microRNA-mediated regulation of microRNA machinery controls cell fate decisions

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Abstract microRNAs associate with Argonaute proteins, forming the microRNA-induced silencing complex (miRISC), to repress target gene expression post-transcriptionally. Although microRNAs are critical regulators in mammalian cell differentiation, our understanding of how microRNA machinery, such as the miRISC, is regulated during development is still limited. We previously showed that repressing the production of one Argonaute protein, Ago2, by Trim71 is important for mouse embryonic stem cells (mESCs) self-renewal (Liu et al., 2021). Here, we show that among the four Argonaute proteins in mammals, Ago2 is the major developmentally regulated Argonaute protein in mESCs. Moreover, in pluripotency, besides the Trim71-mediated regulation of Ago2 (Liu et al., 2021), Mir182/Mir183 also repress Ago2. Specific inhibition of this microRNA-mediated repression results in stemness defects and accelerated differentiation through the let-7 microRNA pathway. These results reveal a microRNA-mediated regulatory circuit on microRNA machinery that is critical to maintaining pluripotency.

Introduction microRNAs (miRNAs) are endogenous ~22 nucleotide (nt) RNAs with critical roles in modulating gene expression under diverse biological contexts (Bartel, 2009; Bartel, 2018). Most miRNAs are produced from long primary transcripts (pri-miRNAs) through successive processing by two double-stranded RNA (dsRNA) endonucleases named Drosha and Dicer, generating pre-miRNAs and ~22 nt dsRNAs, respectively. One RNA strand in the ~22 nt dsRNA, the mature miRNA, is selectively incorporated into the Argonaute (Ago) protein, forming the miRNA-induced silencing complex (miRISC) (Ha and Kim, 2014). In animals, miRISC recognizes its target mRNAs through partial base pairings mediated by the miRNA (Bartel, 2009). The Ago protein recruits GW182 proteins to down-regulate target mRNA expression through mRNA degradation and/or translational repression (Nilsen, 2007). Although miRNAs play critical regulatory roles in mammalian cell differentiation (Ameres and Zamore, 2013; Ebert and Sharp, 2012), our understanding on how miRNA machinery, particularly the miRISC, are regulated during development is still limited.

We recently found that Ago2, a key component in the miRISC, is repressed at the mRNA translation level by an RNA-binding protein named Trim71 in mouse embryonic stem cells (mESCs) (Liu et al., 2021). This repression of Ago2 inhibits stem cell differentiation mediated by the conserved pro-differentiation let-7 miRNAs (Büssing et al., 2008; Liu et al., 2021). These results suggest that Ago2 is developmentally regulated during stem cell self-renewal and differentiation, and beg for characterization of additional regulators of Ago2. Moreover, besides Ago2, there are three additional Ago proteins (Ago1, Ago3, Ago4) in mammals that function redundantly in the miRNA pathway (Meister, 2013). The relative abundance of these Ago proteins and their contribution to miRNA activities during cell differentiation, however, are still unknown.
Here, using mESC fate decisions between pluripotency and differentiation as a mammalian cell differentiation model, we determined that Ago2 is the predominant Ago protein in mESCs, and Ago2 level increases when mESCs exit pluripotency. In the pluripotent state, Mir182 and Mir183, two conserved miRNAs abundantly expressed in mESCs, repress Ago2 and control the stemness of mESCs. Specific inhibition of Mir182/Mir183-mediated repression of Ago2 results in stemness defects and accelerated differentiation of mESCs through the let-7 miRNA pathway. Collectively, these results reveal an miRNA-mediated regulatory circuit on the miRNA machinery that is critical to maintaining pluripotency.

Results

Ago2 is the predominant developmentally regulated Ago protein in mESCs

Mammals have four Ago proteins (Ago1–4) that function redundantly in miRNA-mediated regulations (Meister, 2013). Transcriptomic profiling on mESCs from different laboratories indicated that mESCs express only Ago1 and Ago2 (Figure 1—figure supplement 1A; Liu et al., 2021; Marks et al., 2012). To examine the relative abundance of Ago1 and Ago2 at the protein level, we generated mESCs with a Flag-tag knocked-in at the N-terminus of the Ago1 and Ago2 loci, respectively, via CRISPR/Cas9-mediated genome editing (Figure 1—figure supplement 1B, C). These mESCs with the Flag-tag knocked-in displayed no stemness defects compared to the wild-type (WT) mESCs (Figure 1—figure supplement 1D) and enabled us to use the same antibody (e.g., anti-Flag) to compare the relative abundance of Ago1 and Ago2. Western blotting via an anti-Flag antibody indicated that Ago2 is the predominant Ago protein in mESCs at the protein level (Figure 1A).

To examine whether Ago2 level is regulated during mESCs differentiation, we cultured mESCs under three different conditions that mimic three different developmental stages: ground/naive state (in 2i + Lif), primed state (in 15% FBS+ Lif), and differentiating state (in 15% FBS without Lif), which resulted in decreasing stemness in mESCs, as determined by the colony formation assay (Figure 1B). Western blotting indicated that Ago2 level increased when mESCs exited pluripotency (Figure 1C). This result indicated that Ago2 is developmentally regulated in mESCs, and Ago2 level is repressed in the pluripotent state.

Mir182/Mir183 regulate Ago2 and maintain stemness in mESCs

To determine how Ago2 is regulated in mESCs, we hypothesized that miRNAs expressed in mESCs might contribute to the repression of Ago2 because miRNAs are important negative regulators of gene expression. We identified the conserved miRNA-binding sites in the 3'UTR of Ago2 mRNA through TargetScan (Agarwal et al., 2015) and then examined the expression level of the corresponding miRNAs in mESCs using existing small-RNA-seq datasets (Liu et al., 2021 Figure 1D). This analysis revealed that among the miRNAs that can potentially regulate Ago2, Mir182, and Mir183, two miRNAs from the same miRNA family that are abundantly expressed in stem cells Dambal et al., 2015, have significantly higher expression levels (Figure 1E). Interestingly, Mir182/Mir183 decrease when mESCs transition from the ground state to the primed and differentiating state (Hadjimichael et al., 2016; Wang et al., 2017), which negatively correlates with the Ago2 expression pattern during this transition (Figure 1C). These observations suggest that Ago2 is repressed by Mir182/Mir183 in mESCs. Consistent with this notion, using RNA antisense purification, we found that Mir182 and Mir183 specifically associated with Ago2 mRNA in mESCs (Figure 1—figure supplement 2).

Two lines of evidence indicated that Mir182/Mir183 regulate Ago2 mRNA. First, Ago2 increased when Mir182, Mir183, or both Mir182 and Mir183 were knocked out in mESCs (Figure 2—figure supplement 1A, Figure 2A and B). Second, when either Mir182 or Mir183 was over-expressed in the WT mESCs (Figure 2—figure supplement 1B), the Ago2 level decreased (Figure 2—figure supplement 1C). The results from these loss-of-function and gain-of-function experiments argue that Mir182/Mir183 repress Ago2 expression in mESCs.

Interestingly, Mir182Δ, Mir183Δ, and Mir182Δ/Mir183Δ mESCs displayed defects in self-renewal (Figure 2C), as determined by the colony formation assay in the 15% FBS + Lif medium, where differentiation was not blocked by the two inhibitors in the 2i + Lif medium. Moreover, these miRNA knockout mESCs had accelerated differentiation, as revealed by the exit pluripotency assay (Figure 2D), which
**Figure 1.** Ago2 is the major developmentally regulated Argonaute protein in mouse embryonic stem cells (mESCs). (A) Western blotting in the wild-type (WT), Flag-Ago1, and Flag-Ago2 mESCs. (B) Colony formation assay for the mESCs. The WT mESCs were cultured under the indicated conditions, and the resultant colonies were fixed and stained for AP (alkaline phosphatase activity). The results represent the means (± SD) of four independent experiments. (C) Western blotting in the WT mESCs cultured under the indicated conditions. (D) Outline of identifying miRNAs that can potentially regulate Ago2. (E) Expression levels of the identified miRNAs from (D) in mESCs. CPM: counts per million reads.

The online version of this article includes the following figure supplement(s) for figure 1:

**Source data 1.** Tiff files of raw gel images for Figure 1A and C, Figure 1—figure supplement 1C.

**Figure supplement 1.** Expression of Argonaute proteins in mouse embryonic stem cells (mESCs).

**Figure supplement 2.** Mir182 and Mir183 are associated with Ago2 mRNA in mouse embryonic stem cells (mESCs).
evaluates the rate ESCs exit the pluripotent state (Betschinger et al., 2013), and by the measurement of pluripotency factors through Western blotting on differentiating embryonic bodies (Figure 2E). These cellular phenotypes suggest that Mir182/Mir183-mediated regulation of Ago2 is important to mESCs.

**Mir182/Mir183-mediated repression of Ago2 is required for maintaining pluripotency**

A caveat in interpreting results from miRNA knockout and over-expression experiments is the pleiotropic effects. Because each miRNA can regulate hundreds of mRNAs, when an miRNA is knocked out or over-expressed, hundreds of miRNA:mRNA interactions are altered, making it difficult to determine whether a specific miRNA:mRNA interaction contributes to the phenotypical changes.

To address this issue and specifically examine the functional significance of Mir182/Mir183-mediated regulation of Ago2 in mESCs, we mutated the Mir182/Mir183-binding sites in the 3'UTR of Ago2 mRNA via CRISPR/Cas9-mediated genome editing (Figure 3A and B). Two observations...
indicated that the mutations disrupted the interaction between Ago2 mRNA and Mir182/Mir183. First, similar to the miRNA knockout mESCs (Figure 2B), Ago2 increased in the 3'UTR mutant mESCs (Figure 3C). Second, in contrast to the results in the WT mESCs (Figure 2—figure supplement 1C), over-expression of either Mir182 or Mir183 in the 3'UTR mutant mESCs did not decrease Ago2 (Figure 3—figure supplement 1A, B). Notably, in the Mir182Δ/Mir183Δ mESCs, these mutations did not increase Ago2 (Figure 3C), indicating the increased Ago2 from these mutations in the WT mESCs is dependent on Mir182/Mir183. Moreover, the 3'UTR mutations did not significantly alter the Mir182/Mir183 levels in mESCs (Figure 3—figure supplement 1C). Altogether, these observations indicated that the functional significance of Mir182/Mir183-mediated repression of Ago2 could be specifically evaluated in the 3'UTR mutant mESCs.

When subject to the colony formation assay, the 3'UTR mutant mESCs displayed a defect in maintaining undifferentiated colonies (Figure 3D), indicating compromised self-renewal. When differentiation was evaluated by the exit pluripotency assay, the 3'UTR mutant mESCs had an increased differentiation rate (Figure 3E). Consistent with these findings, differentiating embryonic bodies from the 3'UTR mutant mESCs had a lower amount of pluripotency factors (Figure 3F). Collectively, these results indicate that Mir182/Mir183-mediated repression of Ago2 is important for mESC self-renewal and proper differentiation.
Mir182/Mir183-mediated repression of Ago2 in mESCs inhibits the let-7 miRNA-mediated differentiation pathway

Two observations lead us to the hypothesis that Mir182/Mir183-mediated repression of Ago2 in mESCs counteracts the differentiation pathway controlled by the let-7 miRNAs, a conserved miRNA family that promotes stem cell differentiation (Roush and Slack, 2008). First, in Dgcr8Δ mESCs, where endogenous miRNAs’ biogenesis is blocked, ectopic expression of Mir183 inhibits the stem cell differentiation triggered by exogenous let-7 miRNA (Wang et al., 2017). Second, our recent study indicated that increasing Ago2 levels in mESCs results in stemness defects in a let-7 miRNA-dependent manner. This specificity on let-7 miRNAs is because the pro-differentiation let-7 miRNAs are actively transcribed in mESCs, and the increased Ago2 binds and stabilizes the let-7 miRNAs that are otherwise degraded in mESCs, thereby promoting mESCs differentiation (Liu et al., 2021).

To test this hypothesis, we examined the expression of let-7 miRNAs. The 3’UTR mutant mESCs had significantly higher let-7 miRNAs than the WT mESCs (Figure 4A). This increase is specific to let-7 miRNAs because non-let-7 miRNAs were not elevated (Figure 4A). Moreover, consistent with our previous observation that increased Ago2 stabilizes mature let-7 miRNAs (Liu et al., 2021), the pri-let-7 miRNAs and the pre-let-7 miRNAs were not significantly increased in the 3’UTR mutant mESCs (Figure 4A). To determine whether the increased let-7 miRNAs are responsible for the stemness defects in the 3’UTR mutant mESCs, we inhibited let-7 miRNAs using locked nucleic acid (LNA) antisense oligonucleotides targeting the conserved seed sequence of let-7 miRNAs. When let-7 miRNAs were inhibited, the stemness defects of the 3’UTR mutant mESCs were abolished (Figure 4B), indicating that disruption of Mir182/Mir183-mediated repression of Ago2 in mESCs activates differentiation through the let-7 miRNA pathway.

Mir182/Mir183 and trim71 function in parallel to repress Ago2 mRNA in mESCs

Our previous study indicated that Ago2 mRNA is also repressed by Trim71 in mESCs (Liu et al., 2021). Interestingly, the Trim71-binding site in the 3’UTR of Ago2 mRNA is different from the
Mir182/Mir183-binding sites, suggesting that Mir182/Mir183 and Trim71 function in parallel to repress Ago2 mRNA in mESCs. We performed the following experiments to test this.

At the molecular level, we observed that over-expression of Trim71 still repressed Ago2 in the 3'UTR mutant mESCs (Figure 5A), where Mir182/Mir183-mediated repression is abolished (Figure 3). Moreover, in the 3'UTR mutant mESCs, inhibiting Trim71-mediated repression of Ago2 through deleting the Trim71-binding site in the 3'UTR of Ago2 mRNA (CLIPΔ) (Liu et al., 2021) further increased Ago2 level (Figure 5B, Figure 5—figure supplement 1). These results indicate that Trim71 and Mir182/Mir183 independently repress Ago2 mRNA in mESCs.

At the cell function level, we found that introducing the CLIPΔ in the 3'UTR mutant mESCs further decreased stem cell self-renewal, as determined by the colony formation assay (Figure 5C), and

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Source data 1. Tiff files of raw gel images for Figure 5A and B, Figure 5—figure supplement 1.

Figure supplement 1. Generation of the CLIPΔ in the 3'UTR mutant mouse embryonic stem cells (mESCs).
accelerated differentiation, as measured by the exit pluripotency assay (Figure 5D). These observations argue that Trim71 and Mir182/Mir183 function independently in regulating stemness in mESCs through modulating Ago2 mRNA.

Collectively, these findings indicate that Mir182/Mir183 and Trim71 function in parallel to repress Ago2 mRNA in mESCs.

**Discussion**

Our data reveal that the predominant Ago protein in mESCs, Ago2, is developmentally regulated, with gradually increasing levels when mESCs exit pluripotency. Two miRNAs abundantly expressed in mESCs, Mir182/Mir183, contribute to the repression of Ago2 in the pluripotent state. This miRNA-mediated regulation of Ago2 is critical to maintaining stemness. Our findings raise several interesting aspects of miRNAs in stem cell biology.

First, since Ago2 is the predominant Ago protein in mESCs, the Ago2 expression pattern during mESCs' transition from self-renewal to differentiation argues that although certain individual miRNAs may be required for pluripotency (e.g., Mir182/Mir183), the global miRNA activity is suppressed in the pluripotent state and induced when mESCs initiate differentiation. Consistent with this notion, knocking out key components in global miRNA biogenesis, such as Dgcr8 (Wang et al., 2007), Dicer (Kanellopoulou et al., 2005; Murchison et al., 2005), or Ago2 in the miRISC (Liu et al., 2021), does not negatively affect mESCs self-renewal. However, differentiation in all these mutant mESCs is severely compromised. Thus, at the global level, miRNAs may play more important roles in mESC differentiation.

Second, previous studies indicate that the two components of the miRISC, the Ago protein and its associated miRNA, mutually regulate each other. In the absence of miRNAs, the Ago protein is destabilized (Martinez and Gregory, 2013; Smibert et al., 2013), while miRNAs are also unstable if they are not associated with Ago proteins (Winter and Diederichs, 2011). Thus, the effective miRNA activity depends on the limiting component in the miRISC. Our previous studies indicated that the conserved pro-differentiation let-7 miRNAs are sensitive to Ago2 levels because an increase of Ago2 results in specific stabilization of let-7 miRNAs that are otherwise degraded (Liu et al., 2021). Thus, for let-7 miRISC, Ago2 is possibly the limiting component in mESCs. Repression of Ago2 by either Mir182/Mir183 as we characterized here or Trim71 as we identified previously (Liu et al., 2021) likely limits the effective let-7 miRISCs. Interestingly, the pro-differentiation let-7 miRISCs can positively auto-regulate their own biogenesis through inhibiting Lin28a, a conserved let-7 target, because Lin28a inhibits the biogenesis of let-7 miRNAs through promoting their pre-miRNA degradation (Tsialikas and Romer-Seibert, 2015). Thus, the effective let-7 miRNAs need to be tightly controlled in stem cells. The two repression mechanisms on Ago2 mRNA contribute to limiting the amount of effective let-7 miRISCs and maintaining pluripotency in mESCs. We speculate that similar mechanisms of regulating miRISCs by RNA-binding proteins and miRNAs may exist in other developmental processes. Moreover, Ago2 is dysregulated under many pathological conditions, such as cancer (Adams et al., 2014). Thus, regulating miRISCs through modulating Ago2 levels may also contribute to pathogenesis.

Finally, it is noticed that the Mir182Δ/Mir183Δ mESCs displayed stronger defects in self-renewal and differentiation than the 3'UTR mutant mESCs did (Figure 2C and D versus Figure 3D and E). Thus, besides Ago2 mRNA, Mir182/Mir183 may regulate additional mRNAs that are important for stem cell biology.
| Reagent type (species) or resource | Designation | Source or reference | Identifiers | Additional information |
|-----------------------------------|-------------|---------------------|------------|------------------------|
| Antibody (Rabbit monoclonal) anti-Ago2 | Bimake | Cat# A5701 | WB (1:3000) | |
| Antibody (Mouse monoclonal) anti-Oct-4 | BD Transduction Laboratories | Cat# 611202 | WB (1:5000) | |
| Antibody (Rabbit monoclonal) anti-Nanog (D2A3) | Cell Signaling Technology | Cat# 8822 | WB (1:3000) | |
| Antibody Goat Anti-Rabbit IgG (H L)-HRP Conjugate | Bio-Rad | Cat# 170-6515 | WB (1:5000) | |
| Antibody Goat Anti-Mouse IgG (H L)-HRP Conjugate | Bio-Rad | Cat# 170-6516 | WB (1:5000) | |
| Chemical compound, drug DMEM/F-12 | Gibco | Cat# 12500096 | | |
| Chemical compound, drug nLIF | Millipore | Cat# ESG1107 | | |
| Chemical compound, drug PD0325901 | APEXBio | Cat# A3013 | | |
| Chemical compound, drug N2 | Millipore | Cat# SCM012 | | |
| Chemical compound, drug N27 | Millipore | Cat# SCM013 | | |
| Chemical compound, drug MEM NEAA | Gibco | Cat# 11140-50 | | |
| Chemical compound, drug Penicillin-Streptomycin | Gibco | Cat# 11548876 | | |
| Chemical compound, drug L-Glutamin | Sigma-Aldrich | Cat# G7513 | | |
| Chemical compound, drug β-Mercaptoethanol | Sigma-Aldrich | Cat# M3148 | | |
| Chemical compound, drug Accutase | Millipore | Cat# SF006 | | |
| Chemical compound, drug Fugene6 | Promega | Cat# E2691 | | |
| Chemical compound, drug Puromycin | Sigma-Aldrich | Cat# P9620 | | |
| Chemical compound, drug Doxycycline | Sigma-Aldrich | Cat# D9891 | | |
| Chemical compound, drug Protease inhibitors | Bimake | Cat# B14001 | | |
| Chemical compound, drug Gelatin | Sigma-Aldrich | Cat# G1890 | | |
| Chemical compound, drug One Step-RNA Reagent | Bio Basic | Cat# BS410A | | |
| Chemical compound, drug DNasel | NEB | Cat# M0303L | | |
| Chemical compound, drug SuperScript II Reverse Transcriptase | Invitrogen | Cat# 18064014 | | |
| Chemical compound, drug SsoAdvanced Universal SYBR Green Supermix | Bio-Rad | Cat# 1725270 | | |
| Chemical compound, drug Q5 High-Fidelity DNA Polymerase | NEB | Cat# M0491L | | |
| Chemical compound, drug Control LNA | Qiagen | Cat# 39137 | | |
| Chemical compound, drug anti-let-7 LNA | Qiagen | Cat# YF0450006 | | |
| Commercial assay or kit Alkaline Phosphatase Assay Kit | System Biosciences | Cat# AP100R-1 | | |
| Commercial assay or kit Gibson Assembly Master Mix | NEB | Cat# E2611L | | |
| Commercial assay or kit Pierce BCA Protein Assay Kit | Thermo Fisher Scientific | Cat# 23225 | | |
| Commercial assay or kit Mir-X miRNA First Strand Synthesis Kit | Takara | Cat# 638313 | | |
| Cell line (Mus musculus) ES-E14TG2a mESC | ATCC | CRL-1821 | | |
| Cell line (Mus musculus) FLAG-Ago1 mESC | This paper | | | |
| Cell line (Mus musculus) FLAG-Ago2 mESC | PMID: 33599613 | | | |
| Cell line (Mus musculus) Mir182Δ mESC | This paper | | | |
| Cell line (Mus musculus) Mir183Δ mESC | This paper | | | |
| Cell line (Mus musculus) Mir182Δ/Mir183Δ mESC | This paper | | | |
| Cell line (Mus musculus) 3’UTR Mutant mESC | This paper | | | |

Continued on next page
All the reagents, plasmids, and oligonucleotides used in this study are listed in Supplementary file 1.

**Cell lines**

All the cell lines from this study are based on ES-E14TG2a mESC (ATCC, CRL-1821). They are listed in Supplementary file 1. The ES-E14TG2a mESCs were authenticated through STR profiling and were negative for mycoplasma contamination determined by a PCR-based kit.

**CRISPR/Cas9-mediated genome editing in mESCs**

To generate the FLAG-Ago1, FLAG-Ago2 mESCs, or Ago2 3’UTR mutant mESCs, cells were co-transfected with 2 µg of pWH464 (pSpCas9(BB)-2A-GFP [pX458]) expressing the corresponding targeting sgRNA and 1 µg of the corresponding donor oligo or plasmid using the Fugene6 (Promega). To generate Mir182Δ and Mir183Δ mESCs, cells were transfected with 2 µg of pWH464 expressing a pair of sgRNAs targeting pri-Mir182 or pri-Mir183. The transfected cells were subject to single cell sorting and the resulting clones were subject to genotyping to identify the correct clones.

**qRT-PCR**

For pri- miRNA quantification, reverse transcription was performed using random hexamers and Superscript II Reverse Transcriptase. Pre-miRNA and miRNA quantifications were using the Takara’s Mir-X miRNA quantification method. qPCR was performed in triplicate for each sample using the SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) on a CFX96 real-time PCR detection system (Bio-Rad).

**Western blotting**

Proteins were harvested in RIPA buffer (10 mM Tris-HCl pH 8.0, 140 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, and protease inhibitor cocktail) and quantified with a BCA Protein Assay kit (Thermo Fisher Scientific). Equal amounts of protein samples were resolved by SDS-PAGE, and then transferred to PVDF membranes. Western blotting was performed using a BlotCycler (Precision Biosystems) with the corresponding primary and secondary antibodies. The membranes were then treated with the Western ECL substrate (Bio-Rad), and the resulting signal was detected using an ImageQuant LAS 500 instrument (GE Healthcare).

**Colony formation assay and exit pluripotency assay**

For colony formation assay, 500 cells were plated on a 12-well plate in 2i + Lif media or Lif media (DMEM/F12 supplemented with 15% FBS, 1x penicillin/streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, 0.1 mM 2-mercaptoethanol, and 1000 U/ml Lif). For exit from pluripotency assay, 1000 cells were plated on a gelatin-coated six-well plate in differentiation media (DMEM/F12 supplemented with 15% FBS, 1x penicillin/streptomycin, 0.1 mM non-essential amino acids, 2 mM L-glutamine, and 0.1 mM 2-mercaptoethanol) for 2 days, then cultured in 2i + Lif media for another 5 days. Colonies were stained using AP staining kit and grouped by differentiation status 6–7 days after plating.
Embryoid body formation

For differentiation via embryoid body (EB) formation, $3 \times 10^6$ cells were plated per 10 cm bacterial grade Petri dish and maintained on a horizontal rotator with a rotating speed of 30 rpm in differentiation media. The resultant EBs were harvested at the indicated time points.

RNA antisense purification mESCs were crosslinked with 0.1 % formaldehyde for 5 min at room temperature, and the crosslinking reaction was quenched by adding 1/20 volume of 2.5 M glycine and incubating the mESCs at room temperature for 10 min on a rotating platform. The cells were then harvested and lysed in cell lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA, 10 % glycerol, 1 % Tween-20, with freshly added proteinase inhibitors). The cell lysate was cleared by centrifugation at 20,000 g for 10 min at 4 °C. The resulting supernatant was used for RNA antisense purification; 5 mg lysate in 500 µl lysis buffer was used for each purification. Specifically, a set of 5'-end biotinylated anti-sense DNA oligos and 5 µl RNase inhibitor (NEB) were added to the lysate, resulting in a final concentration of 0.1 µM for each oligo. The lysate was incubated at room temperature for 1 hr on a rotating platform. Then, 100 µl Dynabeads MyOne Streptavidin C1 (Invitrogen) was added and the lysate further incubated for 30 min at room temperature on a rotating platform. The magnetic beads were isolated through a magnetic stand and then subject to four washes, with each wash in 500 µl high salt wash buffer (5× PBS, 0.5 % sodium deoxycholate, 1 % Triton X-100). The washed beads were resuspended in 100 µl DNasel digestion mix (1 × DNasel digestion buffer with 5 µl DNasel [NEB]) and incubated at 37 °C for 20 min, followed by adding 350 µl LET-SDS buffer (25 mM Tris-HCl pH 8.0, 100 mM LiCl, 20 mM EDTA pH 8.0, 1% SDS) and 50 µl proteinase K (20 mg/ml, Thermo Fisher Scientific). The beads were then incubated on a thermomixer at 55 °C 1000 rpm for 2 hr. The RNA was isolated through phenol extraction and isopropanol precipitation with glycoblue (Ambion) as a coprecipitant.

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Author contributions

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Additional files

Supplementary files
- Supplementary file 1. Antibodies, plasmids, and oligonucleotides used in this study.
- Transparent reporting form

Data availability
All data generated or analyzed during this study are included in the manuscript and supporting files. Source data files have been provided for Figures 1–5.

The following previously published datasets were used:

| Author(s) | Year | Dataset title | Dataset URL | Database and Identifier |
|-----------|------|---------------|-------------|------------------------|
| Marks H, Menafra R, Kalkan T, Denissov S, Jones K, Hofemeister H, Nichols J, Kranz A, Stewart AF, Smith A, Stunnenberg HG | 2012 | Epigenome and transcriptome of naive pluripotent mouse embryonic stem (ES) cells cultured in 2i serum-free medium | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE23943 | NCBI Gene Expression Omnibus, GSE23943 |
| Hu W, Liu Q, Zhang H, Chen X, Zhang S | 2021 | Studies on Trim71 in mouse embryonic stem cells | https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE138284 | NCBI Gene Expression Omnibus, GSE138284 |

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