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Review Article

How does the Xist activator Rlim/Rnf12 regulate Xist expression?

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The long non-coding RNA (lncRNA) Xist is crucially involved in a process called X chromosome inactivation (XCI), the transcriptional silencing of one of the two X chromosomes in female mammals to achieve X dosage compensation between the sexes. Because Xist RNA silences the X chromosome from which it is transcribed, the activation of Xist transcription marks the initiation of the XCI process and thus, mechanisms and players that activate this gene are of central importance to the XCI process. During female mouse embryogenesis, XCI occurs in two steps. At the 2–4 cell stages imprinted XCI (iXCI) silences exclusively the paternally inherited X chromosome (Xp). While extraembryonic cells including trophoblasts keep the Xp silenced, epiblast cells that give rise to the embryo proper reactivate the Xp and undergo random XCI (rXCI) around implantation. Both iXCI and rXCI are dependent on Xist. Rlim, also known as Rnf12, is an X-linked E3 ubiquitin ligase that is involved in the transcriptional activation of Xist. However, while data on the crucial involvement of Rlim during iXCI appear clear, its role in rXCI has been controversial. This review discusses data leading to this disagreement and recent evidence for a regulatory switch of Xist transcription in epiblasts of implanting embryos, partially reconciling the roles of Rlim during Xist activation.

Introduction

During embryogenesis X dosage compensation in female mice occurs in two waves. An early, imprinted form of XCI (iXCI), which silences exclusively the paternally inherited X (Xp), follows soon after zygotic genome activation (ZGA) at the end of the two-cell stage. While this pattern of XCI is maintained in extraembryonic tissues including trophoblast and primitive endoderm, epiblast cells which give rise to the embryo proper reactivate the Xp (XCR) and undergo a random form of XCI (rXCI) around implantation [1,2]. The X-linked long non-coding RNA *Xist* plays crucial roles during both forms of XCI and paints the X from which it is expressed [3,4]. Thus, the onset of Xist transcription is considered as the initiation of the XCI process. Because early during pre-implantation development the maternally inherited Xist gene contains an imprint that abolishes its activation [5,6], Xist is only activated from the paternally inherited allele, thus ensuring silencing exclusively of the Xp during iXCI. However, in implanting embryos, this imprint is no longer present and thus either the maternally or paternally inherited X is silenced during rXCI in epiblast cells. To ensure the random and mono-allelic up-regulation of Xist, mathematical modeling has provided strong evidence for the requirement of a trans-acting X-linked activator inducing Xist transcription when expressed from two alleles, with the silencing of one activator allele preventing up-regulation of Xist on the other X chromosome [7]. Therefore, whether or not an X-linked activator requires two alleles to activate Xist transcription is highly significant as to the underlying mechanisms.

The *Rlim* gene (also known as Rnf12), which is localized on the X chromosome encodes a RING H2 zinc finger ubiquitin ligase (E3) [8,9]. In cells, RLIM can shuttle between the nucleus and cytoplasm in a phosphorylation-dependent manner but localizes mainly to the nucleus [10,11], where

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many of its substrates — including transcription factors and transcriptional co-regulators — reside [9,12–15]. Like many other RING finger E3s, RLIM self-ubiquitinates and mutations or deletion of RLIM's RING finger can induce gain of function activities such as stabilization of the mutated Rlim as well as its substrate proteins [9,14,16,17].

RLIM activates Xist via the proteasomal targeting of REX1 (Figure 1), a potent repressor of Xist transcription [12]. Indeed, the early embryonic lethal phenotype observed in Rlim KO females [18,19] is largely reversed in Rlim-Rex1 double KO mice [20], demonstrating a central functional interaction of this module during XCI *in vivo*. However, while data on the crucial involvement of Rlim during iXCI appear clear, its role in rXCI has been controversial. Crucial roles of high, dose-dependent cellular RLIM levels achieved by expression from two alleles for the activation of Xist have been reported in female embryonic stem cells (ESCs). This contrasts dispensable functions of Rlim during rXCI in mice with Xist activation dependent on low nuclear REX1 levels (Figure 1). The different mechanisms of how the Rlim-Rex1 axis promotes XCI have major consequences for iXCI, XCR and rXCI as they lead to divergent biological concepts. This review discusses mechanisms of how

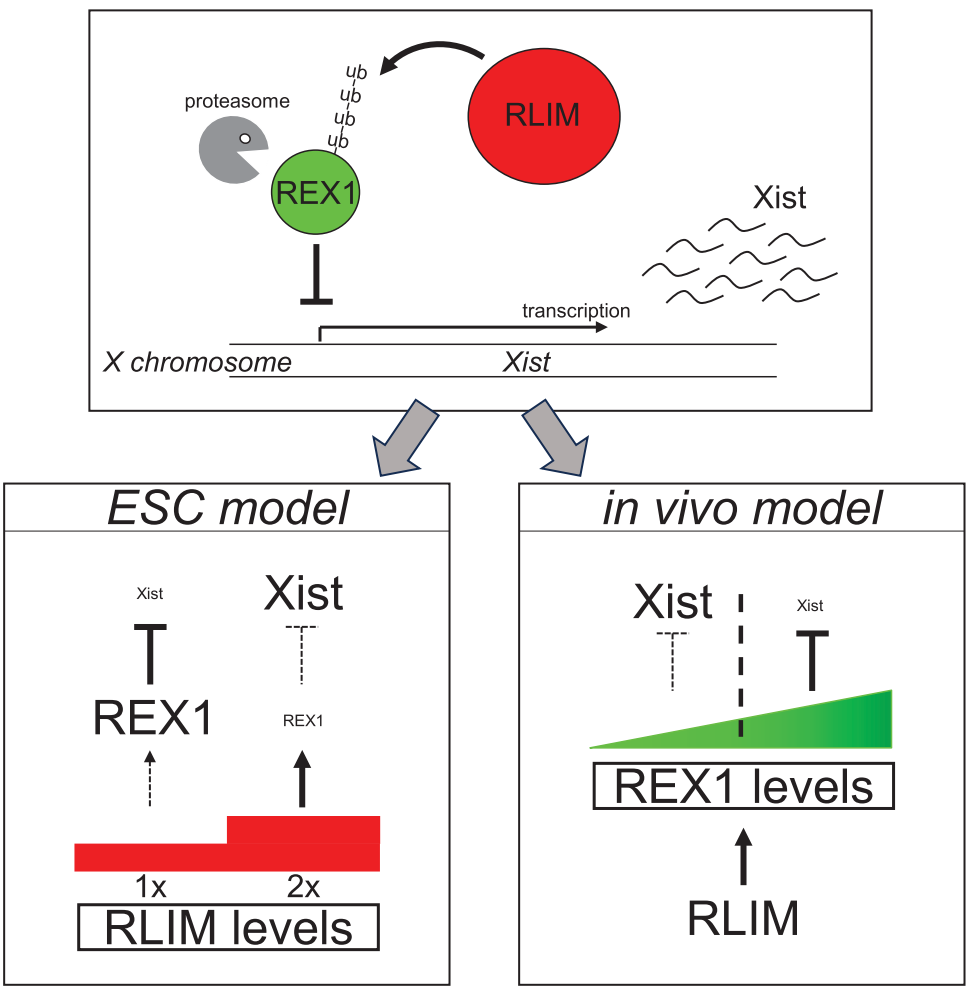

Figure 1. Two distinct models of how RLIM, via proteasomal targeting of REX1, activates Xist transcription.
Upper panel: REX1 binds to regulatory sequences of the Xist gene, thereby repressing transcription and XCI. The E3 ligase RLIM activates Xist transcription by mediating polyubiquitination of REX1 (ub) leading to proteasomal degradation. Lower panel, left: Current model of how Xist is activated by the Rlim-Rex1 module based on ESC data (ESC model). Xist activation depends on RLIM expressed from two alleles. Not shown are postulated additional factors. Lower panel, right: Model of mechanisms underlying Xist activation by the Rlim-Rex1 module in female mice (*in vivo* model). Activation of Xist can occur when REX1 is below threshold levels, if RLIM is present or not. Postulated additional factors regulating Xist transcription are not shown.

Xist transcription is activated by Rlim and its importance during XCI, thereby partially clarifying and reconciling this controversy.

Overcoming transcriptional repression by Rex1: the *in vivo* model

The *in vivo* model of the roles of the Rlim-Rex1 module for Xist activation has been proposed recently [21] and is mostly based on data obtained in female mice with a conditional Rlim KO (cKO). This model assigns a critical role to nuclear levels of Rex1 in the activation of Xist, independently of the Rlim status (Figure 1). We first summarize the published *in vivo* data in historical order, comparing data focusing on those conflicting with the ESC model.

Early on, it was discovered that a Rlim cKO targeted to female oocytes, led to a female-specific parent of origin effect in offspring: a maternally transmitted Rlim KO allele (KO_m) resulted in embryonic lethality of all female offspring at peri-implantation stages, while pre-implantation development appeared normal. KO_m hemizygous males or heterozygous female embryos receiving a paternally transmitted Rlim KO allele (KO_p) developed normally. The lethality of female KO_m embryos was due to failure of iXCI as no signs of XCI could be detected in trophoblast tissues. However, blastocyst outgrowths of female E4.5 embryos with a germline Rlim KO developed Xist clouds in the epiblast region as well as ESCs isolated from these embryos. This prompted the original conclusion that Rlim is important for iXCI but not rXCI in female mice [18]. The claim that Rlim is dispensable for rXCI in mice was confirmed in tetraploid complementation assays in which female ESCs lacking Rlim were able to form post-gastrulation embryos that displayed signs of XCI. This was further corroborated by mouse genetics, targeting the Rlim allele on the Xm in pre-implantation epiblast cells via a paternally transmitted Sox2-Cre transgene, which allowed the efficient generation of adult female animals displaying normal XCI but systemically lacking Rlim [22]. However, these findings of Rlim-independent Xist activation in female epiblast cells or female ESCs *in vitro* were in direct conflict with results obtained from an independent female Rlim KO model in ESCs (see below). Targeting the Rlim cKO in embryos after ZGA by inducing Cre recombinase expression via a paternally transmitted Rosa26-Cre transgene recapitulated the early embryonic lethality in females [19]. Together with data obtained from single embryo RNA-seq experiments on pre-implantation embryos systemically lacking Rlim, the crucial role that Rlim plays for X dosage compensation during iXCI was confirmed [19]. Initial Xist RNA expression, formation of Xist clouds and signs of X-silencing were detected in female embryos lacking Rlim up to morula stages. However, at blastocyst stages these embryos no longer displayed signs of elevated Xist mRNA and clouds as well as X-silencing. It was concluded that during iXCI, Rlim is important for the maintenance but not initiation of Xist transcription [19]. Again, the Rlim-independent activation of Xist was in direct conflict with the ESC model. As REX1 was identified as a target of RLIM in ESCs [12], examining the genetic interaction between Rlim and Rex1 in mouse embryos identified REX1 as the critical *Rlim* target for iXCI as the Rlim phenotype was reversed by additional KO of Rex1 [20]. This finding indicated that Rlim regulates iXCI by targeting the Xist repressor REX1 for proteasomal degradation (Figure 1) and thus both genes work as a functional module during pre-implantation development. Testing the expression profiles of the Rlim-Rex1 module via immunostaining comparing early WT and Rlim KO mouse embryos revealed that low REX1 levels are dependent on the presence of Rlim throughout pre-implantation development. This function of Rlim was independent of sex, occurred in most/all cells of the embryo and was particularly pronounced at blastocyst stages [21]. Such expression profiles are fully consistent with crucial roles of the Rlim-Rex1 module during iXCI (Figure 1). Moreover, as Rex1 mRNA levels are up-regulated from low levels after ZGA to high levels at blastocyst stages [17], such pattern explains the Rlim functions for the maintenance as opposed to initiation of Xist transcription [19], with a stringent requirement of RLIM's E3 ligase functions to ensure low REX1 protein levels at later pre-implantation stages. However, the functional interaction between Rlim and Rex1 dramatically changes with a precipitous Rlim-independent down-regulation of REX1 at implantation specifically in epiblast cells [21,23]. As a consequence, low REX1 protein levels are no longer Rlim-dependent (Figure 2A), allowing for the activation of Xist specifically in epiblast cells [21]. Thus, the functional interaction of the Rlim-Rex1 module is active throughout pre-implantation development but severed specifically in epiblast cells of implanting embryos, indicating a major switch in the regulation of Xist transcription, and explaining as to why Rlim is crucial for iXCI but dispensable for rXCI [21]. Considering the onset of Xist mRNA expression in female embryos lacking Rlim during both iXCI and rXCI at time points when Rex1 protein levels are low, a common theme emerges indicating that it is REX1 levels that critically influence Xist expression, thereby providing a compelling mechanism of iXCI regulation by the Rlim-Rex1 module in mice [21] (Figure 1).

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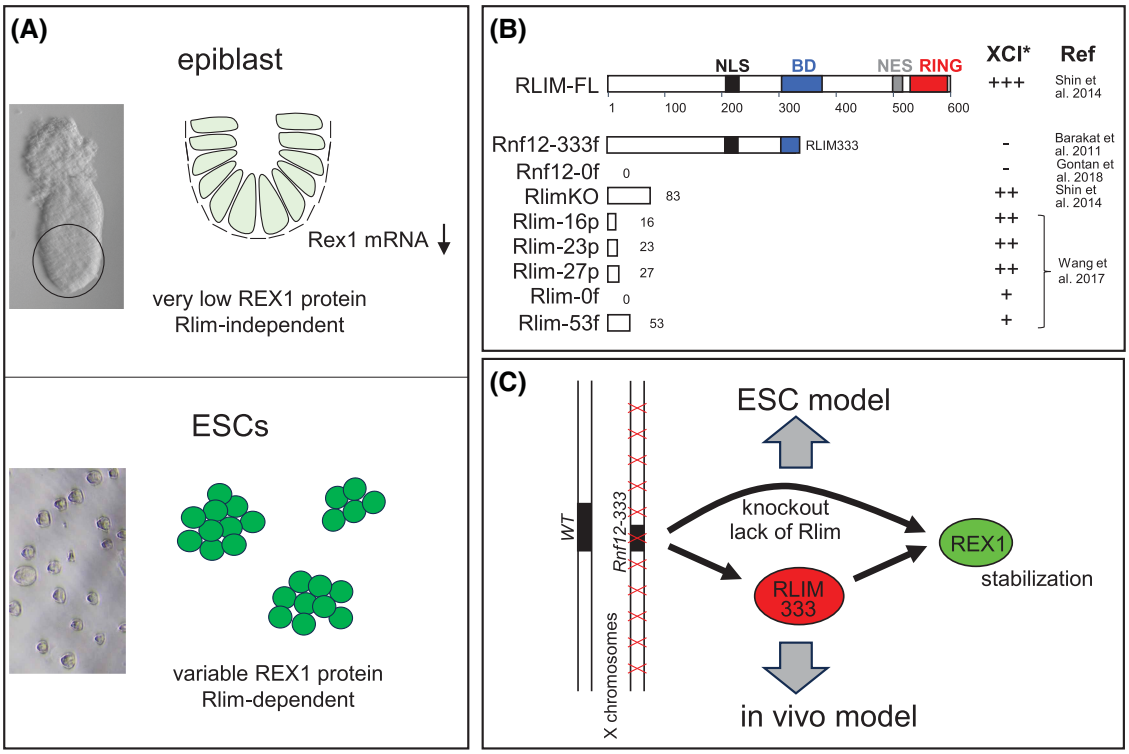

Figure 2. Roles of the RLIM/Rex1 module during random XCI.

(**A**) Difference in REX1 regulation and status in epiblast cells and ESCs at the timepoint when XCI is triggered. Upper panel: In epiblast cells levels of REX1 are very low and Rlim-independent. Lower panel: Different ESC lines display variable REX1 levels. As ESCs represent cells at pre-implantation stages, REX1 is under the control of Rlim. (**B**) Effects of Rlim on XCI in female ESC models. RLIM full length protein with functional domains is shown. NLS, nuclear localization sequence; BD, basic domain; NES, nuclear export sequence; RING, RING finger domain. Background of ESCs (f, F121; p, pGK12.1), expected peptides potentially expressed from each KO locus, and ability to develop Xist clouds are indicated. (**C**) Example of how the presence of RLIM333 can affect/obscure data interpretation. In Rnf12-333f heterozygous ESCs XCI is strongly biased towards the mutated allele. In support of the ESC model this bias has been interpreted from a KO perspective concluding that Rlim regulates X choice. An alternative explanation for this bias is that the presence of RLIM333 inhibits Xist transcription by stabilization of REX1 and XCI can only occur when RLIM333 is removed by silencing of the mutated allele. Such scenario is in support of the *in vivo* model. Within this ESC system these different scenarios cannot be distinguished.

Dose-dependent activation of Xist by Rlim: the ESC model

The ESC model implies that RLIM levels expressed from two alleles reach a critical dose required for down-regulation of REX1 and activation of Xist. XCI leads to silencing of one Rlim allele, thereby the dose of RLIM falls below the threshold ending the XCI process. This model, which is based on early results obtained in ESCs, has been the prevailing model over the last decade [1,24,25] and is attractive as it complies with theoretical considerations about a trans-acting X-linked activator of Xist during rXCI.

This model is derived mostly by data obtained from female ESCs in which the Rlim gene is mutated by the insertion of foreign DNA into the Rlim gene (Rnf12-333f; Figure 2B). In heterozygous Rnf12-333f ESCs, the initiation of Xist transcription and XCI is markedly delayed with a strong bias to inactivate the KO allele [26,27]. Moreover, a female Rnf12-333f ESC derivative carrying the same Rlim mutation on both alleles is unable to activate Xist [27]. This ESC line expresses very high levels of REX1, which, upon induction of differentiation, appears partially resistant to down-regulation [12]. Interpreted as KO effects, it was concluded that high RLIM levels expressed from two Rlim alleles is required for the random, monoallelic up-regulation of Xist, but that other contributing factors must exist [24]. Other data in favor of the ESC model was the finding that the ectopic overexpression of RLIM induces Xist expression in male ESCs causing inactivation of the single X

[26]. As male cells carry only one X this result was interpreted as evidence for RLIM expression from two alleles triggering XCI in females.

Rnf12-333f ESC derivatives were used to generate data resulting in claims concerning the contribution of Rlim and cis-acting activators of Xist towards rXCI [28]. Moreover, Rnf12-333f ESCs were used to generate mice and the homozygous Rnf12-333f mutation inhibits iXCI and to some degree rXCI in female embryos leading to embryonic lethality. Importantly, this phenotype is reversed in Rnf12/Rex1 double KO females, in which both iXCI and rXCI occurred in a normal manner [20].

However, the Rnf12-333f mutation is not a KO, but due to a cloning artifact the 'Rnf12KO' locus expresses a truncated RLIM protein containing the N-terminal 333 aa (RLIM333) [17,29], including the nuclear localization sequence and part of the REX1 interaction domain but lacking C-terminal sequences including the RING finger and the nuclear export sequence (Figure 2B). Thus, the nucleocytoplasmic shuttling as well as the E3 ligase activities of this RLIM mutant protein are defective, trapping RLIM333 to the nucleus and altering its regulation including decreased turnover. Importantly, RLIM333 acquires dominant-negative functions towards target proteins, as its expression in ESCs can lead to the stabilization and accumulation of Rex1 in the nucleus [17,29].

The importance of Rlim for XCI in ESCs has been controversial early on, as primary ESCs isolated from female RlimKO mice developed Xist clouds and gave rise to post-gastrulation embryos [18,22]. Over the years several female ESC lines lacking Rlim other than Rnf12-333f derivatives have been generated including in pGK12.1 [30] and F121 [27] backgrounds (Figure 2B), and data on XCI were variable: While the presence of Rlim generally improves efficiency of developing Xist clouds upon differentiation, in some ESC systems its presence was crucial [20], in others it was not [29]; (Figure 2B). At this stage, the underlying reasons for these discrepancies are unclear due to differences in ESC lines and differentiation protocol used [17]. Moreover, some lines do not develop Xist clouds using one differentiation protocol, but Xist clouds can be detected using another (Rnf12-0f, Rlim-0f; Figure 2B). Thus, the importance of Rlim for XCI in female ESCs is variable and the picture remains somewhat unclear due to lack of rigorous control experiments on ESC status, REX1 levels and differentiation protocols.

Discussion

Mechanisms of Xist activation by the Rlim-Rex1 module

The different mechanisms promoted by the ESC vs the *in vivo* models as to how the Rlim-Rex1 module activates Xist transcription, have important implications for the regulation of iXCI, XCR and rXCI, leading to divergent biological concepts. Comparing data compatibility with models, it is important to emphasize that the *in vivo* model is fully consistent with important functions of Rlim for inducing XCI in specific ESC lines, depending on endogenous REX1 levels (as discussed below). Therefore, to differentiate between the two models, data assigning crucial functions for XCI in female ESCs need to be distinguished from those that demand Rlim to be present in two copies, which have been mostly based on results obtained from Rnf12-333f ESCs. In Rnf12-333f ESCs both the lack of functional Rlim and the presence of truncated RLIM333 stabilize REX1, thereby independently affecting the kinetics of the rXCI process (Figure 2C). Within this system, these enhancing effects cannot be distinguished but only KO effects were considered, and this represents a major problem as many of the results that support the requirement of two Rlim alleles can actually be explained by the *in vivo* model, when stabilizing effects of RLIM333 on REX1 are considered. Concerning the effects of RLIM333 on REX1 dynamics, extremely high levels of REX1 protein have been reported in homozygous Rnf12-333f ESCs, which moreover, are mostly resistant to differentiation-induced down-regulation over a period of 72 h [12]. In contrast, in epiblasts lacking Rlim, REX1 is very low/undetectable 6 h after initiation of rXCI [21]. Thus, in contrast with female ESCs lacking Rlim that retain some ability to activate Xist [22,29], induction of rXCI in Rnf12KO systems is excessively impaired. Concerning the skewed rXCI in female ESCs carrying a heterozygous Rnf12-333f mutation: Rather than the requirement of an active copy of Rlim to induce/maintain Xist transcription, the inhibition of Xist transcription by the presence of RLIM333 might account for the observed X-bias in XCI (Figure 2C), and such scenario would be in support of the *in vivo* model. Consistent with such scenario is the observation that heterozygous female RlimKO systems do not display such bias (22). The presence of RLIM333 may also explain discrepancies observed in mice. Rnf12-333f females are defective in iXCI and die around implantation, a phenotype similar to the Rlim phenotype but more severe in terms of Xist transcription and initiation of iXCI onset at pre-implantation stages [18–20].

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Importantly, a significant portion of male Rnf12-333f animals die during embryogenesis [20], a phenotype not observed in RlimKO mice [31], suggesting additional gain of functions of Rlim333 against other Rlim targets [17]. Concerning the Rnf12/Rex1 double KO female mice that undergo mostly normal XCI [20], even though mice used in this study were the Rnf12KO expressing RLIM333 [29], the additional Rex1 KO eliminates all Rlim effects on Rex1 including effects caused by RLIM333.

Thus, by stabilizing REX1 and inhibiting Xist transcription, the presence of RLIM333 is likely to significantly interfere with the Rlim-Rex1 dynamics and the kinetics of the XCI process. While the deletion of Rlim333 in Rnf12-333f ESC improves their ability to activate Xist [29], in all Rnf12-333f derivative cell lines and mice, the consequences of RLIM333 expression have not been addressed. Therefore, combining the existing published data, it cannot be excluded that the misinterpretation of data caused by a cloning artifact significantly contributed to the ESC model.

Concerning male ESCs undergoing XCI upon RLIM overexpression, while the *in vivo* model cannot explain these results, it is unclear how strong an argument this result represents for the ESC model. This is because like male cells, female cells heterozygous for Rlim contain only one copy of Rlim, yet only the female cells undergo XCI while the male cells do not. This indicates a significant difference in mechanisms underlying Xist activation between sexes and that rXCI in females is not associated solely with the number of Rlim alleles. Moreover, the Rex1 KO in male mice does not result in an up-regulation of Xist, indicating that, unlike in females, REX1 is not involved in repressing Xist in male embryos. While the reasons of why Xist transcription is initiated in male ESCs overexpressing RLIM are unclear, more experiments are needed to clarify the roles of the Rlim-Rex1 axis in male ESCs.

Another argument for the ESC model that has repeatedly been made is that because Rlim is X-linked and regulates XCI in ESCs, its mechanisms of action should comply with the mathematical modeling data [1,7,25]. However, because Rlim is not required for activation of Xist during rXCI in mice [21,22], the theoretical prerequisites assigned for X-linked Xist activators during rXCI namely to be present in two copies do not apply.

In contrast, the combined data from mice demonstrate Xist activation in female animals lacking Rlim both during iXCI and rXCI, and the dynamic expression of REX1 mechanistically explains why this is. The *in vivo* model is attractive because it is consistent with most/all *in vivo* results on Rlim function in mice, including expression patterns, genetics, activation of Xist, X-silencing and phenotypes. Moreover, the functional severance of the Rlim-Rex1 axis and regulatory switch of Xist expression in epiblasts is consistent with the finding that both male and female mice lacking Rex1 do not ectopically up-regulate Xist even after removal of the maternal Xist imprint but undergo normal embryogenesis [32]. From a developmental perspective, with a Rlim-independent down-regulation of REX1 specifically in epiblasts, the *in vivo* model offers a convenient window for XCR in implanting embryos. While the expression profile at late blastocyst stages is not in support of an active role of REX1 in the XCR process [21], the functional interruption of the Rlim-Rex1 axis is likely required to allow for rXCI. Considering the ESC model, it is difficult to see mechanisms of how XCR could be orchestrated in the very short time window that exists between the end of pre-implantation and early post-implantation stages. Because it appears inconceivable that Rlim uses different mechanisms in mouse embryos vs ESCs and XCI occurs only in females, the combined findings favor the *in vivo* model and essentially exclude the ESC model.

Functions of Rlim during XCI

Concerning the importance of Rlim functions for XCI, results that appear contradictory have been published over the years. In mice, by targeting Rex1 for degradation, Rlim plays crucial roles for the maintenance of Xist transcription during iXCI in female pre-implantation embryos. This represents its main *in vivo* function, as Rlim is dispensable for rXCI in the epiblast. However, REX1 levels in epiblast cells at the time point of rXCI initiation are slightly higher in RlimKO embryos when compared with controls [21]. Moreover, early female post-implantation embryos lacking Rlim express somewhat lower Xist levels [22] and Xist cloud detection in the ICM of blastocyst outgrowths is less frequent [29], suggesting that the presence of Rlim may slightly influence REX1 levels in epiblast cells and thus possibly the timing of onset and/or efficiency of the rXCI process. Thus, minor and redundant functions of Rlim during rXCI in mice are likely.

In female ESCs Rlim has variable importance for activating Xist (Figure 2B). Because there is a limited window in time when female cells are competent to undergo XCI, such variability may be partially explained by the combination of two features that converge on REX1 levels. First, ESCs represent cells at pre-implantation stages, when REX1 is under the strict control of Rlim. Second, different ESC lines express various levels of

REX1. Thus, at the time point when XCI is triggered, Rlim is more or less required to adjust REX1 levels allowing for Xist activation, depending on both the initial REX1 levels and the kinetics of Rex1 mRNA down-regulation. In contrast, due to an Rlim-independent down-regulation of Rex1 mRNA that starts at implantation, epiblast cells express very low REX1 protein levels when XCI is triggered (Figure 2A) and thus, Rlim is dispensable. Consistent with such a scenario, differences in Rlim requirement for rXCI have been reported in female ESC pGK12.1 and F121 lines [18,22,27,29] and the ability to activate Xist generally correlates with endogenous levels of REX1 [17,29]. Furthermore, such scenario might also partially explain the observation that the activation of Xist transcription *in vitro* via differentiation into embryoid bodies or upon the addition of retinoic acid occurs with considerably slower kinetics when compared with epiblast cells in mouse embryos. Thus, the importance of Rlim for XCI in ESCs is to decrease REX1 levels to allow for Xist transcription, analogous to its role during iXCI.

Such scenario implies a switch in Xist regulation occurring specifically in differentiating epiblast cells likely involving a separate set of transcriptional regulators. Thus, functions of Rlim during pre-implantation development are restricted to keeping REX1 levels below the threshold allowing for the activation of Xist by other factors, consistent with a recent report that identified up-regulation of Xist during iXCI by GATA transcription factors [33]. Likewise, Rlim is dispensable for XCI in the epiblast, implying that other factors must exist, responsible for activating Xist expression and X choice during rXCI. Indeed, the X-linked histone demethylase Kdm5c has been proposed as a dose-dependent activator of Xist [34]. Even though this gene escapes X-silencing [35], expression levels are higher in female cells before XCI when compared with those after XCI, due to lower expression from the silenced X [36,37]. Thus, at this stage the contribution of Kdm5c for the monoallelic up-regulation of Xist remains unclear but the existence of other X-linked Xist activators appears likely.

The functions of Rlim during rXCI have been controversial for more than a decade and a general problem was the inadequate discussion of published, contradicting data. While data challenging the ESC model were available early on, these results were either systematically dismissed as erroneous without scientific evidence, or ignored by the proponents of the ESC model, even after the revelation of the cloning artifact in Rnf12-333f ESCs. Over the years such biased data representation has significantly contributed to the confusion in the XCI field about the functions of Rlim.

In summary, compelling results show that Rlim is dispensable for Xist transcription during rXCI in female mice, and that the Rlim-Rex1 module is selectively active in pre-implantation embryos during iXCI. In female ESCs, due to remaining REX1, there is various requirement of Rlim for Xist activation, but this is not dependent on the presence of two Rlim alleles. Moving forward, it will be exciting to identify novel X-linked factors that participate in the monoallelic up-regulation of Xist during rXCI and elucidate their mechanisms of action.

Perspectives

- Two conflicting models have been proposed as to how the E3 ligase Rlim activates expression of Xist, the master regulator of XCI.
- Recent results favor one model of how the proteasomal targeting of the transcriptional repressor REX1 by Rlim regulates Xist transcription.
- These findings support research into the identification of novel X-linked Xist activators during rXCI.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations

ESC, embryonic stem cell; iXCI, imprinted XCI; rXCI, random XCI; XCI, X chromosome inactivation; ZGA, zygotic genome activation.

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