RESEARCH ARTICLE

CrossMark

Changes of host DNA methylation in domestic chickens infected with *Salmonella enterica*

FEI WANG, JIANCHAO LI, QINGHE LI, RANRAN LIU, MAIQING ZHENG, QIAO WANG, JIE WEN
and GUIPING ZHAO*

Institute of Animal Sciences, Chinese Academy of Agricultural Sciences, Beijing 100193, People's Republic of China
For correspondence. E-mail: zhaoguiping@caas.cn.

Abstract. Cytosine methylation is an effective way to modulate gene transcription. However, very little is known about the epigenetic changes in the host that is infected with *Salmonella enterica*. In this study, we used methylated DNA immunoprecipitation sequencing to analyse the genomewide DNA methylation changes in domestic chickens after infected with *Salmonella*. The level of DNA methylation was slightly higher in the genomic regions around the transcription start termination sites in a *Salmonella*-infected group compared to the controls. Overall, 879 peaks were differentially methylated between *Salmonella*-infected and control groups, among which 135 were located in the gene promoter regions. Genes including *MHC class IV* antigen, *GABARAPL1*, *MR1* and *KDM1B* were shown to be methylated more heavily after infected with *Salmonella*, whereas *DYNLRB2*, *SEC14L3* and *ANKIB1* tended to have fewer methylated cytosine residues in the promoter regions. Gene interaction network analysis of differentially methylated genes in the promoter regions revealed extensive connections with immune-related genes, indicating the possible impact of infection with *Salmonella* on the epigenetic status of the host.

Introduction

DNA methylation is involved in many biological activities, including regulation of gene expression, genomic imprinting, cell differentiation and silencing of transposons. Bedsides the methylation of cytosine in CpG dinucleotides, CHG or CHH (where H is A, T or C) was found to have methylated cytosine residues. A variety of DNA modifications have been reported in recent years, including 5-hydroxymethylcytosine, 5-formylcytosine and 5-carboxylcytosine (Ito *et al.* 2011). However, methylation of the fifth cytosine residue remains an efficient and dominant DNA modification that regulates gene expression.

Promoter DNA is the main target of methylation for suppressing gene transcription activity. Methylation of promoter DNA usually leads to corresponding suppressive modifications of histones in the same region and, thus, forms a condensed chromatin conformation that suppresses gene expression. Genomewide studies have provided sufficient evidence to show the negative correlation between promoter DNA methylation and the level of gene expression. By contrast, methylation in a gene body is always correlated positively with the level of gene expression.

Salmonella enterica, a major cause of infectious diseases, infects humans and other animals, and is a great threat to public health and animal production worldwide. The domestic chicken (*Gallus gallus domesticus*) is an important host of *S. enterica*, which causes a great loss for avian production every year. Thus, it is important to have a comprehensive understanding of how *S. enterica* infects chickens and the host immune response. The pathogenicity

of *S. enterica* relies heavily on its virulence genes, which are termed *Salmonella* pathogenicity islands (Srikanth *et al.* 2011). *S. enterica* secretes effectors that promote the entry of bacteria and control host inflammatory responses. For example, SipA of the bacteria is activated by caspase-3 and has a crucial role in bacterial entry (Giacomodonato *et al.* 2007), SopE promotes the activation of Rho GTPases and initiates the process of invasion (Hardt *et al.* 1998). Host innate immune responses are triggered by the *S. enterica* infection; NF-KB, ERK and p38 are induced strongly upon infection. Toll-like receptors recognize the infection and activate transcription responses to *S. enterica*, leading finally to the release of inflammatory factors, including interleukin-1β (IL-1β) and tumour necrosis factor α (TNF-α) from the macrophages (Srikanth *et al.* 2011). Using high-throughput approaches, many studies have provided comprehensive transcriptome-wide gene expression analyses in chickens challenged with *Salmonella*. Luan *et al.* (2012) have identified hundreds of differentially expressed genes in *Salmonella*-challenged chickens, which are represented by key genes, including IL-8, chemokine (C–C motif) ligand 5 (CCL5) and B-cell CLL/lymphoma 2 (Luan *et al.* 2012). Avidin immune responsive gene 1 and chemokine ah221 are highly induced in both spleen and cecum in *Salmonella*-infected (SE) chickens (Matulova *et al.* 2012). Genes such as IgG, interferon γ, inducible nitric oxide synthase 2, IL-1β and IL-17 would have inductions even >100-fold in chicken cecum after infected with *Salmonella* (Matulova *et al.* 2013). Although, there have been many investigations of the transcriptional changes in chickens infected with *Salmonella*, there are only a few reports of the molecular mechanisms underlying the transcription variations.

With the development of high-throughput sequencing or DNA array technologies, further analyses have been carried out on the epigenetic changes upon infection by pathogens. Through a genomewide DNA methylation analysis, Xu *et al.* (2015) showed DNA methylation changes in chickens challenged with *Escherichia coli*, the transcription of IL-8, IL 2 receptor β and IL 1 receptor accessory protein-like 1 were found to be epigenetically regulated in response to bacterial infection. Uropathogenic infection by *E. coli* in humans resulted in hypermethylation of CDKN2A and downregulation of its transcription (Tolg *et al.* 2011) and *Legionella pneumophila* induced the expression of IL-8 by promoting acetylation of IL-8 promoter (Schmeck *et al.* 2008). Besides the epigenetic changes induced by bacterial infection, the components of bacterial pathogens can modulate the epigenetic status of host genes. The nuclear targeted protein A (LntA) of *Listeria monocytogenes* prevented binding of the bromo adjacent homology domain containing 1 chromatin silencing complex to upregulate the expression of interferon-stimulated genes (Lebreton *et al.* 2011). *Listeria monocytogenes* increased the acetylation levels of H3K14 and H4K8 of the promoter of IL-8 (Schmeck *et al.* 2008).

The investigations mentioned above have demonstrated the influence of bacterial infection on host epigenetic modifications; however, details of the host epigenetic changes after infection by *Salmonella* are unknown.

Materials and methods

Animals

Eighty one-day-old white leghorn chickens were raised in an isolation tank. At day 12, the chickens were divided randomly into two groups. One group was used as the control and the other was challenged with *Salmonella*. Chickens were sacrificed 24-h postinfection. Three individuals in *articulo mortis* from the infected group and three from the control (NC) groups were used for the isolation of DNA from blood samples. The DNA of three individuals in each group was pooled for methylated DNA immunoprecipitation (meDIP) analysis. This research complied with the animal welfare guidelines for experimental animals established by the Ministry of Science and Technology of China.

Methylated DNA immunoprecipitation sequencing (*MeDIP-seq*)

Genomic DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany). The DNA was sheared into 400–1000 bp fragments by sonication (Bioruptor, Diagenode) and subjected to MeDIP-seq assay as described (Zhang *et al.* 2006; Li *et al.* 2011, 2015). Briefly, 2 mg of sonicated DNA was denatured and incubated with 5 mg of mouse monoclonal anti-5-methylcytosine antibody (Diagenode) in 500 mL of IP buffer (0.5% (v/v) NP40, 1.1% (v/v) Triton X-100, 1.5 mM EDTA, 50 mM Tris-HCl and 150 mM NaCl) at 4°C for 4 h on a rotating wheel. The mixture was incubated with 50 mL of magnetic beads coupled to ant–mouse IgG (Bangs Laboratories, USA) at 4°C for 2 h by end-over-end rotation and washed thrice with 700 mL of IP buffer. Methylated DNA was recovered using proteinase K digestion at 50°C for 3 h and purified by extraction with phenol–chloroform followed by precipitation in ethanol. The methylated DNA was used for the construction of a sequencing library and sequenced by HiSeq 2000 Illumina. The chicken GalGal4 genome release was used for reads alignment. Model-based analysis of ChIP-Seq (MACS) was used to scan the methylated peaks in the genome.

Pathway enrichment analysis

After infection with Salmonella, the genes with differentially methylated promoters were subjected to pathway analysis using the online tool KEGG orthology based annotation system (KOBAS) (Xie *et al.* 2011), which provided pathway enrichment analysis based upon the Kyoto

Table 1. Summary of meDIP-Seq data.

Sample	Total reads number	Number of mapped reads	Percentage of mapped reads (%)	Number of uniquely mapped reads	Percentage of uniquely mapped reads (%)
SE	45,088,386	34,476,442	74.3	30,350,306	67.3
NC	45,177,104	34,369,760	76.1	30,127,376	66.7

Table 2. Distribution of methylation peaks.

Sample	Peak number	Mean length of peak	Peak coverage of genome (%)
SE	65697	1783.2	11.5
NC	58943	1818.6	10.5

Encyclopedia of genes and genomes (KEGG) database. Pathways with only $P < 0.05$ were reserved.

Results

Genomewide DNA methylation analysis by MeDIP-seq

To decipher the variations in DNA methylation after infection with *Salmonella*, DNA from three samples of both SE and NC groups were pooled for MeDIP-seq analysis. A total of 45×10^6 raw reads were generated for both groups, of which >74% could be mapped onto the chicken genome and 66% were mapped uniquely (table 1).

MeDIP-seq is a methylated DNA enrichment-based technique that cannot give single-base resolution; however, it is reliable and is widely used for DNA methylation analysis. Further, methylation peak calling is a well-recognized approach for methylation difference analysis. We used MACS software to scan methylation peaks across the chicken genome. In all, 65,697 and 58,943 methylation peaks were found in the SE and NC groups covering 11.5% and 10.5% of the chicken genome, respectively (table 2).

We further investigated the DNA methylation distribution around the transcription start site (TSS) and termination site (TTS) and part of the gene body. The level of DNA methylation decreased towards TSS (figure 1) and the overall level of DNA methylation of the SE group was slightly higher around both TSS and TTS compared to the NC group (figure 1).

Identification of differentially-methylated genes (DMGs) after infection with Salmonella

With inclusion criteria of fold change >1.5 and $P < 0.05$, we identified 879 peaks methylated differentially between the SE and NC groups. These peaks were further divided according to the location in the promoter, in the gene body or in other genomic positions. We defined the promoter as the genomic regions from TSS to 2 kb upstream. In all, 120 peaks were located in promoters with 71 upregulated and 49 downregulated in the SE group (figure 2; table 3). There were 544 differentially methylated peaks in the gene body regions and more than half were upregulated in the SE group (table 3).

Promoter DNA methylation is the most effective way to downregulate the level of gene expression. We assumed genes with significant DNA methylation changes in promoter regions as crucial genes affected by *Salmonella* infection and termed these as DMGs. In this study, 135 genes were differentially methylated after infection with *Salmonella*. This number was less than the number of differentially methylated peaks because some peaks occurred in the same gene. A large portion of the DMGs were not well annotated in the chicken genome but many genes with important biological functions were included in the DMGs; for example, methylation levels of the promoter of major histocompatibility complex (MHC) class IV antigen, GABA(A) receptor-associated protein like 1 (GABARAPL1), major histocompatibility complex, class I-related (MR1) and lysine (K)-specific demethylase 1B (KDM1B) were increased in the SE group. Dynein light chain, roadblock-type 2 (DYNLRB2), SEC14-like 3 (SEC14L3), and ankyrin repeat and IBR domain containing 1 (ANKIB1) showed decreased levels of methylation in promoter regions.

Expression of DMGs in SE chicken spleen

We undertook transcriptome analysis of SE chicken spleen by high-throughput sequencing (data not shown). We knew DNA methylation and gene expression varied among different tissues even in the same individual; however, we thought it might provide some clues for understanding the mechanism underlying *Salmonella* infection if we
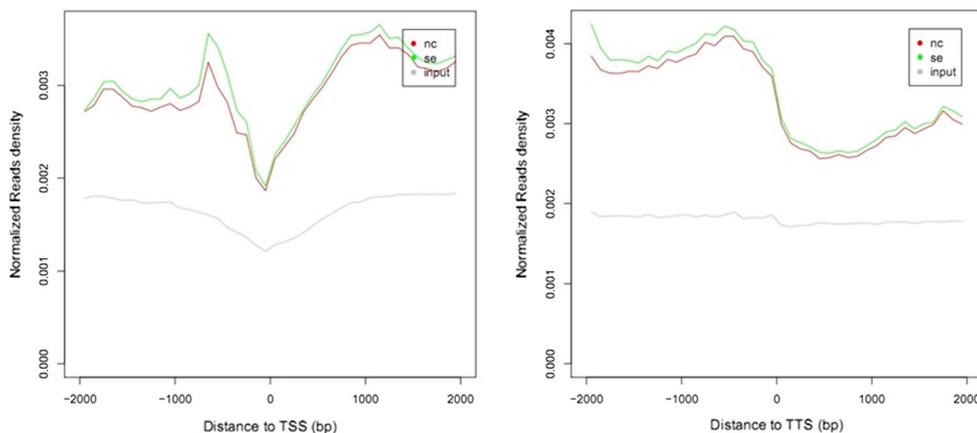
Figure 1. DNA methylation around gene flanking regions. The level of DNA methylation was calculated by the reads that were aligned to the corresponding genomic regions. The 2 kb regions upstream and downstream from the transcription start site and termination site were included in the analysis. The regions were split into 20 regions not overlapped and the average depth of coverage was calculated for each window.
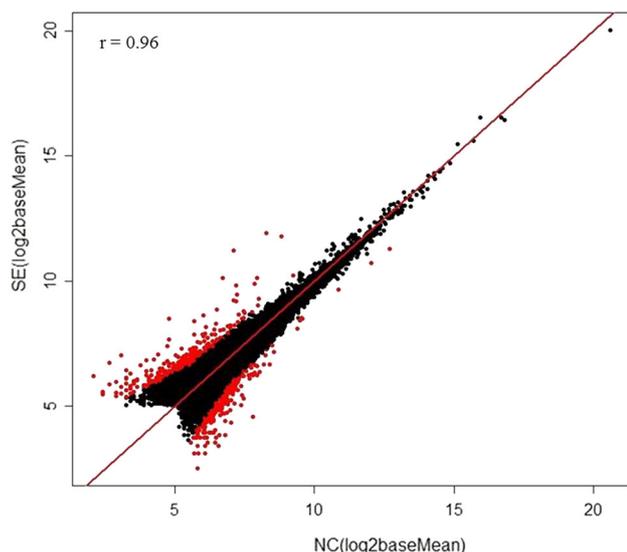

Figure 2. A scatter plot showing the correlation between the control group and the SE group. The red dots indicate differentially methylated peaks.

combined the methylation data from the blood sample with the gene expression data for the spleen. Of the 135 DMGs, 25 had reverse trends of gene expression change in the spleen (Pearson's correlation coefficient $r = -0.51$)

(figure 3; table 1 in electronic supplementary material at http://www.ias.ac.in/jgenet/).

Gene interaction networks of DMGs

We investigated the gene interaction networks of DMGs in blood samples. Only DNA-directed polymerase β (POLB), GABARAPL1, catalase (CAT) and proteasome subunit, β type, 3 (PSMB3) were found to have interaction partners (figure 4). We used data for the genes of these four interaction proteins to construct gene interaction networks. GABARAPL1, CAT and POLB interacted with 10, nine and four genes, respectively. Proliferating cell nuclear antigen (PCNA) was involved in synthesis of the leading strand during DNA replication (Chilkova *et al.* 2007). In this study, it was associated with POLB, which is a DNA polymerase responsible for base excision and repair. GABARAPL1 was associated with phosphoinositide-3-kinase class 3 (PIK3C3), which is the catalytic subunit of the PI3K complex that has a crucial role in the initiation and maturation of autophagosomes (Roberts *et al.* 2013). Fas ligand (FASLG) induced cell apoptosis when triggered by the FAS/FASLG signalling pathway were found to interact with both CAT and GABARAPL1, which were DMGs in the promoter upon Salmonella infection.

Table 3. Summary of differentially methylated peaks between *Salmonella* infected and control groups.

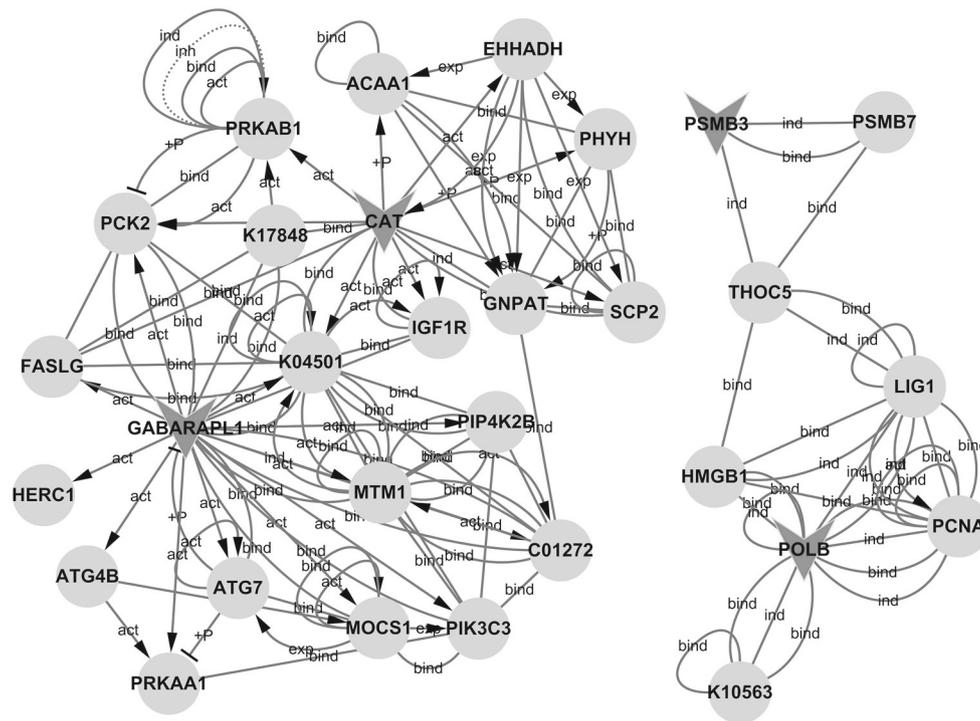| Genomic regions	Upregulated peaks	Downregulated peaks	Total number of peaks
Promoter	71	49	120
Gene body	286	258	544

Figure 3. Gene interaction networks of the DMGs after infection with Salmonella. Act, activate; exp, transcription regulation; ind, indirect interaction; +P, phosphorylate; inh, inhibit.
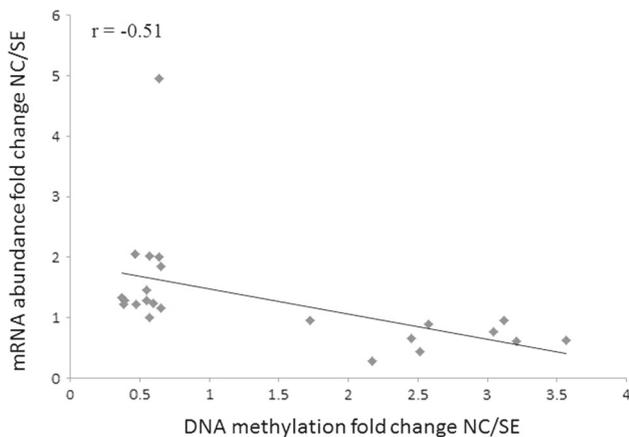

Figure 4. Correlation between the gene expression changes and DNA methylation changes of the DMGs.

Discussion

We present the results of genomewide DNA methylation analysis of SE chicken. We found DNA methylation levels of a substantial number of genes were altered by the infection of *Salmonella*. Methylation of promoter DNA has a crucial impact on gene expression. In this study, we found 135 genes which were differentially methylated in promoter regions, among which many were host immune response-related genes. For example, promoters of MHC and MHC class IV antigen were modified with more methylated cytosine residues compared to the controls. Our results revealed the impact of *Salmonella* infection on the methylation of immune-related genes.

The promoter region of a gene is crucial for regulation of gene expression. After *Salmonella* infection, promoters of MHC class IV antigen, GABARAPL1, MR1, KDM1B, DYNLRB2, SEC14L3 and ANKIB1 showed altered levels of DNA methylation. Transcription of GABARAPL1, which might be involved in maturation of the autophagosome, was upregulated in human enteroendocrine cells following infection with *Chlamydia trachomatis* (Dlugosz *et al.* 2014). The dynein complex is involved in the intracellular transport of various membranous organelles, including lysosomes, endosomes, phagosomes and the Golgi complex (Hirokawa 1998). The dynein complex might also be involved in the delivery of viral or bacterial components after infection (Merino-Gracia *et al.* 2011).

We applied gene interaction network analysis for DMGs in the promoter after infection with *Salmonella*. The gene interaction network reflects the reciprocal relations between genes, such as inhibition, activation, binding or phosphorylation. Owing to the lack of full annotation of the chicken genes, only a small subset of DMGs was included in the gene interaction network. CAT, POLB, PSMB3 and GABARAPL1 were the core of the network. Host factors, including PCNA, PIK3C3 and FASLG were found to interact with the DMGs after infection with *Salmonella*. POLB interacted with PCNA and both were

involved in the replication of DNA. PCNA was shown to have a crucial impact on the replication of herpes simplex virus (Sanders *et al.* 2015). Our results indicated that infection with *Salmonella* might affect the activity of genes involved in DNA replication. Autophagosomes are involved in the intracellular degradation of cytoplasmic contents and invading microorganisms. We found GABARAPL1, which was associated with formation of the autophagosomal vacuole, was methylated differentially after infection with *Salmonella*. Through the gene interaction network, we showed GABARAPL1 was associated with several genes, including FASLG and PIK3C3, with function related to an immune response, indicating a possible impact on the related immune processes of *Salmonella* infection.

Acknowledgements

References

Chilkova O., Stenlund P., Isoz I., Stith C. M., Grabowski P., Lundstroem E. *et al.* 2007 The eukaryotic leading and lagging strand DNA polymerases are loaded onto primer-ends via separate mechanisms but have comparable processivity in the presence of PCNA. *Nucleic Acids Res.* **35**, 6588–6597.

Dlugosz A., Muschiol S., Zakikhany K., Assadi G., D'Amato M. and Lindberg G. 2014 Human enteroendocrine cell responses to infection with Chlamydia trachomatis: a microarray study. *Gut Pathog.* **6**.

Giacomodonato M. N., Uzzau S., Bacciu D., Caccuri R., Sarnacki S. H., Rubino S. *et al.* 2007 SipA, SopA, SopB, SopD and SopE2 effector proteins of Salmonella enterica serovar Typhimurium are synthesized at late stages of infection in mice. *Microbiology-Sgm* **153**, 1221–1228.

Hardt W. D., Chen L. M., Schuebel K. E., Bustelo X. R. and Galan J. E. 1998 S-typhimurium encodes an activator of Rho GTPases that induces membrane ruffling and nuclear responses in host cells. *Cell* **93**, 815–826.

Hirokawa N. 1998 Kinesin and dynein superfamily proteins and the mechanism of organelle transport. *Science* **279**, 519–526.

Ito S., Shen L., Dai Q., Wu S. C., Collins L. B., Swenberg J. A. *et al.* 2011 Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* **333**, 1300–1303.

Lebreton A., Lakisic G., Job V., Fritsch L., To Nam T., Camejo A. *et al.* 2011 A bacterial protein targets the BAHD1 chromatin complex to stimulate type III interferon response. *Science* **331**, 1319–1321.

Li Q., Li N., Hu X., Li J., Du Z., Chen L. *et al.* 2011 Genome-wide mapping of DNA methylation in chicken. *PLoS One* **6**.

Li Q., Wang Y., Hu X., Zhao Y. and Li N. 2015 Genome-wide mapping reveals conservation of promoter DNA methylation following chicken domestication. *Sci. Rep.* **5**.

Luan D. Q., Chang G. B., Sheng Z. W., Zhang Y., Zhou W., Li Z. Z. *et al.* 2012 Analysis of gene expression responses to a Salmonella infection in Rugao chicken intestine using genechips. *Asian-Aust. J. Anim. Sci.* **25**, 278–285.

Matulova M., Rajova J., Vlasatikova L., Volf J., Stepanova H., Havlickova H. *et al.* 2012 Characterization of chicken spleen transcriptome after infection with Salmonella enterica serovar enteritidis. *PLoS One* **7**, e48101.

Matulova M., Varmuzova K., Sisak F., Havlickova H., Babak V., Stejskal K. *et al.* 2013 Chicken innate immune response to oral infection with Salmonella enterica serovar Enteritidis. *Vet. Res.* **44**, 37.

Merino-Gracia J., Garcia-Mayoral M. F. and Rodriguez-Crespo I. 2011 The association of viral proteins with host cell dynein components during virus infection. *FEBS J.* **278**, 2997–3011.

Roberts R., Al-Jamal W. T., Whelband M., Thomas P., Jefferson M., van den Bossche J. *et al.* 2013 Autophagy and formation of tubulovesicular autophagosomes provide a barrier against nonviral gene delivery. *Autophagy* **9**, 667–682.

Sanders I., Boyer M. and Fraser N. W. 2015 Early nucleosome deposition on, and replication of, HSV DNA requires cell factor PCNA. *J. Neurovirol.* **21**, 358–369.

Schmeck B., Lorenz J., N'Guessan P. D., Opitz B., van Laak V., Zahlten J. *et al.* 2008 Histone acetylation and flagellin are essential for Legionella pneumophila-induced cytokine expression. *J. Immunol.* **181**, 940–947.

Srikanth C. V., Mercado-Lubo R., Hallstrom K. and McCormick B. A. 2011 Salmonella effector proteins and host-cell responses. *Cell. Mol. Life Sci.* **68**, 3687–3697.

Tolg C., Sabha N., Cortese R., Panchal T., Ahsan A., Soliman A. *et al.* 2011 Uropathogenic E. coli infection provokes epigenetic downregulation of CDKN2A (p16INK4A) in uroepithelial cells. *Lab. Invest.* **91**, 825–836.

Xie C., Mao X., Huang J., Ding Y., Wu J., Dong S. *et al.* 2011 KOBAS 2.0: a web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res.* **39**, W316–W322.

Xu H., Zhu X., Hu Y., Li Z., Zhang X., Nie Q. *et al.* 2015 DNA methylome in spleen of avian pathogenic escherichia coli-challenged broilers and integration with mRNA expression. *Sci. Rep.* **4**.

Zhang X., Yazaki J., Sundaresan A., Cokus S., Chan S. W. L., Chen H. *et al.* 2006 Genome-wide high-resolution mapping and functional analysis of DNA methylation in Arabidopsis. *Cell* **126**, 1189–1201.

Corresponding editor: Indrajit Nanda