REX1 is the critical target of RNF12 in imprinted X chromosome inactivation in mice

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In mice, imprinted X chromosome inactivation (iXCI) of the paternal X in the pre-implantation embryo and extraembryonic tissues is followed by X reactivation in the inner cell mass (ICM) of the blastocyst to facilitate initiation of random XCI (rXCI) in all embryonic tissues. RNF12 is an E3 ubiquitin ligase that plays a key role in XCI. RNF12 targets pluripotency protein REX1 for degradation to initiate rXCI in embryonic stem cells (ESCs) and loss of the maternal copy of Rnf12 leads to embryonic lethality due to iXCI failure. Here, we show that loss of Rex1 rescues the rXCI phenotype observed in Rnf12−/− ESCs, and that REX1 is the prime target of RNF12 in ESCs. Genetic ablation of Rex1 in Rnf12−/− mice rescues the Rnf12−/− iXCI phenotype, and results in viable and fertile Rnf12−/−;Rex1−/− female mice displaying normal iXCI and rXCI. Our results show that REX1 is the critical target of RNF12 in XCI.
evolution of the eutherian sex chromosomes and the concomitant gradual loss of nearly all ancestral genes from the Y chromosome forced co-evolution of intricate dosage compensation mechanisms including X chromosome inactivation (XCI). XCI leads to equalization of X-linked gene dosage between male and female cells by inactivation of one X chromosome in every female somatic cell. Two different types of XCI have been described in mice. Imprinted X chromosome inactivation (iXCI) takes place during pre-implantation development in the embryo and in the extraembryonic tissues, where the paternal X chromosome is always inactivated. At the blastocyst stage, the inactivated paternal X is reactivated within the pluripotent cells of the inner cell mass (ICM), while extraembryonic tissues such as the placenta and visceral yolk sac endoderm (VYSE) retain an inactive paternal X chromosome. Upon formation of the epiblast, the cells of the embryo inactivate their maternal or paternal X chromosome (Xm and Xp, respectively) through random X chromosome inactivation (XCI). Later during development, the inactive X (Xi) chromosome is reactivated in female primordial germ cells (PGCs) to erase the inactive state prior to conception.

iXCI and rXCI utilize complex regulatory networks to properly induce mono-allelic Xist expression from one X chromosome. Xist is transcribed in a 17-kb-long non-coding RNA that spreads in cis to coat the future Xi chromosome, initiating epigenetic changes including H3K27me3 accumulation, involved in establishment and maintenance of the inactive state (reviewed in ref. 6). Rnf12, located in close proximity to Xist, plays a crucial role in the regulation of iXCI. Transcriptional activation of an Rnf12 mutant allele to daughters is lethal, due to failure of the Xp to inactivate during pre-implantation development. On the other hand, daughters with a paternally transmitted Rnf12 mutant allele are viable and do not show iXCI defects. How RNF12 mechanistically effects iXCI in vivo is still an open question. In addition, rXCI is severely affected upon differentiation of Rnf12−/− embryonic stem cells (ESCs), while Rnf12 heterozygous ESCs manage to inactivate an X chromosome, indicating that one functional copy of Rnf12 is required to properly initiate rXCI in vitro.

Rnf12 encodes an E3 ubiquitin ligase, and pull-down experiments of Rnf12 followed by mass spectrometry identified Rex1 as a partner and target of RNF12 in ESCs. The role of Rex1 in pluripotency of ESCs, in genomic imprinting and in pre-implantation development has been studied in mice. Rxl arose in placental mammals via retrotransposition of the constitutively expressed Y1 transcription factor. In rXCI through Rex1, Rnf22, and REX1 through REX1 is unknown. Also, putative roles for Rex1 in rXCI and X chromosome reactivation (XCR) in vivo have not been studied so far.

Here, we dissect the Rex1−Rnf12 axis in XCI in vivo and in vitro. We show that Rex1 is the prime target of RNF12 in ESCs. We also show that deletion of Rex1 in Rnf12−/− ESCs rescues the XCI phenotype, indicating that, at least in vitro, RNF12 regulates rXCI primarily through Rex1. Moreover, the lethal phenotype of Rnf12−/− (in the +/+ or −/− nomenclature, the maternally inherited allele is shown first) and Rnf12−/− female mice is completely rescued in a mutant Rex1 background, indicating that RNF12-mediated degradation of Rex1 is also a critical event in iXCI. These results highlight the crucial role for RNF12 in facilitating initiation of rXCI and iXCI, by targeting Rex1 for proteasomal degradation.

**Results**

**Rex1 is the prime target of RNF12 in ESCs.** We previously performed an immunoprecipitation of RNF12 and identified Rex1 as an RNF12 interaction partner, which is ubiquitinated by RNF12 to be targeted for degradation. To identify the full spectrum of RNF12 targets in ESCs, we performed quantitative proteomics by stable isotope labelling of amino acids in cell culture (SILAC) and compared protein extracts from Rnf12−/− and wild type (WT) ESCs (Supplementary Fig. 1a; Supplementary Data 1). This analysis revealed REX1 to be the protein with the strongest increase in stability in extracts from Rnf12−/− cells, as compared to WT cells (Fig. 1a; Supplementary Fig. 1b). This indicates that Rex1 is the main target of RNF12 for proteasomal degradation in ESCs. We also compared extracts of WT ESCs cultured in the presence or absence of the proteasome inhibitor MG132 (Supplementary Fig. 1c). REX1 and RNF12 were found to be among the proteins with the largest change in abundance in ESCs upon addition of the proteasome inhibitor MG132 (Supplementary Fig. 1d, e; Supplementary Data 1) highlighting their high turnover in ESCs.

Rex1 deletion rescues the rXCI phenotype of Rnf12−/− ESCs. As Rex1 appears to be the primary substrate of RNF12 in ESCs, genetic removal of REX1 from Rnf12−/− ESCs may complement their XCI phenotype. To address this question, we first generated Rnf12CR+/−/− ESC lines by CRISPR/Cas9-mediated removal of the complete open reading frame of Rnf12 in F1 129/Sv/Cast/Eij (129/esc) ESCs (Supplementary Fig. 2a, b), and compared them to our previously generated Rnf12−/− ESCs which still express the N-terminal 333 amino acids of RNF12, encoding the nuclear localization signal and part of the basic domain but excluding the catalytic Ring finger domain. Targeting and loss of Rnf12 in Rnf12CR+/−/− ESCs was confirmed by PCR analysis on genomic DNA and western blotting (WB) analysis (Fig. 1b, c; Supplementary Fig. 3a). As expected, a marked increase of REX1 protein levels in Rnf12CR+/−/− and Rnf12−/− ESCs was observed by WB analysis (Fig. 1c; Supplementary Fig. 3a). Accordingly, we observed by immunofluorescence (IF) staining that increased REX1 expression is nuclear in both Rnf12CR+/−/− and Rnf12−/− ESCs (Fig. 1d), and in contrast to the homogeneous OCT4-staining, REX1 expression is heterogeneous within individual ESC colonies and overlaps with cells displaying high NANOG expression as previously described (Supplementary Fig. 3b). This indicates that the absence of functional RNF12 causes nuclear accumulation of REX1. Quantitative RT-PCR and Xist RNA-FISH analysis indicated that Xist expression and Xist coating of the Xi was severely compromised in differentiating Rnf12−/− ESCs, both in monolayer and embryoid body (EB) differentiating conditions, confirming our previous observations that Rnf12 is required for rXCI in vitro. We then generated Rnf12CR+/−/−Rex1+/−/− and Rnf12CR+/−/−Rex1−/−/− ESC lines by CRISPR/Cas9-mediated deletion of most of the open reading frame of Rex1 (Supplementary Fig. 2a, b). Targeting was confirmed by PCR analysis on genomic DNA (Fig. 1b). WB and RT-qPCR analysis confirmed loss of Rnf12 and Rex1 expression in Rnf12CR+/−/−Rex1−/−/− ESC lines by CRISPR/Cas9-mediated double-knockout (DKO) ESCs (Fig. 1c; Supplementary Fig. 4a, b). REX1 protein levels were also increased and accumulated in the nucleus in the absence of RNF12 in Rnf12CR+/−/−Rex1−/−/− ESCs (Fig. 1c; Supplementary Fig. 4c). rXCI was rescued in Rnf12CR+/−/−Rex1−/−/− but not in Rnf12CR+/−/−Rex1−/−/− differentiating ESCs (Fig. 1e-g; Supplementary Fig. 4d,e). A slight delay in rXCI in Rnf12CR+/−/−Rex1−/−/− ESCs was observed compared to WT ESCs, which is likely related to the slower differentiation kinetics observed in
Rnf12\textsuperscript{CR}/CR\textsuperscript{-} ESCs (Supplementary Fig. 4f), and not due to a defect in rXCI (Supplementary Fig. 4g). These results illustrate the crucial role for RNF12-mediated degradation of REX1 in the initiation of rXCI in vitro.

REX1 is dispensable for XCR, iXCI and rXCI. Rex\textsuperscript{1} is expressed at all stages during mouse pre-implantation development, where iXCI takes place (2- to 4-cell stage and trophoblast), and in cell types where the Xi is reactivated in the mouse life cycle: in the
epiblast lineage of E4.5 female blastocysts as well as in developing PGCs, at E10.5\(^{14,19,20}\). Although Rex1 mutant mice were reported to be viable, they are born at sub-Mendelian ratios and display defects in imprinted gene regulation\(^{12,13}\). In rXCI, REX1 is an important repressor of \(Xist\)\(^1\) and its expression in the ICM and PGCs makes it a candidate factor in X reactivation.

To investigate the effect of \(Rex1\) ablation on XCI, we generated \(Rex1\) knockout (KO) mice by blastocyst injection of \(Rex1^{+/+}\) F1 129:cas ESCs generated by gene targeting through homologous recombination (Fig. 2a–c, Supplementary Fig. 2a). \(Rex1\) KO mice were backcrossed for at least six generations in two different genetic backgrounds (129/Sv and Cast/EiJ). In agreement with previous studies, we found a reduced litter size for \(Rex1^{-/-}\) crosses, and no significant gender bias against birth of female animals (Fig. 2d). We subsequently isolated blastocysts and established a \(Rex1^{-/-}\) F1 129:cas female ESC line. In ESC monolayer differentiation experiments, we observed by quantitative RT-PCR and \(Xist\) RNA-FISH analysis increased \(Xist\) expression and reduced \(Tsix\) expression (Supplementary Fig. 5a–c) in \(Rex1^{+/+}\) and \(Rex1^{-/-}\) ESCs, with significantly more \(Xist\) clouds in \(Rex1^{+/+}\) and \(Rex1^{-/-}\) ESCs than in WT controls (Fig. 2e, f). More importantly, we also observed significantly more cells with two \(Xist\) clouds (Fig. 2e, f), indicating an important role for \(Rex1\) in repression of \(Xist\), providing the feedback required to prevent XCI of too many X chromosomes.

To test the involvement of \(Rex1\) in the reactivation of the Xi in the ICM or PGCs, we firstly analysed the ICM of E3.5 embryos and epiblast of E4.5 embryos by IF detecting H3K27me3 marking the Xi\(^{21,22}\), together with OCT4 and KLF4, which stain the cells specific to the ICM and epiblast, respectively (Fig. 3a, b; Supplementary Fig. 6a). This revealed no delay in timing of loss of the H3K27me3-coated Xi between \(Rex1^{-/-}\) and WT female embryos, indicating that \(Rex1\) is dispensable for Xi reactivation in the ICM. To confirm these results, we crossed our \(Rex1^{-/-}\) female mice with \(Rex1^{-/-}\) male mice containing an X-linked GFP reporter adjacent to Hprt\(^{23}\) (\(Rex1^{-/-}\):\(Hprt^{GFP}\)). Comparison of \(Rex1^{-/-}\):\(Hprt^{+}\)/\(GFP\) to \(Hprt^{+}\)/\(GFP\) E4.5 female blastocysts showed no difference in the amount of Xp-reactivated GFP-positive cells lacking the H3K27me3 domain (Supplementary Fig. 6b, c), confirming our previous results. Analysis of XCR in PGCs by H3K27me3 IF staining together with OCT4 to map them revealed no difference in the rate of XCR between \(Rex1^{-/-}\) and WT E9.5 and E11.5 female embryos (Fig. 3c, d; Supplementary Fig. 6d, e). Together, these results show that \(Rex1\) is not required for the reactivation of the Xi chromosome in vivo.

We then investigated the role of \(Rex1\) in iXCI and rXCI in vivo. \(Xist\) RNA-FISH analysis of \(Rex1^{-/-}\) blastocyst outgrowths showed no differences in iXCI compared to WT cells (Supplementary Fig. 7a, b). Allele-specific expression analysis in \(Rex1^{+/+}\) and WT E11.5 female embryos of X-linked genes \(Xist\), \(G6pdx\) and \(Mecp2\) indicated normal iXCI and rXCI, with preferential inactivation of the paternally inherited X (cas) in the VYSE of F1 129:cas embryos, and rXCI in embryonic tissues (Fig. 3e, f; Supplementary Fig. 7c). \(Rex1^{-/-}\) ESCL were born at expected Mendelian ratios, confirming our previous results. Analysis of XCR in PGCs by RNA-FISH analysis of WT, \(Rex1^{+/+}\), \(Rex1^{-/-}\) and \(Rex1^{+/+}\):\(Rex1^{-/-}\) ESCs (clones 36 and 27) and \(Rex1^{+/+}\):\(Rex1^{-/-}\):\(Rex1^{+/+}\):\(Rex1^{-/-}\) (clone 17) ESCs at day 6 of differentiation. DNA was stained with DAPI (blue). Scale bar: 20 μm. f Quantification of cells with Xi clouds in WT, \(Rex1^{+/+}\), \(Rex1^{-/-}\) and \(Rex1^{+/+}\):\(Rex1^{-/-}\):\(Rex1^{+/+}\):\(Rex1^{-/-}\) (clone 17) ESCs at day 3 and day 6 of differentiation. g QPCR analysis of \(Xist\) expression in undifferentiated, day 3 and day 6 differentiated WT, \(Rex1^{+/+}\), \(Rex1^{-/-}\) and \(Rex1^{+/+}\):\(Rex1^{-/-}\) (clone 17) ESCs (average expression ± s.d., n = 3 biological replicates).
**Rex1 deletion rescues the lethality of Rnf12^{−/−} mice.** To test whether stabilization of REX1 in Rnf12 mutant embryos might be related to the lethality associated with maternal transmission of Rnf12 mutant alleles, we crossed our Rnf12 KO mice and Rex1 KO mice to generate Rnf12^{−/−}:Rex1^{−/−} DKO mice. In contrast to Rnf12^{−/−} or Rnf12^{−/−} mice that were never obtained in a Rex1 WT or heterozygous background, several Rnf12^{+/−}:Rex1^{−/−} and Rnf12^{−/−}:Rex1^{−/−} female mice were born (Fig. 5a; Supplementary Fig. 9a). In general, litters were smaller, but no gender or allele bias was observed (Fig. 5a). We confirmed these results by crossing Rnf12^{+/−}:Rex1^{−/−} or Rnf12^{−/−}:Rex1^{−/−} females with Rex1^{−/−} males, whose Rnf12^{+/−}:Rex1^{−/−} daughters were viable but their Rnf12^{−/−}:Rex1^{−/−} sisters were not (Supplementary Fig. 9a). IF staining on Rnf12^{−/−}:Rex1^{−/−} E4.5 female blastocysts from compound homozygous crosses confirmed loss of REX1 and showed proper XCR in the epiblast during pre-implantation development, as seen by the loss of H3K27me3 domains corresponding to the Xi (Fig. 5b). Also, IF staining detecting H3K27me3 and OCT4 revealed normal XCR in PGCs (Supplementary Fig. 9b, c), suggesting that reactivation of the Xi is normal in Rnf12^{−/−}:Rex1^{−/−} female blastocysts and embryos.

We then investigated iXCI and nXCI in Rnf12^{−/−}:Rex1^{−/−} blastocysts, embryos and adults. Rex1 and H3K27me3 IF staining of Rnf12^{−/−}:Rex1^{−/−} blastocysts showed normal iXCI (H3K27me3 domains corresponding to the Xi) compared to WT blastocysts (Fig. 5b), in line with Xist RNA-FISH analysis in trophoblast cells of Rnf12^{−/−}:Rex1^{−/−} blastocyst outgrowths (Supplementary Fig. 7a, b), indicating that iXCI in Rnf12^{−/−}:Rex1^{−/−} blastocysts is normal. Allele-specific RT-PCR analysis examining Xist, G6pdx and Mecp2 expression on RNA isolated...
Fig. 3 XCR and imprinted and random XCI in female Rex1/− mice are not compromised. a Representative Z-stack projections of WT and Rex1/− E3.5 and E4.5 female blastocysts immunostained for H3K27me3 (Xi marker, green), the lineage markers OCT4 (E3.5 ICM, red, left panels) and KLF4 (E4.5 epiblast, red, right panels) and DNA (DAPI, blue). Whole embryo and ICM/epiblast higher magnification for OCT4 (PGC marker, red) and H3K27me3 (Xi marker, green). H3K27me3 domains are present in somatic cells and in some E9.5 PGCs, while they are lost respectively. The number of mice analysed is indicated.

b Quantification of H3K27me3 domains in E3.5 and E4.5 PGCs (XCR). Representative PGCs are marked with yellow dashed lines. Scale bars: 5 μm.

c Representative paraffin sections of female WT and Rex1/− E9.5 embryo hindguts and E11.5 embryo trunks immunostained for OCT4 (PGC marker, red) and H3K27me3 (Xi marker, green). Arrowheads mark the Xi in OCT4+ cells. KLF4+ cells display XCR (stars). Scale bars: 20 μm.

d Quantification of H3K27me3 domains in PGCs 208 E9.5 WT and 137 E11.5 WT embryos and VYSE in 102 E9.5 Rex1−/− and 151 E11.5 Rex1−/− embryos. LP, length polymorphism.

e Quantification of Cas (129) and 129Cas (677) gene expression (%). Light/dark colours indicate cas or 129 allelic origin, respectively. The number of mice analysed is indicated.

f Quantification of Xist (green), G6pdx (blue) and Mecp2 (pink) expression in WT and Rex1−/− embryos and VYSE in 4-week-old mice. Light/dark colours indicate cas or 129 allelic origin, respectively. The number of mice analysed is indicated.
from E11.5. Rnf12−/−;Rex1−/− extraembryonic VYSE and embryos confirmed normal initiation of iXCI and rXCI similar to WT embryos, with preferential expression of Xist from the paternal c57X chromosome in VYSE, and skewed rXCI towards inactivation of the 129 X chromosome observed in the embryo (Fig. 5c, d; Supplementary Fig. 9d). Rnf12−/−;Rex1−/− and Rnf12−/−;Rex1−− after Rnf12 adults showed no differences in rXCI with WT adults (Fig. 5e, f; Supplementary Fig. 9e). These results show that the lethality due to the lack of iXCI in Rnf12 KO mice can be fully rescued by knocking out Rex1, indicating that RNF12-mediated targeting of REX1 is crucial for proper regulation of iXCI.

Discussion
The intricate relationship between REX1 and RNF12 during embryonic development is intriguing. REX1 is expressed during all stages of pre-implantation development when iXCI takes place, and decreases rapidly before rXCI initiates in epiblast cells soon after implantation14,19. Although Rex1+/− litters are smaller, possibly due to placental imprinting problems12, no sex-bias was observed in our study. The absence of Rex1 did not affect iXCI, nor XCR, which does not completely rule out a role for Rex1 in XCR, as this process might be regulated by redundant mechanisms possibly including Prdm14 and other putative Xist regulators81.

Our studies indicate that REX1 is the main target of RNF12 in the initiation of iXCI in the mouse. It has been previously shown that RNF12 removal leads to absence of Xist clouds in trophoblast cells, resulting in lack of iXCI and embryonic lethality7. We propose that this absence of Xist clouds is caused by increased REX1 levels in the Rnf12−/− pre-implantation embryo. Lethality associated with maternal inheritance of an Rnf12 mutant allele is bypassed by removing Rex1. In this line, at least one copy of Rnf12 needs to remain active for the iXCI process to proceed by preventing accumulation of REX1 and subsequent silencing of Xist on the paternal X, similar to our findings in rXCI in ESCs10,11. We propose a model where the Rnf12-Rex1 axis regulates initiation of iXCI and the maintenance of the Xi in the pre-implantation embryo (Fig. 6). During pre-implantation development, both Rex1 and Rnf12 are expressed. If the Rnf12 mutant allele is maternally transmitted, Rnf12−/− cells in the pre-implantation embryo will initiate inactivation of their paternal WT X chromosome, effectively leading to an Rnf12 homozygous knock out situation. This results in transient accumulation of REX1 protein, not detectable by IF staining, but high enough to likely repress Xist expression from the paternal X, analogous to its action in preventing rXCI in ESCs11. A small number of cells will escape this situation and inactivate the paternal X chromosome but the general lack of maternal Xi will eventually cause the death of the embryo. If Rex1 is genetically removed, Xist expression can no longer be repressed, allowing the inactivation of the paternal X. Maternal transmission of an Rnf12 mutant allele is in this way no longer lethal as iXCI is rescued.

Overexpression of Rnf12 has also been shown to facilitate erasure of the repressive imprint on the maternally inherited Xist allele in XmXm parthenogenetic embryos25. This effect was eliminated by collective knockdown of Rnf12 and Rex1, indicating that REX1 is normally involved in maintenance of Xist repression on the Xm. These and our results highlight the fact that REX1 expression needs to be precisely regulated. Too much REX1 results in repression of Xist on the Xp and embryonic lethality, whereas too little results in de-repression of Xist in an XmXm parthenogenetic setting. Interestingly, Rex1 KO embryos with bi-parental

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**Fig. 4** Rnf12 KO embryos show REX1 stabilization in embryonic and extraembryonic tissues. a Sex and genotype distribution from different matings of Rnf12-deficient mice in a C57BL/6 background. Number of breedings, number of mice per breeding and total number of mice are indicated. Note that no female embryos were born with a maternally transmitted Rnf12 deleted allele. No significant differences were observed between the number of females with a paternal mutant allele and their WT brothers (χ² test, p > 0.05). A significant slight lethality associated with the mutation in male mice compared to WT brothers was observed (χ² test, p = 1.05E−4). b Representative Z-stack projections of WT, Rnf12−/− and Rnf12−/− E4.5 blastocysts immunostained for REX1 (red), H3K27me3 (X marker, green), the trophectoderm marker CDX2 (grey) and DNA (DAPI, blue). Scale bars: 20 μm.
Xm and Xp chromosomes do not display any iXCI defect indicating that additional mechanisms are in place to facilitate maintenance of iXCI during embryonic development in the absence of REX1. During rXCI, the Xist 5′ regulatory region containing YY1 binding sites becomes asymmetrically methylated. Mono-allelic methylation prevents YY1 binding and activation of that Xist allele, while binding competition of YY1 and REX1 to the unmethylated allele results in activation and repression of Xist, respectively. In iXCI, a similar mechanism might be in place to facilitate Xist expression from the paternal X,
in conjunction with a repressive imprint on the maternal Xist allele. Accumulation of R鑫1 in the absence of Rnf12 might outcompete the binding of YY1 and prevent the transcriptional activation of the paternal Xist.

Our present and previous studies indicate that in vitro, Rnf12-deficient ESCs show a complete loss of R鑫1 in a 129/Sv:Cast/Eij hybrid genetic background^{17}. For the present study, we generated Rnf12<sup>−/−</sup>−/− ESCs through complete removal of the open reading frame of Rnf12, and compared these cells with Rnf12<sup>/−</sup>−/− ESCs that we generated in a previous study, which express a 333 aa N-terminal peptide that does not contain the catalytic Ring finger domain. Both Rnf12<sup>−/−</sup>−/− ESCs and Rnf12<sup>/−</sup>−/− ESCs display stabilization and nuclear localization of R鑫1, contrasting a recent report that suggested a role for this 333 aa N-terminal peptide in the nuclear localization of the stabilized R鑫1^{27}. Our quantitative mass spectrometry analysis revealed R鑫1 to be the main target of RNF12. Moreover, the loss of r鑫1 phenotype observed in differentiating Rnf12<sup>−/−</sup>−/− KO ESCs is rescued in a compound Rnf12<sup>−/−</sup>−/−:R鑫1<sup>ΔΔ</sup>−/− ESC background. This result indicates that R鑫1 accumulation instigated by the loss of RNF12 is key to the absence of Xist upregulation. In addition, loss of R鑫1 leads to a significant increase of cells with two Xist clouds in differentiated R鑫1<sup>−/−</sup>−/− and R鑫1<sup>ΔΔ</sup>−/− ESCs. These findings and the high turnover of these proteins, mediated by proteasomal degradation, provides a powerful feedback mechanism preventing XCI of both X chromosomes during ESC differentiation. In vivo, the role of the Rnf12-R鑫1 axis in r鑫1 is taking place^{19}, possibly allowing the embryos to overcome a R鑫1-mediated block of r鑫1 in Rnf12<sup>−/−</sup>−/− epiblasts as was observed in tetraploid complementation assays^{8}. In addition, in embryos the regulation of r鑫1 seems more robust than in vitro, which might explain why we observe a subpopulation of R鑫1<sup>−/−</sup>−/− and R鑫1<sup>ΔΔ</sup>−/− ESCs with two Xist clouds whereas in R鑫1<sup>−/−</sup>−/− and R鑫1<sup>ΔΔ</sup>−/− embryos and adults rXCI appears unaffected. If XCI of two X chromosomes happens in the developing epiblast, we anticipate that these cells will quickly restore the XaXa status to allow reinitiation of XCI, or will be counter selected and eliminated from the embryo. Intriguingly, the fact that Rnf12<sup>−/−</sup>−/−:R鑫1<sup>ΔΔ</sup>−/− DKO mice are born, and that r鑫1 takes place in Rnf12<sup>−/−</sup>−/−:R鑫1<sup>ΔΔ</sup>−/− DKO ESCs, highlights the existence of additional regulatory mechanisms that act in concert with the Rnf12-R鑫1 axis to properly and timely execute XCI.

**Methods**

**SILAC labelling of ESCs.** For the SILAC experiments, undifferentiated female WT line F1 2-1 and Rnf12<sup>−/−</sup>−/− ESCs^{27} were used. ESC lines were metabolically labelled by culturing either in “light” or “heavy” media for at least five passages (around 2 weeks), in order to achieve maximum incorporation of the isotope labelled amino acids into the proteins. For the protein stability experiment, cells were treated with either vehicle (dimethyl sulfoxide, DMSO) or proteasome inhibitor (15 μm MG132, Sigma, C2211) for 3 h prior to harvesting.

SILAC medium containing DEMEM High Glucose (4.5 g l<sup>−1</sup>) devoid of arginine and lysine (PAA Cell Culture Company) supplemented with 15% dialyzed foetal bovine serum (Invitrogen), 100 μM l−1 penicillin, 100 μg ml<sup>−1</sup> streptomycin, 200 mM GlutaMAX (Invitrogen), 0.1 mM non-essential amino acids (NEAA), 1000 U ml<sup>−1</sup> IF, 0.1 mM 2-mercaptoethanol (Sigma) and 200 mg l<sup>−1</sup> l-proline (Sigma, P0380) to avoid arginine-to-proline conversion^{28}. Either naturally occurring isotopes of lysine and arginine (Argo, Sigma, A5006; Lys6, Sigma, L5501) (light media) or the heavy isotopes (Arg10, Cambridge Isotope Laboratories, CNLM-539; Lys8, Cambridge Isotope Laboratories CNLM-291) (heavy media) were added to the medium at a concentration of 100 mg l<sup>−1</sup> for lysine and 40 mg l<sup>−1</sup> for arginine.

Medium was refreshed every day, and the cells were split every other day. The ESC lines were cultured for three passages on feeder cells and for an additional two

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**Fig. 5** Rnf12<sup>−/−</sup>−/−:R鑫1<sup>−/−</sup>−/− double knockout mice are viable and have normal iXCI and rXCI. a Sex and genotype distribution from different Rnf12 mutant crossings in an R鑫1-deficient background. Number of breedings, number of mice per breeding and total number of mice are indicated. Note that female embryos were born with a maternally transmitted Rnf12 deleted allele in an R鑫1<sup>−/−</sup>−/− background. No significant bias in gender or genotype was observed (χ², p > 0.05). b Representative Z-stack projections of WT, Rnf12<sup>−/−</sup>−/−:R鑫1<sup>−/−</sup>−/− and Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/− E4.5 blastocysts immunostained for R鑫1 (red), H3K27me3 (Xi marker, green), the trophectoderm marker CDX2 (grey) and DNA (DAPI, blue). Scale bars: 20 μm. WT samples are same control samples as in Fig. 4b. c Xist, G6pdx and Mecp2 allele-specific RNA expression analysis in E11.5 female Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/− embryos (E) and corresponding VYSE. d Quantification of the average allelic Xist (green), G6pdx (blue) and Mecp2 (pink) expression in E11.5 female WT and Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/− embryos and VYSE in e. Light/dark colours indicate cas or 129 allelic origin, respectively. WT samples are same control samples as in Fig. 3f. The number of mice analysed is indicated. e Xist, G6pdx and Mecp2 allele-specific RNA expression analyses in heart (H), liver (L), stomach (St), brain (Br) and lung (Lu) in two Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/−−/− and Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/−−/− 4-week-old female mice. f Quantification of the average allelic Xist (green), G6pdx (blue) and Mecp2 (pink) expression in WT and Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/−−/− and Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/−−/− 4-week-old female mice in e. Light/dark colours indicate cas or 129 origin, respectively. WT samples are the same control samples as in Fig. 3h. The number of mice analysed is indicated.

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**Fig. 6** Model for iXCI regulation by the R鑫1-Rnf12 axis. In a WT background, R鑫1 is expressed during the early stages after fertilization, but is degraded by RNF12. This leads to the upregulation of the paternal Xist allele, whilst its maternal counterpart is prevented from upregulation by an imprint. In Rnf12<sup>−/−</sup>−/− embryos, the RNF12 level is sufficient to prevent R鑫1 stabilization, allowing for paternal Xist upregulation. However, this will result in inactivation of the paternal Rnf12 copy, which leads to an Rnf12 KO situation, in its turn resulting in brief R鑫1 stabilization and preventing further paternal Xist upregulation. This feedback loop will prevent proper iXCI in trophoblast cells leading to Rnf12<sup>/−</sup>−/− embryo death. In Rnf12<sup>−/−</sup>−/− embryos, in the absence of RNF12, R鑫1 accumulates, preventing upregulation of Xist from the paternal allele. Xist is absent and the embryos die. In Rnf12<sup>/−</sup>−/−:R鑫1<sup>−/−</sup>−/− embryos, the entire R鑫1-Rnf12 axis is absent and iXCI can proceed normally as in WT embryos.

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passages feeder-free, on 0.2% gelatin-coated cell culture plates. The SILAC media in a Q Exactive mass spectrometer (Thermo) or on an 1100 series capillary liquid chromatography system (Agilent Technologies) was performed on an EASY-nLC 1000 system coupled to a Q Exactive Q-Exactive mass spectrometer (Thermo) or on an 1100 series capillary liquid chromatography system (Agilent Technologies) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo), both operating in positive mode. Peptides were trapped on a ReproSil C18 reversed phase column (Dr. Maisch, 1.5 cm x 100 μm) at a rate of 8 μl min⁻¹ and separated on a ReproSil C18 reversed-phase column (Dr. Maisch; 15 cm x 50 μm) using a linear gradient of 0–80% acetonitrile (in 0.1% formic acid) during 120–170 min at a rate of ~200 nl min⁻¹ using a splitter (LTQ-Orbitrap XL) or not (Q Exactive). The elution was directly sprayed into the electrospray ionization (ESI) source mass spectrometer. Spectra were acquired in continuous mode, with the intention to introduce the data in a de novo database using CID (LTQ-Orbitrap XL) or HCD (Q Exactive).

Raw mass spectrometry data were analysed with MaxQuant software (version 1.5.6.0) as described before[55]. A false discovery rate of 0.01 for proteins and peptides and a minimum peptide length of 6 amino acids were set. The Andromeda search engine[56] was used to search the MS/MS spectra database (taxonomy: Mus musculus, release October 2016) concatenated with the reversed versions of all sequences. A maximum of two missed cleavages was allowed. The peptide tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.6 Da for CID spectra and to 20 mnu for HCD spectra. The enzyme specificity was set to trypsin and cysteine. Carbamidomethylation was set as a fixed modification, while protein N-acetylation and GlyGly (K) were set as variable modifications. MaxQuant automatically quantified peptides and proteins based on standard SILAC settings (multiplicity = 2, RR10). SILAC protein ratios were calculated as the median of all peptide ratios assigned to the protein. In addition a posterior error probability (PEP) threshold for MS/MS spectra was required. In case the identified peptides of two proteins were the same or the identified peptides of one protein included all peptides of another protein, these proteins were combined by MaxQuant and reported as one protein group. Before further statistical analysis, known contaminants and reverse hits were removed. For experiments 1 and 3, proteins with ‘razor = unique peptides’ <1 were selected. Further data analysis was performed using the Perseus software suite. SILAC H/L ratios of two replicate experiments from the MaxQuant proteingroups.txt output table were imported into Perseus and statistical outliers were determined using standard one-sample two-sided t-testing (parameters: p-value < 0.05, SD: 0). Proteins displaying averaged H:L ratios >1.5 were selected for further analysis.

**Cell culture.** 129/Sv-Cast/Ei[57] female ESCs were cultured as previously described[41]. In brief, ESCs were grown on feeder cells in DMEM (GIBCO), 15% standard foetal calf serum (FCS), 100 U ml⁻¹ penicillin/streptomycin, 0.1 mM NEAA, 0.1 mM 2-mercaptoethanol and 5000 μM LIF.

For embryoid body differentiation, we plated 0.25 × 10⁶ ESCs (day 0) in a 10-cm bacterial dish and let them differentiate in normal ESC medium with serum. During the next days, blastocysts were allowed to attach on gelatin-covered coverslips and grown at 37 °C, 5% CO₂. The differentiation was followed by regular checks. No randomization of animals was performed. No blinding of the experimenters was used. The investigators were not blinded to group allocation of mice during the experiments.

**Generation of Rex1 and Rnf12 deficient mice.** All animal experiments were performed according to the legislation of the Erasmus MC Rotterdam Animal Experimental Commission.

The Rex1 KO mice were generated from Rex1−/−(129/−/−) heterozygous KO ESC made by the bacterial artificial chromosome (BAC) targeting method. The targeting vector was designed to replace part of exon 4, the only coding exon of Rnf12−/−(129/−/−)-target 2; 5′-GGAAACAAAGTACTCTAAA-3′ for Rnf12-target 2; 5′-CCGTGAATCACACCACTCC-3′ for Rex1-target 1; 5′-TCCACTCTGATGATGCGAC-3′ for Rex1-target 2; 5′-AAACCTTCTCCTGAGCTG-3′ for Rex1-target 3; 5′-CTACTCCGAAACCTGGTCGA-3′ for Rex1-target 4.

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Protein extraction and western blot. To obtain nuclear extracts, cells were harvested in 1 ml ice-cold PBS plus complete protease inhibitor (Roche, 04693132010) and 15 μM MG132 (Sigma, C2211). Cell pellets were incubated with 400 μl buffer A (10 mM Hepes, 1.5 mM MgCl2, 10 mM KCl, 0.5 mM DTT, protease inhibitor, and 15 μM MG132) for 10 min on ice, vortexed for 20 s and centrifuged (2000 rpm/5 min/4 °C). Nuclei were lysed by adding the pellet volume of buffer C (20 mM Hepes, 5% glycerc, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor and 15 μM MG132) for 20 min on ice, centrifuged (14,000 rpm/2 min/4 °C) and the supernatant was used as nuclear protein. Protein concentrations were determined using NanoDrop (Thermo Scientific). Western blotting was performed as described above.

RNA Fish. In brief, ESCs or differentiating ESCs were fixed for 10 min at RT with 4% PFA in PBS. Cells were subsequently washed three times with 70% EtOH for 3 min and permeabilized with 0.2% BSA at 75 °C for 30 min and fixed for 20 min with 4% PFA in PBS. Coverslips were then put on slides carrying the Xist hybridization probe. The probe was made as follows: 2 μg of a 5.5-kb BglII cDNA fragment covering exons 3–7 of mouse Xist was DIG-labeled (DIG Nick-Translation Kit, Roche) following the manufacturer’s instructions. About 1 μl of the probe was diluted in hybridization mix (40% formamide, 5% glycerc, 1 mg/ml yeast tRNA, 2× SSC, 4 μg/ml yeast RNA, 30 mM Hepes, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, protease inhibitor and 15 μM MG132) for 20 min on ice, centrifuged (14,000 rpm/2 min/4 °C) and the supernatant was used as protein extract. Probes were used at a concentration of 10 ng/μl. Confocal images were collected using a Zeiss LSM700 microscope (Carl Zeiss, Jena) and processed with Fiji and Adobe CC Photoshop software (Adobe). Cell counts were performed using Image J (Fiji) software.

When necessary, blastocysts were genotyped by PCR after FI/FISH and confocal microscopy. Briefly, embryos were individually recovered, washed in PBS and lysed in 10 μl of lysis buffer (AM1722, Cells-to-cDNA II Kit, Thermo Fisher Scientific) for 15 min at 75 °C. After 1 μl of the lysis solution was directly used in a 25 μl PCR reaction. Primer pairs used for the genotyping are listed in Supplementary Table 1.

PGCs IF staining. After embryo isolation from the uteri, regions containing the developing germ cells were dissected from E9.5 and E11.5 embryos. E9.5 embryo hindguts and E11.5 embryo trunk were fixed in ice cold 4% PFA for 3 h, followed by two washes in PBS. Immunofluorescence was performed using standard histology procedures and 5 μm paraffin sections were dissected with a Cryostat HM 560. Heat-mediated (900 W in a microwave for 20 min) epitone retrieval in citrate buffer pH 6.0 was performed on paraffin sections. After cooling down, sections were blocked with blocking solution (2% BSA, 5% donkey serum in PBS) for 30 min at RT, followed by primary and paraffin embedding antibody incubation at 4 °C overnight. The next day, slides were washed in PBS and incubated with secondary antibodies for 1 h at RT. Slides were then washed in PBS and mounted with ProLong® Gold Antifade Mountant with DAPI (Thermo Fisher Scientific). Confocal imaging was performed on a Zeiss LSM700 microscope (Carl Zeiss, Jena). The following primary antibodies were used: anti-Oct4 (Santa Cruz, sc-46227, 1:250), anti-Thy1.2 (BD, 554502, 1:250), anti-rabbit anti-H3K27me3 (Diagenode, C15310069, 1:500). Embryos were then blocked three times in blocking buffer, incubated briefly in increasing concentrations of Vectashield with DAPI (Vector laboratories) before mounting on polyly-sine slides in small drops of concentrated Vectashield with DAPI.

RT-PCR analysis of mice tissues and embryos. To assess XCI skewing, hybrid female mice (129/Sv-Cast/EJ) were sacrificed by cervical dislocation. Parts of organs were collected, snap-frozen and triturated using micro-pistoles in 1 ml of Trizol reagent (Invitrogen). RNA was purified following manufacturer’s instructions; 1 μg RNA was DNase-treated (Invitrogen, #186806015) and reverse-transcribed with SuperScript II (Invitrogen, #18080051), using random hexamers. Allele-specific Xist expression was analysed by RT-PCR amplification using a pair of primers specific to the Rnf12 or G6pdx 3′ expression. PCR primer sequences are described below. PCR products were digested with the indicated restriction enzymes and analysed on a 2% agarose gel stained with ethidium bromide. Allele-specific expression was determined by measuring relative band intensities using a Typhoon image scanner (GE healthcare) and ImageQuant TL software.

E11.5 embryos were obtained by natural matings and dissected from decidual tissues. The endoderm (VYSE, imprinted XCI) and mesoderm (VYSM, random XCI) layers of the visceral yolk sac (VYS) were isolated by enzymatic approach described in ref. 34. Briefly, yolk sacs were dissected from E13.5–E14.5 embryos and incubated at 4 °C for 1.5 h with gentle shaking in a mixture of 0.5% trypsin (Sigma, 59427 C) and 2.5% pancreatin (Sigma, P7545) in Ca2+-free HANS buffer (154 mM NaCl, 5 mM KCl, 2 mM MgCl2, and 0.8 mM CaCl2). After 15 min, sections were blocked with mounting medium with DAPI (Molecular Probes). Images were acquired using a confocal Zeiss LSM700 microscope (Carl Zeiss, Jena) with Zen image acquisition software and processed with Fiji and CC Photoshop software (Adobe).

Blastocysts IF staining. To collect blastocysts, 5- to 8-week-old female mice were superovulated by intraperitoneal (i.p.) injection of 5 IU of pregnant mare serum gonadotropin (Folligon; Inviron) followed by an i.p. injection of 5 IU of human chorionic gonadotropin (Chorulon; Inviron) 48 h later. The supere ovulated female mice were mated with selected males. Embryos at the blastocyst stage were harvested by flushing the uteruses at 3.5 days post-coitum in M2 medium. When E4.5 blastocysts were needed, E4.5 blastocysts were isolated from the uteruses, washed in M2 medium and fixed in 4% PFA in PBS for 20 min at RT. Subsequently, embryos were rinsed in PBS containing 0.1% w/v Tween-20 (P3179, Sigma) (PBS-T), permeabilized in PBS containing 0.2% Triton X-100 (23,472-9, Sigma) for 15 min on ice and blocked in blocking buffer (PBS-T, 2% w/v bovine serum albumin (BSA, fraction V), 5% v/v normal goat serum) for 4 h at RT and incubated with appropriate primary antibodies diluted in blocking buffer at 4 °C overnight. The following antibodies were used in this study: goat anti-REX1 (Santa Cruz, sc-50670, 1:100), goat anti-KLF4 (R&D, AF1358, 1:400), goat anti-OCT4 (Santa Cruz, sc-8628, 1:1000), mouse anti-β-Actin (Sigma, A5452, 1:500), mouse anti-CDX1 (BioGenex, MU392A-UC, 1:400), rabbit anti-NANOG (Calbiochem, SC1000, 1:100) and rabbit anti-H3K27me3 (Diagenode, C15310069, 1:500). Rxi

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Data availability
The mass spectrometry proteomics data have been deposited to the ProteomXChange Consortium via the PRIDE[35] partner repository with the dataset identifier PXD010944. All other relevant data supporting the key findings of this study are available within the article and its Supplementary Information files or from the corresponding author upon reasonable request.

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Author contributions
C.G. and J.G. conceived the study. C.G., H.M.-B. and J.G. designed the experiments. C.G. and H.M.-B. performed most experiments, assisted by E.R. for the generation of the Rex1 knockout mice. C.G. and J.D. performed the mass spectrometry work. J.G., C.G. and H.M.-B. wrote the manuscript.

Additional information
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