RLIM is dispensable for X-chromosome inactivation in the mouse embryonic epiblast

JongDae Shin1, Mary C. Wallingford2, Judith Gallant3, Chelsea Marcho2, Baowei Jiao†, Meg Byron1, Michael Bossenz4, Jeanne B. Lawrence3, Stephen N. Jones3, Jesse Mager2 & Ingolf Bach1,5

In female mice, two forms of X-chromosome inactivation (XCI) ensure the selective silencing of female sex chromosomes during mouse embryogenesis. Beginning at the four-cell stage, imprinted XCI (iXCI) exclusively silences the paternal X chromosome. Later, around embryogenesis, epiblast cells of the inner cell mass that give rise to the embryo reactivate the paternal X chromosome and undergo a random form of XCI (rXCI)1-3. Xist, a long non-coding RNA crucial for both forms of XCI, is activated by the ubiquitin ligase RLIM (also known as Rnf112)-4. Although RLIM is required for triggering iXCI in mice, its importance for rXCI has been controversial. Here we show that RLIM levels are downregulated in embryonic cells undergoing rXCI. Using mouse genetics we demonstrate that female cells lacking RLIM from pre-implantation stages onwards show hallmarks of XCI, including Xist clouds and H3K27me3 foci, and have full embryogenic potential. These results provide evidence that RLIM is dispensable for rXCI, indicating that in mice an RLIM-independent mechanism activates Xist in the embryo proper.

The X-linked gene Rlim encodes the RING-finger ubiquitin ligase RLIM5, which functions as a sex-specific epigenetic regulator of nurturing tissues in female mice. In the mammary glands of pregnant and lactating females, RLIM expressed from the paternal X chromosome (Xp) serves as a survival factor for milk-producing alveolar cells6-8. In contrast, female embryos with a maternal deletion of Rlim in the germ line (Am) show defective Xist cloud formation and subsequent Xp silencing during iXCI, and die between embryonic day (E)5.5 and E10.5 due to a lack of extraembryonic trophoblast tissues in the placenta. Using an embryonic stem (ES)-cell model, evidence has been provided that RLIM can serve as a dose-dependent activator of XCI: the overexpression of RLIM induces ectopic Xist clouds in male and female ES cells9, and, in this system, homozygous disruption of Rlim results in a failure to initiate Xist transcription during rXCI10. Thus, current models of rXCI present RLIM as a crucial activator of Xist11,12, although some evidence suggests that it has a less important role in mice13.

During mouse development, RLIM-encoding messenger RNA is ubiquitously expressed, whereas its protein expression profile seems more restricted13-15. Thus, to investigate in vivo functions of RLIM for rXCI, we first analysed its protein expression and co-stained early embryos with antibodies against RLIM and the pluripotency factor Oct4, which identifies undifferentiated cells in the inner cell mass (ICM) undergoing rXCI. Whereas RLIM seemed to be uniformly expressed in the cells of E3.5 embryos (Fig. 1a), at E4.5 and E5.5 only low levels were detected in Oct4-positive cells, in contrast to extraembryonic cell types (Fig. 1b, c and Extended Data Fig. 1a; data not shown). Likewise, at E6.5 and E7–7.5 we detected low levels of RLIM in embryonic epiblast tissues and amnion tissues, whereas levels were high in many extraembryonic tissues, including cells of the ectoplacental cone, extraembryonic ectoderm and visceral endoderm (Fig. 1d and Extended Data Fig. 1b; data not shown).

Indeed, owing to low levels of RLIM, it was difficult to distinguish wild-type embryos from those lacking RLIM in embryonic tissues by immunostaining (Extended Data Fig. 1b). Although we did not genotype E5.5 embryos for technical reasons, in all ten wild-type embryos analysed we detected no/very low levels of RLIM in Oct4-positive nuclei. Moreover, we observed similarly low RLIM levels in genotyped males and female embryos at E4.5 and E7–7.5 of various genetic backgrounds (Fig. 1 and Extended Data Fig. 1; data not shown). As rXCI occurs around E5–E5.5, our data indicate that levels of RLIM are downregulated in mouse epiblast cells before rXCI.

To induce the conditional knockout of Rlim (cKO) in female embryos after the occurrence of iXCI but before induction of rXCI, we used Sox2-Cre (SC) transgenic mice14,15, as Sox2 is specifically expressed in embryonic epiblast cells of E3.5 blastocysts16, and a paternally transmitted SC transgene is robustly transcribed in ICM cells of pre-implantation blastocysts17. Indeed, from a fl/fl × WT/Y-SC (where fl indicates floxed, WT indicates wild type, and Y-SC indicates a male that carries both a Y chromosome and the SC transgene) cross, female and male pups with a paternal cKO (cKOm) are born in Mendelian sex ratios, and the percentages of male and female pups carrying cKOm were similar (Fig. 2a, confirmed normal iXCI. Importantly, female pups carrying a cKOm and a paternal germline deletion of Rlim (cKOm/Ap) generated via a fl/fl × cKO/Y-SC cross are born at Mendelian ratios (Fig. 2b), and, except for a mammary phenotype, adult cKOm/Ap females appear normal and are fertile (data not shown). In genotyping these animals using polymerase chain reaction (PCR) we detected only the knockout allele but no longer the floxed allele (Fig. 2c), and RLIM protein was undetectable in somatic tissues (Fig. 2d). Moreover, in matings using cKO/Y-SC males we never observed the transmission of an unrecombined floxed allele (data not shown). Consistent with published data14,15, these results indicate complete penetrance of the SC-mediated cKO. Analysing embryos from fl × WT/Y-SC crosses, the recombination of the floxed allele was robustly detectable using PCR in blastocysts as early as E3.75 (Fig. 2e), well before initiation of rXCI.

To test whether an essential function of RLIM in rXCI might be masked by the C57BL/6 mouse background, we generated first filial generation (F1) parents in mixed C57BL/6–SV129 and C57BL/6–FVB hybrid backgrounds suitable for generating SC-mediated cKOm offspring. Although the numbers of cKOm F2 pups were slightly underrepresented compared with maternal wild type (WTm) pups, there was no difference between the numbers of male and female cKOm pups (Extended Data Fig. 2a, b), indicating that this effect is XCI independent. Sequencing analyses of cKOm/Ap pups in mixed C57BL/6–SV129 backgrounds using 156 strain-specific single nucleotide polymorphisms (SNPs) distributed among all chromosomes revealed no general bias towards the C57BL/6 background (Extended Data Fig. 2c). Together with strain-independent low RLIM protein levels in the embryonic epiblast (Fig. 1 and Extended Data Fig. 1), these data indicate that the genetic background has little or no influence on pup numbers.

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We next examined XCI using RNA fluorescence in situ hybridization (RNA-FISH) in mouse embryonic fibroblasts (MEFs) isolated from E12.5 embryos, co-hybridizing with probes specific for Xist\(^{18}\) and Rlim\(^{4}\). As expected, male WT/Y MEFs only showed Rlim transcription foci but no Xist clouds (Fig. 3a). In contrast, the majority of female WT/WT, cKO\(^\Delta m\)/WT, WT/cKO\(^\Delta p\) and cKO\(^\Delta m\)/\(\Delta p\) MEFs showed monoallelic expression of both genes, with Xist painting the inactive X and foci of Rlim transcription marking the active X (Xi and Xa, respectively) (Fig. 3a, b). XCI in cKO\(^\Delta m\)/\(\Delta p\) MEFs was verified by co-staining cells with antibodies directed against Rlim and H3K27me3, an XCI marker downstream of Xist\(^{18}\) (Extended Data Fig. 3). We observed a similar pattern for primary mammary epithelial cells isolated from 3-month-old adult females (Fig. 3c, d; data not shown). We also examined X skewing in MEFs isolated from female embryos with either a maternal or paternal cKO allele, using transgenic mice containing an X-linked green fluorescent protein (XGFP) transgene\(^{14}\) on the wild-type X chromosome. MEFs of both cKO\(^\Delta m\)/XGFP\(_m\) and XGFP\(_p\)/cKO\(^\Delta p\) embryos showed approximately 1:1 ratios of GFP-positive:GFP-negative cells (Extended Data Fig. 4). These results indicate normal rXCI in somatic tissues.

Focusing our analyses on embryonic stages E5.5–E7.5, when rXCI occurs in vivo\(^{15-17}\), we dissected blastocystic tissue of E6–6.5 WT/WT and cKO\(^\Delta m\)/\(\Delta p\) embryos and measured mRNA levels of Rlim and Xist by quantitative PCR with reverse transcription (RT–qPCR). Although levels of Rlim were greatly diminished in cKO\(^\Delta m\)/\(\Delta p\) embryos, those of Xist were only slightly reduced (Fig. 4a). The residual levels of Rlim in cKO\(^\Delta m\)/\(\Delta p\) embryos are probably due to minor amounts of contaminating extra-embryonic tissue in blastocystic dissections. Consistent with the kinetics of developing H3K27me3 marks on the Xi\(^{18}\), we first detected H3K27me3

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**Figure 1** Downregulation of Rlim levels in early embryonic tissues before and after implantation. a, b, Co-stainings of whole WT/WT pre-implantation embryos at E3.5 (a) and E4.5 (b) (n = 5 and 8, respectively) using antibodies against Rlim (red) and Oct4 (green). Embryos were first photographed and then processed for genotyping. DAPI, 4',6-diamidino-2-phenylindole. c, Co-stainings of sections of wild-type E5.5 post-implantation embryos (arrow; n = 10) within placental tissues. Note the lack of Rlim staining (red) in nuclei of Oct4-positive cells (green). d, Co-stainings of sections of WT/WT E7–7.5 embryos (n = 5) within decidual tissues using antibodies against Rlim (red) and H3K27me3 (green). Boxed regions are shown in bottom panels. d1, ectoplacental cone region; d2, amnion region; d3, embryonic epiblast region. Note the low Rlim staining intensity in cells in the amnion and epiblast regions. Scale bars, 15 μm (a, b), 30 μm (c) and 75 μm (d).

**Figure 2** Rlim is dispensable at post-implantation stages in female mice. a, b, Schematic diagram showing parental genotypes, the total number (n) of born and genotyped F1 offsprings. Number, gender and genotypes of offspring are indicated. m (maternal) and p (paternal) indicate the origin of the floxed Rlim allele. Maternally transmitted cKO alleles are indicated in red. c, High penetrance of the SC-mediated cKO in somatic tissues. PCR analysis of genomic DNA isolated from tail tissue samples using primer combinations that detect floxed and knockout Rlim alleles. Parental genotypes are indicated. d, Lack of Rlim in somatic tissues of adult cKO\(^\Delta m\)/\(\Delta p\) females in western blots hybridized with Rlim antibodies. e, Recombination via paternally transmitted Sox2-Cre occurs in pre-implantation embryos. PCR genotyping of E3.75 blastocysts using primers that detect wild-type and knockout Rlim alleles. Parental genotypes are indicated.
foci in WT/WT embryonic tissues at stages E7–E7.5 but not in earlier post-implantation embryos (Fig. 4b; data not shown). These signals were indistinguishable from those of E7–7.5 cKOm/Δp embryos, indicating similar XCI kinetics in the absence of RLIM. Moreover, cKOm/Δp and WT/WT embryos were similar in size, and in immunostainings using antibodies directed against cleaved caspase 3, no increase in cells undergoing apoptosis was detected in cKOm/Δp embryos (data not shown). To obtain definitive evidence that rXCI does not require the presence of RLIM in cells, we performed tetraploid complementation assays in which tetraploid WT/WT embryos were aggregated with low-passage Rlim Δ/Δ (ES-cell line IB8 or IB11), and control βcr/Δp (IB6) or male Δp/WT ES cells (and IB13), freshly isolated from blastocysts. These ES-cell lines were generated by a βcr/Δp × Δ/Δ-Y-SC cross, and are capable of developing H3K27me3 foci upon differentiation in culture, indicating XCI (Extended Data Fig. 5a, b). In tetraploid injections, the development of embryos derived from Δ/Δ ES cells was indistinguishable from matings. c, Tetraploid complementation assay using WT/WT blastocysts complemented with low-passage Δ/Δ and, as a control, with Δ/Y and βcr/ΔES cells. Embryos were harvested at E9–9.5 and image was recorded before genotyping. Scale bars, 0.5 mm. d, Δ/Δ ES cells are able to undergo XCI in vivo. RNA-FISH experiments on MEFs isolated from E10.5 embryos generated via tetraploid complementation using Δ/Δ ES line IB11 reveals monoallelic expression of both Xist (green) and Rlim (red). Arrows indicate specific signals. Scale bars, 3 μm.
control ES cells at E9–9.5 (Fig. 4c), and no significant differences in complementation efficiencies between these ES-cell lines were detected (Extended Data Fig. 5c). Examining rXCI in MEFs isolated from E10.5 embryos generated from Δ/Δ and Δ/β ES cells revealed that more than 75% of Δ/Δ MEFS developed H3K27me3 foci and Xist clouds (Fig. 4d and Extended Data Fig. 5d, e). Combined with the presence of a single Rlim transcription focus in cells, this indicated that ES cells lacking Rlim are capable of undergoing XCI in vivo. Together, our results provide strong evidence that Rlim is dispensable for rXCI in mice. Moreover, because maternal Rlim is crucial for rXCI and the Δ/Δ ES-cell line IB11 is SC negative (Extended Data Fig. 5a) — therefore containing maternal and paternal alleles — these data indicate that the process of rXCI is not required in pre-implantation embryos for the epiblast precursor-cell lineage that will give rise to the embryo proper.

Clearly, Rlim can serve as an activator of Xist in some systems. The fact that in cKOα/Δ embryos, Xist levels are only mildly affected and H3K27me3 signals are established with kinetics similar to WT/WT (Figs 4a, b), combined with the finding that Rlim levels are downregulated in wild-type embryos (Fig 1, Extended Data Fig. 1), suggests that in female mouse embryos an Rlim-independent mechanism is used to activate/upregulate Xist transcription during rXCI. This is further underscored by our results that outgrowths of E4.5 blastocyst-stage female embryos with a maternal and paternal germline Rlim knockout can develop Xist clouds in cells of the ICM. Because rXCI occurred with low frequency in the Rlim-knockout ES-cell system, it seems likely that two independent mechanisms for Xist activation exist in mice: one that is independent of Rlim and may be inactive or downregulated in certain ES-cell systems in culture, and another that is Rlim dependent. Such a model is consistent with the presence of Xist activators other than Rlim, such as Jpx RNA20,21, and would explain why Rlim is necessary for XCI in specific ES cells but not in embryos. A similar case in which ES-cell systems do not accurately reflect the embryonic functions, as phenotypes in morphogenesis upon Rlim mutation have been observed in zebrafish24 but not in mice5.

METHODS SUMMARY

To investigate the functions of Rlim during rXCI in mice, the conditional Rlim knockout2 was targeted to the ICM in pre-implantation blastocystas using a paternally transmitted Sox2-Cre transgene14 as a driver. Analyses of cells and embryos at pre- and post-implantation stages was carried out as described previously using RNA-FISH3, immunological staining methods3,4, western blotting5 and RT–qPCR6. Rlim Δ/Δ ES-cell lines were newly generated28 and used for tetraploid injections. All authors analysed the data. I.B. wrote the manuscript with input from J.S.

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1. Augui, S., Nora, E. P. & Heard, E. Regulation of X-chromosome inactivation by the X-inactivation centre. Nature Rev. Genet. 12, 429–442 (2011).
2. Lee, J. T. Gracefully ageing at 50, X-chromosome inactivation becomes a paradigm for RNA and chromatin control. Nature Rev. Mol. Cell Biol. 12, 815–826 (2011).
3. Barakat, T. S. et al. RNF12 activates Xist and is essential for X chromosome inactivation. PLoS Genet. 7, e1002001 (2011).
4. Jonkers, I. et al. RNF12 is an X-encoded dose-dependent activator of X chromosome inactivation. Cell 139, 999–1011 (2009).
5. Shin, J. et al. Maternal Rnf12/LIM is required for imprinted X-chromosome inactivation in mice. Nature 467, 977–981 (2010).
6. Bach, I. et al. Rlim inhibits functional activity of LIM homedomain transcription factors via recruitment of the histone deacetylase complex. Nature Genet. 22, 394–399 (1999).
7. Ostendorff, H. P. et al. Ubiquitination-dependent cofactor exchange on LIM homedomain transcription factors. Nature 416, 99–110 (2002).
8. Jiao, B. et al. Functional activity of Rnf12/Lim12 is regulated by phosphorylation-dependent nucleocytoplasmic shuttling. Mol. Biol. Cell 24, 3085–3096 (2013).
9. Gontani, C. et al. Rnf12 initiates X-chromosome inactivation by targeting Rex1 for degradation. Nature 485, 386–390 (2012).
10. Dupont, C. & Gribnau, J. Different flavors of X-chromosome inactivation in mammals. Curr. Opin. Cell Biol. 25, 314–321 (2013).
11. Schulz, E. G. & Heard, E. Role and control of X chromosome dosage in mammalian development. Curr. Opin. Genet. Dev. 23, 109–115 (2013).
12. Ostendorf, H. P. et al. Dynamic expression of LIM cofactors in the developing mouse neural tube. Dev. Dyn. 235, 786–791 (2006).
13. Hayashi, S., Lewis, P., Perry, L. & McMahon, A. P. Efficient gene modulation in mouse epiblast using a Sox2Cre transgenic mouse strain. Mech. Dev. 119 (suppl. 1), S97–S101 (2002).
14. Hayashi, S., Tenzen, T. & McMahon, A. P. Maternal inheritance of Cre activity in a Sox2Cre deleter strain. Genesis 37, 51–53 (2003).
15. Avilion, A. A. et al. Multipotent cell lineages in early mouse development depend on Sox2 function. Genes Dev. 17, 126–140 (2003).
16. Panning, B. X inactivation in mouse ES cells: histone modifications and FISH. Methods Enzymol. 376, 419–428 (2003).
17. Plath, K. et al. Role of histone H3 lysine 27 methylation in X inactivation. Science 300, 131–135 (2003).
18. Hadjantonakis, A. K., Cox, L. L., Tam, P. P. & Nagy, A. An X-linked FGF transgene reveals unexpected paternal X-chromosome activity in trophoblastic giant cells of the mouse placenta. Genesis 29, 133–140 (2001).
19. Tan, D., Sun, S. & Lee, J. T. The long noncoding RNA, Jpx, is a molecular switch for X chromosome inactivation. Cell 143, 390–403 (2010).
20. Sun, S. et al. Jpx RNA activates Xist by evoking CTCF. Cell 153, 1537–1551 (2013).
21. Mitsu, K. et al. The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells. Cell 113, 631–642 (2003).
22. Chambers, I. et al. Nanog safeguards pluripotency and mediates germline development. Nature 450, 1230–1234 (2007).
23. Zhang, L. et al. RNF12 controls embryonic stem cell fate and morphogenesis in zebrafish embryos by targeting Smad7 for degradation. Mol. Cell 46, 650–661 (2012).
24. Hall, L. L. et al. An ectopic human Xist gene can induce chromosome inactivation in post differentiation human HT-1080 cells. Proc. Natl Acad. Sci. USA 99, 8677–8682 (2002).
25. Griffith, G. J. et al. Yin-Yang1 is required in the mammalian oocyte for follicle expansion. Biol. Reprod. 84, 654–663 (2011).
26. Tursun, B. et al. The ubiquitin ligase Rnf6 regulates LIM kinase 1 axes in axonal growth cones. Genes Dev. 19, 2307–2319 (2005).
27. Robertson, E. J. Teratocarcinomas and Embryonic Stem Cells—A Practical Approach Ch. 4, 711–712 (RL, 1987).
28. Nagy, A. et al. in Manipulating the Mouse Embryo—a Laboratory Manual 3rd edn, 8395–397 (Cold Spring Harbor, 2003).

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Author Contributions J.S. and I.B. conceived and designed the experiments. All authors performed the tetraploid injections. M.B. generated the flow Rlim mice, I.G. and S.N.J. generated the ES-cell lines and performed the tetraploid injections. M.B. and J.B.L. carried out RNA-FISH experiments and M.C.W. and J.M. performed immunohistochemistry on early embryos. All authors analysed the data. I.B. wrote the manuscript with input from J.S.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to I.B. (Ingrid.bach@umassmed.edu).
METHODS

Mice. Mice used in this study and genotyping have been previously described: Rlim<sup>fl</sup> (ref. 5), Sox2-Cre<sup>+</sup>, XGFP<sup>−</sup>, Sox2-Cre, XGFP, wild-type type SV129 and FVB mice were purchased from the Jackson Laboratories. Rlim<sup>−/−</sup> mice were generally bred in a congenic C57BL/6 background. However, to examine background effects on XCI, the floxed Rlim and Sox2-Cre alleles were introduced into C57BL/6–SV129 and C57BL/6–FVB hybrid backgrounds. The extent of C57BL/6 versus SV129 background of hybrid F2 cKO<sup>M</sup>/Y (IB13) were generated as described<sup>28</sup>. Apart from embryos generated via tetraploid fusion at the two-cell stage using a CF-150B cell sorter, all blastocysts and embryos were generated by naturally mating 8–14 ES cells were microinjected into the tetraploid-fused embryos, and the injected embryos that had undergone tetraploid fusion at the two-cell stage using a CF-150B cell sorter. ES cell lines Rlim<sup>Δ/Δ</sup> (IB8 and IB11), flm<sup>/−</sup><sup>/−</sup>, ΔY<sup>−</sup> (IB6) and ΔY<sup>−/−</sup> (IB13) were generated as described<sup>36</sup>. Briefly, E3.5 blastocyst-stage embryos from a female mice with males. Embryos were collected at stages E3.5 to E12.5 for ploid injection, all blastocysts and embryos were generated by naturally mating 8–14-week-old female mice with males. Embryos were collected at stages E3.5 to E12.5 for further processing. Genotyping of embryos was carried out as described<sup>1</sup>. Analyses of dissected epiblast tissues of E6.5 embryos using RT–qPCR including primer sequences as described<sup>1</sup>. Embryos were genotyped after image recording. Primary mammary epithelial cells were isolated from adult virgin female mice (12 weeks), cultured as described<sup>6</sup> and then processed for RNA–FISH or immunostainings.

RNA–FISH. RNA–FISH experiments on MEFs and primary mammary epithelial cells were performed as described previously<sup>19</sup>. For the synthesis of specific Xist probes, we used plasmids containing mouse Xist exon 1 and 6 that recognize Xist and Tsix<sup>−/−</sup>. For the Rlim probe, we used a plasmid containing genomic Rlim sequences upstream of the knockout site that detects specific Rlim mRNAs transcribed from both wild-type and knockout alleles<sup>7</sup>. Antibodies and western blots. Primary antibodies used for immunostainings were rabbit Rlim<sup>−/−</sup>, guinea pig Rlim<sup>−/−</sup>, Oct4 (Santa Cruz, sc-5279; Abcam, ab27985), H3K27me3 (Abcam, ab6002; Millipore, 07–447), GFP (Rockland, 600–101–215), cleaved caspase 3 (Cell Signaling, 5A1E) and actin (Sigma, A 4700). Secondary antibodies were Alexa Fluor-488 donkey anti-rabbit 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). Western blots were carried out as reported<sup>19</sup>. Immunohistochemistry. Immunohistochemical staining of embryonic sections was carried out essentially as described<sup>25</sup>. After dissection and removal of the eutocoplacental cone for genotyping, embryos were gently placed into a small section of uterus, in order to facilitate the embedding protocol and maintain orientation for sectioning. Embryos/uteri were fixed for histology in 4% paraformaldehyde (PFA) for 2 h at room temperature or overnight at 4 °C. Embryos in uteri were dehydrated in methanol washes; 20 min at 25%, 50%, 75% methanol in PBS/0.01% Tween-20 (PBT), followed by two 100% methanol washes. After overnight incubation in 100% Xylenes, embryos were incubated for 2 h in molten paraffin, before embedding and sectioning<sup>36</sup>. Sections were mounted and dried on superfrost plus slides. Wax was removed through three 10 min xylene washes and rehydrated with three 5 min washes in 100% ethanol, followed by successive washes in 90%, 80%, 70% ethanol and finally water (1 min each). Antigen retrieval consisted of boiling for 5 min in 0.01 M Tris Base pH 10.0 with 0.05% Tween-20. After cooling to room temperature, slides were washed twice in PBT for 2 min before blocking with 0.5% milk in PBT (2 h at room temperature) in a humidified chamber. Primary antibodies were incubated in 0.05% milk/PBT overnight at 4 °C. Three 15 min PBT washes were done before 1 h secondary antibody incubation (also in 0.05% milk/PBT) in a humidiﬁed chamber at room temperature. Slides were washed twice in PBT for 15 min and once in PBS. Nuclei were stained using DAPI (Roche or Molecular Probes) 1:10,000 in PBS for 2 min and rinsed once with PBS. Prolong Gold (Invitrogen) was used to seal and coverslip the slides. Images of sectioned embryos were taken with a Nikon Eclipse TE2000-S inverted fluorescence microscope and QImaging Retiga Exi Fast 1394 camera using NIS-Elements BR Software.

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Extended Data Figure 1 | Strain-independent downregulation of RLIM in the mouse embryonic epiblast. **a**, Test of specificity of the RLIM antibody (compare with Fig. 1b). Shown is an E4.5 Δ/Y blastocyst outgrowth stained with RLIM (red). Increasing the general signal levels (RLIM*) reveals augmented unspecific staining mainly in the cytoplasm of cells. **b**, Co-stainings of sections of E7–7.5 embryos within decidual tissues using antibodies directed against RLIM (red) and H3K27me3 (green). Representative C57BL/6 and SV129 embryos are shown (out of at least three that were stained). Scale bars, 75 μm.
Extended Data Figure 2 | Genetic background has little or no influence on RLIM dispensability during rXCI. a, b, C57BL/6–SV129 (a) and C57BL/6–FVB (b) hybrid F1 parents were generated by crossings of fl/Y (C57BL/6) males with WT/WT (SV129 or FVB) females and WT/Y (SV129 or FVB) males with cKO/D-SC (C57BL/6) females. F1 WT/fl females and cKO/Y-SC males were then backcrossed to generate F2 cKOm offspring. Percentages of offspring (grouped into female and male) and their genotypes with respect to Rlim and SC are indicated in the abscissa and ordinate, respectively, and the total number (n) of born and genotyped F2 pups is shown. Maternally transmitted cKO alleles are indicated in black. c, No discrimination against SV129 in born cKOm/Dp pups with a mixed C57BL/6–SV129 background. Sequencing analyses of genomic DNA isolated from eight hybrid cKOm/Dp pups using 156 strain-specific SNPs distributed among all chromosomes (blue/red) or ten SNPs distributed on the X chromosome (green/orange). Note that the SV129 contribution in born cKOm/Dp pups is up to 70% (total) and 80% (on the X).
Extended Data Figure 3 | Co-stainings of MEFs isolated from cKO_m/D_p and WT/WT embryos using antibodies directed against H3K27me3 (green) and RLIM (red). a, Representative images are shown. b, Summary graph of cells showing H3K27me3 foci is shown on the right. Numbers of counted cells from independent biological duplicates are 111/112 (WT/WT) and 110/104 (cKO_m/D_p).
Extended Data Figure 4 | Random XCI in female mice heterozygous for the Sox2-Cre-mediated deletion of Rlim. Female mice were generated carrying either a paternal or maternal Rlim deletion and a GFP transgene on the other X chromosome (XGFP). Mating schemes to generate these females are indicated. Female littermates without the Sox2-Cre transgene were used as controls. Numbers of cells counted from independent biological duplicates are indicated.
Extended Data Figure 5 | Female ES cells lacking RLIM are able to undergo rXCI in vivo. a, Parental cross used for the generation of ES-cell lines is indicated on top. Newly generated ES-cell lines were genotyped using PCR for the presence of wild-type, floxed or knockout Rlim alleles, as well as Cre driver (SC) and Y chromosome (Zfy2). b, Newly isolated female ES cells were differentiated for 5 days in culture and stained with the H3K27me3 antibody. c, Summary of the tetraploid complementation assays showing the injected ES-cell lines, genotypes, number of deciduas and embryos obtained (see also Fig. 4c). All embryos generated via tetraploid complementation were genotyped for the presence of wild-type, floxed and knockout Rlim alleles as well as Zfy2. d, Left, MEFs isolated from an E10.5 embryo generated via tetraploid injection of Δ/Δ line IB11 ES cells were cultured for 24 h before staining with antibodies against H3K27me3. Right, summary of H3K27me3 stainings. Numbers of counted cells from independent biological duplicates are 60/62 (WT/WT) and 78/75 (cKOm/Δp). e, Summary of RNA-FISH experiments on MEFs isolated from embryos generated via tetraploid injection of Δ/Δ line IB11 ES cells (n = 109) (see Fig. 4d). MEFs isolated from WT/WT embryos (n = 106) served as control.