International Journal of Biochemistry and Cell Biology 110 (2019) 103–110

Contents lists available at ScienceDirect

International Journal of Biochemistry and Cell Biology

journal homepage: www.elsevier.com/locate/biocel

The natural antisense transcript NATTD regulates the transcription of decapping scavenger (DcpS) enzyme

Zhu-Zhong Mei*, Hongwei Sun, Xiaoli Ou, Lei Li, Junwei Cai, Shuiwang Hu, Juan Wang, Haihua Luo, Jinghua Liu*, Yong Jiang*

From Guangdong Provincial Key Laboratory of Proteomics, State Key Laboratory of Organ Failure Research, Department of Pathophysiology, Southern Medical University, Guangzhou 510515, China

ARTICLE INFO

Keywords:
Natural antisense transcript
NATTD
DcpS
Epigenetic regulation

ABSTRACT

Natural antisense transcripts (NATs) are transcribed from the opposite strand of other genes. Most of them are noncoding RNAs. They have been reported to play important roles in a variety of biological processes. In this study, we identified a novel NAT, NATTD, which is partially complementary to both the TIRAP/Mal and DcpS genes. Interestingly, NATTD only positively regulates the expression of DcpS, a decapping scavenger enzyme which is a promising therapeutic target for spinal muscular atrophy. But it has no obvious effects on the expression of TIRAP/Mal gene. The NATTD transcript primarily resides in the nucleus and does not alter the mRNA stability of DcpS. Instead, it is required for the recruitment of RNA polymerase II at the mouse DcpS promoter. Chromatin immunoprecipitation assays revealed that knocking-down NATTD transcript with shRNA enhanced the H3K27-Me3 modification at the DcpS promoter. In summary, our studies identified NATTD as a regulator of DcpS transcription through epigenetic mechanisms.

1. Introduction

Natural antisense transcripts (NATs) are typically a class of long noncoding RNAs (lncRNAs) transcribed from the opposite strand of some genes, especially protein-coding genes. Thus, they are partially complementary to their counterpart sense transcripts. Antisense RNA transcription is a common theme in mammalian genomes (Katayama et al., 2005; Yelin et al., 2003). It is estimated that more than 70% of protein-coding genes have NATs in the human and mouse genomes (Werner et al., 2009; Kiyosawa et al., 2003; Okazaki et al., 2002). Thousands of sense-antisense gene pairs are conserved between human and mouse (Engstrom et al., 2006), suggesting that they might play important roles in biological processes. Indeed, NATs have been reported to be involved in the regulation of a variety of processes, including physiological responses, metabolism, and pathogenic processes (Magistri et al., 2012). Dysregulated expression of NATs is associated with a variety of pathological conditions, such as Alzheimer's disease, Parkinson's disease, and cancers (Faghihi et al., 2008; Scheele et al., 2007; Balbin et al., 2015). Nevertheless, the functions of most NATs remain largely unknown.

In the past decades, multiple reports have indicated that NATs are functional elements and regulate the expression of their cognate genes in a *cis*- or *trans*- manner through diverse transcriptional and/or post-transcriptional mechanisms (Balbin et al., 2015; Carrieri et al., 2012). *Cis*-NATs regulate the expression of the sense genes transcribed from the same genomic locus, while *trans*-NATs regulate the expression of genes from different loci. NATs are generally expressed in low abundance that is more than 10-fold lower than their corresponding sense transcripts (Pelechano and Steinmetz, 2013). Unlike mature mRNAs that are mainly located in the cytoplasm, NATs preferentially accumulate in the nucleus. Most NATs function in the nucleus in a *cis*-regulatory manner through epigenetic changes of target genes, which leads to gene silencing or activation. They were originally believed to downregulate the expression of their sense counterparts (Osato et al., 2007), but a recent report revealed that the majority of *cis*-NATs are activating regulatory elements (Conley and Jordan, 2012).

Decapping scavenger (DcpS) enzyme belongs to the Histidine Triad (HIT) superfamily of pyrophosphatases. It hydrolyzes the cap structure into 7-methylguanosine monophosphate and nucleoside diphosphate (NDP) in mRNA turnover (Milac et al., 2014). Thus, it prevents the

Abbreviations: NAT, natural antisense transcript; NATTD, natural antisense Transcript to both TIRAP/Mal and DcpS; DcpS, decapping scavenger; shRNA, short hairpin RNA; TLR, toll-like receptor; DRB, 5,6-Dichloroben- zimidazole 1-β-D- ribofuranoside; FISH, fluorescence in situ hybridization

* Corresponding authors.
E-mail addresses: mzhuong@126.com (Z.-Z. Mei), liujhua@163.com (J. Liu), jiang48231@163.com (Y. Jiang).

https://doi.org/10.1016/j.biocel.2019.03.005
Received 16 November 2018; Received in revised form 7 March 2019; Accepted 8 March 2019
Available online 08 March 2019

potentially toxic accumulation of capped mRNA fragments that may competitively bind to other cap-binding proteins. The reduction of DcpS activity might lead to dysfunctions in cap-dependent processes, such as mRNA splicing, translation initiation and mRNA degradation. Furthermore, DcpS has also been reported to regulate miRNA turnover in *C. elegans* and human cells independently of its decapping activity (Meziane et al., 2015; Bosse et al., 2013). A recent report from M Kildedjian's group revealed that DcpS is also a transcript-specific modulator of RNA in mammalian cells (Zhou et al., 2015). Thus, the DcpS enzyme plays important roles in the regulation of RNA metabolism.

In the present study, we identified a head-to-head NAT of the DcpS gene, NATTD (**N**atural **A**ntisense **T**ranscript to both **T**IRAP/Mal and **D**cpS), in mouse cells. NATTD is a spliced and polyadenylated transcript that is transcribed from exon 1 of the mouse DcpS gene in the antisense direction. NATTD is also partially complementary to TIRAP/Mal (TIR domain containing adaptor protein/MYD88 adapter-like), a critical adaptor in Toll-like receptors (TLRs) signaling pathway. Unexpectedly, NATTD only positively regulates the expression of DcpS by regulating the local epigenetic status of its promoter in a *cis*-regulatory manner. In contrast, it has no obvious effect on the expression of TIRAP/Mal in mouse macrophage-like RAW264.7 cells.

2. Materials and methods

2.1. Antibodies, chemicals and kits

A mouse monoclonal antibody against DcpS (OTI1E6, No. TA505420) was purchased from Origene (Rockville, MD, USA). Rabbit anti-β-tubulin (No. 10068-1-AP, lot#1001352) polyclonal antibodies were purchased from Proteintech Biotechnology (Wuhan, China). A rabbit monoclonal antibody against TIRAP/MAL (ab133332) was obtained from Abcam (Cambridge, MA, USA). Rabbit monoclonal antibodies against Rpb1 (No. 2629), H3K27-Me3 (No. 9733), H3K9-Ac (No. 9649), and H3K9-Me3 (No. 13969) were purchased from Cell Signaling Technology (Beverly, MA, USA). The specificity of all antibodies was confirmed by western blotting. The SimpleChIP Enzymatic Chromatin IP Kit (Magnetic Beads) (No. 9003) was purchased from Cell Signaling Technology (Beverly, MA, USA). GeneRacer kit (No. L1502-1), FISH Tag™ RNA Green Kit and Trizol reagent were obtained from Life Technology (Carlsbad, CA, USA). LPS (from *Escherichia coli* 0111: B4) was obtained from Sigma-Aldrich (St. Louis, MO, USA). The mouse TLR1-9 agonist kit (No. tlrl-kit1mw) was obtained from Invivogen (San Diego, CA, USA).

2.2. Cell culture and stimulation

RAW264.7 macrophage-like cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Life Technology) supplemented with 10% fetal bovine serum in a humidified atmosphere of 5% CO2 at 37 °C. Bone marrow-derived macrophages (BMDM) were prepared as adherent cultures using previously described procedures (Mei et al., 2016). In selected experiments, cells were treated with 100 ng/ml LPS, 100 ng/ml pam3CSK4, 5 μM ODN1826, 10 μg/ml poly (I:C), 1 μg/ml ST-FLA or 10 μg/ml SSRNA40 to activate different TLRs.

2.3. Rapid amplification of cDNA ends (RACE) and quantitative real-time PCR analysis

Total RNA was extracted by Trizol reagent according to the manufacturer's instructions. The 5′ and 3′ regions of mouse NATTD were amplified using a RACE kit based on the sequence of EST BI455134 according to the manufacturer's protocol. For qRT-PCR, RNA was reverse transcribed using a ReverTra Ace qPCR RT kit. qRT-PCRs were performed using FastStart Universal SYBR Green Master (Rox) (Roche) and a 7500 Real-Time PCR System (Applied Biosystems). All primers are listed in Table 1.

2.4. RNA fluorescence in situ hybridization (FISH) assay

RNA FISH assay was performed using the FISH Tag™ RNA Green Kit, with Alexa Fluor™ 488 dye according to the manufacturer's protocol (Life Technology). In brief, RAW264.7 cells seeded on coverslips were fixed for 30 min with 4% formaldehyde and permeabilized for 5 min with 0.1% Triton X-100 in 1 × PBS at room temperature. The cells were washed twice with 1 × PBS and once with 2×saline-sodium citrate (SSC). They were then incubated with hybridization solution (50% formamide, 5XSSC, 50 μg/ mL heparin, 0.1% Tween-20, 100 μg/ml yeast tRNA) containing fluorescently labeled RNA probes at 55 °C for overnight. The cells were washed twice with pre-warmed wash buffer (0.2 × SSC and 50% formamide) at 55 °C for 30 min each. After a post-hybridization treatment, the cells were mounted using Duolink® In Situ Mounting Medium with DAPI (Sigma-Aldrich). Confocal microscopy images were obtained by using Zeiss LSM 700 confocal microscope (Carl Zeiss, Germany). The acquired images were processed in ZEN 2012. Primers for generating DNA templates for RNA probe synthesis are listed in Table 1.

2.5. RNA interference with short hairpin RNA (shRNA)

DNA fragments encoding shRNAs were subcloned downstream of the human U6 promoter and confirmed by DNA sequencing. The plasmids were transfected into RAW264.7 cells with FugeneHD (Promega, Madison, WI, USA) according to the manufacturer's protocol. Stable cell lines expressing the shRNA targeting NATTD (RAW264.7-shNATTD) were established by Zeocin selection. Knockdown efficiency was assessed by qRT-PCR. Sequences/primers are listed in Table 1.

2.6. Chromatin immunoprecipitation assay

ChIP was performed following the manufacturer's instructions (CST Signaling) with antibodies against Rpb1, H3K27-Me3, H3K9-Ac, and H3K9-Me3. Immunoprecipitated DNA was analyzed by qRT-PCR and normalized to input DNA. All primers are listed in Table 1.

2.7. mRNA stability assay

RAW 264.7-shNATTD cells were exposed to 50 μM DRB (5,6-Dichlorobenzimidazole 1-β-D- ribofuranoside) to inhibit transcription. Total RNA was extracted and reverse transcribed into cDNA. NFKBIA, TNFα, TNFAIP3 and CXCL10 cDNAs were quantitated by qRT- PCR. RAW264.7 cells expressing a negative control shRNA were used as a control.

2.8. Computational analysis

The largest open reading frame (ORF) of NATTD was analyzed using NCBI ORF finder (http://www.ncbi.nlm.nih.gov/gorf/gorf.htsml), Coding Potential Calculator (CPC) (http://cpc.cbi.pku.edu.cn/) and a manual approach of annotating long noncoding RNA genes. CPC predicated that NATTD is a noncoding RNA with a calculated coding potential score of -1.11003 (Kong et al., 2007). Manual approaches to assess the protein coding capacity of NATTD followed the workflow described in Ref (Jia et al., 2010). There was no protein-coding reference transcript of NATTD in the UCSC genome database. The longest ORF proposed by the NCBI ORF finder was 66 aa for NATTD, which is different from the CPC analysis (69 aa). No hit of the 66 aa (NCBI ORF finder) or 69 aa (CPC analysis) putative protein was provided by BLASTP analysis. No conserved protein domains are found in either putative protein. The putative proteins did not align with any known proteins in the database. Thus, NATTD is a non-coding transcript.

Table 1
Primer list for all experiments.

Gene name	Forward primer	Reverse primer
qRT-PCR primers		
NATTD	CTGTGGCACACGCAGACATC	TCCGTCCGAGCCATTAGG
DcpS	GCTGAAGCTGACCGGATTGT	TGGCGATCAAATACAGGTCATC
TIRAP/Mal	CAATGCCTGCTCTTTCATGGT	GGCATCTTCTTGGGCTTCTTC
NFKBIA	GAAGAGAAGCCGCTGACCAT	CAGAAGTGCCTCAGCAATTCC
β-actin	ACGGCCAGGTCATCACTATTG	AGAGGTCTTTACGGATGTCAACGT
TNFα	GACCCTCACACTCAGATCATCTTCT	TCCTCCACTTGGTGGTTTGC
TNFAIP3	AGCTCGTGGCTCTGAAAACC	TTCCTCAGGACCAGGTCAGTATC
CXCL10	AATCATCCCTGCGAGCCTATC	TGATGGTCTTAGATTCCGGATTC
Atoh7	ATTGCCTCCCTATCTCCACTTCT	TTTCACAGCAGTCCAAAGTCATTC
TRADD	GGTGGAGCCATACAGGTAGCTT	GAGCTGCCCGTGGAACAG
ChIP Q-PCR primers		
DcpS	AGTTCTTGTGTGAGACCCTGATTTAG	CTGGCTATAGAGAACAAAGAGCTTCTT
GAPDH	CAGTTCGGAGCCCACACG	TAGCTGGGCCTCTCTCATTTCC
Primers for FISH probes		
DcpS	CCTGCATGGGAAGGTGAATG	TAATACGACTCACTATAGGGATGCCTTGGAGGAAACAGGT*
NATTD	CAAAGGAAGAGTGCTGGAGT	TAATACGACTCACTATAGGGTCAGTGGTCAGAGGTACAG*
TIRAP/Mal	CCAGGAAAGCCACCTCTATG	TAATACGACTCACTATAGGGTACGGCTCTCCGGTGAACTG*
U6 snRNA	GTGCTCGCTTCGGCAGCACAT	TAATACGACTCACTATAGGGCGAATTTGCGTGTCATCCTTG*
β-actin	AGGGAAATCGTGCGTGACAT	TAATACGACTCACTATAGGGGAACCGCTCGTTGCCAATAG*
RT-PCR primers for NATTD splicing variants		
Primer 1	AGACTCCCTGAGCACTTTTTGTACTC	
Primer 2	GGACGGAGGCTTTTACCAAGTCAAG	
Primer 3	CTGTGGCACACGCAGACATC	
Primer 4	TGTGCATATGTCTATGTAAGTATATGC	
shRNA target sequences		
shNC-For	CACCGTCTTCCGAACGTGTCACTTTCAAGAGAAGTGACACGTTCGGAAGACTTTTTT	
shNC-Rev	AAACAAAAAAGTCTTCCGAACGTGTCACTTCTCTTGAAAGTGACACGTTCGGAAGAC	
shNATTD1-For	ACCGCTCAATGCTGAACAGAGATTCAAGAGATCTCTGTTCAGCATTGAGCTTTTTT	
shNATTD1-Rev	AAACAAAAAAGCTCAATGCTGAACAGAGATCTCTTGAATCTCTGTTCAGCATTGAG	
shNATTD2-For	ACCGCTGGAGTCAGAGGAGATATTCAAGAGATATCTCCTCTGACTCCAGCTTTTTT	
shNATTD2-Rev	AAACAAAAAAGCTGGAGTCAGAGGAGATATCTCTTGAATATCTCCTCTGACTCCAG	

* The underline denotes the T7 promoter sequence.

2.9. Statistical analysis

Unless otherwise indicated, the experiments were performed in triplicate and repeated at least three times. The figures show the results from one representative experiment. The intensity of the protein bands was determined by densitometry using ImageJ. All data are presented as the means ± SEM. Datasets were tested using a two-tailed Student's t-test. Statistical analyses were performed using Origin 8 software (Origin Lab Corporation). Differences were considered statistically significant for values of $p < 0.05$.

3. Results

3.1. Identification of a natural antisense transcript of DcpS

By searching the FANTOM3 database, we identified expressed sequence tags (EST, BI455134 for mouse and BX508466 for human) as one sense-antisense pair conserved between the human and mouse genomes (engstrom et al., 2006). These ESTs are partially complementary to the TIRAP/Mal gene. To obtain the full-length cDNA sequence of this antisense transcript, RACE PCR was performed based on the sequence of mouse EST BI455134. Six potential splicing variants, ranging from 1014-1217 nt, were identified in murine macrophage-like RAW264.7 cells. DNA sequencing results revealed that they contain poly-A tails and cap structures, indicating that they are fully processed transcripts of RNA polymerase II. DNA blast analysis results revealed that the genomic DNA encoding this transcript spans approximately 18 kb, consisting of 6 exons (Fig. 1A). To determine which variant of this transcript is expressed in RAW264.7 cells, a PCR amplification strategy was designed to amplify different fragments of the cDNA. Splicing variants 1, 2, 4 and 5 were detected in RAW264.7 cells (Fig. 1A). While exon 1 of all NATTDs overlaps with the first exon of DcpS by 56 bp in an antisense fashion, exons 3 (variants 1 and 2) and 4 (all variants) of NATTD overlap exons 7 and 6 of TIRAP/Mal by 44 bp and 447 bp, respectively (Fig. 1B). Thus, we designated this transcript as NATTD for **N**atural **A**ntisense **T**ranscript to both **T**IRAP and **D**cpS. The NATTD transcript was also expressed in BMDM and NIH3T3 cells (data not shown). The sequences of the NATTD variants have been submitted to GenBank (accession numbers: MK071705, MK071706, MK071707, MK071708, MK071709 and MK071710). The largest ORF in the longest NATTD variant (NATTD1) spans 205 bp and encodes 69 amino acids (from CPC analysis). Using the CPC (coding potential score = −1.11003) and manual approach for annotating long noncoding mouse RNA genes, we classified NAaTTD as a long non-coding RNA.

3.2. Expression analysis of the NATTD transcript

By generating standard curves using DNA plasmids containing cDNA of TIRAP/Mal, DcpS and NATTD, the absolute expression levels of these transcripts in RAW264.7 cells were analyzed using qRT-PCR. The RNA level of NATTD is approximately 1/8 of TIRAP/Mal mRNA and 1/100 of DcpS mRNA. This result is consistent with the reports that the RNA level of NATs is far lower than that of their sense transcripts (Modarresi et al., 2012; Pelechano and Steinmetz, 2013). The expression of NATTD, DcpS and TIRAP/Mal in mouse tissues was also analyzed by qRT-PCR. It revealed that TIRAP/Mal, DcpS and NATTD were all expressed in the mouse tissues analyzed, with the highest expression of NATTD in liver and muscle (Fig. 2A).

Given that the intracellular localization of the antisense transcript is essential for its function, the intracellular localization of the NATTD transcript, DcpS mRNA and TIRAP/Mal mRNA were assessed by RNA-FISH assay. U6 snRNA and β-actin mRNA were also included as control.

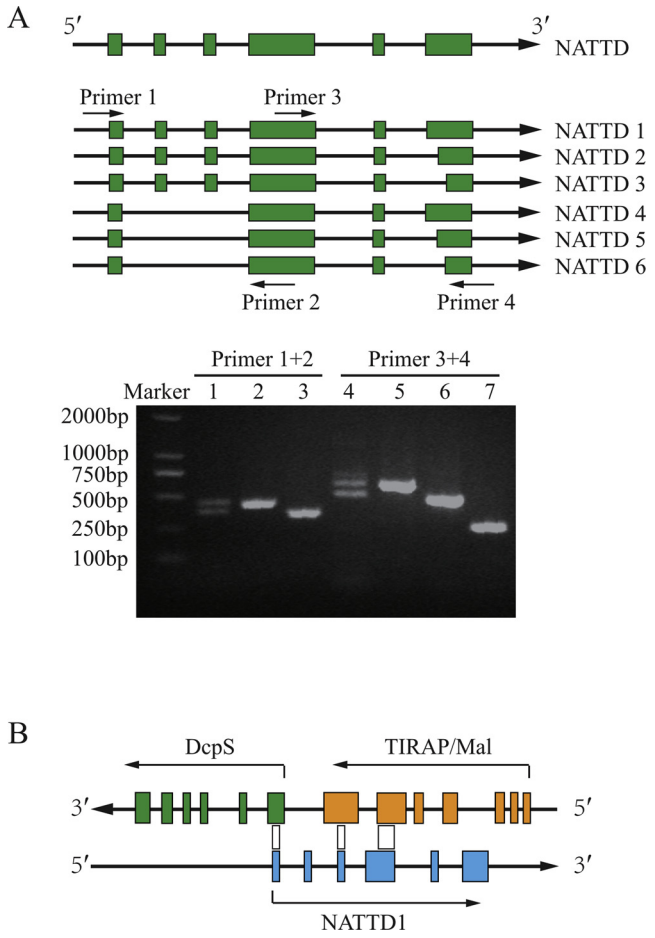

Fig. 1. Identification of NATTD in mouse cells.
A. Schematic diagram of NATTD in RAW264.7 cells. Upper panel shows potential alternative splicing variants of mouse NATTD (NATTD 1–6) identified in RAW264.7 cells by RACE PCR. Primers 1, 2, 3 and 4 have been designed to identify the variants expressed in RAW264.7 cells. The green boxes represent exons. The lower panel is the RT-PCR results of NATTD splicing variants expressed in RAW264.7 cells amplified with the indicated primer pairs. Template for lanes 1 and 4: cDNA of RAW264.7 cells; lanes 2 and 5: NATTD1; lane 3: NATTD4; lane 6: NATTD2; and lane 7: NATTD3.
B. Genomic organization of the NATTD1, DcpS and TIRAP/Mal genes on mouse chromosome 9. While exon 1 of NATTD1 overlaps with the first exon of DcpS by 56 bp in an antisense fashion, exons 3 and 4 of NATTD1 overlap with exons 7 and 6 of TIRAP/Mal by 44 bp and 447 bp, respectively. Colored boxes represent exons; arrows show the direction of transcription. Overlapping regions are indicated by open bars.

As expected, U6 RNA was confined in the nucleus, whereas TIRPA/Mal, DcpS and β-actin mRNA were localized in both the nucleus and cytosol. As can be seen from Fig. 2B, we could observe the nuclear localization of NATTD transcript. It suggests that NATTD transcript functions in the nucleus.

3.3. NATTD transcript positively regulates DcpS transcription

To analyze whether NATTD plays a regulatory role in the expression of TIRAP/Mal and/or DcpS, RAW264.7 cells were infected with a lentivirus expressing NATTD1 transcript. Overexpressing NATTD1 moderately upregulated the expression of DcpS while it had no effect on the expression of TIRAP/Mal (Fig. 3A). To further characterize NATTD's regulation of DcpS expression, we designed two independent short hairpin RNAs (shRNAs) that targeted NATTD exons that did not overlap with that of DcpS or TIRAP/Mal. Then, two stable cell lines expressing NATTD shRNAs were generated in mouse macrophage RAW264.7 cells (RAW264.7-shNATTD 1 and 2). We confirmed that the expression of NATTD was significantly reduced by both shRNAs. While the expression of TIRAP/Mal mRNA and protein were not affected by knocking down NATTD, depletion of NATTD led to moderate downregulation of the mRNA and protein levels of DcpS (Fig. 3B). Consistent with previous report (Zhou et al., 2015), downregulation of DcpS in RAW264.7 cells led to the upregulation of TRADD genes (Fig. 3C).

Some NATs influence the expression of their sense genes by regulating their mRNA stability (Faghihi et al., 2008; Zong et al., 2016; Li et al., 2010). To investigate whether NATTD regulates the expression of DcpS by influencing its mRNA stability, RAW264.7-shNATTD1 cells were treated with 50 μM DRB to inhibit transcription. Total RNA was collected at 0, 1 h and 2 h post-DRB treatment. The mRNA levels of TIRAP/Mal and DcpS were analyzed by qRT-PCR. Compared with RAW264.7 control cells, knocking down NATTD had no significant effects on the mRNA stability of TIRAP/Mal or DcpS (Fig. 3D). These results demonstrated that NATTD does not regulate the expression of DcpS in a mRNA stability-dependent manner.

RNA polymerase II is the enzyme responsible for the transcription of protein-coding genes. RNA polymerase II binding at the promoter is reduced when gene expression is inhibited. To determine whether knocking down NATTD downregulates the expression of DcpS at the transcriptional level, we analyzed the occupancy of RNA polymerase II at the region surrounding the transcription start site of mouse DcpS. A ChIP assay showed that the recruitment of Rpb1, a subunit of RNA polymerase II, at the DcpS promoter was decreased in NATTD knockdown cells. However, the recruitment of Rpb1 at the GAPDH promoter region was not different between RAW264.7 cells and RAW264.7-shNATTD1 cells, confirming a specific inhibitory effect on the transcription of DcpS after knocking down NATTD (Fig. 3E).

Because epigenetic mechanisms, especially histone modifications, play important roles in gene expression, we surmised that NATTD may regulate the transcription of DcpS by regulating the epigenetic marks at its promoter. We measured the association of active (H3K4-Me3 and H3K9-Ac) and repressive (H3K27-Me3) chromatin marks at the DcpS promoter by ChIP assays. The qRT-PCR results showed that depleting NATTD had no obvious effects on H3K4-Me3 and H3K9-Ac modifications at mouse DcpS promoter; however, it enhanced the inhibitory epigenetic mark H3K27-Me3 at the promoter. The effects of NATTD shRNA was specific to the DcpS promoter because the recruitment of Rpb1 and H3K27-Me3 to the GAPDH promoter was unaffected in RAW264.7 cells expressing NATTD shRNA (Fig. 3E). These results indicate that the NATTD transcript is required to facilitate the recruitment of RNA polymerase II to the DcpS promoter.

3.4. The expression of NATTD and DcpS mRNA is downregulated in macrophages exposed to proinflammatory stimuli

TIRAP/MAL is an important adaptor for TLR signaling (Bonham et al., 2014), and its expression is dynamically regulated in macrophages upon LPS treatment (Mansell et al., 2006). Although DcpS has not yet been reported to be involved in the regulation of inflammation, we found that the mRNA level of DcpS was also dynamically regulated in macrophages exposed to LPS (Murray et al., 2005) in the GEO database (GEO accession number: GDS1043). To uncover the possible function of NATTD transcript in inflammation, we first analyzed the expression of NATTD in LPS-treated RAW264.7 cells by qRT-PCR. The results revealed that LPS treatment dynamically downregulates the expression of NATTD, similar to that of DcpS mRNA (Fig. 4A). The concordant expression pattern of NATTD and DcpS mRNA is consistent with our previous observation that NATTD positively regulates the transcription of DcpS (Fig. 3A and B). To investigate whether the downregulation of NATTD and DcpS mRNA is unique to LPS treatment, we treated RAW264.7 cells with other TLR agonists. Our qRT-PCR results showed that exposing to pam3CSK4 (TLR1/TLR2 agonist) and ODN1826 (TLR9 agonist) also led to the downregulation of both DcpS

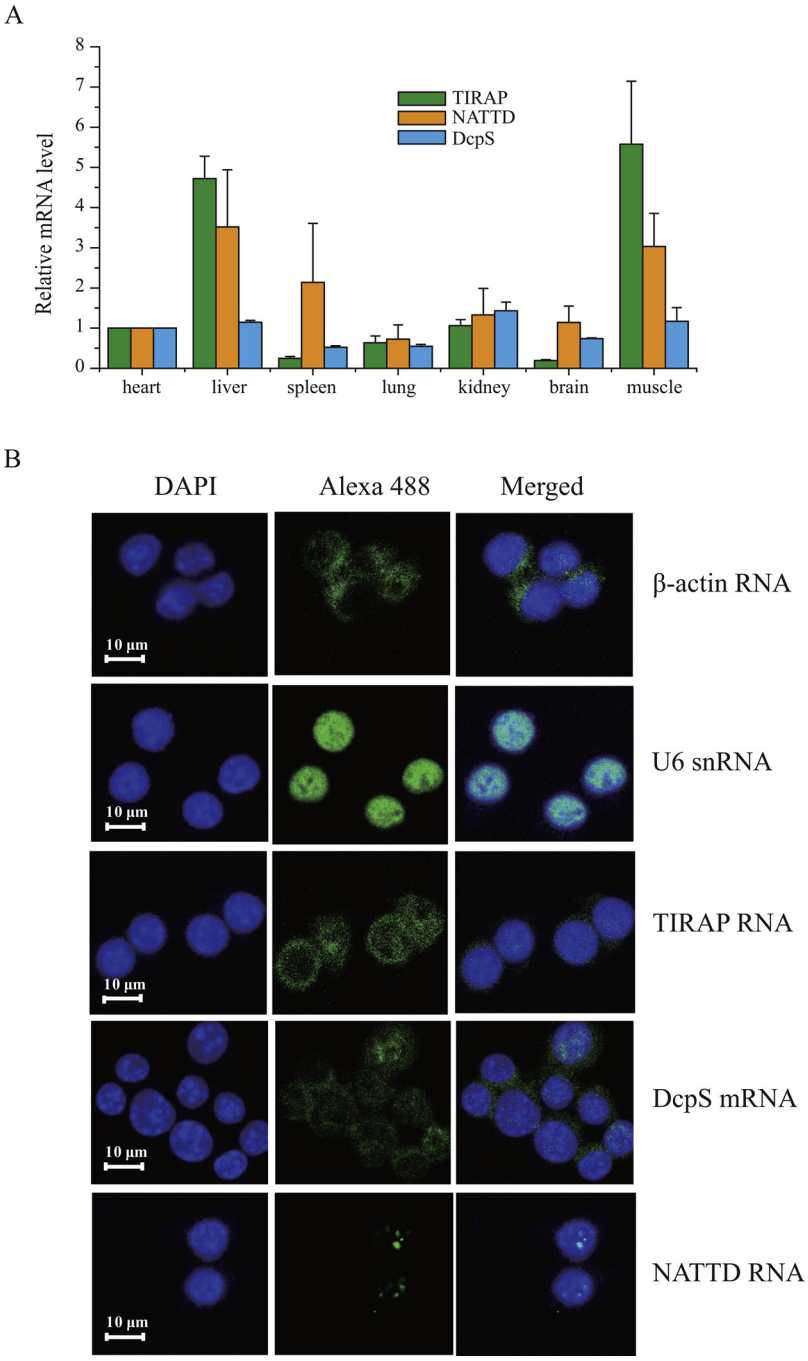

Fig. 2. Expression profiles of NATTD in mouse cells and tissues.
A. Quantitative PCR analysis of NATTD, DcpS and TIRAP in mouse tissues. The relative expression level of these transcripts in different tissues are normalized to those in the heart. (n = 3).
B. Intracellular localization of NATTD transcript, DcpS and TIRAP/Mal mRNA. RNA-FISH was carried out in RAW264.7 cells. The cells were fixed and hybridized with Alexa488-labeled antisense RNA probes prepared by in vitro transcription. Cell nuclei were counterstained by DAPI dye. The subcellular distributions of in cells were examined by confocal microscopy. The U6 snRNA and β-actin mRNA were also assessed as control.

mRNA and NATTD in RAW264.7 cells. However, activating TLR3, TLR5 or TLR7 with their specific agonists Poly (I:C) (TLR3), ST-FLA (TLR5) and ssRNA40 (TLR7) had no obvious effects on the expression of both DcpS mRNA and NATTD in RAW264.7 cells (Fig. 4B). These results suggest that NATTD may be involved in the regulation of inflammation under certain circumstances. Next, we analyzed whether knocking down NATTD affected the LPS-induced expression of NF-κB target genes. The qRT-PCR results revealed that knocking-down NATTD had no obvious effects on the LPS-induced expression of the tested NF-κB target genes (NFKBIA, TNFAIP3, CXCL10 and TNFα) in RAW264.7 cells (Fig. 4C).

4. Discussion

The DcpS enzyme plays important roles in RNA metabolism and is required for hydrolyzing the cap structure of degraded mRNAs. It has been also reported to be required for the expression of SMN protein in humans. The molecular basis for this regulation is presently unknown. But targeting DcpS with the specific inhibitor RG3039 is already in preclinical trials for spinal muscular atrophy (SMA) treatment (Gogliotti et al., 2013). A recent report from T Maeda's group showed that DcpS is also a promising target for acute myeloid leukemia (AML) therapy (Yamauchi et al., 2018). Nevertheless, our knowledge of the regulation of DcpS expression is scarce. In this study, we identified a novel antisense transcript, NATTD, of the mouse DcpS gene. NATTD positively regulates the expression of DcpS by regulating the local epigenetic status of the mouse DcpS promoter in a *cis*-regulatory manner.

Although the complementary region between NATTD and TIRAP/Mal is much larger than that of DcpS (491 bp *Vs* 56 bp), NATTD has no

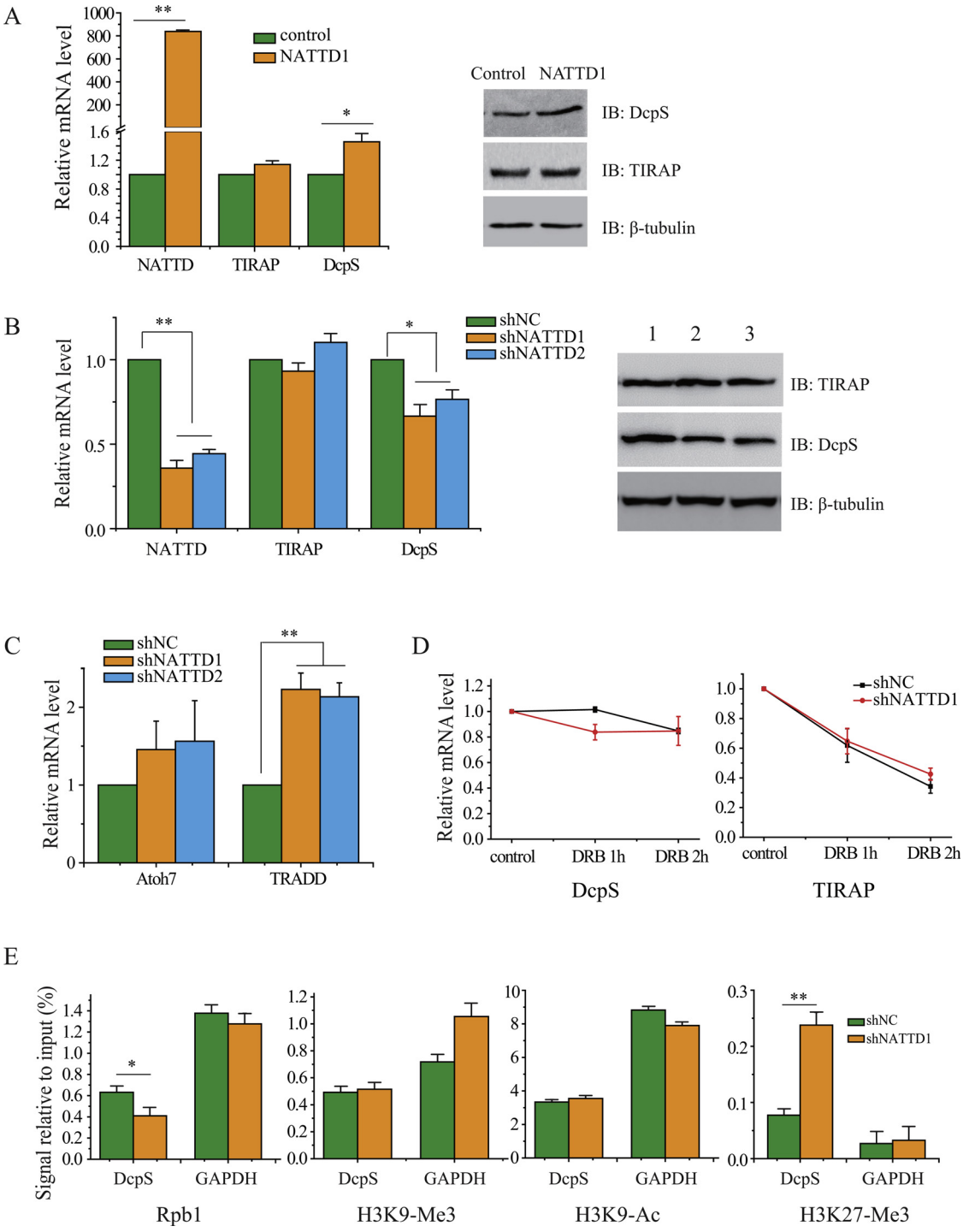

Fig. 3. NATTD regulates the expression of DcpS by epigenetic mechanisms.

A. Overexpressing NATTD1 moderately upregulated the expression of DcpS in RAW264.7 cells. RAW264.7 cells were infected with a lentivirus expressing NATTD1 and selected with blasticidin to generate stable cell lines. The mRNA and protein levels of NATTD, TIRAP and DcpS were analyzed by qRT-PCR (left panel) and western blotting (right panel), respectively. (n = 3; *: $p < 0.05$; **: $p < 0.01$).

B. Knocking down NATTD downregulated the expression of DcpS in RAW264.7 cells. Two independent shRNA plasmids targeting all NATTD splicing variants were transfected into RAW264.7 cells and selected with zeocin to generate stable cell lines (RAW264.7-shNATTD). The mRNA and protein levels of NATTD, TIRAP and DcpS were analyzed by qRT-PCR (left) and western blotting (right) respectively. (n = 3; *: $p < 0.05$; **: $p < 0.01$).

C. Knocking-down NATTD upregulated the expression of TRADD gene. The expression of Atoh7 and TRADD were analyzed in RAW264.7-shNATTD cells by qRT-PCR. (n = 3; **: $p < 0.01$)).

D. RAW264.7-shNATTD1 cells were treated with 5 μM DRB to inhibit transcription. Then, the cells were collected at 1 h and 2 h post-treatment. The mRNA levels of DcpS and TIRAP were analyzed by qRT-PCR. RAW264.7 cells expressing a negative shRNA (shNC) were used as a control. (n = 3).

E. Knocking down NATTD decreased the recruitment of RNA polymerase II and promoted H3K27-Me3 modification at the mouse DcpS promoter. Genomic DNA was immunoprecipitated using antibodies against Rpb1, H3K9-Me3, H3K9-Ac and H3K27-Me3 and quantitated by qRT-PCR with primers specific to the NATTD transcription start region. GAPDH was used as a control. All values represent the mean ± SEM from triplicate samples (n = 3). *$p < 0.05$, **$p < 0.01$ compared with the respective control.

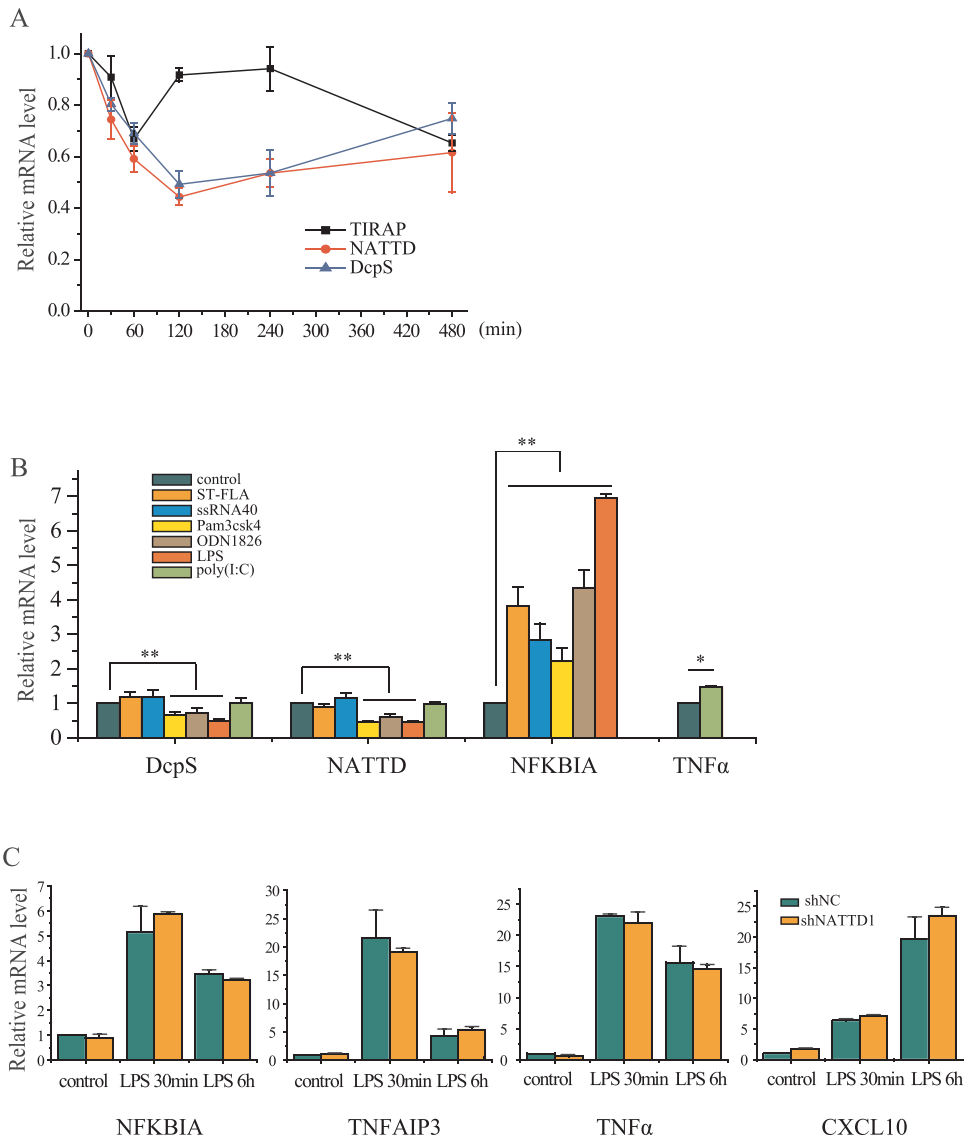

Fig. 4. Dynamic expression of NATTD in macrophages exposed to proinflammatory stimuli.

A. Mouse macrophage-like RAW264.7 cells were treated with 100 ng/ml LPS. The cells were collected at 0, 30 min, 1 h, 2 h, 4 h and 8 h post-treatment. The expression of NATTD, DcpS and TIRAP was analyzed by qRT-PCR (n = 3).

B. RAW264.7 cells were exposed to 100 ng/ml pam3CSK4, 5 μM ODN1826, 10 μg/ml poly (I:C), 1 μg/ml ST-FLA, 100 ng/ml LPS or 10 μg/ml SSRNA40. The cells were collected at 2 h post-treatment. The expression of NATTD and DcpS were analyzed by qRT-PCR. NFKBIA and TNFα were also included as positive controls. All values represent the mean ± SEM from triplicate samples (n = 3). *$p < 0.05$, ** $p < 0.01$ compared with the respective control.

C. Knocking down NATTD did not affect the LPS-induced expression of NF-κB target genes. RAW264.7-shNATTD1 cells were treated with 100 ng/ml LPS and collected at 30 min and 6 h post-treatment. The expression of NFKBIA, TNFAIP3, CXCL10 and TNFα was analyzed by qRT-PCR (n = 3).

obvious effects on the expression of TIRAP/Mal in RAW264.7 cells. The reason for this difference is presently unclear. We also cannot exclude the possibility that NATTD may regulate the expression of TIRAP/Mal under other conditions or in other cells. Similar to other lncRNAs, NATs might also function differently in different tissues. In this paper, we have identified 4 alternative splicing variants of NATTD in RAW264.7 cells. The specific functions of each variant remain unknown. These variants might have different functions to finely tune the expression of the DcpS gene under specific conditions. Furthermore, the variants may have functions other than regulating the expression of DcpS, such as regulating the expression of the TIRAP/Mal gene under other circumstances.

Because NATTD is expressed at a much lower level than that of DcpS mRNA, whether it has measurable effects on DcpS transcription in vivo cannot be assessed using current techniques. The low level of its expression might not limit its function in modulating H3K27 methylation at the mouse DcpS promoter because targeting chromatin may not need many RNA molecules, suggesting that its effects could be catalytic rather than stoichiometric. Pairing the NATTD transcript with exon 1 and the upstream region of the mouse DcpS gene is likely the mechanism allowing NATTD to specifically modulate the DcpS promoter. However, how the NATTD transcript interacts with DNA is still unknown. The nascent NATTD transcript may interact with the sense strand of the DcpS promoter immediately after it is transcribed because unwinding dsDNA in the DcpS promoter is required for it to pair with the NATTD transcript.

The flexibility of RNA molecules means that NATs are capable of folding into complex tertiary structures in vivo, allowing them to interact with DNA, other RNAs or proteins to form specific functional complexes. Multiple modes of action have been reported for NATs, including epigenetic regulation, steric clashes with the transcriptional machinery, splicing regulation and RNA interference. While some reported NATs reside in the cytoplasm, most NATs localize in the nucleus where they regulate the expression of their sense genes in a *cis* manner. In the nucleus, NATs generally bridge the interaction between proteins and DNA, facilitating the formation and modulation of chromatin effector complexes that are crucial for epigenetic signaling. Epigenetic regulation has been reported as a common theme of NAT action (Magistri et al., 2012). For example, AS1DHRS4, an NAT of the DHRS4 gene, not only mediates the deacetylation of histone H3 and the demethylation of H3K4 *in cis* but also induces H3K9me2 and H3K27me3 at the promoters of the DHRS4L1 and DHRS4L2 genes *in trans* by interacting with the histone methyltransferases G9a and EZH2 (Li et al., 2012). It is now widely accepted that NATs are important regulatory elements of gene expression.

Although DcpS has not been reported to be involved in the

regulation of inflammation, we found that both DcpS and NATTD RNA are downregulated in macrophages exposed TLR4, TLR9 and TLR1/2 agonists. However, activating TLR3, TLR5 or TLR7 had no obvious effects on their expression. The reasons for this difference remain unknown. One possibility may be the different associated signaling pathways activated by these TLR receptors (Blasius and Beutler, 2010; Lee et al., 2012). The dynamic downregulation of both DcpS and NATTD in macrophages exposed to proinflammatory stimuli suggests that they may play roles in the regulation of inflammation. Our preliminary results showed that NATTD had no obvious effects on the LPS-induced expression of the tested NF-κB target genes. However, we cannot exclude the possibility that NATTD and DcpS may regulate inflammation in a manner other than by regulating the expression of proinflammatory cytokines. Further experiments are needed to determine their function in inflammation.

In summary, we identified a novel NAT, NATTD, which is partially complementary to both the DcpS and TIRAP/Mal genes. NATTD only positively regulates the expression of DcpS, while it has no obvious effects on TIRAP/Mal in unstimulated RAW264.7 cells. A chromatin immunoprecipitation assay revealed that NATTD promotes the transcription of the DcpS gene by modifying the epigenetic mark H3K27-Me3 at the mouse DcpS promoter. Our data suggest that the interaction of an NAT and a promoter is one mechanism to regulate the expression of its sense gene.

Conflict of interest

None.

Acknowledgments

This work was supported by grants from the NSFC-Guangdong Joint Foundation of China (No. U1601225) and the Guangdong Natural Science Foundation (2015A030313294).

References

Balbin, O.A., Malik, R., Dhanasekaran, S.M., Prensner, J.R., Cao, X., Wu, Y.M., Robinson, D., Wang, R., Chen, G., Beer, D.G., Nesvizhskii, A.I., Chinnaiyan, A.M., 2015. The landscape of antisense gene expression in human cancers. Genome Res. 25 (7), 1068–1079.

Blasius, A.L., Beutler, B., 2010. Intracellular toll-like receptors. Immunity 32 (3), 305–315.

Bonham, K.S., Orzalli, M.H., Hayashi, K., Wolf, A.I., Glanemann, C., Weninger, W., Iwasaki, A., Knipe, D.M., Kagan, J.C., 2014. A promiscuous lipid-binding protein diversifies the subcellular sites of toll-like receptor signal transduction. Cell 156 (4), 705–716.

Bosse, G.D., Ruegger, S., Ow, M.C., Vasquez-Rifo, A., Rondeau, E.L., Ambros, V.R., Grosshans, H., Simard, M.J., 2013. The decapping scavenger enzyme DCS-1 controls microRNA levels in Caenorhabditis elegans. Mol. Cell 50 (2), 281–287.

Carrieri, C., Cimatti, L., Biagioli, M., Beugnet, A., Zucchelli, S., Fedele, S., Pesce, E., Ferrer, I., Collavin, L., Santoro, C., Forrest, A.R., Carninci, P., Biffo, S., Stupka, E., Gustincich, S., 2012. Long non-coding antisense RNA controls Uchl1 translation through an embedded SINEB2 repeat. Nature 491 (7424), 454–457.

Conley, A.B., Jordan, I.K., 2012. Epigenetic regulation of human cis-natural antisense transcripts. Nucleic Acids Res. 40 (4), 1438–1445.

Engstrom, P.G., Suzuki, H., Ninomiya, N., Akalin, A., Sessa, L., Lavorgna, G., Brozzi, A., Luzi, L., Tan, S.L., Yang, L., Kunarso, G., Ng, E.L., Batalov, S., Wahlestedt, C., Kai, C., Kawai, J., Carninci, P., Hayashizaki, Y., Wells, C., Bajic, V.B., Orlando, V., Reid, J.F., Lenhard, B., Lipovich, L., 2006. Complex Loci in human and mouse genomes. PLoS Genet. 2 (4) e47.

Faghihi, M.A., Modarresi, F., Khalil, A.M., Wood, D.E., Sahagan, B.G., Morgan, T.E., Finch, C.E., St Laurent 3rd, G., Kenny, P.J., Wahlestedt, C., 2008. Expression of a noncoding RNA is elevated in Alzheimer's disease and drives rapid feed-forward regulation of beta-secretase. Nat. Med. 14 (7), 723–730.

Gogliotti, R.G., Cardona, H., Singh, J., Bail, S., Emery, C., Kuntz, N., Jorgensen, M., Durens, M., Xia, B., Barlow, C., Heier, C.R., Plasterer, H.L., Jacques, V., Kiledjian, M., Jarecki, J., Rusche, J., DiDonato, C.J., 2013. The DcpS inhibitor RG3039 improves survival, function and motor unit pathologies in two SMA mouse models. Hum. Mol. Genet. 22 (20), 4084–4101.

Jia, H., Osak, M., Bogu, G.K., Stanton, L.W., Johnson, R., Lipovich, L., 2010. Genome-wide computational identification and manual annotation of human long noncoding RNA genes. RNA 16 (8), 1478–1487.

Katayama, S., Tomaru, Y., Kasukawa, T., Waki, K., Nakanishi, M., Nakamura, M., Nishida, H., Yap, C.C., Suzuki, M., Kawai, J., Suzuki, H., Carninci, P., Hayashizaki, Y., Wells, C., Frith, M., Ravasi, T., Pang, K.C., Hallinan, J., Mattick, J., Hume, D.A., Lipovich, L., Batalov, S., Engstrom, P.G., Mizuno, Y., Faghihi, M.A., Sandelin, A., Chalk, A.M., Mottagui-Tabar, S., Liang, Z., Lenhard, B., Wahlestedt, C., 2005. Antisense transcription in the mammalian transcriptome. Science 309 (5740), 1564–1566.

Kiyosawa, H., Yamanaka, I., Osato, N., Kondo, S., Hayashizaki, Y., 2003. Antisense transcripts with FANTOM2 clone set and their implications for gene regulation. Genome Res. 13 (6b), 1324–1334.

Kong, L., Zhang, Y., Ye, Z.Q., Liu, X.Q., Zhao, S.Q., Wei, L., Gao, G., 2007. CPC: assess the protein-coding potential of transcripts using sequence features and support vector machine. Nucleic Acids Res. 35 (Web Server issue), W345–349.

Lee, C.C., Avalos, A.M., Ploegh, H.L., 2012. Accessory molecules for Toll-like receptors and their function. Nat. Rev. Immunol. 12 (3), 168–179.

Li, K., Blum, Y., Verma, A., Liu, Z., Pramanik, K., Leigh, N.R., Chun, C.Z., Samant, G.V., Zhao, B., Garnaas, M.K., Horswill, M.A., Stanhope, S.A., North, P.E., Miao, R.Q., Wilkinson, G.A., Affolter, M., Ramchandran, R., 2010. A noncoding antisense RNA in tie-1 locus regulates tie-1 function in vivo. Blood 115 (1), 133–139.

Li, Q., Su, Z., Xu, X., Liu, G., Song, X., Wang, R., Sui, X., Liu, T., Chang, X., Huang, D., 2012. AS1DHRS4, a head-to-head natural antisense transcript, silences the DHRS4 gene cluster in cis and trans. Proc. Natl. Acad. Sci. U. S. A. 109 (35), 14110–14115.

Magistri, M., Faghihi, M.A., St Laurent 3rd, G., Wahlestedt, C., 2012. Regulation of chromatin structure by long noncoding RNAs: focus on natural antisense transcripts. Trends Genet. 28 (8), 389–396.

Mansell, A., Smith, R., Doyle, S.L., Gray, P., Fenner, J.E., Crack, P.J., Nicholson, S.E., Hilton, D.J., O'Neill, L.A., Hertzog, P.J., 2006. Suppressor of cytokine signaling 1 negatively regulates Toll-like receptor signaling by mediating Mal degradation. Nat. Immunol. 7 (2), 148–155.

Mei, Z.Z., Chen, X.Y., Hu, S.W., Wang, N., Ou, X.L., Wang, J., Luo, H.H., Liu, J., Jiang, Y., 2016. Kelch-like protein 21 (KLHL21) targets IkappaB kinase-beta to regulate nuclear factor kappa-light chain enhancer of activated B cells (NF-kappaB) signaling negatively. J. Biol. Chem. 291 (35), 18176–18189.

Meziane, O., Piquet, S., Bosse, G.D., Gagne, D., Paquet, E., Robert, C., Tones, M.A., Simard, M.J., 2015. The human decapping scavenger enzyme DcpS modulates microRNA turnover. Sci. Rep. 5, 16688.

Milac, A.L., Bojarska, E., Wypijewska del Nogal, A., 2014. Decapping Scavenger (DcpS) enzyme: advances in its structure, activity and roles in the cap-dependent mRNA metabolism. Biochim. Biophys. Acta 1839 (6), 452–462.

Modarresi, F., Faghihi, M.A., Lopez-Toledano, M.A., Fatemi, R.P., Magistri, M., Brothers, S.P., van der Brug, M.P., Wahlestedt, C., 2012. Inhibition of natural antisense transcripts in vivo results in gene-specific transcriptional upregulation. Nat. Biotechnol. 30 (5), 453–459.

Murray, R.Z., Wylie, F.G., Khromykh, T., Hume, D.A., Stow, J.L., 2005. Syntaxin 6 and Vti1b form a novel SNARE complex, which is up-regulated in activated macrophages to facilitate exocytosis of tumor necrosis Factor-alpha. J. Biol. Chem. 280 (11), 10478–10483.

Okazaki, Y., Furuno, M., Kasukawa, T., Adachi, J., Bono, H., Kondo, S., Nikaido, I., Osato, N., Saito, R., Suzuki, H., Yamanaka, I., Kiyosawa, H., Yagi, K., Tomaru, Y., Hasegawa, Y., Nogami, A., Schonbach, C., Gojobori, T., Baldarelli, R., Hill, D.P., Bult, C., Hume, D.A., Quackenbush, J., Schriml, L.M., Kanapin, A., Matsuda, H., Batalov, S., Beisel, K.W., Blake, J.A., Bradt, D., Brusic, V., Chothia, C., Corbani, L.E., Cousins, S., Dalla, E., Dragani, T.A., Fletcher, C.F., Forrest, A., Frazer, K.S., Gaasterland, T., Gariboldi, M., Gissi, C., Godzik, A., Gough, J., Grimmond, S., Gustincich, S., Hirokawa, N., Jackson, I.J., Jarvis, E.D., Kanai, A., Kawaji, H., Kawasawa, Y., Kedzierski, R.M., King, B.L., Konagaya, A., Kurochkin, I.V., Lee, Y., Lenhard, B., Lyons, P.A., Maglott, D.R., Maltais, L., Marchionni, L., McKenzie, L., Miki, H., Nagashima, T., Numata, K., Okido, T., Pavan, W.J., Pertea, G., Pesole, G., Petrovsky, N., Pillai, R., Pontius, J.U., Qi, D., Ramachandran, S., Ravasi, T., Reed, J.C., Reed, D.J., Reid, J., Ring, B.Z., Ringwald, M., Sandelin, A., Schneider, C., Semple, C.A., Setou, M., Shimada, K., Sultana, R., Takenaka, Y., Taylor, M.S., Teasdale, R.D., Tomita, M., Verardo, R., Wagner, L., Wahlestedt, C., Wang, Y., Watanabe, Y., Wells, C., Wilming, L.G., Wynshaw-Boris, A., Yanagisawa, M., Yang, I., Yang, L., Yuan, Z., Zavolan, M., Zhu, Y., Zimmer, A., Carninci, P., Hayatsu, N., Hirozane-Kishikawa, T., Konno, H., Nakamura, M., Sakazume, N., Sato, K., Shiraki, T., Waki, K., Kawai, J., Aizawa, K., Arakawa, T., Fukuda, S., Hara, A., Hashizume, W., Imotani, K., Ishii, Y., Itoh, M., Kagawa, I., Miyazaki, A., Sakai, K., Sasaki, D., Shibata, K., Shinagawa, A., Yasunishi, A., Yoshino, M., Waterston, R., Lander, E.S., Rogers, J., Birney, E., Hayashizaki, Y., 2002. Analysis of the mouse transcriptome based on functional annotation of 60,770 full-length cDNAs. Nature 420 (6915), 563–573.

Osato, N., Suzuki, Y., Ikeo, K., Gojobori, T., 2007. Transcriptional interferences in cis natural antisense transcripts of humans and mice. Genetics 176 (2), 1299–1306.

Pelechano, V., Steinmetz, L.M., 2013. Gene regulation by antisense transcription. Nat. Rev. Genet. 14 (12), 880–893.

Scheele, C., Petrovic, N., Faghihi, M.A., Lassmann, T., Fredriksson, K., Rooyackers, O., Wahlestedt, C., Good, L., Timmons, J.A., 2007. The human PINK1 locus is regulated in vivo by a non-coding natural antisense RNA during modulation of mitochondrial function. BMC Genomics 8, 74.

Werner, A., Carlile, M., Swan, D., 2009. What do natural antisense transcripts regulate? RNA Biol. 6 (1), 43–48.

Yamauchi, T., Masuda, T., Canver, M.C., Seiler, M., Semba, Y., Shboul, M., Al-Raqad, M., Maeda, M., Schoonenberg, V.A.C., Cole, M.A., Macias-Trevino, C., Ishikawa, Y., Yao, Q., Nakano, M., Arai, F., Orkin, S.H., Reversade, B., Buonamici, S., Pinello, L., Akashi, K., Bauer, D.E., Maeda, T., 2018. Genome-wide CRISPR-Cas9 screen identifies leukemia-specific dependence on a Pre-mRNA metabolic pathway regulated by DCPS. Cancer Cell 33 (3), 386–400 e385.

Yelin, R., Dahary, D., Sorek, R., Levanon, E.Y., Goldstein, O., Shoshan, A., Diber, A., Biton, S., Tamir, Y., Khosravi, R., Nemzer, S., Pinner, E., Walach, S., Bernstein, J., Savitsky, K., Rotman, G., 2003. Widespread occurrence of antisense transcription in the human genome. Nat. Biotechnol. 21 (4), 379–386.

Zhou, M., Bail, S., Plasterer, H.L., Rusche, J., Kiledjian, M., 2015. DcpS is a transcript-specific modulator of RNA in mammalian cells. RNA 21 (7), 1306–1312.

Zong, X., Nakagawa, S., Freier, S.M., Fei, J., Ha, T., Prasanth, S.G., Prasanth, K.V., 2016. Natural antisense RNA promotes 3' end processing and maturation of MALAT1 lncRNA. Nucleic Acids Res. 44 (6), 2898–2908.