



Dynamic Transcriptome Profiling Reveals LncRNA-Centred Regulatory Networks in the Modulation of Pluripotency

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Long noncoding RNAs (lncRNAs) have emerged as vital regulators of gene expression during embryonic stem cell (ESC) self-renewal and differentiation. Here, we systemically analyzed the differentially regulated lncRNAs during ESC-derived cardiomyocyte (CM) differentiation. We established a perspicuous profile of lncRNA expression at four critical developmental stages and found that the differentially expressed lncRNAs were grouped into six distinct clusters. The cluster with specific expression in ESC enriches the largest number of lncRNAs. Investigation of lncRNA-protein interaction network revealed that they are not only controlled by classic key transcription factors, but also modulated by epigenetic and epitranscriptomic factors including N⁶-methyladenosine (m⁶A) effector machineries. A detailed inspection revealed that 28 out of 385 lncRNAs were modified by methylation as well as directly recruited by the nuclear m⁶A reader protein Ythdc1. Unlike other 27 non-coding transcripts, the ESC-specific lncRNA *Gm2379*, located in both nucleus and cytoplasm, becomes dramatically upregulated in response to the depletion of m⁶A or Ythdc1. Consistent with the role of m⁶A in cell fate regulation, depletion of *Gm2379* results in dysregulated expressions of pluripotent genes and crucial genes required for the formation of three germ layers. Collectively, our study provides a foundation for understanding the dynamic regulation of lncRNA transcriptomes during ESC differentiation and identifies the interplay between epitranscriptomic modification and key lncRNAs in the regulation of cell fate decision.

Keywords: lncRNA, m⁶A modification, embryonic stem cell, *Gm2379*, cell fate

INTRODUCTION

Embryonic stem cells (ESCs) are pluripotent stem cells derived from the inner cell mass of the blastocyst. They possess dual ability to self-renew unlimitedly and differentiate into multiple tissue types found in the adult animal. Both ESC proliferation and differentiation are complex processes requiring the synergetic regulation by transcription factors (TFs), epigenetic factors, histone modification and chromatin remodelers (Martello and Smith, 2014; Atlasi and Stunnenberg, 2017; Chen et al., 2017; Zhao and Jin, 2017). Apart from protein-mediated transcriptional and epigenetic networks, long non-coding RNAs (lncRNAs) emerge as new players which guide the fate regulation of pluripotency and stem cell differentiation (Fico et al., 2019; Chen et al., 2020; Constanty and

Shkumatava, 2021). They are a major group of non-coding RNAs with over 200 nucleotides in length but generally lack of translated open reading frames and obvious protein coding capacity (Statello et al., 2021). Many lncRNAs are dynamically regulated during ESC differentiation and expressed highly at specific developmental stage (Luo et al., 2016; Li et al., 2017). Therefore, establishment of lncRNA expression profiles at different developmental stages will facilitate our understanding of gene regulatory events during normal development at additional layers.

Distinct from coding mRNAs, lncRNAs themselves serve as the functional unit to modulate the expression of target genes depending on their subcellular localization and interacting molecules (Carlevaro-Fita and Johnson, 2019; Yao et al., 2019). In the cytoplasm, lncRNAs generally function to regulate mRNA stability, mediate mRNA translation and act as competing endogenous RNAs (Statello et al., 2021). In comparison, the functional roles of nucleus-localized lncRNAs have been more extensively studied. The specific interacting proteins present in the nucleus endow lncRNAs with modulation of chromatin architecture and transcriptional activity (Bergmann and Spector, 2014; Rutenberg-Schoenberg et al., 2016; Statello et al., 2021). For example, *XIST* lncRNA is able to recruit the polycomb repressive complex (PRC) to trigger the PRC1-guided mono-ubiquitylation of histone H2AK119 and the PRC2-dependent H3K27me3, resulting in silencing of gene transcription (Plath et al., 2003; Fang et al., 2004). In addition, nuclear lncRNA also participate in establishment of the nuclear compartment organization via scaffolding. For example, *MALAT1* lncRNA functions as a scaffold molecule in nuclear speckles to regulate splicing *via* recruiting serine/arginine (SR) splicing factors (Tripathi et al., 2010). Thus, investigation of the interactions between lncRNAs with RNA-binding proteins (RBPs) are crucial for deciphering their functional roles in diverse biological processes.

In addition to the structural features, the modifications embedded in non-coding RNAs are also able to regulate target gene expression through recruiting a subgroup of specific RBPs (Liu et al., 2020, 2021; Chen et al., 2021; Lee et al., 2021; Wei and He, 2021). N⁶-methyladenosine (m⁶A), the most abundant modification identified in mRNA, has been recently found to exist on lncRNAs and associate with specific “reader” proteins to modulate their functionality. m⁶A residues on the *XIST* are able to recruit nuclear RBP YTH domain containing 1 (YTHDC1) and required for *XIST*-mediated transcriptional gene silencing (Patil et al., 2016). Besides, the interaction of YTHDC1 with methylated adenosines on *MALAT1* has been identified to regulate homeostasis of nuclear speckles and cancer metastasis (Wang et al., 2021). During heat shock stress response, a novel lncRNA *Heat* silences stress gene expression via the m⁶A-YTHDC1 association (Ji et al., 2021). Despite the emergence of certain studies for m⁶A-guided lncRNA functionality, how the interplay between m⁶A and lncRNA regulate stem cell pluripotency and differentiation remains largely unknown.

In this study, we systematically analyzed the lncRNA expression at different stages during ESC-derived cardiomyocyte (CM) differentiation. We found that the largest group of lncRNAs enriched was expressed specifically in ESC. They were not merely modulated by cell fate-associated TFs, but also able to recruit RNA m⁶A epigenetic factors. Indeed, a subgroup of these

lncRNAs were obviously modified by m⁶A and bound with the reader protein YTHDC1. Intriguingly, only one ESC-specific lncRNA *Gm2379* was sensitive to the dysfunction of m⁶A-regulatory proteins. Depletion of m⁶A-modified *Gm2379* resulted in dysregulated expressions of crucial genes required for ESC pluripotency maintenance and differentiation.

MATERIALS AND METHODS

Cell Lines and Reagents

J-1 male mouse embryonic stem cells (ESCs) were purchased from the Cell Bank of Chinese Academy of Sciences. Wild-type and *Mettl3*-KO J-1 mouse ESCs were provided by Howard Y. Chang in Stanford University (Batista et al., 2014). *Ythdc1*^{flox/flox} (Control) and *Ythdc1*-cKO (*Ythdc1*-KO) mouse ESCs were provided by Yawei Gao in Tongji University (Chen et al., 2021). Mycoplasma testing was performed routinely and cells tested negative were used in further experiments. Tissue culture plates were initially treated with 0.2% gelatin for at least 30 min at 37°C. ESCs were subsequently grown in gelatinized plates containing 15% KnockOut Serum Replacement (Gibco), 1% non-essential amino acids (Gibco), 1% Glutamax (Gibco), 1% Penn-Strep (Life technologies), 0.1 mM β-mercaptoethanol (Invitrogen) and 1000 U/ml ESGRO Recombinant Mouse leukemia inhibitory factor (LIF) Protein (Millipore).

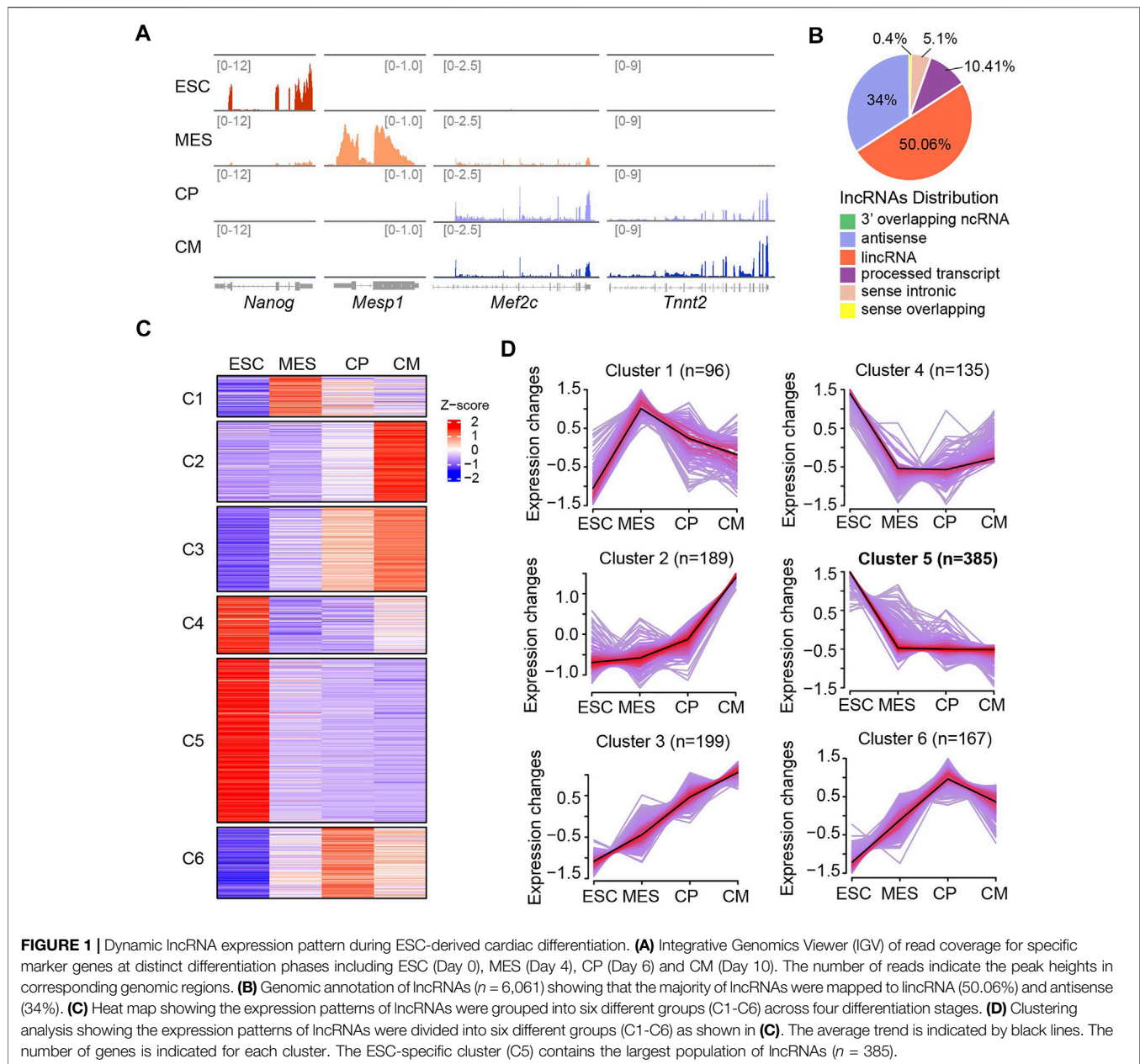
Cardiomyocyte Differentiation

To generate embryoid bodies (EBs), suspension of each cell line at a concentration of 10 × 10⁴ cells per milliliter in differentiating medium (Knock-out DMEM supplemented with 15% FBS (Gibco), 1% non-essential amino acids, 1% Glutamax, 1% Penn-Strep, 0.1 mM β-mercaptoethanol) was deposited in 20 μL hanging drops in Petri dishes. After 2 days, the suspension-cultured cells were plated onto 0.2% gelatin-coated dishes in cardiomyocyte differentiation media (CMD) containing DMEM (BI), 15% FBS (Gibco), 1% Penn-Strep, 1% non-essential amino acids (Gibco), 1% Glutamine (Gibco), 0.1 mM β-mercaptoethanol and 1 mM Ascorbic Acid (Sigma) for continued differentiation. Cell culture media was changed every other day and contracting patches of cells were collected at indicated time points.

Targeted Knockdown of *Gm2379* Long Noncoding RNAs in Mouse Embryonic Stem Cells

The shRNA targeting sequences were cloned into pRSI9-U6-(sh)-UbiC-TagRFP-2A-Blasticidin. Lentiviral particles were packaged using Lenti-X 293T cells. Virus-containing supernatants were collected at 48 h after transfection and filtered to eliminate cell debris. Wild-type and *Mettl3*-KO mESCs were infected by the lentivirus for 48 h and stable mESC knockdown lines were generated using blasticidin (MCE) selection (10 μg/ml). shRNA targeting sequences are listed below.

Gm2379 (target sequence 1): 5'- GCACGGCATTACAGA ATTTC A-3'; *Gm2379* (target sequence 3): 5'- GAAAGCCTT CATAGGAAACAG-3'; Scramble Target Sequence: 5'- AATTCT CCGAACGTGTACGT-3'.



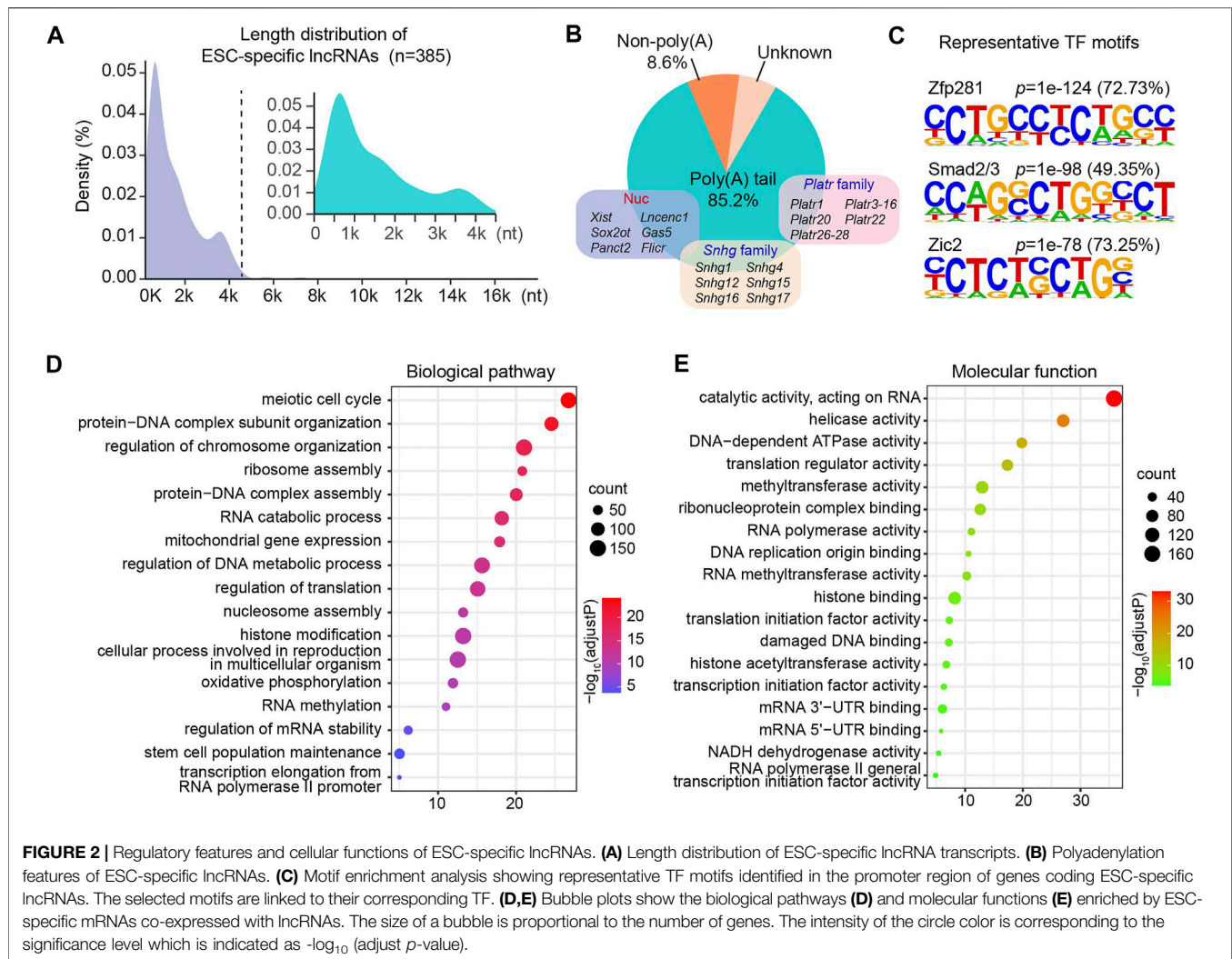
Real-Time Quantitative PCR

Total RNA from different cells was isolated from PBS-washed cells using Trizol reagent (Invitrogen). Isolated RNAs were used as templates for reverse transcription based on the manual for HiScript III 1st Strand cDNA Synthesis Kit plus gDNA wiper (Vazyme Biotech). Real-time PCR analysis was conducted with ChamQ SYBR qPCR Master Mix (Vazyme Biotech) and carried on a QuantStudio three Real-Time PCR System (Applied Biosystems). Primers for amplifying each target were listed in **Supplementary Table S1**.

Cell Fractionation Assay

ESCs were pelleted by centrifugation at 500 g for 3 min at 4°C. After washing with phosphate-buffered saline (PBS) buffer twice,

cells were lysed in ice-cold cytoplasmic lysis buffer (10 mM HEPES pH 7.5, 0.05% TritonX-100, 5 mM MgCl₂, 10 U SUPERase-In and 1x protease inhibitor cocktail) on ice for 5 min. Samples were centrifuged at 2,000 g at 4°C for 10 min. The supernatant was collected as cytoplasmic fraction. The remaining nuclear pellets were washed with PBS twice and centrifuged at 2,000 g at 4°C for 2 min. After removing the supernatant, the nuclear pellet was resuspended in RIPA buffer (50 mM Tris-HCl, pH7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 10 U SUPERase-In and 1 × protease inhibitor cocktail). This sample was collected as nuclear fraction. RNA from different fractions was isolated using TRIzol reagent (Invitrogen), and the protein sample was prepared by adding SDS loading buffer.



RNA-Seq

Total RNA was isolated using TRIzol reagent (Invitrogen). Ribosomal RNAs depletion, strand-specific library preparation and sequencing were performed at the Annoroad Gene Technology (Beijing, China). Two biological replicates were prepared for each differentiation phases. The libraries were sequenced on an Illumina Novaseq 6,000 system using the 2 × 150-bp paired-end read mode.

Long Noncoding RNA-Seq Data Processing

The lncRNA-seq data has been deposited in NCBI's Gene Expression Omnibus and is accessible through GEO Series accession number GSE197205. Trim Galore (v.0.6.6) was used to trim the low quality sequences (Martin, 2011). The paired-end reads were aligned to mouse genome (GRCm38) with HISAT2 aligner (v2.2.1) with `--rna-strandness RF` parameters and Ensemble genes (GRCm38.102) transcriptome annotation (Pertea et al., 2016). Then gene expression were quantified using featureCounts (v2.0.1) with `-s 2` parameter (Liao et al., 2014), and differentially expressed genes were identified using DESeq2 (v1.34.0) R package (Love et al., 2014). The FoldChange

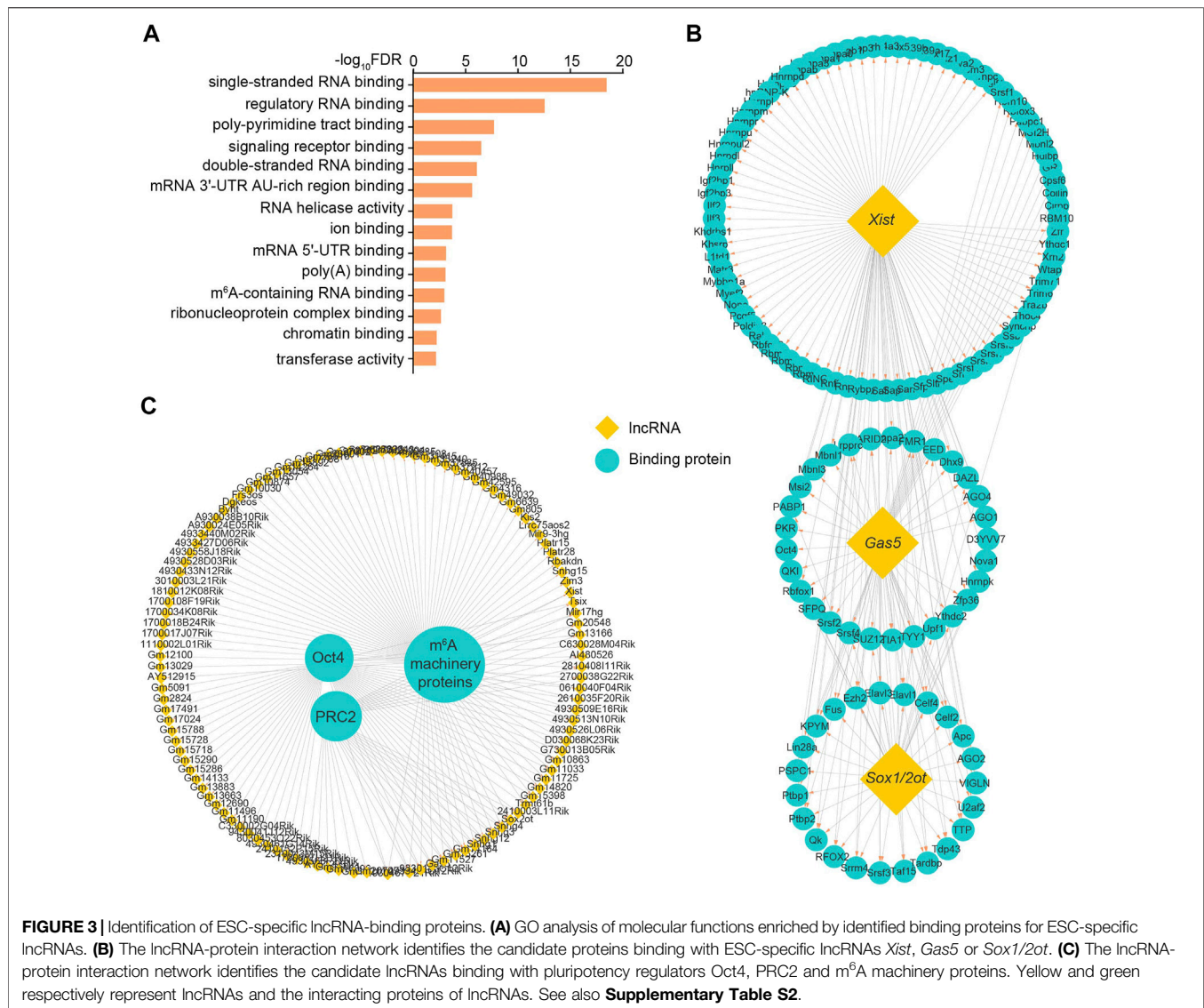
> 2 or < 0.5 and adjusted p -value < 0.05 genes were considered as differentially expressed genes. The TPM (Transcripts Per Kilobase of exonmodel per Million mapped reads) was used to produce heatmap.

Fuzzy C-Means Clustering

lncRNAs with average TPM expressions in replicates from four differentiation stages were grouped into six different clusters using Mfuzz (v2.54.0) package in R with fuzzy c -means algorithm (Kumar and Futschik, 2007).

Regulatory Motif and Functional Analysis of Embryonic Stem Cells-specific Long Noncoding RNAs

HOMER's findMotifs.pl program was used to identify motifs in the promoters of lncRNA genes (encompassing regions 2000 bp upstream and 500 bp downstream of the transcription start site) from each cluster. If one lncRNA has multiple transcript isoforms, the longest transcript isoform was used for analysis and bedtools getfasta (v2.29.2) was used to extract sequences.



Enrichment analysis was performed on Gene Ontology website (Ashburner et al., 2000). Network plot was produced by Cytoscape (v3.8.2) (Paul Shannon et al., 1971).

MeRIP-Seq and YTH Domain Containing 1 RIP-Seq Data Analysis

MeRIP-seq and RIP-seq datasets were obtained from the Gene Expression Omnibus (GEO) public database under accession number GSE145315 and GSE157265, respectively (Chen et al., 2021; Liu et al., 2021). Quality control of MeRIP-seq and RIP-seq data were performed using FastQC (v.0.11.9). Low-quality bases and adaptor contamination were removed by Trim Galore. Remaining reads were aligned to mouse genome version GRCm38 with HISAT2 aligner with default parameters. For RIP-seq data SAMtools (v1.13) was used to remove the reads with mapping quality below 30 (Li et al., 2009).

m⁶A peaks and differential m⁶A peaks were identified using exomePeak2 (v1.6.1) R package with $p_{\text{cutoff}} = 0.00001$ and $\log_2 \text{FC}_{\text{cutoff}} = 1$ parameters (Meng et al., 2014). $\log_2 \text{FoldChange} < -1$ and adjusted p value < 0.05 was considered as significantly downregulated peaks. For biological replicates, bedtools (v2.29.2) was used to intersect peaks with overlapped proportion > 0.5 (Meng et al., 2014). Peaks were annotated by HOMER's annotatePeaks.pl program. The distribution of m⁶A peaks was plotted by R package Guitar (v.1.5.0) (Cui et al., 2016). The final bam files were transformed to bigwig format by using Deeptools bamCoverage (v3.5.1) with $--\text{normalizeUsing RPKM}$ parameters (Ramírez et al., 2014). IGV (Integrative genomics viewer, v2.11.1) was used to visualize the aligned data (Thorvaldsdóttir et al., 2013). DESeq2 was used to identify Ythdc1 binding genes. The $\text{FoldChange} > 2$ and adjusted p -value < 0.05 genes were considered as Ythdc1 binding RNAs.

RNA-Seq Data Analysis

Ythdc1-KO and *Mettl3*-KO RNA-seq datasets were obtained from the Gene Expression Omnibus (GEO) public database under accession number GSE157262. The expression analysis was performed based on the available processed fpkm-matrix files in the database.

RESULTS

Dynamic Long Noncoding RNA Transcriptome During Embryonic Stem Cells-Derived Cardiac Differentiation

To investigate the global expression profiles of lncRNA transcriptome during ESC-derived cardiac differentiation, we collected the samples at four different phases including undifferentiated ESC, mesoderm (MES), cardiac progenitor (CP), and CM. Purified RNA after removal of ribosomal RNA was utilized for sequencing. The reproducible data revealed that 18,749 mRNAs and 6,061 lncRNAs were expressed during cardiac differentiation (**Supplementary Figure S1A**). As stage-specific marker genes, *Nanog* and *Mesp1* were highly expressed at ESC and MES stages, while *Mef2c* and *Tnnt2* were expressed at higher levels in the CP and CM phases (**Figure 1A**), indicating the high effectiveness of lncRNA-seq datasets. Comparative analysis of the expression patterns of mRNAs and lncRNAs showed that the expression of lncRNAs was lower than that of mRNAs. Over 50% of mRNAs had TPM values > 20 at four distinct stages, whereas only less than 10% of lncRNAs had similar TPM values (**Supplementary Figure S1B**). Mapping of these lncRNAs to genomic regions revealed that about half of the lncRNAs were derived from intergenic regions (**Figure 1B**).

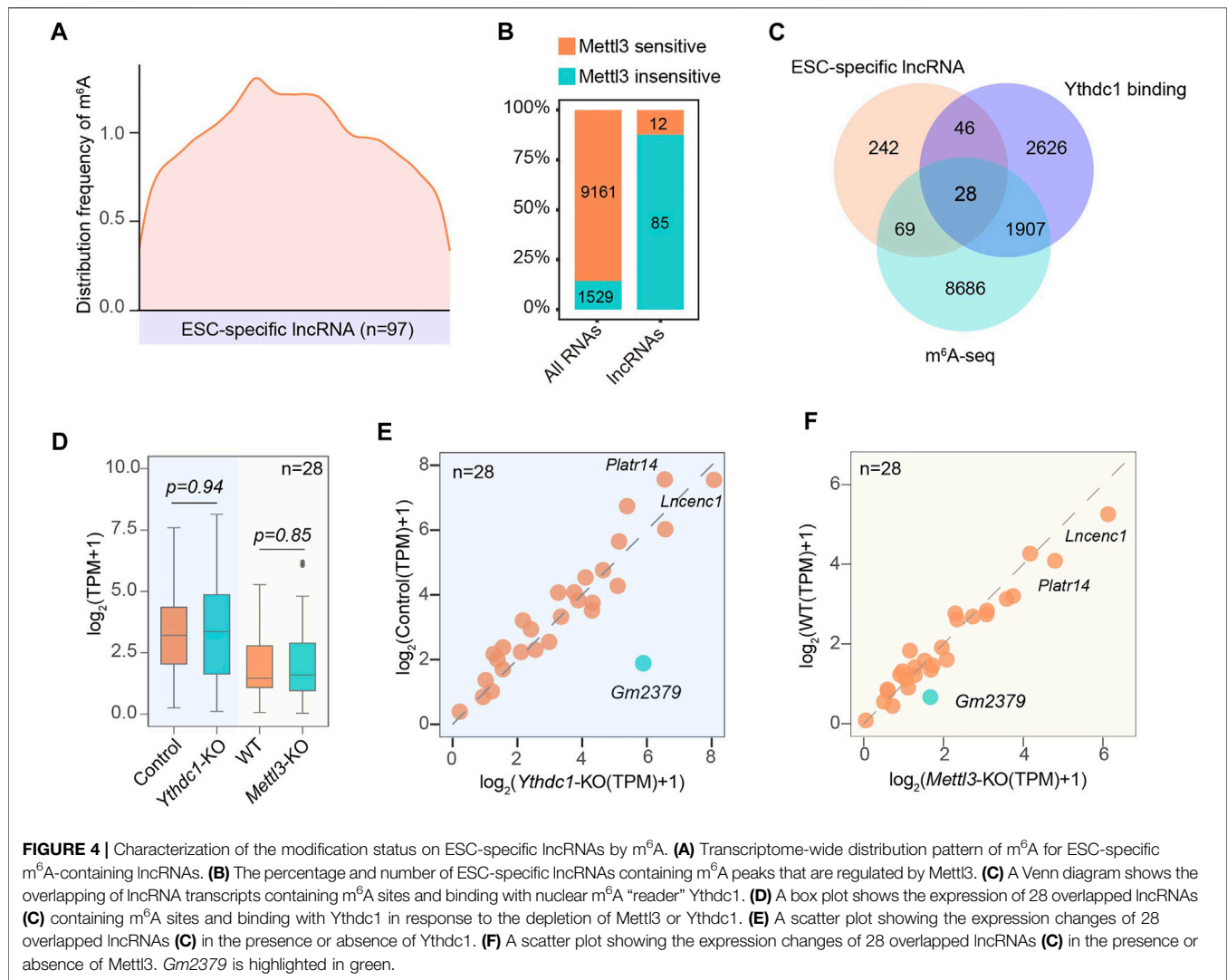
To detect expression changes of lncRNAs during ESC-derived cardiac differentiation, we performed Fuzzy C-means Clustering based on the TPM values. This analysis led to the identification of six different gene clusters with differential lncRNA expression patterns across the differentiation phases (**Figures 1C,D**). Cluster one and six contained a batch of lncRNAs that were specifically expressed at MES and CP stages, respectively. Both cluster two and three comprised genes that were specifically expressed at the CM stage. However, lncRNAs in cluster two showed mildly increased expression from the ESC to CP stage but dramatically upregulated expression from the CP to CM stage. In contrast, the expressions of lncRNAs in cluster three were increased gradually with the progression of cardiac differentiation (**Figures 1C,D**). lncRNAs in cluster four showed decreased expressions from the ESC to CP stage and slightly recovered expression at the CM stage, while cluster five contained largest population of lncRNAs ($n = 385$) which expressed at highest levels in ESC and considerably downregulated afterwards. Altogether, our results suggested lncRNA transcriptome underwent dynamic regulation during ESC-derived cardiac differentiation and identified a batch of potential non-coding regulators involved in cardiogenesis.

Regulatory Properties and Cellular Functions of Embryonic Stem Cells-specific Long Noncoding RNAs

It has been well documented that pluripotent genes such as *Nanog* and *Sox2*, are restricted to pluripotent cells and downregulated upon differentiation (Zhao and Jin, 2017). Similar as the regulatory pattern of pluripotent genes, we identified that 385 lncRNAs were expressed specifically in ESC and downregulated upon differentiation. Close examination of these lncRNAs revealed that the majority of them were less than 4,000 nucleotides in length (**Figure 2A**). Furthermore, 85.2% of ESC-specific lncRNAs harbored poly(A) tails, which might be associated with their stabilization in the cellular compartment. As canonical non-coding regulators of cell fate in the nucleus, *Xist*, *Lncenc1*, and *Gas5*, were included in the subgroup of polyadenylated lncRNAs specifically expressed in ESC (**Figure 2B**), further suggesting the accuracy and validity of our lncRNA-seq data. Intriguingly, this cluster also contained a large number of members from two lncRNA families, *Platr* and *Shhg*, some of which have been reported to regulate the stem cell fate transition (Bergmann et al., 2015; Lu et al., 2019; Li et al., 2020; Du et al., 2021; Yan et al., 2021).

To understand the key regulators that control the gene expressions of these ESC-specific lncRNAs, we next assessed the TF-binding cis-elements imbedded in the promoter region of their coding genes. We observed that about 72.73% of the lncRNAs could be bounded by Zfp281 (**Figure 2C**), which has been reported to be not required for maintenance of ESCs but indispensable for proper differentiation by targeting core pluripotent genes (Fidalgo et al., 2011). In addition, Smad2/3 and Zic2 have been implicated in the self-renewal and ESC specification (Sakaki-Yumoto et al., 2013; Luo et al., 2015), and our analysis suggested that approximately 49.35 and 73.25% of ESC-specific lncRNAs were enriched for the Smad2/3 and Zic2 motif, respectively (**Figure 2C**).

The clustering analysis also led to identification of ten distinct expression patterns of protein-coding genes across four differentiation phases (**Supplementary Figure S2**). We then performed gene ontology (GO) enrichment analysis of ESC-specific protein-coding genes in cluster two that were co-expressed with specifically expressed lncRNAs in ESC. Our analysis revealed that they were significantly enriched in biological processes including meiotic cell cycle, protein-DNA complex assembly, ribosome assembly and protein translation. Interestingly, these co-expressed lncRNAs were also involved in RNA methylation and histone modification (**Figure 2D**). Consistent with their potential biological processes, these ESC-specific lncRNAs were co-expressed with genes encoding proteins with translation regulator activity, methyltransferase activity and histone acetyltransferase activity (**Figure 2E**). These results suggest that the ESC-retained lncRNAs are forming regulatory networks with stage-specific TFs and protein-coding genes to modulate ESC fate determination. Supporting this notion, a human ESC-specific lncRNA, *lncPRESS1*, has been reported to



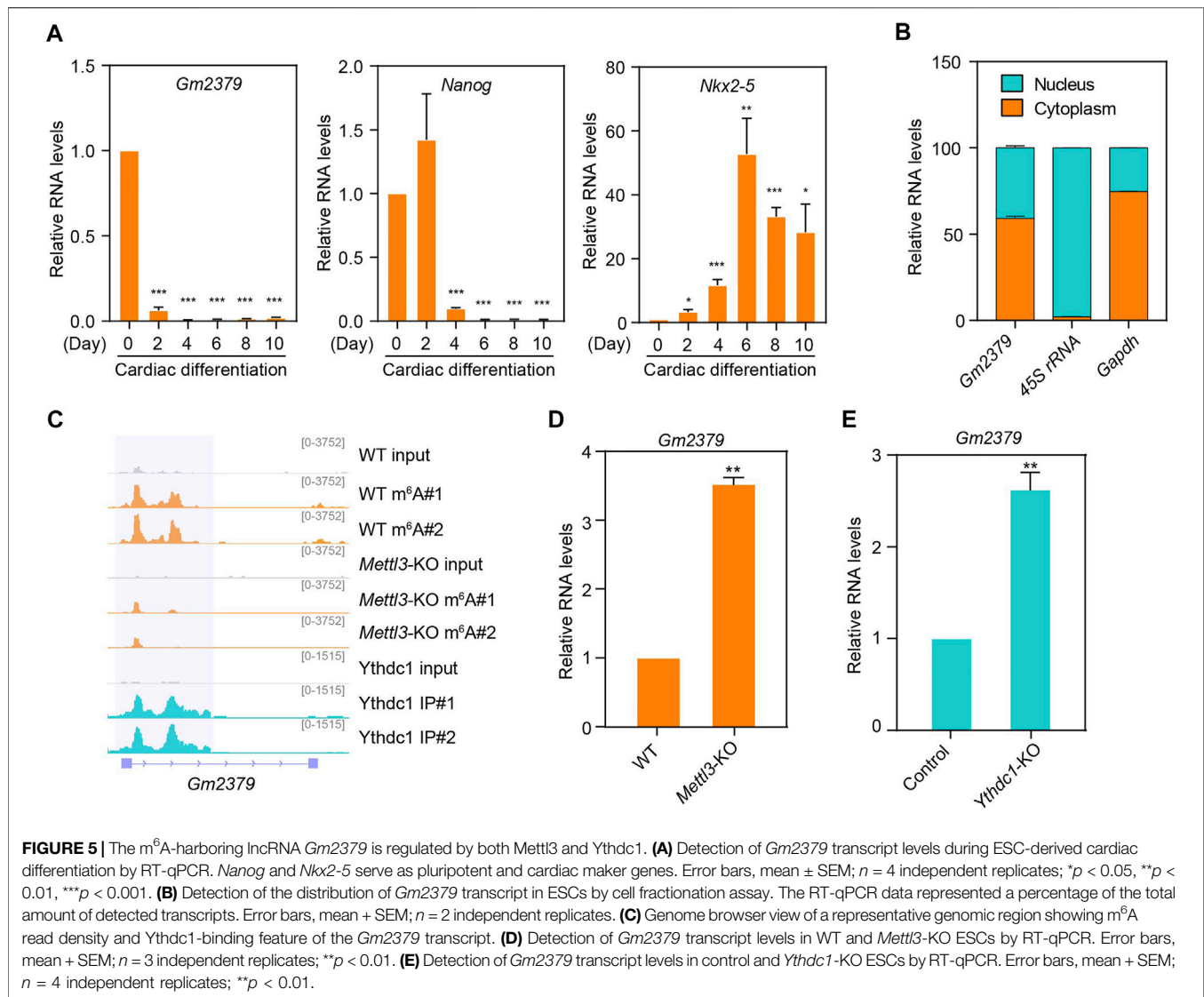
be not only directly regulated by transcription factor P53, but also interact with SIRT6 and modulate histone H3K56 and H3K9 acetylation to effect ESC state (Jain et al., 2016).

Identification of Embryonic Stem Cell-specific Long Noncoding RNA-Binding Proteins

lncRNA generally modulates target gene expression through recruiting specific functional proteins (Statello et al., 2021). We therefore searched for ESC-specific lncRNA-binding proteins by integrative analysis with two RNA-binding protein databases from both NPinter and RNAinter (Teng et al., 2020; Kang et al., 2022). Eventually we identified 1719 pairs of lncRNA-protein interactions. GO analysis of these binding proteins revealed that they harbored molecular activities of single- and double-stranded RNA binding, RNA helicase and chromatin binding. Surprisingly, m⁶A-containing RNA

binding activity was also found in these lncRNA-interacting proteins (Figure 3A).

For individual lncRNAs, *Gas5* and *Sox1/2ot*, which are linked to the regulation of pluripotency and specific lineage differentiation (Knauss et al., 2018; Tu et al., 2018; Xi et al., 2022), were able to recruit many functional factors, such as Ago and Srsf family proteins (Figure 3B). Consistent with multifaceted roles reported previously (Plath et al., 2003; Fang et al., 2004; Patil et al., 2016), *Xist* lncRNA could guide the regulation of gene expression through coupling with a variety of proteins including the key components of PRC and m⁶A machineries (Figure 3B). Among the identified proteins, the most representative ones are the core pluripotency component Oct4, histone modification activator PRC2 and m⁶A machinery proteins such as *Igf2bp1/2/3* and *Ythdc1/2*, which modulate lncRNA functions potentially through direct interaction (Figure 3C). These results suggest that these ESC-specific lncRNAs mediate multi-layered regulation of gene expression



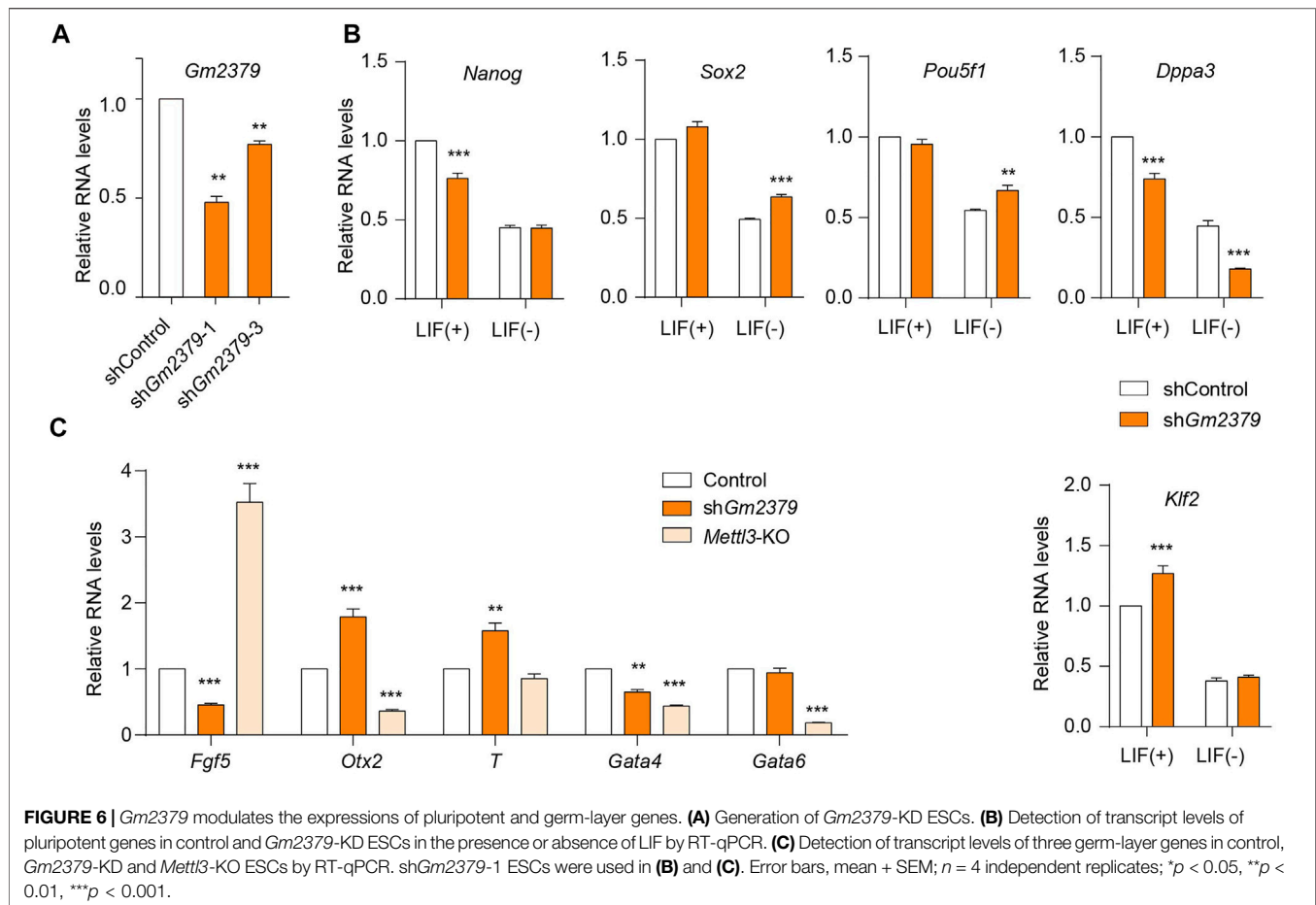
through transcription, epigenetic and epitranscriptomic modifications.

Modification of Embryonic Stem Cell-specific Long Noncoding RNAs by m⁶A

Given that the ESC-specific lncRNAs are directly recognized by m⁶A machinery proteins, we therefore hypothesized that at least some of them could be modified by RNA methylation. Indeed, 97 out of 385 lncRNAs contained typical m⁶A peaks. Distinct from m⁶A distribution in the vicinity of the stop codons of mRNAs (Dominissini et al., 2012; Meyer et al., 2012), m⁶As in lncRNAs were highly enriched in close proximity to the middle of the transcript body (Figures 4A,C). In addition, over 85% of mRNA m⁶As were subject to the regulation of core methyltransferase *Mettl3*. While, only less than 13% of m⁶As in ESC-associated lncRNAs were regulated by *Mettl3* (Figure 4B), suggesting the

existence of additional methyltransferase catalyzing the m⁶A formation of these lncRNAs.

Since *Ythdc1* has recently been identified to regulate the functionality of a number of non-coding RNAs (Wei and He, 2021), we next analyzed whether these m⁶A-containing lncRNAs could recruit *Ythdc1* as the “reader” protein. As shown in Figure 4C, 28 out of 97 lncRNAs harbored m⁶A peaks that were targeted for regulation by *Ythdc1* (Figure 4C). The remaining 69 lncRNAs may be regulated by other “reader” proteins. Surprisingly, although methylated adenosines of the 28 lncRNAs were directly bound by *Ythdc1*, almost all of them did not significantly change their expressions upon loss of *Mettl3* or *Ythdc1* (Figures 4D–F). A recent study has indicated that the RNA transcribed from long interspersed nuclear element-1 (LINE1) acts as a binding scaffold for *Ythdc1* to regulate transcription activities in early embryo and mouse ESCs (Chen et al., 2021; Liu et al., 2021). It is possible that *Ythdc1* may



facilitate the formation of lncRNA-scaffold complex rather than regulate its abundance to modulate the nuclear gene expression in ESC. Intriguingly, one out of the 28 lncRNAs, *Gm2379*, was dually regulated by *Mettl3* and *Ythdc1*, as loss of either m⁶A machinery protein resulted in obviously increased expression of *Gm2379* (Figures 4E,F), suggesting that m⁶A may form different regulatory mode with lncRNAs to modulate their functionality.

Characterization of the Long Noncoding RNA *Gm2379*

We next focused on the m⁶A-containing transcript *Gm2379*, as it is the only lncRNA that responds to the loss of the m⁶A machinery proteins. We initially validated the expression pattern of *Gm2379* during ESC-derived cardiac differentiation. As shown in Figure 5A, its expression was dramatically decreased at day 2 when embryonic bodies (EBs) were formed. Comparatively, the abundance of core pluripotency gene *Nanog* still maintained at high levels at this stage and declined after EBs generation. *Nkx2-5* as one of the earliest markers of the cardiac differentiation, was found to be obviously upregulated at day 6. To decipher the function of *Gm2379*, we next probed its expression in separated nuclear and cytoplasmic fractions from cultured ESCs. Different from the nuclear 45S rRNA, *Gm2379*

was localized in both compartments (Figure 5B). Consistent with the global pattern of 97 ESC-specific lncRNAs, the two main m⁶A peaks of *Gm2379* were distributed in proximity to the middle of the transcript body as well as subject to the regulation of *Mettl3* and *Ythdc1*, as m⁶A signals of *Gm2379* were overlapped with *Ythdc1* RIP-seq signals and almost disappeared upon knockout of *Mettl3* (Figure 5C). In addition, the transcript levels of *Gm2379* were significantly increased in ESCs depleting *Mettl3* or *Ythdc1* (Figures 5D,E), which is in good agreement with previous data analysis. Altogether, our findings indicate that *Gm2379* is specifically expressed in ESCs and dynamically regulated by nuclear “reader” protein *Ythdc1* in a m⁶A-dependent manner.

Gm2379 Modulates the Expressions of Pluripotent and Germ-Layer Genes

m⁶A has been implicated in the regulation of ESC self-renewal and differentiation. Depletion of *Mettl3* results in m⁶A erasure on target pluripotent mRNAs, facilitating ESC self-renewal and impairing directed differentiation (Batista et al., 2014; Geula et al., 2015). The nuclear “reader” *Ythdc1* has been found to be required for the maintenance of ESC pluripotency in a m⁶A-dependent manner (Chen et al., 2021; Liu et al., 2021). We have revealed that the expression of *Gm2379* lncRNA is

regulated by both *Mettl3* and *Ythdc1*, serving as a potential target of m⁶A in ESC. To determine whether *Gm2379* is directly involved in the regulation of pluripotency, we generated stable ESC lines with shRNA-mediated gene knockdown of *Gm2379* (Figure 6A). LIF is indispensable to maintain the pluripotent state of ESC (Martello and Smith, 2014). In the presence of LIF, depletion of *Gm2379* selectively affected the expression of pluripotency genes. The transcript levels of *Nanog* and *Dppa3* were significantly decreased in *Gm2379*-knock down (KD) cells, while *Klf2* was upregulated in *Gm2379*-KD cells (Figure 6B). However, in the absence of LIF, the expression of *Nanog* and *Klf2* rather than *Dppa3* was comparable in control and *Gm2379*-KD ESCs, suggesting the selective regulation of pluripotency genes by *Gm2379* lncRNA in a LIF-dependent manner.

In addition to the pluripotent genes, the crucial genes involved in the formation of three germ layers (ectoderm, mesoderm and endoderm) were dramatically dysregulated in *Gm2379*-KD ESCs (Figure 6C). Specifically, the ectoderm gene *Fgf5* and endodermal gene *Gata4* were significantly downregulated while the transcripts of the mesoderm gene *T* and ectoderm gene *Otx2* were obviously increased in response to *Gm2379* depletion. *Mettl3*, as the core m⁶A methyltransferase, has been demonstrated to regulate ESC pluripotency and differentiation (Batista et al., 2014; Geula et al., 2015). In contrast to *Gm2379* KD, loss of *Mettl3* resulted in increased expression of *Fgf5* and decreased expression of *Otx2* (Figure 6C). Considering the negative regulation of *Gm2379* by *Mettl3* shown above, it is very possible that *Mettl3* directly targets *Gm2379* lncRNA to modulate the expression of ectodermal genes.

DISCUSSION

lncRNA as genomic “dark matter” has been implicated to play crucial roles in multiple cellular processes by forming signaling regulatory networks with other functional molecules. In the process of cell fate regulation, only a small fraction of lncRNAs has been characterized (Fico et al., 2019; Chen et al., 2020). Therefore, it could be expected that many more non-coding transcripts will participate in the regulation of pluripotency and differentiation processes. In this study, we performed a global investigation of lncRNA expression profiles across four different phases of ESC-derived cardiac differentiation. Our findings showed that the lncRNA transcriptome changed dramatically during cardiac differentiation (Figures 1C,D), revealing the dynamic and complex regulation of cell differentiation at additional layers.

Among the six lncRNAs clusters with distinct expression patterns we identified, the cluster five contained the largest number of non-coding transcripts which expressed at most abundant levels in ESC and dramatically downregulated at MES stage and subsequent phases during cardiac differentiation (Figures 1C,D), suggesting the crucial roles of lncRNA in the regulation of ESC gene expression. Careful inspection revealed that many reported lncRNAs in the

regulation of cell fate were included in this subgroup, such as *Gas5* and *Lncenc1*. Depletion of *Gas5* has been found to dysregulate a number of critical genes involved in self-renewal and endodermal differentiation through forming interaction networks with pluripotent transcriptional factors and epigenetic factors (Tu et al., 2018). *Lncenc1* maintains the self-renewal of naive ESCs through cooperation with PTBP1 and HNRNPK to regulate the expression of glycolytic genes (Sun et al., 2018). Therefore, it is reasonable to speculate that the highly specific expression of lncRNAs in ESC is tightly associated with their molecular functions in the regulation of pluripotency and differentiation. In addition, our analysis also revealed a number of previously unappreciated transcripts from same lncRNA families (Figure 2B), which will facilitate our understanding of dynamics and complexity of lncRNA regulatory networks governing cell fate determination.

The function of lncRNAs is accurately exerted dependent on the formation of both cis-regulatory modules with TFs and trans-regulatory modules with RBPs (Statello et al., 2021). An increasing number of studies have been absorbed in deciphering how lncRNA regulates target gene expression, while few researches investigate how lncRNA itself is transcriptionally regulated (Tompkins et al., 2016). Consistent with the stage-specific transcriptional features, our analytic data showed that a large portion of ESC-specific lncRNAs were directly bounded and regulated by the pluripotency TF regulators *Zfp281*, *Smad2/3* and *Zic2* (Figure 2C). In addition, a number of these non-coding transcripts were found to cooperate with stage-specific trans RBPs in the regulation of ESC gene expression, such as *Oct4* and *PRC2* (Figure 3C). Surprisingly, m⁶A machinery proteins were also identified as direct interaction partners of ESC-specific lncRNAs. These observations collectively suggest that the ESC-retained lncRNAs exhibit subtle signaling networks with crucial TF regulator and trans RBPs to modulate cell pluripotency and differentiation progression.

Considering the m⁶A machineries identified as representative lncRNA-binding proteins, we thus evaluated the methylation status of these ESC-specific lncRNAs. Approximately 25% of these non-coding transcripts were modified by m⁶A. Whereas, only a small portion of m⁶A-containing lncRNAs were subject to the regulation of m⁶A-catalyzer *Mettl3* (Figure 4B). Consistent with this observation, a recent study also found a subgroup of chromatin-associated LINE1 regulatory RNAs harbored m⁶A peaks that are insensitive to *Mettl3* (Chen et al., 2021). It is possible that other methyltransferases such as *Mettl16*, mediate m⁶A formation for those *Mettl3*-insensitive ESC-specific lncRNAs. An increasing number of studies have been identified *Ythdc1* as a primary m⁶A “reader” to regulate molecular functions of non-coding transcripts (Patil et al., 2016; Liu et al., 2020, 2021; Chen et al., 2021; Ji et al., 2021; Lee et al., 2021; Ninomiya et al., 2021; Wang et al., 2021). However, our survey revealed that only about 28% of m⁶A-containing lncRNAs were directly targeted by *Ythdc1*. More interestingly, only one out of the 28 *Ythdc1*-binding m⁶A-containing lncRNAs, *Gm2379*, dramatically altered its expression in response to the deletion of *Mettl3* or *Ythdc1*

(Figures 4E,F), suggesting that majority of these non-coding transcripts may act as dynamic structural scaffolds for recruiting Ythdc1, which in turn does not regulate their expressions.

We next focused on the characterization of the novel lncRNA *Gm2379*. Consistent with the lncRNA-seq data, the m⁶A-containing lncRNA *Gm2379* was dramatically downregulated during ESC-derived cardiac differentiation (Figures 5A,C). Furthermore, depletion of *Mettl3* or *Ythdc1* leads to significantly increased expression of *Gm2379* (Figures 5D,E). In addition to the nuclear “reader” *Ythdc1*, it is very likely subject to the regulation of additional m⁶A-binding proteins in the cytoplasm, as *Gm2379* was found in both nuclear and cytoplasmic compartments (Figure 5B). Functional identification showed that depletion of *Gm2379* resulted in limited but significant alteration of pluripotent gene expressions. Instead, the key genes required for the formation of three germ layers, were more dramatically dysregulated upon knockdown of *Gm2379* (Figure 6). The opposite regulation of ectodermal genes by *Mettl3* and *Gm2379* suggest that *Gm2379* lncRNA might function as a direct target of *Mettl3* to govern the formation of ectoderm. Further investigation should focus on the examination of additional cooperating factors that are required for *Gm2379*-mediated ESC differentiation.

In summary, our findings provide global identification and characterization of lncRNA expression profiles during ESC differentiation. Investigation of cis and trans regulatory network suggest that ESC-specific lncRNAs are governed by key TFs as well as m⁶A effector machineries. Subsequent identification of a specific m⁶A-containing lncRNA on the molecular level further demonstrates the crucial interplay between epitranscriptomic modification and key lncRNAs in the regulation of cell state maintenance and transition.

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DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: GEO, accession number GSE197205 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE197205>).

AUTHOR CONTRIBUTIONS

Conceptualization, X-ML; Methodology, SW and JZha; Investigation, SW, YD, HZ, XW, LH, and JH; Data Analysis, JZha; Writing, X-ML; Review and Editing, X-ML, SW, and JZha; Funding Acquisition, X-ML and JZho; Resources, X-ML and JZho; Supervision, X-ML and JZho. All authors read and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fcell.2022.880674/full#supplementary-material>

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