution of rabbits will be the focus of our next study.

In addition, several studies have indicated that intragenic DNA methylation has a significant impact on the regulation of alternate splicing[34]. A number of genes have been found in this study with different splices involved in hair color dilution. A strongly truncated protein caused by an MLPH splice variant leads to a form of hair color dilution in rabbits[35]. The deletion of SLC7A11 in the region from the 11th intron to the adjacent Pcdh18 gene, which changes the amino acid sequence, reduces the formation of melanin, and thus affects coat color[36]. The exon 30 deletion of MYO5A in horses (g.138235715del) produces a frameshift mutation leading to the early termination of transcription, resulting in abnormal

vesicle trafficking that affects the function of melanocytes[37, 38]. The manner in which methylation affects the formation of alternate splicing in DMGs requires further investigation. These DMGs were mostly located in intron and distal intergenic regions in the genome. The results suggest that intragenic DNA methylation may be involved in the regulation of gene function.

## Conclusions

In summary, we have investigated the global DNA methylation pattern of rabbit hair follicles associated with standard Chinchilla and diluted Chinchilla groups. A number of related DMRs were revealed to explain the epigenetic regulation of rabbit inherited coat-color dilutions.

## Methods

### Animals and tissue collection

Five-month-old Rex rabbits were provided by Zhejiang Yuyao Xinnong Rabbit Industry Co., Ltd., including Chinchilla (Ch, n=3) and diluted Chinchilla (DCh, n=3) varieties. The rabbits were anesthetized by intra-articular injection of 0.7% sodium pentobarbital. Hair follicle samples were harvested from the back of each rabbit, immediately snap-frozen in liquid nitrogen then stored at  $-80^{\circ}\text{C}$  until required for use. Iodophor was administered to tissue explant sites to avoid bacterial infection. The rabbits have made a full recovery from the operation, and was free to move in an hour.

### Total DNA extraction and DNA library construction

Genomic DNA was extracted from hair follicle samples of Ch and DCh rabbits using a genomic DNA kit (TIANamp, China). DNA purity and concentration were measured using a NanoPhotometer® spectrophotometer (Implen, CA, USA) and a Qubit® DNA assay kit in a Qubit® 2.0 fluorometer (Life Technologies, CA, USA), respectively.

Genomic DNA was sonicated with Covaris S220 to obtain a 200-300 bp fragment with end repair and adenylation. A cytosine methylated barcode was ligated to the sonicated DNA, and these DNA fragments were treated twice with bisulfite in accordance with the instructions of an EZ DNA Methylation-Gold™ kit. Subsequently, the single-stranded DNA fragment obtained in this way was amplified by PCR using KAPA HiFi Hot-Start Uracil+ ReadyMix (2x). Concentration was determined using a Qubit® 2.0 Fluorometer (Life Technologies, CA, USA) and the size of the insert measured using an Agilent Bioanalyzer 2100 system. Cluster generation was accomplished using a cBot Cluster Generation System using a TruSeq PE cluster kit v3-cBot-HS (Illumina). The library was then sequenced using an Illumina HiSeq 2500 platform and 125bp paired-end reads generated.

### Mapping reads to known genome

The raw reads were filtered to obtain clean reads stored in FASTQ, by removing adapters, Ns and low quality reads. Alignment analysis of the reference genome (OryCun 2.0) was performed and methylation data extracted using Bismark software (version 0.16.1)[39]. Both C-T and G-to-A (reverse complementation) transformations on the sequenced results from the reference genome were ascertained for pairwise alignment using bowtie2[40]. The results were visualized in bigWig format using an IGV (Integrative Genomics Viewer) browser. The non-conversion rate of bisulfite was calculated using the percentage of cytosine sequenced at the cytosine reference site. Based on coverage  $\geq 5\times$  and false discovery rate (FDR)  $< 0.05$ , we performed a binomial distribution test for each C site to identify methylated Cs[41, 42].

## Estimating methylation levels and the identification of DMRs

To confirm methylated sites, we modeled the sum of methylated counts as a binomial (Bin) random variable with a methylation rate:

The sequence was then divided into multiple bins with a size of 10kb to calculate the methylation level. We calculated the sum of methylated and non-methylated read counts in each window. Methylation level (ML) for each window or C site indicates the proportion of methylated Cs, defined as:

$$ML(C) = \frac{reads(mC)}{reads(mC) + reads(C)}$$

DMRs were identified using swDMR software with a sliding-window approach. The window was set to 1000bp and step length to 100bp.

## Gene Ontology and KEGG pathway analysis of DMGs

DMGs were analyzed based on DMRs that overlapped gene functional regions, such as promoter, 5'-UTR, exon, intron and 3'-UTR regions, with at least 1 bp. The DMGs were screened and annotated by gene ontology (GO) and KEGG enrichment analysis. GO enrichment analysis was achieved using the GOseq R software package[43], in which GO items with a corrected P-value  $< 0.05$  were considered significantly enriched. KEGG can be used to analyze the advanced functions and biological systems (such as cells, organisms and ecosystems) at a molecular level (<http://www.genome.jp/kegg/>)[44]. KOBAS software was used to analyze the statistical enrichment of DMGs in KEGG pathways[45].

## Bisulfite sequencing (BSP)

Five hundred ng of genomic DNA were modified and purified using an EpiTect Fast DNA bisulfite kit (Qiagen, Germany). Converted DNA was stored at -20°C until required for use. BSP primers were designed using Meth-Primer software (<http://www.urogene.org/methprimer>), as presented in Table S5. A 50 ng quantity of converted DNA was used in a 50 µL reaction system. These PCR products were cloned into a pMD19-T vector (Takara, Dalian, China). Ten clones per sample were sequenced. Methylation levels were evaluated by calculating the percentage of converted cytosines to the total number of cytosines. BSP results were aligned using MegAlign software, and analysis conducted at the MSR website (<http://www.msrcall.com/MSRcalcalate.aspx>).

## Quantitative real-time PCR (qRT-PCR)

qRT-PCR was conducted using ChamQ™ SYBR® qPCR Master Mix (Vazyme) on an Applied Biosystems® QuantStudio® 5 Real-Time PCR system using the following parameters: Pre-denaturation stage: 95°C for 30 s; PCR reaction: 95°C for 10s, 60°C for 30s (40 cycles); dissolution at 95°C for 15 s, 60°C for 1 min and 95°C for 15 s. The results were normalized to GAPDH expression. The relative expression of DMGs was calculated using the  $\Delta\Delta Ct$  method  $= 2^{(\Delta Ct_{\text{experimental}} - \Delta Ct_{\text{control}})}$ .

## Statistical analysis

Each experiment was repeated at least three times and the data analyzed using an independent-sample test. Significant methylation sites were ascertained using a Fisher's exact test. All results are presented as means  $\pm$  SD, at two levels of significance,  $*P < 0.05$  and  $**P < 0.01$ .

## Abbreviations

Ch: Chinchilla group; DCh: Chinchilla group; DMRs: Differentially methylated regions; DMGs: DMR-associated genes; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BSP: Bisulfite treatment; LTR: Long terminal repeat; TSS: Transcription start site.

## Declarations

### Ethics approval and consent to participate

This study was carried out in accordance with the recommendations of Animal Care and Use Committee at Yangzhou University. The experimental procedures was approved by the Animal Care and Use Committee at Yangzhou University. Operational procedures were stringently conducted in accordance with Laboratory Animal Requirements of Environment and Housing Facilities (GB14925-2001)..

## Consent for publication

Not applicable.

## Availability of data and materials

WGBS data were submitted to National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) database under the accession number SRP107834.

## Competing interests

No potential conflict of interest was reported by the authors.

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## Authors' contributions

All authors read and approved the manuscript. Y.C. was responsible for the collection and analysis of results and wrote the manuscript. Y.C., S.H., M.L., B.Z., N.Y., and J.L. performed experiments. Q.C., J.Z., and G.B prepared figures and/or tables. Y.C. and X.W. designed the study. All authors read and approved the final manuscript.

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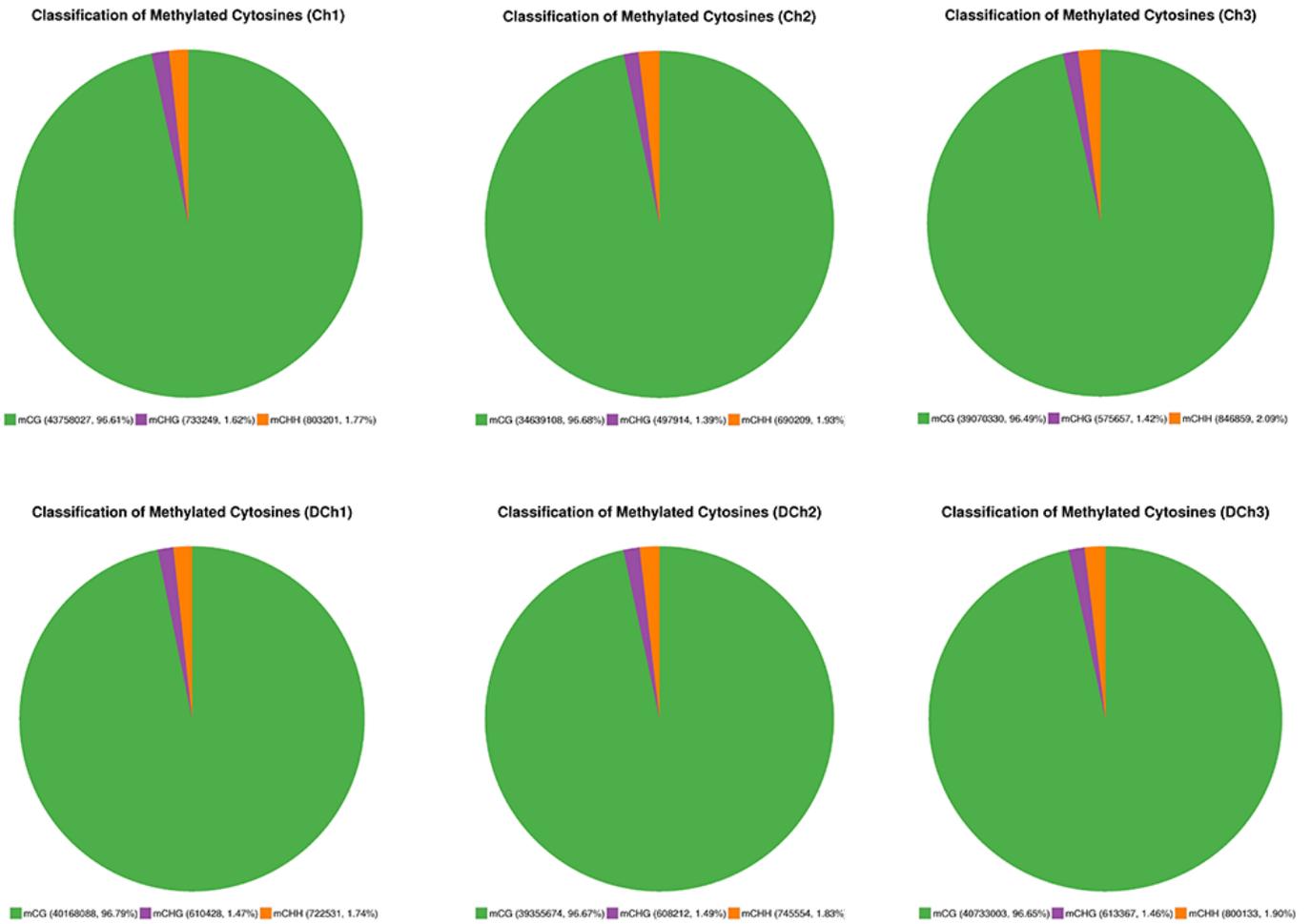
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## Figures



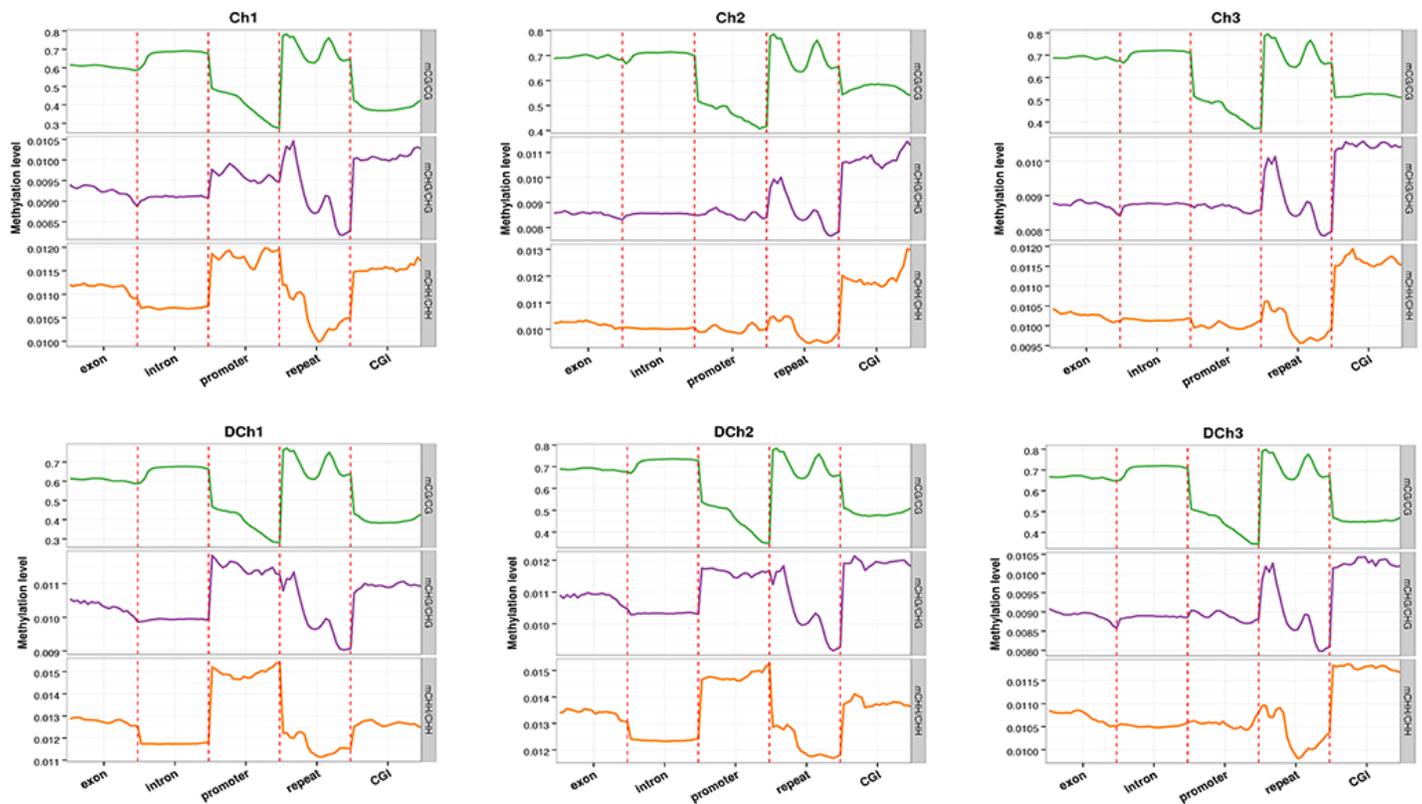
### Figure 1

Individual phenotypic appearance of Chinchilla and diluted Chinchilla rabbits.



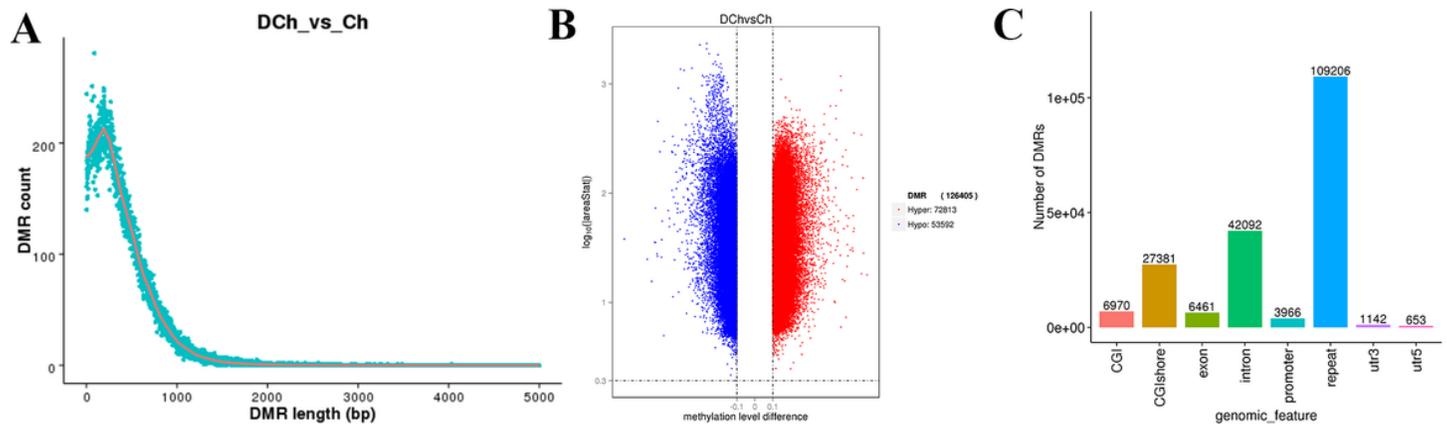
**Figure 2**

Distribution of mCG, mGHG and mCHH in all methylated cytosine residues.



**Figure 3**

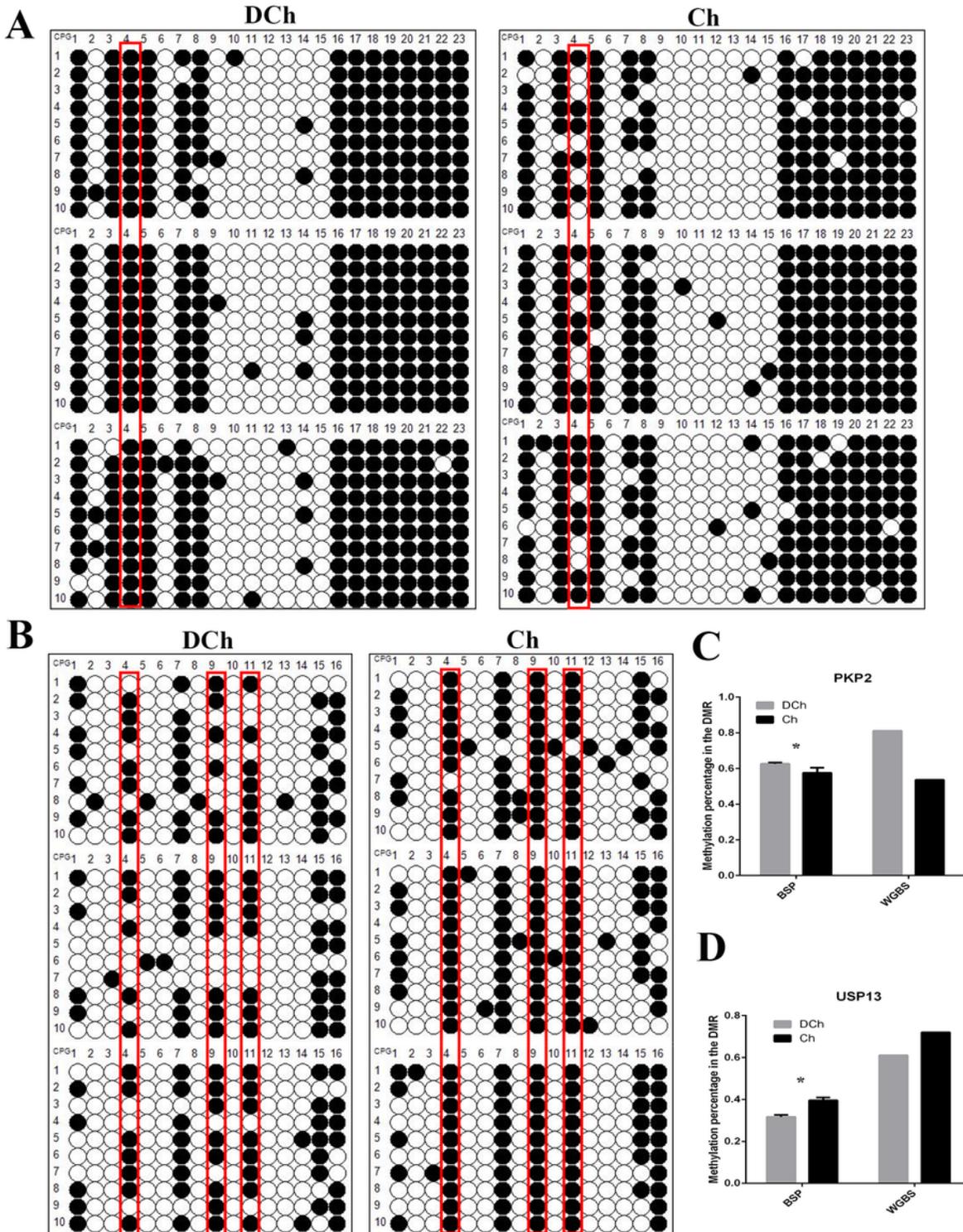
DNA methylation levels in different genomic elements. The abscissa represents the different genomic elements and the ordinate the methylation level. The functional regions of each gene were divided equally into 20 bins then averaged at the C site level of the methylation corresponding bin. Different colors represent different sequence contexts (CG, CHG or CHH).



**Figure 4**

Identification and structural annotation of DMRs. (A) Distribution of DMR length. The abscissa represents DMR length, the ordinate displaying DMR frequency for each corresponding length. (B) DMR volcano maps of two groups. The abscissa represents the difference in methylation level, the ordinate representing differences in methylation levels. areaStat is the sum of the T statistic of all C sites in the

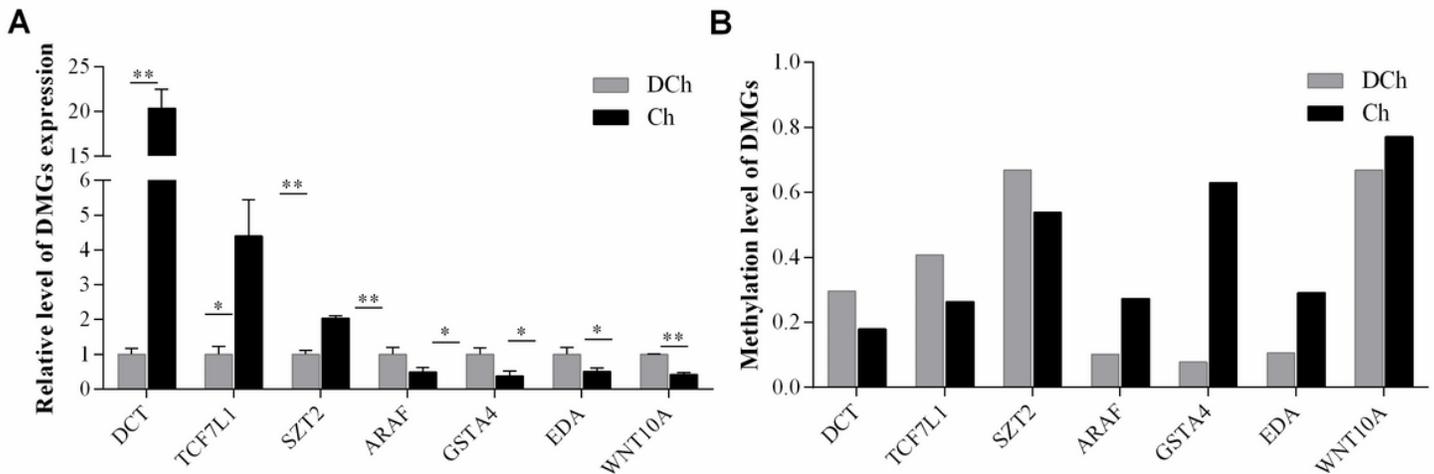
DMR region. (C) Functional region distribution of the DMRs. The abscissa represents different functional regions, the ordinate indicating the number of DMRs in different functional regions.



**Figure 5**

Verification of DMGs by bisulfite sequencing. (A) BSP analysis in the DMR\_8\_8510614 (PKP2) region. Black and white circles indicate methylated and non-methylated C sites, respectively. Each row represents a separate clone. Red boxes indicate significant methylation sites as identified using a Fisher's exact test.

(B) BSP analysis in the DMR\_14\_75103993 (USP13) region. (C) Comparison of the degree of methylation of DMR\_8\_8510614 (PKP2) between BSP and WGBS. For BSP, the ordinate represents the mean methylation rate of 3 samples in each group, while for WGBS, it represents the methylation level of the mean normalized DMR. The results represent means  $\pm$  SD, at two levels of significance, \* $P < 0.05$  and \*\* $P < 0.01$ . (D) Comparison of the extent of methylation of DMR\_14\_75103993 (USP13) between BSP and WGBS.



**Figure 6**

Verification of DMGs by qRT-PCR. (A) mRNA expression levels of 7 DMGs by qRT-PCR. (B) DNA methylation levels of 7 DMGs based on WGBS data.

## Supplementary Files

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