RESEARCH PAPER

Enterotoxigenic *Escherichia coli* infection promotes enteric defensin expression via FOXO6-METTL3-m⁶A-GPR161 signalling axis

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ABSTRACT

The production of natural antimicrobial peptides has emerged as an important mechanism of innate immunity in animals. Defensins, members of a large family of antimicrobial peptides, have been suggested as effector molecules in host defence against bacteria, fungi, protozoa and enveloped viruses. However, the molecular mechanism underlying defensin upregulation in bacterial infection remains poorly understood. The modification of mRNA by N6-adenosine methylation (m⁶A) on internal bases influences gene expression in eukaryotes. Here, we show that β-defensin production triggered by Enterotoxigenic *Escherichia coli* K88 (*E. coli* K88) infection is controlled by the cellular m⁶A methyltransferase METTL3. Adding back with METTL3 robustly stimulated the re-expression of defensin, which further supports the conclusion. Furthermore, using a MeRIP-seq approach, we identified a functional connection between m⁶A dependent GPR161 signalling and the expression of defensins. Mechanistically, we found that the transcription factor FOXO6 interacted with METTL3 to trigger the transcription of GPR161 and the subsequent regulation of β-defensin expression. The study has shed light on the mechanisms by which enterotoxigenic *Escherichia coli* infection promotes enteric defensin expression.

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Introduction

The intestinal epithelium is exposed to myriad of enteric pathogens and provides barrier protection against enteric pathogens [1]. It is becoming increasingly evident that antimicrobial peptides (AMPs) increase the potential of intestinal epithelial cells (IECs) to prevent bacterial uptake and help maintain intestinal homeostasis [2]. For mammals, among the various AMPs produced in the gastrointestinal tract, the two main antimicrobial peptide families are defensins and cathelicidins. In particular, defensins are widely distributed in mammalian epithelial cells and phagocytes and are often present at high concentrations [3,4]. Defensins are cysteine-rich, cationic peptides with β-pleated sheet structures [5]. They are further classified as α-defensins or β-defensins, depending on the position of their disulphide bonds [6]. Compared to that of the α-defensins, the relative expression level of the β-defensins is more variable throughout the gastrointestinal tract [7].

In response to infection, target cells can produce defensins to defend against pathogens in a way that is similar to the manner in which cytokine is induced. Enteropathogenic *Escherichia coli* infection promotes the rapid induction of hBD2 and other proinflammatory molecules in a type three-

secretion system (TTSS)-dependent manner [8]. In previous studies, we showed that the expression of intestinal β-defensins and other AMPs was upregulated in *Escherichia coli* O157:H7 [9] or enterotoxigenic *Escherichia coli* K88 (*E. coli* K88) infected weaned piglets [10]. However, the genetic basis and the mechanisms of defensin expression upon infection remain largely unexplored.

Posttranscriptional modification of mRNA has emerged as an important mechanism for gene regulation. More than 150 distinct chemical marks on cellular RNA have been identified to date [11]. Among these modifications, N6-methyladenosine (m⁶A) has been identified as the most prevalent and abundant posttranscriptional RNA modification in eukaryotic mRNA [12], and occurring primarily in two consensus sequence motifs, G m⁶A C (~70%) and A m⁶A C (~30%) [13,14]. Recent transcriptome-wide m⁶A site mapping revealed its preferential distribution around stop codons and its enrichment at 3' untranslated regions (3' UTRs) in the transcriptomes of humans, mice and even pigs [15–17]. m⁶A in mRNA is a dynamic and reversible modification [18]. Deposition of m⁶A occurs cotranscriptionally through a large protein complex ('writers'), comprising the catalytic subunit methyltransferase-like (METTL) 3 and cofactors such as METTL14 and Wilms' tumour 1-associated protein (WTAP), KIAA1429, RNA binding motif protein 15

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(RBM15) and zinc finger CCCH domain-containing protein 13 (ZC3H13) [19–22]. METTL3 possesses an active methyltransferase domain that catalyzes the conversion of adenosine (A) to m⁶A, METTL14 acts as the RNA-binding platform, and therefore, the METTL3-METTL14 forms a stable heterodimer and highly conserved in mammals. The removal of an m⁶A is facilitated by ‘eraser’ enzymes, including fat mass and obesity-associated protein (FTO) and alkB homologue 5 (ALKBH5) [18,23]. Building on the knowledge of the widespread prevalence of the m⁶A mRNA modification, researchers have recently linked a variety of fundamental cellular processes involving mRNA export, splicing, stability, translation and even gene transcription to m⁶A [24–26]. Remarkably, increasing evidences have revealed that m⁶A is widely involved in the immune response, including T cell homeostasis [27], dendritic cell activation [28], type I IFN production [29] and viral infection [30]. However, the role of m⁶A modification in intestinal innate immunity, especially in the defensin secretion, is unknown.

The G protein-coupled receptors (GPCRs) constitute a large family of heptahelical plasma membrane-spanning proteins that have evolved to transduce extracellular signals into intracellular responses [31]. G proteins and/or their downstream signalling pathways have been implicated in many diseases, and pharmacological modulation of GPCRs has been successfully used in a wide range of diseases, such as those of the cardiovascular, endocrine, neural and immune systems [32,33]. GPR43 has been reported to trigger the expression of defensins through short-chain fatty acids [34]. GPR161 has been identified as a negative regulator of sonic hedgehog (Shh) signalling [35]. The role of GPR161 in the immune response still remains largely unexplored. In this study, using MeRIP-seq, we identified a functional connection between m⁶A-dependent GPR161 signalling and the expression of defensins in intestinal epithelial cells during *E. coli* K88 infection. Moreover, we connected the mechanism of this expression to the transcriptional initiation of *GPR161* gene. Thus, we describe an m⁶A-dependent molecular mechanism that determines β -defensin expression via GPR161 signalling, paving the way to the development of more effective novel therapeutic strategies to treat intestinal bacterial infections.

Materials and methods

Reagents

The following antibodies were used for immunoprecipitation (IP) and immunoblot analysis (IB): GPR161 (13398-1-AP, rabbit, 1:1000), FOXO6 (19122-1-AP, rabbit, 1:1000), GFP (50430-2-AP, rabbit, 1:1000), FLAG (20543-1-AP, rabbit, 1:1000), METTL3 (15073-1-AP, rabbit, 1:1000) and β -actin (60008-1-Ig, mouse, 1:1000) were purchased from Proteintech (Wuhan, China); m⁶A antibody (202111, mouse, 1:1000) was obtained from Synaptic Systems (Germany, Goettingen, Germany). The enterotoxigenic *Escherichia coli* K88 strain used was the standard strain C83907 (O149:K91B:F4ac) obtained from the China Institute of Veterinary Drug Control (Beijing, China). This strain was confirmed to be positive for heat-labile toxin (LT) and heat-stable enterotoxin b (STb), and expressed fimbriae F4ac, as determined by PCR

genotyping (Supplementary Figure S1a and b). The specific primers are presented in Supplementary Table S1.

Cell culture, transfection and *E. coli* K88 infection

Intestinal porcine epithelial cell line J2 (IPEC-J2) cells were purchased from the CBCAS (Cell Bank of the Chinese Academy of Sciences, Shanghai, PR China) and cultured as described previously [36]. All cells tested negative for mycoplasma contamination. The cells were cultured in six-well culture dishes until they reached approximately 80% confluence and then infected with *E. coli* K88 (MOI = 10:1) for 0, 1, 2, 3 h as described previously [37]. Cells without infection were used as control. Plasmids were transfected into cells with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Molecular cloning of related genes

All plasmids, including METTL3, inactive mutant METTL3 (D395A), DEFb1 and DEFb2 were generated by GenScript (Nanjing, China) according to their transcripts of pig in NCBI (<https://www.ncbi.nlm.nih.gov>), which were cloned into Flag-EGFP-pcDNA3.1 vector. Flag and defensin are fusion-expressed through a promoter independent of the promoter of GFP. All plasmids were confirmed by DNA sequencing.

Lentiviral shRNAs and siRNA

Lentiviral shRNAs were performed as described previously [38]. shRNA and siRNA targeting sequences are listed below:

METTL3: 5'- CAGAAATGCAGTATGATATAT -3'

GPR161: 5'- GATCTATGGACTCTGGAATAA -3'

FOXO6: 5'- GTGCCCTACTTCAAGGATAAA -3'

Scramble control sequence: 5'-

AACAGTCGCGTTTGCGACTGG-3'.

siRNA for human *METTL3*: 5'- GCAAGAATTCTGTGACTAT-3'.

All the shRNA targeting sequences were cloned into DECIPHER™ pRSI9-U6-(sh)-UbiC-TagRFP-2A-Puro (obtained from professor Shubing-Qian, Cornell University, USA). Lentiviral particles were packaged using Lenti-X 293 T cells (obtained from professor Shubing-Qian, Cornell University, USA). Virus-containing supernatants were collected at 48 h after transfection and filtered to eliminate cells. IPEC-J2 cells were infected by the lentivirus for 48 h before selection by 3 μ g/ml puromycin. Constant puromycin selection pressure is required for cell line maintenance.

Quantitative real-time PCR analysis (q-PCR)

Total RNA was isolated from pig alveolar macrophage cells by using TRIzol (Invitrogen) according to the manufacturer's instructions. 1 μ g of total RNA was reverse transcribed using the MMLV Reverse Transcriptase (Thermo scientific, USA) following the supplier's protocol. The reactions were incubated at 25°C for 20 min, then at 42°C for 60 min, and terminated at 70°C for 10 min. Real-time PCR reactions were conducted in triplicate on a Step one Real-Time PCR System (ABI Step one

Plus, Applied Biosystems, Foster City, CA, USA) using FastStrat Universal SYBR Green Master (ROX) (Roche, USA). The specific primers are presented in Supplementary Table S2. Real-time PCR reactions were performed as follows: a precycling stage at 95°C for 30 s, then 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 34 s. Fluorescence was measured at the end of each annealing step, and the melting curves were monitored to confirm the specificity of the PCR products. mRNA expression levels were determined using the $2^{-\Delta\Delta C_t}$ method with porcine Gapdh as a reference.

Immunoblot analysis

The whole protein was obtained by Whole Protein Extraction Kit (KeyGEN Biotech, Nanjing, China) according to the manufacturer's instruction. Equal amounts of proteins were loaded and separated by 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes. The membrane was then washed with 1× PBST buffer, blocked with 5% non-fat milk in PBST, and incubated with anti-indicated antibody (1:1,000) overnight at 4°C. Specific bands were visualized with an ECL detection kit (Biosharp, Hefei, China) and scanned using a ChemiScope series 3400 mini system (Clinx Science Instruments, Shanghai, China). Band densities were quantified and normalized to β -actin using Image J v1.47 software (<https://imagej.nih.gov/ij>).

Immunofluorescence staining

After treatment indicated in each experiment, the cells were washed once in PBS and then fixed in 4% paraformaldehyde at room temperature for 15 min. Immunofluorescence was performed as described previously [39]. The images were captured by a confocal microscope (Zeiss LSM710).

m⁶A dot blot

The m⁶A dot blot assay was conducted as previously described [39]. In briefly, mRNA was purified from total RNA using GenElute™ mRNA Miniprep Kit (Sigma). Equal amounts of mRNA were spotted onto an Amersham Hybond-N⁺ membrane (GE Healthcare), followed by UV crosslinking. After incubation with m⁶A antibody (1:1000) overnight at 4°C, the membrane was incubated with antibody anti-mouse (HA1006, HuaAn, Hangzhou, China; 1:5000) for 1 h and visualized by using ECL. Methylene blue staining was used as loading control.

Chromatin immunoprecipitation (ChIP)

ChIP reactions were performed essentially as previously described [40]. Cells were washed and added 1% formaldehyde, and then 0.125 M glycine was added and washed with cold PBS. Cells were lysated in buffer (10 mM Tris-Cl, pH 8.0, 5 mM EDTA, 0.5% NP40, 0.25% triton, 85 mM KCl) with proteinase inhibitor (APExBIO, K1007, Houston, USA). DNA was sonicated and added the agarose beads with IgG control or with FOXO6 antibody at 4°C overnight. Beads were washed, eluted, reversed crosslinking. Primer used for the GPR161 promoter

was listed in supplementary Table 2. Results were normalized to reactions performed with 1% of input.

Coimmunoprecipitation (Co-IP)

At 3 h after infection, IPEC-J2 cells were washed in PBS, harvested and lysed on ice for 30 min in lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM KCl, 10% glycerol, 5 mM MgCl₂, 0.1% NP-40 and protease inhibitors). Supernatants were incubated for 2 h with the immunoprecipitating antibody along with protein A or protein G agarose beads at 4°C followed by three washes of the beads with lysis buffer. Immunoprecipitate on protein A or G beads was eluted by boiling. IP eluates were processed for western blot analysis.

Methylated RNA immunoprecipitation library construction and sequencing

m⁶A-specific-methylated RNA immunoprecipitation (MeRIP) with next-generation sequencing was performed by Novogene (Beijing, China) as previously described [41]. Briefly, m⁶A precipitated RNA fragments were used for library construction with the NEBNext Ultra RNA library Prep kit from Illumina and were sequenced with Illumina HiSeq 2000. Sequencing reads were aligned to the Sscrofa11.1 by Burrows-Wheeler Aligner (<http://bio-bwa.sourceforge.net>), and the m⁶A peak calling by MACS2 (<https://pypi.org/project/MACS2/>). The accession number for the m⁶A-seq reported in this paper is SRA BioProject: PRJNA645290.

mRNA sequencing and analysis

mRNA sequencing and analysis were performed by Novogene (Beijing, China). Briefly, total RNA was first isolated using Trizol reagent followed by fragmentation using freshly prepared RNA fragmentation buffer (10 mM Tris-HCl, pH 7.0, 10 mM ZnCl₂). Illumina TruSeq stranded RNA libraries were prepared from 500 ng of poly(A)-selected total RNA by Novogene (Beijing, China) using the NEBNext Ultra II RNA library preparation kit (New England Biolabs). Twelve libraries were multiplexed and sequenced on an Illumina HiSeq 2000. Gene expression levels were measured using the criteria of reads per kb per million mapped reads. DEGs were detected using the DESeq software [42]. Genes with an adjusted P-value <0.05 were assigned as differentially expressed. Kyoto Encyclopaedia of Genes and Genomes (KEGG) enrichment was performed by the Clusterfile software. Significance analysis was performed by Fisher's Exact Test. The accession number for the RNA-seq reported in this paper is SRA BioProject: PRJNA645284.

Statistical analysis

Results were expressed as means ± SEM. For multiple groups, One-way ANOVA was used to determine statistical differences, and t-test was employed between two groups. **P* < 0.05, ***P* < 0.01. Data were analysed with GraphPad Prism Software. Each experiment was performed at least in triplicate unless otherwise stated.

Results

E. coli K88 promoted the expression of β -defensin and RNA m⁶A methylation in intestinal epithelial cells

To broadly define the effects of *E. coli* K88 on the expression of β -defensin, we first analysed the mRNA levels of β -defensin-1 (*DEFb1*) and -2 (*DEFb2*). As shown in Fig. 1A, *E. coli* K88 infection induced the expression of *DEFb1* and *DEFb2* in a time-dependent manner. Unlike β -defensin, the mRNA level of *Cathelicidins*, especially *PAMP37*, was barely affected by *E. coli* K88 infection (Figure S1c). To further confirm this conclusion, we determined their protein levels using the constructed Flag fusion expression vector. Consistent with the mRNA levels, the protein levels of Flag-*DEFb1* and Flag-*DEFb2* increased after *E. coli* K88 infection in a time-dependent manner (Fig. 1B). In addition, we also found that *E. coli* K88 infection notably increased global m⁶A abundance in a time-dependent manner in IPEC-J2 cells, as determined by m⁶A dot blot assay with poly(A)⁺ RNA (Fig. 1C). To explain the change in m⁶A level, we measured the level of m⁶A-related enzymes. Interestingly, there was no change in the protein and mRNA levels of the 'writers' (METTL3 and METTL14) and 'erasers' (FTO and ALKBH5) during *E. coli* K88 infection (Figure S2a and b). However, the distribution of METTL3 was mainly in the nucleus, no matter with or without infection (Figure S2c). These results indicated that RNA m⁶A methylation may play an important role in *E. coli* K88 infection induced the expression of the β -defensin.

METTL3-deficient intestinal epithelial cells hypoproduced β -defensin in a m⁶A dependent manner

To investigate the role of RNA m⁶A, knocking down of METTL3 cell lines that we established previously were used [39]. In scramble control cells, the expression of *DEFb1* and *DEFb2* was increased in a time-dependent manner upon *E. coli* K88 infection. However, in the cells without METTL3, *E. coli* K88 infection failed to induce the expression of β -defensin (Fig. 2A and B). The phenotype was also validated in the human Caco2 cells (Figure S3a). Consistently, the levels of Flag-*DEFb1* and -*DEFb2* were decreased markedly at 3 h postinfection in the METTL3 depleted cells compared to the wild-type cells (Fig. 2C). As an independent validation, we transfected METTL3 or vector plasmids into METTL3-knockdown cells, we found that the expression of β -defensin in the cells transfected with the METTL3-expressing plasmid was much higher than it was in the cells transfected with the vector plasmid (Fig. 2D and E). Similarly, forced expression of METTL3 robustly stimulated the re-expression of Flag-*DEFb1* and -*DEFb2* (Fig. 2F), which indicates that METTL3 is a major contributor to the expression of β -defensin.

Additionally, to ascertain whether the METTL3 regulation of β -defensin expression was dependent on its m⁶A methyltransferase activity, we constructed the catalytically inactive mutant plasmid of METTL3 (D395A). As shown in Figure S3b-c, different than in cells with the wild-type METTL3 plasmid, the decrease in β -defensin induction was not suppressed after the catalytically inactive D395A mutant plasmid was transfected. Therefore, m⁶A modification appears to regulate the expression of β -defensin during *E. coli* K88 infection.

Figure 1. *E. coli* K88 simultaneously promotes the expression of β -defensin with m⁶A methylation. IPEC-J2 cells infected with *E. coli* K88 (MOI = 10:1) were analysed at various times, and the cells without infection constituted the control group. (A) The mRNA levels of *DEFb1*, *DEFb2* and β -actin were measured by q-PCR, and the results are presented relative to those of *Gapdh*. (B) Immunoblotting was used to analyse the protein levels of *DEFb1* and *DEFb2* after transfection of the Flag fusion expression vector. The right panel shows the relative protein levels quantified by densitometry and normalized to the level of GFP. (C) m⁶A Dot blot was to measure the m⁶A levels with purified mRNA. Methylene blue staining was used as a loading control. The right panel shows the relative levels quantified by densitometry and normalized to the level of the control. The data are expressed as the mean $\pm$ SEM; statistically significant difference relative to the control: **P* < 0.05, ***P* < 0.05, *n* = 3 biological replicates.

Analysis of downstream target of METTL3 in intestinal epithelial cells after *E. coli* K88 infection

To explore the mechanism by which METTL3 regulates β-defensin expression, we first used m⁶A sequencing (m⁶A-seq) with independent biological replicates to analyse the downstream targets of METTL3 through a comprehensive study of gene expression. In total, m⁶A-seq identified 10,148 and

9,741 m⁶A peaks in the control and *E. coli* K88-infected cells, respectively (Figure S4a). We searched for consensus motifs and discovered that the GGACU motif was highly enriched in m⁶A sites in both the control and the infected cells (Fig. 3A). We further investigated the m⁶A distribution patterns within both total and unique peaks. A similar pattern of common m⁶A distribution in the control and the *E. coli*

Figure 3. RNA m⁶A methylome profiles of IPEC-J2 cells after *E. coli* K88 infection. (A) Sequence motifs within m⁶A sites identified by using Homer software. (B) Pie chart depicting the m⁶A peaks as fractions. (C) Enrichment of m⁶A peaks in transcripts. Each transcript was divided into three parts: 5'UTR, CDS and 3'UTR. (D) The differentially m⁶A modified transcripts. (E) Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of m⁶A differentially modified genes. (F) Peaks indicate the relative abundance of m⁶A sites in GPR161 mRNA. The m⁶A motif sequences that corresponded to an immunoprecipitate-enriched region are marked in green.

K88-infected cells was observed when the RNA species were divided into the 5'UTR, CDS and 3'UTR regions of the mRNAs and the noncoding RNAs (Fig. 3B). Interestingly, in contrast to the control group, m⁶A peaks in coding sequences (CDSs) (47.83% and 48.13%, respectively), and 3'UTRs (27.32% and 27.53%, respectively) increased a little. However, *E. coli* K88 barely affect the distribution of m⁶A peaks in 5'UTRs (7.47% and 6.24%, respectively) (Fig. 3C). Then, we identified 232 m⁶A differentially modified transcripts. Among these transcripts, 142 genes were upregulated (log₂ fold change >1), and 90 genes were downregulated (log₂ fold change <-1) (Fig. 3D).

To predict the functions associated with m⁶A-modified genes, we conducted a Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis. Top pathways enriched among genes differentially modified upon *E. coli* K88 infection included those controlling signalling by digestion, Toll-like receptor, infection and influenza, many of which impact infection biology and immune responses (Fig. 3E). Notably, pathway analysis of genes up-regulated by *E. coli* K88 infection included those controlling transcriptional misregulation, TGF- β , hedgehog and anti-infection response-related receptors (Figure S4b). In contrast, top pathways enriched among genes down-regulated upon *E. coli* K88 infection included those involved in vitamin digestion, p53, metabolic processes and acid secretion (Figure S4c).

Among the upregulated genes, we identified *GPR161* as a potential candidate substrate of METTL3 that regulates the expression of β -defensin. As shown in Fig. 3F, there was a statistically significant m⁶A peak in the *GPR161* transcripts. Compared with the control group, the modified level of *GPR161* in the infected group increased by 50%, suggesting a functional relevance between m⁶A and antimicrobial response. Therefore, we hypothesized that METTL3 induced the expression of β -defensin via *GPR161* signalling to defend against *E. coli* K88 infection.

The METTL3-mediated *GPR161* was essential for *E. coli* K88 induced β -defensin expression

Since *GPR161* could be a direct target of METTL3 and a driver of the altered expression of β -defensin, we first investigated the change in the expression levels of *GPR161* when METTL3 was knocked down. As expected, we found that loss of METTL3 suppressed the expression of *GPR161* in a time-dependent manner upon *E. coli* K88 infection, a finding that differed from that of the scramble control cells (Fig. 4A). Interestingly, the suppressed *GPR161* induction was accompanied by a marked decrease in mRNA levels, as determined by q-PCR (Fig. 4B). These results indicated that METTL3 regulated the expression of *GPR161* directly by affecting its transcription. To substantiate this finding further, we conducted rescue experiments in cells lacking METTL3. *E. coli* K88 infection induced notably increased *GPR161* expression in the cells rescued by the METTL3 plasmid, in a finding opposite of that in the cells rescued by the vector (Fig. 4C). Consistent with the protein expression data, the mRNA level qualified by q-PCR indicated an ~70% recovery in the cells transfected with the METTL3 plasmid (Fig. 4D).

However, transfection with the inactive METTL3-mutant plasmid failed to rescue the inhibition of *GPR161* expression that had been induced by METTL3 deficiency under *E. coli* K88 infection (Figure S5a-b). Together, these results demonstrated that METTL3 regulated the transcription of *GPR161* according to its methyltransferase activity.

To test whether the altered defensin level could be a consequence of METTL3-mediated *GPR161* expression, we first silenced *GPR161* in IPEC-J2 cells. Compared with the DEFb1 and DEFb2 expression levels in the scramble control, knocking down *GPR161* significantly reduced the expression of DEFb1 and DEFb2 challenged with *E. coli* K88 infection (Fig. 4E). In contrast, we found that the expression of DEFb1 and DEFb2 was notably increased in cells with overexpressed *GPR161*, confirming the role of *GPR161* in the expression of β -defensin regulation (Fig. 4F). Collectively, these findings suggest that, in *E. coli* K88 infection conditions, METTL3 directed the transcription of *GPR161* to control the expression of β -defensin.

METTL3 directed the expression of *GPR161* in a FOXO6-dependent manner

To further investigate the mechanism by which METTL3 is correlated with *GPR161*, we used mRNA-seq analysis to compare the gene expression profiles following the knock down of METTL3. A total of 124 genes that differentially expressed by at least twofold (59 genes that were upregulated and 65 genes that were downregulated) were selected for KEGG pathway analysis (Fig. 5A). We found that the top pathways associated with enriched differentially expressed genes were primarily involved with 'transcription misregulation' followed by the 'PI3K-AKT signalling pathway' and the 'FOXO signalling pathway', and many had an impact on the immune response and transcription (Fig. 5B, and Figure S6a-b). By directing the transcription of *GPR161*, METTL3 regulates *GPR161* expression. We focused on the differentially expressed genes related to transcription and identified FOXO6 as a potential regulator in METTL3-*GPR161* regulation. FOXO6 is a pivotal transcription factor in cell fate decisions and plays vital roles in bacterial infection [43].

Next, we examined the correlation between METTL3 and FOXO6 in IPEC-J2 cells after *E. coli* K88 infection. Consistent with the mRNA-seq data, we found that the expression of FOXO6 decreased significantly in METTL3-silenced cells upon *E. coli* K88 infection (Fig. 5C). The qPCR result further confirmed this conclusion (Fig. 5D). Additionally, we found that transfection with the METTL3 plasmid rescued the suppressed expression of FOXO6 during *E. coli* K88 infection (Fig. 5E and f). These results further indicated that the decreased expression of FOXO6 was due to METTL3 deficiency. Interestingly, our data revealed that the correlation between METTL3 and FOXO6 was independent of m⁶A RNA methylation, as determined when the inactive METTL3 mutant was able to restore the effect of METTL3 depletion on the expression of FOXO6 (Figure S7). Furthermore, to confirm whether the correlation between METTL3 and FOXO6 is involved in *GPR161* expression, we determined the mRNA level of *GPR161* after knocking down FOXO6. We found that knocking down FOXO6 almost

Figure 4. METTL3-mediated GPR161 is essential for β -defensin induction in intestinal epithelial cells. (A-B) METTL3-expressing and METTL3-knockdown IPEC-J2 cells were infected with *E. coli* K88 (MOI = 10:1) and analysed at various times. (A) The protein level of GPR161 was determined by immunoblot analysis. The right panel shows the relative protein levels quantified by densitometry and normalized to the level of β -actin. (B) Results of q-PCR used to quantify the mRNA level of GPR161 are presented relative to the level of sh-Scramble 0 h group, and normalization to Gapdh. (C-D) Transfected Mettl3 plasmid or vector into METTL3-depleted IPEC-J2 cells, followed by *E. coli* K88 infection for various times. Immunoblot analysis of the protein level of GPR161. The right panel shows the relative protein levels quantified by densitometry and normalized to the level of β -actin (C). Results from the q-PCR analysis of the mRNA level of GPR161 are presented relative to the level of sh-Scramble 0 h group, and normalization to Gapdh (D). (E) The mRNA levels of DEFb1 and DEFb2 in GPR161-expressing and GPR161-knockdown IPEC-J2 cells after *E. coli* K88 (MOI = 10:1) infection and analysed at various times. (F) The mRNA levels of DEFb1 and DEFb2 in GPR161-expressing and GPR161-overexpressing IPEC-J2 cells after *E. coli* K88 (MOI = 10:1) infection and analysed at various times. The data are expressed as the mean $\pm$ SEM; **P* < 0.05, ***P* < 0.05, n = 3 biological replicates.

abolished the expression of *GPR161* compared with the expression level of the control (Fig. 5G). Thus, the correlation between METTL3 and FOXO6 affects *GPR161* expression independent of m⁶A modification.

FOXO6 interacted with METTL3 to initiate the transcription of GPR161

Based on the above findings, we hypothesized that FOXO6 interacted with METTL3 to trigger the transcription of *GPR161*, which could activate the signalling that led to upregulated β -defensin. In the FOXO6 and METTL3 complex, FOXO6 initiates transcription, while METTL3 contributes to the m⁶A modification of the nascent *GPR161* transcripts. To test these hypotheses, we first proved the direct relationship between β -defensin expression and FOXO6. Notably, similar to the findings for the *METTL3*-knockdown cells, loss of FOXO6 significantly inhibited the expression of *DEFb1* and *DEFb2* upon *E. coli* K88 infection (Fig. 6A and b). Second, we performed a co-IP assay and found that METTL3 and FOXO6 interacted with each other after *E. coli* K88 infection (Fig. 6C). Finally, we

conducted ChIP assays using a FOXO6-specific antibody. In scramble control cells, the FOXO6 binds to the GPR161 promoter rapidly upon *E. coli* K88 infection. However, in cells lacking *METTL3*, approximately one-half as much FOXO6 was associated with the promoter (Fig. 6D and E). These results support a role for *METTL3* as an *in vivo* regulator of FOXO6 DNA binding activity and *GPR161* expression. Therefore, the increase in *GPR161* induction upon *E. coli* K88 infection appears to be a direct result of the interaction of *METTL3* with FOXO6.

Discussion

Defensins comprise a major subclass of a family of antimicrobial peptides. Increasing evidence suggests that decreased levels or malfunctioning defensins in the intestine might lead to disturbances in homeostasis, which results in intestinal diseases such as Crohn's disease and ulcerative colitis in humans [44,45]. Furthermore, the upregulation of intestinal defensins is considered an early response to bacterial infections in the intestine [46]. However, the molecular mechanism underlying defensin upregulation in bacterial infection

Figure 5. FOXO6 is involved in the correlation between METTL3 and GPR161. (A, B) Control of genome-wide responses to *E. coli* K88 by m⁶A methylase METTL3. (A) Volcano plots show differentially expressed genes (log₂ fold change > 1) identified from RNA-seq of polyadenylated RNA collected from cells with or without METTL3. (B) Path-way analyses (KEGG) of significantly differentially expressed genes from (a) were conducted using DAVID and filtered according to a Benjamini-Hochberg procedure (<0.05). (C, D) IPEC-J2 cells with or without METTL3 knockdown were infected with *E. coli* K88 (MOI = 10:1) for indicated times. Immunoblot analysis of FOXO6 protein levels. The right panel shows the relative protein levels quantified by densitometry and normalized to β -actin (C). q-PCR analysis of the mRNA level of FOXO6, and the results are presented relative to the level of sh-Scramble 0 h group, and normalization to Gapdh (D). (E-F) Transfected Mettl3 plasmid or vector into METTL3-depleted IPEC-J2 cells, followed by *E. coli* K88 infection for various times. (E) Immunoblot analysis of FOXO6 protein levels. The right panel shows the relative protein levels quantified by densitometry and normalized to the level of β -actin. (F) Results from the q-PCR analysis of the mRNA level of FOXO6 are presented relative to the level of sh-Scramble 0 h group, and normalization to Gapdh. (G) The mRNA level of GPR161 in FOXO6-expressing and FOXO6-knockdown IPEC-J2 cells after *E. coli* K88 (MOI = 10:1) infection and analysed for indicated times. The data are expressed as the mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.05$, n = 3 biological replicates.

remains unclear. Here, on the basis of the correlation found between defensin expression and m⁶A methylation in the course of infection, we identified a FOXO6-METTL3-m⁶A-GPR161 signalling axis that induces β -defensin induction in intestinal epithelial cells upon *E. coli* K88 infection.

Emerging evidence indicates that METTL3-mediated m⁶A modification plays a crucial role in innate immunity [28]. Similar to findings from our previous study [39], the activation of the immune response was accompanied by an elevated level of m⁶A modification, whereas the expression of 'writers' and 'erasers' did not differ. Indeed, similar to several RNA processing factors, including ASF/SF2, hnRNPA1, HuR and TDP-43 [47,48], the subcellular distribution of the m⁶A methyltransferases was altered in response to different stimuli of cell stress [49]. A recent study conducted in human dental pulp cells indicated that inhibition of METTL3 led to a decrease in the induction of inflammatory cytokines after lipopolysaccharide stimulation [50]. Our previous results also revealed that silencing METTL3 could sustain LCFA absorption by blocking the TRAF6-dependent inflammation

response [39]. Therefore, we hypothesized that METTL3-mediated m⁶A methylation acts as a regulator in defensin expression to defend against bacterial infection. As expected, in the current study, we found that METTL3 deficiency in IPEC-J2 cells resulted in decreased defensin induction. The overexpression of wild-type METTL3, but not the mutant D395A, was able to restore defensin expression, indicating that METTL3 mainly affected defensin expression through its methylation activity.

Since METTL3 is the methyltransferase of RNA m⁶A, we believe that it works through adding m⁶A modification to its substrates. Therefore, we performed m⁶A-seq and focused on the differentially modified genes involved in innate immunity. The GPR161 transcript was identified as a substrate of METTL3, which significantly modified it. Feigin *et al.* [51] demonstrated that overexpression of GPR161 in human mammary epithelial cells increased cell proliferation, migration, intracellular accumulation of E-cadherin and formation of multiacinar structures in 3D culture. The transcription activity of GPR161 changed in colonic mucosa in patients

Figure 6. FOXO6 interacts with METTL3 to initiate the transcription of *GPR161*. (A, B) The mRNA levels of DEFb1 (A) and DEFb2 (B) in FOXO6-expressing and FOXO6-knockdown IPEC-J2 cells after *E. coli* K88 (MOI = 10:1) infection and analysed at indicated times. (C, –D) Immunoprecipitation was performed with antibodies against METTL3 (C) or FOXO6 (D), with immunoglobulin G (IgG) serving as the control. The samples were analysed by immunoblot assay using the indicated antibodies. (E–F) ChIP analysis was performed using an FOXO6 antibody (E) and q-PCR (F). The data are expressed as the mean $\pm$ SEM; * P < 0.05, ** P < 0.05, n = 3 biological replicates.

with *Campylobacter jejuni*-induced postinfectious irritable bowel syndrome [52]. In a recent study, METTL3 was found to be stably recruited by CEPBZ to promoters of a specific set of active genes, resulting in m⁶A methylation of the respective mRNAs [53]. We proposed a model in which METTL3 participated in the transcriptional activation of

GPR161 to regulate defensin expression, in which METTL3 was responsible for m⁶A RNA methylation. As expected, *GPR161* protein synthesis was reduced in the absence of METTL3. The comparable *GPR161* mRNA levels in METTL3-expressing and METTL3-knockdown cells indicated that the reduced *GPR161* synthesis was the result of

transcription deficiency. On the basis of the findings, we believed that a transcription factor interacted with METTL3 to form a transcription initiation complex of the *GPR161* gene.

Forkhead transcription factors play key roles in the induction of various downstream genes involved in diverse pathways, including those involved in cell survival, proliferation, differentiation, oxidative stress and metabolism in mammals [54]. According to the model proposed above, based on the mRNA-seq data from the *METTL3*-expressing and *METTL3*-knockdown IPEC-J2 cells, we identified transcription factor FOXO6 as the coregulator with METTL3 in the response to *E. coli* K88 infection. The results from the studies on *FOXO6*-silenced cells confirmed its role in defensin induction. Similarly, *FOXO6*-knockout mice showed reduced PAR2 signalling with a decrease in inflammatory cytokine expression [54]. Interestingly, we found that the correlation between METTL3 and FOXO6 was independent of m⁶A methylation. A previous study revealed that m⁶A methylation was essentially completed upon the release of mRNA into the nucleoplasm [55]. Thus, in our opinion, in the METTL3 and FOXO6 complex, FOXO6 is responsible for the transcriptional activation, while METTL3 is responsible for the m⁶A methylation to *GPR161* nascent transcripts. However, the structural basis of the interaction between METTL3 and FOXO6 needs further study.

In conclusion, we show that METTL3 plays an important role in defensin induction upon bacterial infection via GPR161 signalling and that FOXO6 is a key component that mediates METTL3-dependent GPR161 expression. Our findings suggest that the development of m⁶A-modulating agents may lead to novel therapeutic approaches to a range of bacterial infectious diseases.

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