**Rlim-Dependent and -Independent Pathways for X Chromosome Inactivation in Female ESCs**

**Highlights**
- rXCI in epiblast cells does not require *Rlim*
- XCI in RA-differentiated female ESCs depends on *Rlim*
- *Rlim*-independent XCI occurs in EB-differentiated female ESCs
- Culturing ESCs under physiological O₂ levels promotes *Rlim*-independent XCI

**Authors**
Feng Wang, Kurtis N. McCannell, Ana Bošković, ..., Oliver J. Rando, Thomas G. Fazzio, Ingolf Bach

**Correspondence**
ingolf.bach@umassmed.edu

**In Brief**
Embryonic stem cells are widely used for investigating X chromosome inactivation, the silencing of one X chromosome in females. Wang et al. show that this process is driven by two independent pathways, one that requires the ubiquitin ligase *Rlim* and another that functions independently of *Rlim* and is sensitive to environmental conditions.

**Data and Software Availability**
GSE101838
**Rlim-Dependent and -Independent Pathways for X Chromosome Inactivation in Female ESCs**

Feng Wang,1 Kurtis N. McCannel,1 Ana Bošković,2 Xiaochun Zhu,1 JongDae Shin,1,6 Jun Yu,1 Judith Gallant,3 Meg Byron,3 Jeannie B. Lawrence,3 Lihua J. Zhu,1,4,6 Stephen N. Jones,3,7 Oliver J. Rando,2 Thomas G. Fazzio,1,4 and Ingolf Bach1,4,6,*

1Department of Molecular, Cell and Cancer Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
2Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA 01605, USA
3Department of Cell and Developmental Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
4Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA
5Program in Bioinformatics and Integrative Biology, University of Massachusetts Medical School, Worcester, MA 01605, USA
6Present address: Department of Cell Biology, College of Medicine, Konyang University, Daejeon, Korea
7Present address: Frederick National Laboratory for Cancer Research, Frederick, MD 21702, USA
8Lead Contact
*Correspondence: ingolf.bach@umassmed.edu

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**SUMMARY**

During female mouse embryogenesis, two forms of X chromosome inactivation (XCI) ensure dosage compensation from sex chromosomes. Beginning at the four-cell stage, imprinted XCI (iXCI) exclusively silences the paternal X (Xp), and this pattern is maintained in extraembryonic cell types. Epiblast cells, which give rise to the embryo proper, reactivate the Xp (XCR) and undergo a random form of XCI (rXCI) around implantation. Both iXCI and rXCI depend on the long non-coding RNA Xist. The ubiquitin ligase Rlim is required for iXCI in vivo and occupies a central role in current models of rXCI. Here, we demonstrate the existence of Rlim-dependent and Rlim-independent pathways for rXCI in differentiating female ESCs. Upon uncoupling these pathways, we find more efficient Rlim-independent XCI in ESCs cultured under physiological oxygen conditions. Our results revise current models of rXCI and suggest that caution must be taken when comparing XCI studies in ESCs and mice.

**INTRODUCTION**

Female mammalian embryogenesis and reproduction critically depend on a process called X chromosome inactivation (XCI), which silences one of the two sex chromosomes to achieve dosage compensation. XCI serves as a paradigm to study the epigenetic regulation, whereby gene expression states are maintained independent of DNA sequence. In mice, an imprinted form of XCI (iXCI) is initiated in embryos at the 4-cell stage, silencing exclusively the paternal X (Xp), and this XCI pattern is maintained in extraembryonic tissues. However, epiblast cells, which give rise to the embryo proper, experience a major epigenetic switch around implantation: these cells reactivate the Xp and undergo a random form of XCI (rXCI), in which the Xp or the maternal X (Xm) is inactivated in each cell with equal probability (Payer, 2016). Both forms of XCI require the long non-coding Xist RNA, which forms clouds on the inactive X chromosome (Xi) from which it is transcribed, leading to X silencing. The X-linked gene Rlim (also known as Rnf12) has emerged as a critical mediator of Xist activity. Rlim encodes a ubiquitin ligase (E3) (Ostendorf et al., 2002) that is involved in transcriptional regulation (Bach et al., 1999; González et al., 2012; Güngör et al., 2007) and shuttles between the nucleus and the cytoplasm (Jiao et al., 2013). In mice, a maternally transmitted Rlim knockout (KO) allele (Δm) results in early lethality of female embryos in a sex-specific parent-of-origin effect due to a failure to maintain iXCI and Xist clouds (Shin et al., 2010, 2014). In contrast, loss of Rlim in female epiblast cells has minimal effect on the rXCI process. Rlim protein levels are downregulated specifically in epiblast cells of implanting embryos, consistent with the lack of rXCI phenotype in Rlim mutant females (Shin et al., 2014). These data identify Rlim-dependent and Rlim-independent mechanisms of XCI in vivo that separately act in pre-implantation embryos and epiblasts, respectively. However, Rlim is crucial for XCI in female embryonic stem cells (ESCs) differentiated in culture (Barakat et al., 2011, 2014).

To further investigate mechanisms of rXCI, we generated female ESCs with a homozygous RlimKO. We found that these cells undergo XCI in vivo but that XCI in vitro is strongly influenced by culture conditions, including both method of differentiation and O2 levels. Our results demonstrate Rlim-dependent and Rlim-independent pathways for XCI exist in ESCs and, together with published data, profoundly change current models of X dosage compensation.

**RESULTS**

**Female ESCs Lacking Rlim Undergo XCI In Vivo**

Genetic evidence indicates that iXCI in female pre-implantation embryos requires Rlim but that activation of Xist during rXCI in the female epiblast is Rlim-independent (Shin et al., 2010, 2014). Xist clouds form specifically in the inner cell mass (ICM) of female blastocyst outgrowths with a maternal Rlim deletion (Δm) (Shin et al., 2010), consistent with a critical role for Rlim
in iXCI but not rXCI. To exclude any influence of RLIM on rXCI, we examined Dm/Dp (paternally transmitted Rlim KO allele) female blastocysts generated by crossing ∆/Y males with either SC-cKO/m or fl/fl females. The boxed area (∆/∆ embryo) is shown in higher magnification in the right panel. Focus in images is on cells in the ICM. Embryos were genotyped after image recording.

E4 blastocysts generated by this cross were cultured for 3 days and analyzed by RNA fluorescence in situ hybridization (FISH). Xist clouds were readily detectable specifically in cells of the ICM in female blastocyst outgrowths (Figure 1A), consistent with Rlim-independent induction of rXCI in vivo.

Newly isolated primary female ESCs that lack RLIM activate Xist and form Xist clouds upon differentiation in vitro (Shin et al., 2010, 2014). However, in a female ESC line with a
homzygous \textit{Rlim} KO (\textit{Rnf12KO}), XCI was blocked upon ESC differentiation (Barakat et al., 2011), suggesting that XCI is induced in a context-dependent manner. Because primary female \textit{Rlim}KO ESCs proved unstable upon prolonged culture, to investigate mechanisms of XCI, we generated three independent ESC lines lacking RLIM via CRISPR/Cas9 technology (Figure 1B) using the established mouse PGK12.1 female ESC model (Norris et al., 1994). Sequencing confirmed homozygous frameshift mutations in exon 3, the first coding exon in model (Norris et al., 1994). Sequencing confirmed homozygous

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formation, whereas levels of RLIM by day 6 were similar to those

in undifferentiated ESCs (Figure 2A). ssRT-qPCR analyses

revealed that day 6 EB-differentiated Wt ESCs displayed

less than 3-fold increased \textit{Xist} levels when compared to

RA-differentiated WT cells (Figure 2B). However, \textit{Xist} levels in

EB-differentiated \textit{Rlim}KO ESCs were >50-fold higher under EB
differentation relative to RA, with \textit{Xist} clouds developing in

a significant number of cells (Figures 2C and 2D). Combined,

these results provide the first evidence of \textit{Rlim}-independent XCI in an established female ESC line and show that differentiation 

conditions play a major role in \textit{Rlim}-independent induction of \textit{Xist}. Moreover, unlike in differentiating epiblast cells in vivo (Shin et al., 2014), differentiation of ESCs in vitro does not induce significant RLIM downregulation.

In another female ESC model, the \textit{Rlim}KO (\textit{Rnf12KO}) resulted in 

complete inhibition of XCI, leading to far-reaching conclusions 

regarding \textit{Rlim} function in rXCI (Barakat et al., 2011, 2014). Because 

these results did not match our results (Figure 2), we directly compared our \textit{Rlim}KO ESC lines with the \textit{Rnf12KO} cell line (Barakat et al., 2011). The \textit{Rnf12KO} cell line was achieved via insertion of foreign DNA into the \textit{Rlim} gene at a position that might allow expression of a truncated RLIM protein consisting of the N-terminal 340 amino acids (Figure S2A) (Jonkers et al., 2009). Western blots using two independent RLIM antibodies detected a prominent band migrating around 45 kDa (RLIM340) in these ESCs (Figures 3A and S2B). Because of their F121 

ESC background, we refer to this ESC line as \textit{Rlim}340f. In agreement 

with published results (Gontan et al., 2012), REX1 levels in 

\textit{Rlim}340f ESCs were increased (Figure 3A). \textit{Rlim}Kop ESCs 

displayed similar REX1 levels to those of Wt ESCs (Figure 3A), indicating that in PGK12.1 ESCs, RLIM is not solely responsible 

for regulating cellular REX1 levels. The predicted RLIM340 protein lacks the RING finger and nuclear export signal (NES) but retains part of the basic domain that mediates interactions with many substrate proteins, including REX1 (Figure S2A) (Gontan et al., 2012; Ostendorff et al., 2002). ICC on undifferentiated 

\textit{Rlim}340f ESCs revealed predominantly nuclear localization of the truncated RLIM protein (Figure S2C), consistent with find-

ings that RLIM lacking the NES is trapped in the nucleus (Jiao et al., 2013). Moreover, forced expression of Myc-tagged 

RLIM340 in \textit{Rlim}Kop ESCs via transient transfections revealed 

accumulation of endogenous REX1 protein in nuclei of transfected cells (Figure 3B). However, in WT PGK12.1 cells, expression of Myc-RLIM340 did not lead to REX1 accumulation, suggesting that the presence of the partial basic domain is not able to block interactions between full-length RLIM and REX1.

To investigate the effects of RLIM340 expression on XCI, we 

induced frameshift mutations in \textit{Rlim}340f ESCs via CRISPR/ 

Cas9, using the same guide RNAs as for the \textit{Rlim}Kop ESCs and 

yielding, in independent ESCs carrying a homozygous KO of 

\textit{Rlim}340, lines \textit{Rlim}53f and \textit{Rlim}10f (Figures S2A) (data not shown). Analysis of these lines via western blot corroborated the KO and showed that the presence of \textit{Rlim}340 did not significantly affect overall REX1 levels in undifferentiated ESCs (Figure S2D). However, ICC staining confirmed that \textit{Rlim}340 was critical to trap REX1 in the nucleus, because nuclear REX1 was significantly reduced in both KO lines relative to the \textit{Rlim}340f line (Figure 3C). Moreover, these KO ESCs performed XCI upon EB differentiation at a higher rate than RLIM340f cells, as measured by formation of \textit{Xist} clouds (Figure 3D). Combined, these results indicate gain-of-function activity of the truncated
RLIM340, as opposed to dominant-negative functions. Consistent with findings that substrate proteins are often targeted by multiple E3 ligases mediated by interactions via the same or a similar binding site (Bach and Ostendorff, 2003; Gungor et al., 2007), these results provide additional evidence that varying levels or the repertoire of cellular competence factors in different ESC systems influences cellular REX1 levels and XCI.

**Efficiency of Rlim-Independent XCI In Vitro Is Influenced by Culture Conditions**

Although the lack of RLIM does not affect overall rXCI efficiency in vivo (Figures 1 and S1), when compared to WT, RlimKO ESCs undergo XCI with reduced efficiency upon EB differentiation in vitro (Figure 2), suggesting suboptimal culture conditions. In utero, ESCs differentiate in the context of extraembryonic cells and mammalian embryos are exposed to low O₂ levels (2%–8%) (Fischer and Bavister, 1993). We therefore tested whether XCI efficiency of RlimKO ESCs could be improved by mimicking these natural conditions. To test possible influences of extraembryonic cell types on XCI, we co-cultured EB-differentiating female GFP-RlimKOp ESCs in the presence of a trophoblast stem cell (TSC) line and/or a primitive endoderm (XEN) cell line (Niakan et al., 2013). However, this approach yielded no signs of improved XCI (data not shown). To examine influences of O₂ levels on XCI, we compared Xist RNA levels and formation of clouds in RlimKO ESCs with WTp ESCs, differentiated by EB and cultured in 7.5% O₂. EB differentiation in 7.5% O₂ resulted in a general increase of around 2-fold in Xist levels and Xist cloud formation.

**Figure 2. Rlim-Dependent and Rlim-Independent XCI Pathways Exist in ESCs**

(A) WTp ESCs were split and in vitro differentiated in parallel by either EB or RA. Protein extracts were prepared at various time points during differentiation. The same western blot was hybridized with antibodies against RLIM, OCT4, and β-actin. OCT4 levels drop dramatically by day 3, whereas RLIM levels are not significantly downregulated by day 6.

(B) Comparison of Xist RNA levels in undifferentiated ESCs and after day 6 RA or EB differentiation via qRT-PCR (control, E14 male ESCs; Xist levels of undifferentiated WTp cells are set to 1). Data represent three independent experiments. Xist levels are <3x higher in EB-differentiated WTp ESCs when compared to RA differentiation but >50x for all RlimKOp ESCs.

(C) Formation of Xist clouds in (day 6) EB-differentiated RlimKOp ESCs in RNA FISH using Xist as the probe. Representative images are shown.

(D) Summary of three independent experiments as shown in (C). Xist clouds in ESCs of 10 EBs were evaluated, with >100 cells counted per EB. Error bars indicate SEM.
development in all \textit{Rlim}KO ESC lines (Figures 4A–4D) (data not shown). Although at day 6 of differentiation \textit{Rlim}16p ESCs appeared to develop similar XCI efficiencies when compared to WT, efficiency was lower at day 3 of EB differentiation (Figure S3A). No effects of 7.5% O$_2$ on XCI efficiency were observed on day 6 RA-differentiated \textit{Rlim}KO ESCs (data not shown).

Transcriptome analyses of undifferentiated and day 6 EB-differentiated WTp, \textit{Rlim}16p, and E14 male ESCs via RNA sequencing (RNA-seq) confirmed similar \textit{Xist} levels in female ESCs (Figure S3 B), and comparisons of total X-linked transcripts versus total autosomal transcripts in differentiating WTp and mutant female ESC lines (relative to male E14) revealed similar global X silencing in \textit{Rlim}KO and WTp ESCs (Figures 4E and S3C). Because iXCI in female mice is \textit{Rlim}-dependent, whereas rXCI occurs in an \textit{Rlim}-independent fashion, the identification of \textit{Rlim}-dependent and \textit{Rlim}-independent pathways for XCI in female ESCs illuminates mechanisms underlying X dosage compensation in female mice, including the epigenetic switch from iXCI to rXCI in epiblast cells (Figure 4F).

\textbf{DISCUSSION}

Investigating XCI in female ESCs, we found that in an \textit{in vivo} context, XCI in ESCs lacking \textit{Rlim} occurs with efficiencies similar to those in WT ESCs (Figure 1), but \textit{in vitro} \textit{Rlim}-independent XCI is highly sensitive to the differentiation protocol, as well as cell culture conditions (Figures 2 and 4). In particular, XCI in RA-differentiated ESCs is strictly \textit{Rlim}-dependent (Figure 2), indicating that this type of differentiation is not compatible with \textit{Rlim}-independent XCI. The formation of EBs more closely mimics the situation in blastocysts, and the finding that \textit{Rlim} levels slightly increase during EB differentiation (Figure 2A) (Marks et al., 2015) is reminiscent of the increase in \textit{Rlim} mRNA levels observed in early blastocysts, when the ICM forms (Wang et al., 2016). Moreover, we found that culturing differentiating ESCs in 7.5% O$_2$ levels had a general positive effect on \textit{Rlim}-independent XCI efficiency (Figure 4). In utero, mammalian embryos are naturally exposed to low 2%–8% O$_2$ levels (Fischer and Bavister, 1993), and atmospheric O$_2$ levels negatively influence development, global gene expression, and XCI in cultured embryos or ESCs (Harvey et al., 2004; Lengner et al., 2010; Orsi and Leese, 2001). However, even in 7.5% O$_2$, the kinetics of XCI in \textit{Rlim}KO cells is still slower than in WT ESCs (Figure S3A), suggesting that these remain suboptimal XCI conditions. An alternative possibility is that the \textit{Rlim}-independent XCI occurs more slowly upon induction of ESC differentiation. In this scenario, the presence of RLIM facilitates the more rapid XCI kinetics in WT ESCs.
Combined, our results indicate elevated XCI efficiencies by the Rlim-independent pathway under conditions that more closely parallel conditions found in vivo. Thus, it will be interesting to identify the factor or factors and conditions that orchestrate rXCI in epiblasts.

In contrast to epiblast cells of embryos undergoing rXCI, Rlim expression is maintained in differentiating ESCs (Figure 2A). Thus, the Rlim-dependent pathway likely contributes to XCI to varying degrees in vitro, depending on differentiation, culture conditions, and likely the specific ESC model used. The findings that (1) REX1 levels are not significantly affected by the Rlim deletion in PGK12.1 ESCs (Figure 3A), (2) REX1 levels rapidly drop to undetectable levels within 24 hr of RA differentiation in ESCs (Gontan et al., 2012) (data not shown), and (3) the development of Xist clouds and H3K27me3 foci upon RA differentiation is strictly Rlim-dependent (Figures 1B and 1C) indicate that at least some functions of Rlim for XCI occur independent of REX1. However, the findings that REX1 levels in F121 ESCs are affected by the presence or absence of Rlim (Gontan et al., 2012) and that Rlim-independent XCI in these ESCs is generally less efficient when compared to PGK12.1 ESCs (Figures 4A–4D) suggests that the cellular repertoire of expressed competency factors (e.g., E3 ligases) in different female ESC models has an important impact on XCI in vitro.

Rlim340f ESCs exhibit very low XCI activity, and our results suggest that expression of the truncated Rlim340 might be a contributing factor, because it traps REX1 in the nucleus (Figure 4A–4D). Therefore, Rlim regulates a variety of factors by both RING-finger-dependent and RING-finger-independent mechanisms (Her and Chung, 2009; Kraemer et al., 2003; Ostendorff et al., 2002), it is likely that the activities of other nuclear proteins are altered in Rlim340f ESCs, with a potential effect on XCI. Moreover, the specific epigenetic background in individual ESC lines may contribute to XCI activity, because RlimKO ESC lines undergo XCI with variable efficiencies. Although it is clear that Rlim promotes XCI, our results indicate that in vivo, additional factors must be involved in proper counting of X chromosomes, as previously proposed (Barakat et al., 2011; Jonkers et al., 2009). However, secondary roles for Rlim in rXCI in counting X’s in mice with X chromosome abnormalities...
cannot be ruled out. In this context, it is important to point out the possibility that the gain-of-function activity of Rlim340 might contribute to the skewed inactivation of the X harboring the mutated Rlim allele in Rlim340f heterozygous ESCs (Jonkers et al., 2009).

During mouse embryogenesis, Rlim protein is detectable throughout preimplantation development, consistent with its functions in iXCI maintenance. However, in contrast to differentiating ESCs in culture (Figure 2A), Rlim protein levels are downregulated in nuclei of epiblast cells of implanting embryos to levels that are undetectable by immunofluorescence (Shin et al., 2014). Rlim levels continue to remain low at early post-implantation stages through E7.5. At E8.5, Rlim protein levels are slowly upregulated in specific embryonic cell types, and by E11.5, Rlim protein is widely detectable in many tissues (Ostendorff et al., 2006) (data not shown). Thus, functions of Rlim in Xci maintenance at later embryonic stages and/or in mature tissue types are likely. The developmental expression pattern, combined with the finding of Rlim-dependent and Rlim-independent XCI pathways in ESCs in vitro, suggests a model for X dosage compensation in which Rlim occupies a major role to maintain Xist clouds and iXCI in cells of female embryos before X chromosome reactivation (XCR) (Figure 4F).

Although this role continues in extraembryonic tissues, Rlim is specifically downregulated in the epiblast lineage shortly before implantation, thereby likely contributing to XCR, followed by induction of rXCI by an Rlim-independent pathway (Figure 4F). This scenario is consistent with findings that Rlim is essential for the maintenance of iXCI but dispensable for rXCI in epiblast cells. Moreover, it explains the precocious rXCI in epiblast cells of ΔΔ blastocyst outgrowths (Figure 1A), because with lack of iXCI, XCR is not required before induction of rXCI. Thus, iXCI in early female embryos and rXCI in epiblast cells are regulated by distinct pathways, and the existence of Rlim-dependent and Rlim-independent pathways for XCI in female ESCs is likely the consequence of persistent Rlim expression upon differentiation in vitro.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Generation of PGK12.1 Cell Lines Lacking Rlim**

Female PGK12.1 and male E14 ESCs were cultured as described (Hooper et al., 1987; Norris et al., 1994). For RA differentiation, cells were plated at 2.5 × 10^5/cm^2^ and cultured minus Leukemia inhibitory factor (LIF) with 100 nM RA (Sigma R2625) for 6 days. EBs were formed in suspension by incubating cells in medium lacking LIF for 3 days on bacteriological Petri dishes, 100 nM RA (Sigma R2625) for 6 days. EBs were formed in suspension by incubating cells in medium lacking LIF for 3 days on bacteriological Petri dishes.

**Generation of Chimeric Mouse Embryos and Preparation of MEFs**

E3.5 blastocysts (C57BL/6J-Tyrc-22J/J strain) were microinjected with 12–15 individual ESCs. Injected blastocysts were surgically transferred into the uteri of pseudo-pregnant recipient SWR/J mouse strain (SW) female mice. After recovery, the females were housed under standard vivarium conditions. Pregnant dams were sacrificed 10 days post-surgery, and chimeric E12.5 embryos were recovered for preparation of MEFs (Shin et al., 2010). All mice were housed in the animal facility of the University of Massachusetts Medical School (UMMS) and used according to NIH guidelines and those established by the UMMS Institute of Animal Care and Usage Committee.

**Blastocyst Outgrowths and RNA FISH**

Mice were maintained on a C57BL/6J background, and parental genotypes to generate embryos for blastocyst outgrowths were based on described mouse models (Shin et al., 2010, 2014). E4 blastocysts were generated by natural mating, cultured for 48–96 hr before RNA FISH, and genotyped after image recording. All mice were housed in the animal facility of UMMS and used according to NIH guidelines and those established by the UMMS Institute of Animal Care and Usage Committee. RNA FISH experiments including probes have been described (Shin et al., 2010, 2014). The Rlim probe detects mRNAs transcribed from both WT and KO alleles (Shin et al., 2010).

**Antibodies, Western Blots, Immunostaining, and Transient Transfections**

Primary antibodies were rabbit and guinea pig Rlim (Ostendorff et al., 2002, 2006), H3K27me3 (Abcam ab6002 and Millipore 07-447), GFP (Rockland 800-101-215), REX1 (Abcam ab28141), Myc (Sigma 9E10), OCT4 (Santa Cruz sc-8628), and β-actin (Sigma A1978 and A5316). Secondary antibodies were Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) (Invitrogen, A21206), Alexa Fluor 488 goat anti-mouse IgG (Invitrogen, A11029), Alexa Fluor 546 goat anti-guinea pig IgG (Invitrogen, A11074), Alexa Fluor 568 goat anti-rabbit IgG (Invitrogen, A11011), goat anti-rabbit IgG-horseradish peroxidase (HRP) (Bio-Rad 170-6515), goat anti-mouse IgG-HRP (Bio-Rad 170-6516), and donkey anti-goat IgG-HRP (Santa Cruz sc-2020). Whole-cell lysates for western blots were prepared by lysis cells in WE16th lysis buffer (25 mM Tris [pH 7.5], 125 mM NaCl, 2.5 mM EDTA, 0.05% SDS, 0.5% NP-40, 10% glycerol). Transient transfections of RLIM340 (in pCS2MT) were carried out using FuGENE HD Transfection Reagent (Promega).

**RNA-Seq and Data Analyses**

RNA-seq on ESC lines was essentially performed as described (Vallaster et al., 2017), and libraries were sequenced on a NextSeq 500 platform from illumina. Quality-controlled reads were aligned to the mouse genome (Mus musculus/mm10) using TopHat (v.2.0.12) (Trapnell et al., 2009), with default settings, except that parameter read mismatches were set to 2, followed by running HTSeq (v.0.6.1p1) (Anders et al., 2015) and Bioconductor packages edgeR (v.3.10.0) (Robinson et al., 2010; Robinson and Smyth, 2007), for differential gene expression analysis, and ChiPpeakAnno (v.3.2.0) (Zhu, 2013), for annotation. edgeR and trimmed mean of M value (TMM) were used as described (Wang et al., 2016).

**Statistical Analyses**

Student's t tests were used to calculate statistical differences between individual groups via Microsoft Excel. p values < 0.05 were considered statistically significant.
DATA AND SOFTWARE AVAILABILITY

The accession number for the RNA-seq data reported in this paper is GEO: GSE101838.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2017.12.004.

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AUTHOR CONTRIBUTIONS


DECLARATION OF INTERESTS


REFERENCES


