The Long Noncoding RNA Lncenc1 Maintains Naive States of Mouse ESCs by Promoting the Glycolysis Pathway

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SUMMARY

The naive embryonic stem cells (nESCs) display unique characteristics compared with the primed counterparts, but the underlying molecular mechanisms remain elusive. Here we investigate the functional roles of Lncenc1, a highly abundant long noncoding RNA in nESCs. Knockdown or knockout of Lncenc1 in mouse nESCs leads to a significantly decreased expression of core pluripotency genes and a significant reduction of colony formation capability. Furthermore, upon the depletion of Lncenc1, the expression of glycolysis-associated genes is significantly reduced, and the glycolytic activity is substantially impaired, as indicated by a more than 50% reduction in levels of glucose consumption, lactate production, and extracellular acidification rate. Mechanistically, Lncenc1 interacts with PTBP1 and HNRNPK, which regulate the transcription of glycolytic genes, thereby maintaining the self-renewal of nESCs. Our results demonstrate the functions of Lncenc1 in linking energy metabolism and naive state of ESCs, which may enhance our understanding of the molecular basis underlying naive pluripotency.

INTRODUCTION

Pluripotent stem cells (PSCs) can self-renew indefinitely and harbor the capability to generate almost all cells of an organism, thus holding a great promise in medical applications (De Los Angeles et al., 2015; Martello and Smith, 2014). Mouse embryonic stem cells (ESCs), the first derived PSCs, were obtained from the inner cell mass of the blastocyst (Evans and Kaufman, 1981; Martin, 1981). Epiblast stem cells (EpiSCs), a newer type of PSCs, were derived from the post-implantation embryo of the mouse (Brons et al., 2007; Tesar et al., 2007). It was proposed that ESCs and EpiSCs represent two different states of pluripotency: the naive and primed state, corresponding to pre- and post-implantation epiblasts in vivo, respectively (Marks et al., 2012). ESCs cultured in conventional serum/feeder systems are heterogenous cells with multiple pluripotent states. By contrast, ESCs cultured in 2i/LIF, i.e., ground-state ESCs or naive ESCs (nESCs), are relatively homogeneous. The nESCs harbor unique characteristics including high capability of colony formation, expression of naive pluripotency genes, global DNA hypomethylation, reactivation of two X chromosomes in female cells, and uses of both glycolysis and oxidative metabolism, mostly reflecting naive pluripotency in vivo (Hackett and Surani, 2014). Furthermore, nESCs can be converted into epiblast-like cells (EpiLCs) in vitro (Hayashi et al., 2011), representing an intermediate state between nESCs and EpiSCs (Kalkan and Smith, 2014; Kurimoto et al., 2015).

The naive-state PSCs were initially derived in rodents including mouse (Li et al., 2008) and rat (Huang da et al., 2009). Recently, human naive-like PSCs were generated by overexpression of naive pluripotency factors (Takahshima et al., 2014; Theunissen et al., 2014) or by isolating directly from early embryos (Guo et al., 2016). Human naive PSCs are similar to rodent PSCs on global transcriptional state, core transcription factor networks, and metabolism properties; however, there are still significant discrepancies regarding signaling profile and epigenetic identity (Bates and Silva, 2017), suggesting that our understanding of the molecular mechanisms of the naive state remains incomplete.

Long noncoding RNAs (lncRNAs), transcripts longer than 200 nucleotides without protein-coding capability, can regulate gene expression at transcriptional and post-transcriptional levels. They are emerging as important players in many biological processes including embryonic development, stem cell self-renewal, and differentiation (Flynn and Chang, 2014; Luo et al., 2016; Rosa and Ballardino, 2016). As one of the most commonly used cell models for lncRNA studies, PSCs have been extensively profiled for RNA expression, epigenetic modifications, and RNA-protein interactions (Guttman et al., 2009, 2011; Kelley and Rinn, 2012). Functional studies have indicated that...
IncRNAs are involved in the self-renewal of PSCs through regulating key pluripotency factors (Kaneko et al., 2014; Perry and Ulitsky, 2016; Sheik Mohamed et al., 2010), mediating chromatin modifications (da Rocha et al., 2014; Jain et al., 2016; Lin et al., 2014), or sponging/counteracting microRNAs (Liu et al., 2017; Wang et al., 2013). However, the lists of potentially functional IncRNAs in ESCs that have been reported by different studies overlap poorly (Guttman et al., 2011; Kaneko et al., 2014), which suggests that the functions of IncRNAs in PSCs are still not yet fully understood. Here we profiled genome-wide IncRNA expressions in mouse nESCs and their derived EpiLCs, and investigated the functions of one of the highly expressed IncRNAs in nESCs.

RESULTS

The Profiling of IncRNAs in Mouse nESCs and EpiLCs

To investigate the transcripts of nESCs and EpiLCs, we performed strand-specific, ribosomal RNA-depleted RNA sequencing (RNA-seq) experiments in two independent mouse ESC lines (Figure 1A). Through differential expression (DE) analysis between nESCs and EpiLCs, 2,227 DE genes are detected (Figure 1B), including 2,025 mRNAs (1,196 upregulated and 829 downregulated) and 202 IncRNAs (67 upregulated and 135 downregulated). The full list of DE IncRNA genes is provided in Table S1.

To examine the functions of IncRNAs preferentially expressed in nESCs, we tested seven IncRNAs in the lists through small hairpin RNA (shRNA)-based RNAi. We selected these IncRNAs because they are relatively enriched in nESCs, and their functions remain unknown. We achieved a significant depletion of greater than 40% with both shRNAs targeting four of seven IncRNAs tested (Lncenc1, Panct2, GM13110, and GM805). Depletion of each of these IncRNAs was associated with a significant reduction in Oct4 and/or Nanog mRNA levels, along with visible flattening of clone morphologies, suggesting that these IncRNAs may play roles in maintaining the state of nESCs (Figure S1).

Lncenc1 Is Specifically Expressed in nESCs and Is Highly Dynamic during Differentiation

Among these IncRNAs, we decided to focus on Lncenc1, as (1) it is the most abundant IncRNA that is differentially expressed in nESCs (Table S1); (2) its gene locus overlaps a super-enhancer domain (Figure 1C), suggesting that it is potentially functional; and (3) it is specifically expressed in nESCs relative to somatic tissues (Figure 2A). We noticed a published mouse work, which knocked out a IncRNA named as lincenc1 (Sauvageau et al., 2013), but the reported sequence transcribes from the opposite direction and does not overlap with the Lncenc1 gene we studied here (Figure 1C).

To test the completeness of the Lncenc1 transcript, we performed northern blot analysis. The data showed that the transcript is about 3,400 nucleotides in size in nESCs, but was almost undetectable in EpiLCs (Figure 2B). According to our RNA-seq data (Figure 1C) and RT-PCR analysis (Figure S2A), the shorter isoform (NR_110430) expresses in nESCs rather than the longer one (NR_110432). Furthermore, both RNA FISH and cellular fraction experiments indicated that transcripts of Lncenc1 located in both the nucleus and cytoplasm of mouse nESCs (Figures 2C and 2D).

To study dynamic changes of Lncenc1 expression during the nESC-to-EpiLC transition, we measured its expression over a 72-hr time window in E14 nESCs. The results showed that the expression level is relatively stable during the first 24 hr, but then decreases dramatically and becomes nearly undetectable after 72 hr (Figure 2E). Similar dynamics were observed in spontaneous differentiation induced by the withdrawal of 2i/LIF from the medium, and the decline of Lncenc1 expression happened earlier at around hour 6 (Figure 2F). Interestingly, the decline of Lncenc1 expression occurred after Nanog but before Oct4 in both cases; this suggests that Lncenc1 could be involved in pluripotency networks, consistent with a recent observation that the expression profile of Lncenc1 is associated with known pluripotency genes (Bergmann et al., 2015). Similar expression dynamics were also observed in the R1 cell line (Figures S2B and S2C), indicating a general role of Lncenc1 in nESCs. Functional experiments thereafter were performed only in E14 cells unless otherwise stated.

Lncenc1 Is Required for Self-Renewal in nESCs

To investigate the function of Lncenc1, we knocked down its expression in nESCs by two independent shRNAs, which can deplete 70%–80% of Lncenc1 transcripts as shown by qRT-PCR. As a result, the expression of six pluripotency genes decreases significantly, although the magnitude of deduction is usually less than 50% (Figure 3A). Consistently, protein levels of OCT4, NANOG, and SOX2 are decreased in Lncenc1 knockdown cells as shown by western blot and immunofluorescence (Figures S3A and S3B). Moreover, clone morphologies changed from “round and tight” to “flat and loose,” and alkaline phosphatase staining became weaker in Lncenc1-knockdown cells (Figures 3B and S3C). As Lncenc1 transcripts locate in both nucleus and cytosol, we also conducted antisense oligonucleotide (ASO)-mediated gene silencing, which showed higher knockdown efficiency compared with RNAi for nuclear RNAs (Lennox and Behlke, 2016). Consistently, when Lncenc1 was suppressed by two independent ASOs, expression levels of core pluripotency genes were significantly decreased (Figure S3D).
To further rule out potential off-target effects of shRNA- or ASO-based gene silencing, we generated Lucenc1 knockout (KO) cell lines through CRISPR/Cas9-based genome editing technology. We successfully established four KO cell lines by deleting the entire gene locus or the largest exon, and Lucenc1 transcripts were almost completely depleted in these cells. Compared with wild-type cells, the expression of pluripotency genes is significantly reduced in all these KO cell lines (Figure 3C). More important, colony formation capability, a proxy for self-renewal efficiency (Martello and Smith, 2014), was significantly decreased in knockdown and KO cells (Figure 3D).

To test whether Lucenc1 is sufficient to maintain the naive state, we overexpressed Lucenc1 (OE) in nESCs and
Figure 2. Characterization of Lncenc1 and Its Expression Dynamics during ESC Differentiation

(A) Expression of Lncenc1 in mouse nESCs and somatic tissues as measured by qRT-PCR. Expression levels were normalized against Actb. Results are shown as means ± SD of three independent experiments.

(B) The Lncenc1 transcript detected by northern blot analysis. The agarose gel imaging (black background) underneath the blot (gray background) shows total RNA used for analysis.

(C) RNA in situ hybridization (RNAscope) targeting Lncenc1 in mouse nESCs and EpiLCs. Ppib was used as a positive control. Nuclei were stained with DAPI. Scale bars, 5 μm.

(D) Relative expression levels of Lncenc1 in cytoplasm, nucleoplasm, or chromatin fractions as determined by qRT-PCR analysis of RNA extracted from each fraction. Neat1 and Actb were used as controls. Data are the means ± SD of three independent experiments. Statistical significance of t test: *p < 0.05, **p < 0.01, ***p < 0.001.

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then withdrew 2i/LIF for 2 days. The morphology of OE cells is more “naive-like” compared with the control (Figure 3E). In the 2i/LIF culture (day 0), the expression levels of pluripotency genes tested are all significantly increased in the OE cells (Figure 3F). After the withdrawal of 2i/LIF for 1 day, the expression levels of pluripotency genes, especially naive-state genes (Nanog, Tbx3, Rex1, and Esrrb), remain significantly higher, whereas those of post-implantation genes (Fgf5 and Dmnt3b) are significantly lower in the OE cells, suggesting that the overexpression of Lncenc1 in nESCs delays their differentiation. On day 2, the expression levels of pluripotency genes decrease further and are comparable between OE and control cells. Therefore, Lncenc1 is required but not sufficient to keep the ESCs in a naive state.

**Lncenc1 Controls the Glycolysis Pathway in nESCs**

To reveal global gene expression changes upon Lncenc1 depletion, we conducted RNA-seq experiments with control and knockdown cells and identified 101 downregulated and 182 upregulated genes (Figure 4A; Table S2). Functional annotation of these affected genes indicated that they are highly enriched in the glycolysis/glucogenesis pathway (Figure 4B). We then examined mRNA expression with qRT-PCR in knockdown (by shRNAs and ASOs) and KO nESCs and confirmed that these glycolysis genes detected by RNA-seq are all significantly decreased upon depletion or deletion of Lncenc1 (Figures 4C, 4D, and S3E). Consistently, protein levels of these glycolytic enzymes are markedly decreased in Lncenc1 knockdown and KO cells as determined by western blotting (Figure 4E).

Furthermore, upon the overexpression of Lncenc1 in nESCs, the mRNA levels of glycolysis genes are upregulated significantly; however, after spontaneous differentiation, the control and OE ESCs display similar dynamic changes on these glycolysis genes (Figure 4F).

To test whether the functions of Lncenc1 can be rescued in its depleted or deleted cells, we have re-expressed Lncenc1 in the knockout and knockdown ESCs. Unfortunately, although Lncenc1 can be re-expressed markedly, the expression of glycolysis and pluripotency genes still cannot be restored (Figure S4). These data suggest that increased expression alone might not be sufficient for Lncenc1 activity. Other factors might also contribute toward making Lncenc1 functional, such as the location of expression in the genome, and the localization of the ectopic Lncenc1 RNAs.

Next, we examined the glycolytic activity through functional assays. First we measured the glucose consumption and lactate production, the “in and out” of glycolysis, and found that they are both significantly decreased in Lncenc1 knockdown (Figure 5A) and KO (Figure 5B) cells compared with the controls. We further examined glycolytic activity through glycolysis stress tests. As expected, the level of glycolytic activity was significantly decreased in knockdown cells as indicated by the measurement of the extracellular acidification rate (ECAR), which is largely the result of glycolysis (Figure 5C). We measured the initial pH in glucose-free culture medium (non-glycolysis). Then, to activate glycolysis, we sequentially added oligomycin (a mitochondrial ATP synthase inhibitor) to the culture medium. Both before and after activation, the glycolytic rate of control cells was significantly higher than that of knockdown cells (Figure 5D). It is notable that, when knockdown experiments were conducted with shLncenc1-1, which displayed better efficiency, glucose consumption, lactate production, and ECAR were all reduced by 50% or more. These data indicate that the glycolysis activity was severely impaired after the downregulation of Lncenc1.

**Lncenc1 Functionally Interacts with HNRNPK and PTBP1**

To identify potential partners of Lncenc1, we performed the RNA pull-down assay using biotin-labeled probes. Compared with the antisense control, the pull-down sample with sense probe showed two specific bands on the SDS-PAGE gel, which were identified as PTBP1 and HNRNPK by mass spectrometry and further validated by western blotting (Figure 6A). Moreover, results of RNA immunoprecipitation against PTBP1 and HNRNPK antibodies showed that Lncenc1 RNAs are highly enriched compared with the other six IncRNAs tested (Figure 6B), which further confirmed interactions between Lncenc1 and these two proteins. The yeast two-hybrid assay showed that PTBP1 and HNRNPK interact with each other (Kim et al., 2000), and they interact as part of a complex in murine ESCs (Lin et al., 2014). We then investigated whether PTBP1 and HNRNPK are functionally related to Lncenc1. As expected, knockdown of Ptbp1 or Hnmpk resulted in a significant downregulation of pluripotency and glycolysis genes (Figures 6C and 6D) and a significant reduction of glucose consumption and lactate production (Figure 6E), which is consistent with phenotypes observed in the Lncenc1 knockdown or KO cells. These data indicated that Lncenc1,

(E and F) Expression dynamics of two pluripotency genes (Oct4 and Nanog) and Lncenc1 during the nESC-to-EpiLC transition (E), and during spontaneous differentiation (F). Expression levels were normalized against Actb. Results are shown as means ± SD of three independent experiments. Data are compared with 0 hr. Statistical significance of t test: *p < 0.05, **p < 0.01, ***p < 0.001. See also Figures S2B and S2C.
Figure 3. Lncenc1 Is Required to Maintain the Self-Renewal of Mouse nESCs

(A) Expression levels of six pluripotency genes as measured by qRT-PCR in nESCs upon shRNA-mediated knockdown of Lncenc1. Expression levels were normalized against Actb. Results are shown as means ± SD of three independent experiments. Statistical significance of t test: *p < 0.05, **p < 0.01, ***p < 0.001.

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PTBP1, and HNRNPK are interacting physically and functionally.

To test whether the Lncenc1-PTBP1-HNRNPK complex regulates the expression of pluripotency and/or glycolysis genes, we performed chromatin immunoprecipitation (ChIP) experiments in Lncenc1 knockdown and control nESCs. Indeed, HNRNPK binds to the promoters of pluripotency and glycolysis genes. However, only the occupancy of HNRNPK on glycolytic genes, but not pluripotency genes, were significantly decreased in knockdown cells compared with the control cells (Figure 6F). Furthermore, the occupancy of RNA polymerase II on glycolysis gene promoters decreased significantly upon Lncenc1 or PTBP1 knockdown (Figures 6G and S5). In addition, we performed chromatin isolation by RNA purification (ChIRP) assay in Lncenc1 knockout and control nESCs. Consistent with our ChIP data, Lncenc1 occupied the promoters at four of the five glycolytic genes (Figure 6H). These data suggested that Lncenc1 guides HNRNPK and PTBP1 binding to glycolysis genes; therefore promoting their levels of transcription.

Interplays among Lncenc1, Core Pluripotency Networks, and Glycolytic Genes

Given that Lncenc1 can affect self-renewal and glycolysis, we were curious about the regulatory relationships among them. First, upon the knockdown of the individual pluripotency gene (Oct4, Sox2, Nanog, or Klf4) (Figure S6A), Lncenc1 and all examined glycolysis genes were downregulated consistently (Figure 7A). Second, a recent study showed that core pluripotency factors directly regulate expression of glycolysis genes (Kim et al., 2015), and that linc-ROR promotes reprogramming of a positive feedback loop with Oct4 (Sheik Mohamed et al., 2010), and TUNA maintains self-renewal of mESCs through promoting transcriptional activities of pluripotent genes including Nanog, Sox2, and Fgf4 (Kaneko et al., 2014). However, an Oct4 pseudogene IncRNA guides SUV39H1, a repressive chromatin modifier, to the promoter and epigenetically silences the Oct4 gene in differentiated cells (Scarola et al., 2015). Similarly, in somatic cells, lincRNA-p21 interacts with SETDB1, which silences pluripotency genes by depositing histone H3K9me3 on their promoters (Bao et al., 2015). Furthermore, linc-ROR promotes reprogramming by repressing pathways including P53 responses (Loewer et al., 2010), and this lncRNA maintains self-renewal of human ESCs through sponging miR-145, which targets mRNAs of core pluripotency genes (Wang et al., 2013). Recently, Austin Smith and colleagues reported that the IncRNA Ephemeron modulates the exit from naive pluripotency by connecting microRNA and de novo DNA

DISCUSSION

Functional studies indicated that some IncRNAs regulate self-renewal or pluripotency through integrating or disrupting core pluripotency networks. For example, Gomafu regulates Oct4 and Nanog expression through the formation of a positive feedback loop with Oct4 (Sheik Mohamed et al., 2010), and TUNA maintains self-renewal of mESCs through promoting transcriptional activities of pluripotent genes including Nanog, Sox2, and Fgf4 (Kaneko et al., 2014). However, an Oct4 pseudogene IncRNA guides SUV39H1, a repressive chromatin modifier, to the promoter and epigenetically silences the Oct4 gene in differentiated cells (Scarola et al., 2015). Similarly, in somatic cells, lincRNA-p21 interacts with SETDB1, which silences pluripotency genes by depositing histone H3K9me3 on their promoters (Bao et al., 2015). Furthermore, linc-ROR promotes reprogramming by repressing pathways including P53 responses (Loewer et al., 2010), and this lncRNA maintains self-renewal of human ESCs through sponging miR-145, which targets mRNAs of core pluripotency genes (Wang et al., 2013). Recently, Austin Smith and colleagues reported that the IncRNA Ephemeron modulates the exit from naive pluripotency by connecting microRNA and de novo DNA
Figure 4. Lncenc1 Regulates the Glycolysis Pathway in nESCs

(A) The heatmap of differentially expressed genes upon Lncenc1 knockdown as identified by RNA-seq experiments.

(B) Significantly enriched pathways (p < 0.05) of differentially expressed genes upon Lncenc1 knockdown as revealed by KEGG pathway analysis. Lower panel: the schematic showing of the glycolysis pathway and significantly downregulated genes (in red) upon Lncenc1 depletion.

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methylation (Bates and Silva, 2017). In this study, we
demonstrate that Lncenc1 maintains self-renewal by regu-
lating the glycolysis pathway. To our knowledge, Lncenc1
is the first lncRNA linking self-renewal and energy meta-
bolism in PSCs.

We showed that Lncenc1 interacts with two RNA binding
proteins (RBPs), PTBP1 and HNRNPK. Interestingly, the
lncRNA TUNA also interacts with PTBP1 and HNRNPK,
activating pluripotency genes directly (Kaneko et al.,
2014). However, linc-p21 interacts with HNRNPK and
SETBD1, silencing rather than activating its target genes
in somatic cells (Bao et al., 2015). PTBP1 is required for em-
bryonic development (Suckale et al., 2011), and HNRNPK
could interact with multiple proteins through its K interac-
tive region to play various roles in diverse cellular processes
(Keuens et al., 2016). Based on these data, we propose that
a class of RBPs such as HNRNPK could form complexes with
different lncRNAs and/or proteins that regulate specific
classes of genes in different cell types. Further investiga-
tions are needed to reveal the functions of lncRNA-RBP
complexes in various biological systems.

Recently, energy metabolism has been closely linked to
stem cell fates. For example, during the ESC-to-EpiSC tran-
sition, energy metabolism switched from bivalent (utiliza-
tion of both glycolysis and oxidative phosphorylation) to
exclusively glycolytic, and hypoxia inducible factor 1a
was sufficient to drive ESC to an EpiSC-like stage (Zhou
et al., 2012). A recent metabolome analysis further indi-
cated that, although early metabolites are increased, down-
stream glycolysis metabolites are decreased in primed
hESCs (Sperber et al., 2015). Interestingly, when mESCs
adapted from serum to 2i conditions, the glycolysis
pathway was significantly upregulated as detected by
RNA-seq (Marks et al., 2012). Similarly, protein levels of
glycolysis enzymes are significantly increased in naive
ESCs compared with ESCs cultured in serum (Taleahmad
et al., 2015). Our data further demonstrate that the
downregulation of glycolysis by Lncenc1 depletion impairs
A RNA pull-down analysis showing the presence of PTBP1, HNRNPK, and H3 proteins in anti-sense and sense RNA samples.

B Western blot analysis showing the differential expression of HNRNPK, PTBP1, and IgG in various cell lines.

C Bar graph comparing the relative expression levels of Oct4, Sox2, Nanog, ErbB, and KIt in different cell lines.

D Bar graph comparing the relative expression levels of Pphp, Alkoc, Tphi, Pkp1, and Llina in different cell lines.

E Glucose uptake concentration (mmol/l) and lactate production concentration (mmol/l) for shCtrl, shPtbp1-1, shPtbp1-2, shHnmpk-1, and shHnnmpk-2.

F HNRPK ChIP analysis showing the IP/input ratio for different cell lines.

G RNA Pol II ChIP analysis showing the IP/input ratio for different cell lines.

H Lncenc1 CHIRP analysis showing the normalized input for different cell lines.

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self-renewal, which suggests that a high level of glycolysis is vital for naive ESCs. However, it remains unclear how glycolysis regulates naive pluripotency. It is possible that a faster generation of ATPs by glycolysis benefits highly proliferative cells including stem cells and cancer cells, or that glycolysis-generated metabolites are essential for...
The future. Finally, as exemplified by Lncenc1 activity of cytoplasmic Lncenc1, it would be interesting to explore further functional roles of different lncRNAs in regulating energy metabolism (Dowen et al., 2014; Geuens et al., 2016; Rupaimoole et al., 2015), yet the specific glycolysis metabolites.

Moreover, it would be possible to optimize culture systems for PSCs by establishing the epigenetic modifications required for the maintenance of PSCs (Lin et al., 2016; Moussaieff et al., 2009). Two independent ASOs targeting Lncenc1, and developing Lncenc1-deficient mouse models, would be critical in addressing this question.

Overexpression and Knockdown

Overexpression experiments were performed with electroporation, according to the manufacturer's instructions. The transient transfections were conducted as described (Fan et al., 2018). For differential gene expression analysis, we used phosphorothioate-modified oligodeoxynucleotides (Ideue et al., 2009). Two independent ASOs targeting Lncenc1 were provided in Table S3. For ASO-mediated knockdown, we used ASOs of 200-bp RNA (pair1: GAGCCAATCTCTAGGCAAGT and GTATGACAAAGG) and one control ASO (pair2: GTGCTTTTGTGTTATCCCGG and GGTCC). The qRT-PCR experiments were conducted as described (Bao et al., 2015). The validated shRNA sequences were provided in Table S1. For shRNA-mediated knockdown, we used shRNAs targeting Lncenc1 (ATTATGTAACCACCT) were used to target the locus in E14 ESCs, we applied CRISPR/Cas9.

Gene Knockout

Gene knockouts were verified by genomic DNA PCR and sequencing. Our described region (15 pg/ml, and 4 pg/ml; Nagai et al., 2015). We performed 1 × 106 cells for each treatment (n = 3). Gene knockouts were verified by genomic DNA PCR and sequencing. The transient transfections were conducted as described (Fan et al., 2018)
RNA Fluorescence In Situ Hybridization
RNA fluorescence in situ hybridization for the detection of Lncenc1 and Ppib (positive control) was performed using an RNAscope (Advanced Cell Diagnostics, Hayward, CA, 323100), according to the protocol provided by the manufacturer.

Alkaline Phosphatase Detection
ESCs were fixed with 4% paraformaldehyde and stained with the Alkaline Phosphatase Staining Kit (Millipore) according to the manufacturer's protocol.

Colony Formation
ESCs were plated at a density of 100 per cm² in laminin-coated 6-well plates in 2i/LIF. The medium was changed every 3 days. After 6 days, colonies were fixed and stained for alkaline phosphatase. Colonies were counted using the ImageJ software.

The Measurement of Glucose Uptake and Lactate Production
The same amount (1 × 10⁶) of control and knockdown or knockout ESCs were seeded on a well of 12-well plates in 0.5 mL of N2B27 medium containing 2i/LIF. After 6 hr, the culture medium was collected for glucose and lactate measurement, using the Cobas C311 Chemistry Analyzer (Roche), according to the manufacturer's instructions. The intracellular glucose consumption and lactate production were calculated by the concentration differences between cultured medium and the blank medium.

Seahorse XF Bioenergetic Analysis
SeaHorse plates were pre-treated by coating with 0.1% gelatin. ECAR in mESCs were measured using the Seahorse XF96 biolalyzer according to the manufacturer's protocols. Cells were seeded in an XF96 microplate at a density of 20,000 cells per well. Culture medium was changed to unbuffered XF assay medium at pH 7.4 (Seahorse Biosciences), supplemented with 5.5 mM glucose (Sigma) and 1 mM pyruvate for assay of glycolysis rates. Before assay, the microscope plate was transferred into a non-CO₂ incubator at 37°C for 60 min. For the glycolysis stress test, after collecting baseline acidification rate data, cells were sequentially treated with 10 mM glucose, 1 mM oligomycin, and 50 mM 2-DG, and the subsequent changes were quantified. All metabolic profiles were normalized to cell numbers.

RNA Pull-Down Assay
RNA pull-down experiments were performed as described previously (Tsai et al., 2010). Specific gel bands were excised and subject to mass spectrometry analysis.

RNA Immunoprecipitation
Mouse nESCs (1 × 10⁷) were subjected to nuclei isolation as described (Doi et al., 2009). Nuclei were then resuspended in 1 mL of cold RNA immunoprecipitation (RIP) buffer (150 mM KCl, 25 mM Tris [pH 7.4], 5 mM EDTA, 0.5 mM DTT, 0.5% NP40, 100 U/mL RNAase inhibitor, 1× Complete) and sheared by sonication. For immunoprecipitation (IP), 2 μg of specific antibody (HNRNPK [Abcam, ab39975]; PTBP1 [Invitrogen, 324800]) or the control IgG (Cell Signaling Technologies, 2,729) was incubated with 6–10 mg supernatant at 4°C for 2 hr, then 20 μL of protein G magnetic beads (Invitrogen) were added to each IP sample and incubated at 4°C for 1 hr. The beads were collected and washed three times with RIP buffer. RNAs were extracted with TRIzol, and enrichment for each gene was determined by qRT-PCR.

ChIP
ChIP experiments were performed with 1 × 10⁷ mESCs as described previously (Irizarry et al., 2008). The following antibodies were used for ChIP: anti-HNRNPK (Abcam, ab39975) and anti-RNA PolII (Abcam, ab817). Primers for ChIP-PCR were provided in Table S5.

ChIRP
ChIRP experiments were performed as described previously (Chu et al., 2011). Probe sequences are provided in Table S6.

ACCESSION NUMBERS
The RNA-seq datasets from this study have been submitted to the NCBI GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GEO: GSE116602.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and six tables and can be found with this article online at https://doi.org/10.1016/j.stemcr.2018.08.001.

AUTHOR CONTRIBUTIONS
Z.S., M.Z., L.C., and B.W. conceived and designed the study. Z.S., M.Z., L.C., Q.W., P.T., and Z.Y. performed the experiments. P.L. performed bioinformatics analysis. All authors reviewed and approved the manuscript.

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Supplemental Information

The Long Noncoding RNA Lncenc1 Maintains Naive States of Mouse ESCs by Promoting the Glycolysis Pathway

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The Long Noncoding RNA Lncenc1 Maintains Naïve States of Mouse ES Cells by Promoting the Glycolysis Pathway

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This document includes Figures S1-6 and Tables S3-6.
Figure S1. Identification of lncRNAs involved in the maintenance of nESC self-renewal (Related to Figure 1)

(A) Work flow showing the strategy to identify candidate lncRNAs required for nESC self-renewal. nESCs were transduced with the shRNA containing lentivirus and selected with puromycin for 4 days, and then total RNAs were harvested for analysis.

(B) Knockdown efficiency was determined by qRT-PCR. Expression levels were normalized against Actb. Data are the mean ± SD of three independent experiments.

(C) Micrographs of ESCs after infection with a control shRNA or shRNAs targeting Oct4 and seven lncRNAs. Scale bars, 200µm. (D,E) Relative expression of Oct4 (D) and Nanog (E) mRNA after knockdown these lncRNAs. Expression levels were normalized to Actb. Data are the mean ± SD of three independent experiments.
Figure S2. Lncenc1 isoform validation and expression dynamics of Lncenc1 in R1 nESC differentiation (Related to Figure 2)

(A) Schematic illustration of the RT-PCR primers of Lncenc1 isoforms referring to Refseq Accessions (left) and RT-PCR products (right).

(B, C) Expression dynamics of two pluripotency genes (Oct4 and Nanog) and Lncenc1 during the nESC-to-EpiLC transition (B) and during spontaneous differentiation (C) in R1 ESCs. Expression levels were normalized against Actb. Results are shown as mean ± SD of three independent experiments. The expression at all time points is significantly changed compared to 0h, statistical significance of T-Test: *** p < 0.001.
Figure S3. Expression of pluripotency and glycolysis genes upon Lncenc1 knockdown or overexpression (Related to Figure 3, 4).

(A) Images of immunofluorescence with OCT4 and NANOG antibodies in control and knockdown nESCs. Scale bars, 200µm.

(B) Western Blotting with OCT4 and SOX2 antibodies in control and knockdown nESCs (up), relative protein level (down).

(C) Bright-field micrographs (upper panels) and alkaline phosphatase staining in control nESCs and Lncenc1 knockdown R1 ESCs. Scale bars, 200µm.

(D, E) Expression levels of six pluripotency and five glycolysis genes upon Lncenc1 knockdown by ASOs. Expression levels were detected by qRT-PCR. Data were normalized against Actb and are shown as mean ± SD of three independent experiments. Statistical significance of T-Test: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure S4. Expression of pluripotency and glycolysis genes upon \textit{Lncenc1} overexpression in KO or KD cells (Related to Figure 3,4).

(A,B) Expression of pluripotency and glycolysis genes upon \textit{Lncenc1} overexpression in KO (A) and KD (B) cells. Data were normalized against \textit{Actb} and are shown as mean ± SD of three independent experiments. Statistical significance of T-Test: * \( p < 0.05 \), ** \( p < 0.01 \), *** \( p < 0.001 \).
Figure S5. The occupancy of RNA Polymerase II upon Ptbp1 depletion (Related to Figure 6).

(A) The efficiency of depletion of Hnmpk and Ptbp1 mRNA.

(B) Chromatin immunoprecipitation (ChIP) experiments with RNA Polymerase II antibody. Relative enrichments of ChIP and input DNA were determined by qPCR. Primers were designed at promoters of glycolysis genes and control genes (Actb and intergenic region) were used as negative controls. Data were shown as mean ± SD of three independent experiments. Statistical significance of T-Test: * p < 0.05, ** p < 0.01, *** p < 0.001.
Figure S6. The regulation between self-renewal and glycolysis (Related to Figure 7).

(A, B) The knockdown efficiency of pluripotency and glycolysis genes.

(C, D) The expression of pluripotency genes and unrepressed genes (Ptbp1, Hnmpk and Nono) upon depletion of five glycolysis genes.

(E) Bright-field micrographs of E14 nESCs treated or untreated with 2-DG (left). Scale bars, 200µm. The expression of pluripotency genes upon 2-DG treatment (right). Data were shown as mean ± SD of three independent experiments. Statistical significance of T-Test: * p < 0.05, ** p < 0.01, *** p < 0.001.
## Table S3. Target sequences of shRNAs in this article.

| Gene Name | shRNA | Hairpin sequence (5’ to 3’) |
|-----------|-------|-----------------------------|
| Control   | shRNA1 | ACTCGACACTATAGTATCTCA       |
|           | shRNA2 | CGCAACTAATACTGATATCA        |
| Lncenc1   | shRNA1 | GGTAAAGGGTGCTTTTAGATC       |
|           | shRNA2 | GCATCTATTGGAAGAATATAT       |
| Panct2    | shRNA1 | GCTTGAGAAGCGAAGAAACT        |
|           | shRNA2 | GTTCTGTAGTGATCAATATAT       |
| 2310043M15Rik | shRNA1 | GTCTTCATCGACATCTTCAGA     |
|           | shRNA2 | GTTCTCTACCTACAAATATTC       |
| Gm13110   | shRNA1 | GTATGCTATGAGCATATAAT        |
|           | shRNA2 | GCTCGAAACCTCTGTAACACT       |
| Halr1     | shRNA1 | GAAGAAAGCAAGAAATATGA        |
|           | shRNA2 | CACATCAAGCCTTTGGAIAAAG      |
| Gm805     | shRNA1 | GTATACCCACAGATGAATAT        |
|           | shRNA2 | GTTACTCAGGCCCATATATAT       |
| 4930461G14Rik | shRNA1 | GCATTGTGGAGATGAACACTGG      |
|           | shRNA2 | GAATTCTCTGGTCTCCTAAACC      |
| Ptbp1     | shRNA1 | AAGGGTGAAGAATCTTCAAT        |
|           | shRNA2 | CTCAATGTCAAGTACAAAT         |
| Hnrnpk    | shRNA1 | AAGGGGATCTAATGGCTTATT       |
|           | shRNA2 | CCAAGTTTGGCTGGAACTA         |
| Ldha      | shLdha | GTGAAACATCTTCAAGGGCTC       |
| Aldoc     | shAldoc | CAAAGGTTGCCAGGTATAT        |
| Tpi1      | shTpi1 | GCTGAAATCCAATGCAATG        |
| Pgk1      | shPgk1 | CAATAATATGCTAGACAAAGT       |
| Pfkp      | shPfkp | CCAAGTGATCTTATATATATG       |
| Oct4      | shOct4 | GCCGACAACATGACAACTTT       |
| Sox2      | shSox2 | GCCCACCTACAGCATGCTT         |
| Nanog     | shNanog | GCCAACCAGTACTATGTTTAA       |
| Klif4     | shKlif4 | ACCTATACCAAGGATTCCTAT       |
Table S4. Primers used for qRT-PCR in this study.

| Gene name | Primer name | Sequence (5’ to 3’) |
|-----------|-------------|---------------------|
| Lncenc1   | Lncenc1_f   | CCAGCGTCTGTTGGAAGAG |
|           | Lncenc1_r   | GATTCTCATCCCTGCTAT  |
| Panc2     | Panc2_f     | GACAAGGAGATGCGGCTTT |
|           | Panc2_r     | CCCAACCGCTTCTTCTCA  |
| 2310043M15Rik | 2310043M15Rik_f | AGGAACACAAGCACAATCAAG |
| Gm13110   | Gm13110_f   | ACCCTCGAATCATGAGCACA |
|           | Gm13110_r   | GTAGGTCAGGGGACAGCTG  |
| Hlal1     | Hlal1_f     | GCAGCCTAGTTCAACGACAC |
|           | Hlal1_r     | GGAACCTGTGAAATCGGCT |
| Gm805     | Gm805_f     | AGGAGAAGGAATGGCCCAAA |
|           | Gm805_r     | AGCTCTTGGTCTGGGATCC |
| Actb      | Actb_f      | GTGTCATCACTATGGGAACG |
|           | Actb_r      | AGGGATGTCACAGTCACACT |
| B2m       | B2m_f       | GACCGGCTGTATGATC    |
|           | B2m_r       | TGTTGAGTCTCCATTCTCC |
| Ptbp1     | Ptbp1_f     | CCACAAAGAGCTACGAGCC |
|           | Ptbp1_r     | CATGGCCATTTCTGCATCCA |
| Hnrnpk    | Hnrnpk_f    | CCCAGAGCGCTACTGAGAT |
|           | Hnrnpk_r    | CCACAGCCTGCATTGAGCC |
| Ldha      | Ldha_f      | TGGCTTGAAATACAGTGGC |
|           | Ldha_r      | AAGAGACTTCCAGGAGACG |
| Tpi1      | Tpi1_f      | AGAAGTGCCCTGGGAGACT |
|           | Tpi1_r      | TGGCAAAATGCTGTAAGCG |
| Aldoc     | Aldoc_f     | ACCATGACCTAAAGAGGTC |
|           | Aldoc_r     | TTAGCAGAGTGACCCTTGAG |
| Pfkp      | Pfkp_f      | ATCAAGGAGGATCAGTGGC |
|           | Pfkp_r      | TGCTCTGCGATCTCTTCCAA |
| Pgk1      | Pgk1_f      | AAGACTGCCAACAGCTACT |
|           | Pgk1_r      | AATCTGCTTAGCTCGACC |
| Oct4      | Oct4_f      | GTGGGAGAAAGGGTGAACAA |
|           | Oct4_r      | CTCCCTTGCAGGGGTCTT |
| Sox2      | Sox2_f      | GTATCAGGAGTTGTCAGGCA |
|           | Sox2_r      | CTCTTCTTCTCCAGCCCT |
| Nanog     | Nanog_f     | TTCTTGCTTACAGGGGCTTCG |
|           | Nanog_r     | AGAGGAAGGGCAGGAGA |
| Klf4      | Klf4_f      | CCAACTCCCTCTTCTTG   |
|           | Klf4_r      | TGGGATGACCTTGATGATA |
| Esrrb     | Esrrb_f     | CAGGCAAGGATGACAGCC |
|           | Esrrb_r     | GAGACAGCAGAAGGGACTG |
| Tbx3      | Tbx3_f      | TTATTTCCAGGTCAGGAGATG |

Note: The table lists primers used for qRT-PCR with their specific sequences.
|      | Forward (Stella) | Reverse (Rex1) |
|------|-----------------|----------------|
| Tbx3 | GGTCGTTTGAAACCAAGTCCCTC | TAACGTATATTTCTGCCGTATGC |
| Stella | AGGCTCGAAGGAAATGAGTTTGG | TCCATGGCATAGTTCCAACAG |
| Stella_r | TCCTAATTCTTCCGCATTTCG | TAACGTATATTTCTGCCGTATGC |
| Gene name     | Primer name | Sequence (5’ to 3’) |
|--------------|-------------|---------------------|
| Intergenic_1 | Intergenic-ChIP-F1 | AGGAATGGCAGAGTGGAGAC |
|              | Intergenic-ChIP-R1 | AACCTGACGTGAATTGCGCC |
| Intergenic_2 | Intergenic-ChIP-F2 | GCATTGTAGGTGGCAGTGA |
|              | Intergenic-ChIP-R2 | CCTAGTAGCCACGCTCTCTC |
| B2m         | B2M-ChIP-F    | CCAAACTCAAGAGCACACC |
|             | B2M-ChIP-R    | CTGAACCTCACAGCAATCCG |
| Actb        | Actb-ChIP-F   | CATGGTGCTCCCTAGTGTG |
|             | Actb-ChIP-R   | ACAGCTTTCTTTCAGCTCT |
| Pfkp        | Pfkp-ChIP-F   | ATAGGGTAATGGCAGCGCAG |
|             | Pfkp-ChIP-R   | GCTCTACGTGACAGCTCTC |
| Tpi1        | Tpi1-ChIP-F   | CTGGTGATACGGGGTCTTT |
|             | Tpi1-ChIP-R   | GCAAATGAAGGACCTAGGGC |
| Aldoc       | Aldoc-ChIP-F  | ACCAGGCTTAATCCCAACA |
|             | Aldoc-ChIP-R  | TTAGAGACGATGCCCTCCC |
| Pgk1        | Pgk1-ChIP-F   | CGGCACCTTACTGCTCC  |
|             | Pgk1-ChIP-R   | TACCAGCTTCCATTGCTCA |
| Ldha        | Ldha-ChIP-F   | GAGCTTACCTTATGGCCCT |
|             | Ldha-ChIP-R   | CCCAAATCTGAACACCCTTC |
| Oct4        | Oct4-ChIP-F   | TTAGAAATGAGGCCCTCTT |
|             | Oct4-ChIP-R   | AGCGCTATCTGCTGCTTTC |
| Sox2        | Sox2-ChIP-F   | AACTCGGAGATCAGCAAG  |
|             | Sox2-ChIP-R   | AGCGTGTACCTTCCCTTTT |
| Nanog       | Nanog-ChIP-F  | TAGGGTAGGAGGCTTGGAG |
|             | Nanog-ChIP-R  | GACACCAACCAAACAGC  |
| Klf4        | Klf4-ChIP-F   | TATACCGGCTGACATCCA  |
|             | Klf4-ChIP-R   | CAATCACCCGGCCTCAC  |
| Rex1        | Rex1-ChIP-F   | AGAAACCTGACCTGTACC |
|             | Rex1-ChIP-R   | TAAGTGGCAGGACAGAG  |
| Tbx3        | Tbx3-ChIP-F   | AGAGCCATCAACAAGCTC |
|             | Tbx3-ChIP-R   | CCTCCCTCGCTCTAGAATT |
| Primer name                  | Sequence (5’ to 3’)          |
|-----------------------------|-----------------------------|
| *Lncenc1* ChIRP probe-1     | aaggtgcttcagttcaagt         |
| *Lncenc1* ChIRP probe-2     | tcattacctataccgacga         |
| *Lncenc1* ChIRP probe-3     | gatcaccatgacctaaaggg        |
| *Lncenc1* ChIRP probe-4     | actggtacagcagaaaccac        |
| *Lncenc1* ChIRP probe-5     | aaggtggttcagcatgga          |
| *Lncenc1* ChIRP probe-6     | caccacacagatttaggact        |
| *Lncenc1* ChIRP probe-7     | caggcaaggttcagttgca         |
| *Lncenc1* ChIRP probe-8     | cagtgatagtgtgtggtg          |
| *Lncenc1* ChIRP probe-9     | caggccaacagaaacatgaa        |
| *Lncenc1* ChIRP probe-10    | aacccacatgaaaggctaca        |
| *Lncenc1* ChIRP probe-11    | aatttaggggtctaggacc         |
| *Lncenc1* ChIRP probe-12    | agtggagaatcatgcatgc         |
| *Lncenc1* ChIRP probe-13    | acattgcacccaagactctt        |
| *Lncenc1* ChIRP probe-14    | tccaggacagtggaatg          |
| *Lncenc1* ChIRP probe-15    | tctagtgtgcgtgctactg         |
| *Lncenc1* ChIRP probe-16    | ttcggcacacacactcagc         |
| *Lncenc1* ChIRP probe-17    | atgcctttcgaagagctca         |
| *Lncenc1* ChIRP probe-18    | cttatatccacccacctgc         |
| *Lncenc1* ChIRP probe-19    | cataacacccagagggagaa        |
| *Lncenc1* ChIRP probe-20    | cagcagtgacccacattacact      |