Cell Research (2012) 22:1524-1526.

www.nature.com/cr

RESEARCH HIGHLIGHT

Releasing the break on X chromosome inactivation: Rnf12/RLIM targets REX1 for degradation

Ingolf Bach[1, 2]

[1]*Program in Gene Function & Expression,* [2]*Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA 01605, USA*

Cell Research (2012) **22**:1524-1526. doi:10.1038/cr.2012.98; published online 26 June 2012

One of the two X chromosomes in cells of female mammals is transcriptionally silenced in a process known as X chromosome inactivation (XCI). Initiation of XCI is regulated by the ubiquitin ligase Rnf12/RLIM, but the mechanisms by which Rnf12/RLIM mediates this process has been a mystery. A recent study by Gontan *et al*. shows that Rnf12/RLIM targets REX1, an inhibitor of XCI, for proteasomal degradation, providing an answer to this question.

To achieve dosage compensation, female mammals silence one of their two sex chromosomes in a process called X chromosome inactivation (XCI). In mice two forms of XCI exist: In 2-4 cell staged embryos, imprinted XCI (iXCI) results in exclusive silencing of the paternal X (Xp). Around implantation, female epiblast cells that give rise to somatic tissues reactivate the silenced Xp and undergo random XCI (rXCI), which inactivates the Xp or the maternal X (Xm) with equal probability. Both forms of XCI require the long non-coding *Xist* RNA, which coats the inactive X chromosome (Xi) from which it is transcribed [1, 2].

The X-linked gene *Rnf12* encodes the RING finger protein Rnf12/RLIM, a transcriptional coregulator [3]. Rnf12/RLIM has been identified as a ubiquitin ligase [4] that regulates activities of different classes of transcription factors [5, 6] in part by adjusting cellular levels of various nuclear cofactors via the ubiquitin/proteasome system (UPS) [4, 7]. In female mice, sex-specific parent-of-origin effects have been reported upon *Rnf12* deletion. Deletion of the maternal *Rnf12* allele in oocytes results in early embryonic lethality specifically in female embryos due to a failure to develop extraembryonic trophoblast tissues in the placenta [8]. In contrast, in pregnant and lactating adult females Rnf12/RLIM expressed from the paternal allele serves as a survival factor specifically for milk-producing alveolar cells in mammary glands [9]. Thus, *Rnf12* functions as a sex-specific epigenetic regulator in female mouse nurturing tissues.

In an embryonic stem cell (ESC) model, Rnf12/RLIM has been identified as a major regulator for the initiation of rXCI. Indeed, forced overexpression of Rnf12/RLIM triggers ectopic *Xist* clouds in male and female ESCs [10], and homozygous deletion of *Rnf12* resulted in a failure to initiate rXCI [11]. In mice, the early lethality of female embryos upon deletion of the maternal *Rnf12* allele was shown to be the consequence of defective initiation of iXCI [8]. Strong evidence has been provided that Rnf12/RLIM exerts its functions for XCI initiation via promoting *Xist* expression [8, 10, 11] in a dose-dependent manner [10]. Intriguingly, cellular Rnf12/RLIM levels increase at the onset of rXCI [10, 11], likely due to a release from transcriptional repression by pluripotency factors [12]. Together, these studies indicate crucial functions of Rnf12/RLIM for the initiation of XCI via activation of *Xist*.

However, these studies left unanswered the question of how Rnf12/RLIM promotes the activation of *Xist*. A recent study has addressed this point [13]. Gontan *et al*. used affinity purification followed by mass spectrometry to identify the transcription factor REX1 as a major interaction partner of Flag-tagged Rnf12/RLIM in ESC nuclei. In these cells, REX1 appears to repress genes that promote differentiation [14] and its expression correlates with the pluripotent ESC state [15]. In an elegant series of experiments, the authors showed that endogenous Rnf12/RLIM interacts with, polyubiquitinates, and targets REX1 for degradation via the UPS, thereby dynamically adjusting levels of REX1 in various cell types including ESCs. In agreement with these results, the upregulation of Rnf12/RLIM upon differentiation of ESCs was accompanied with a downregulation of REX1. While Rnf12/RLIM appears to be a major regulator of REX1 dur-

Correspondence: Ingolf Bach
E-mail: ingolf.bach@umassmed.edu

ing differentiation, the authors also observed downregulation of REX1 in ESCs lacking Rnf12/RLIM, indicating that other proteins contribute to the reduction of REX1 levels upon ESC differentiation. Interestingly, the authors showed that the RING finger of RLIM, which mediates its ubiquitin ligase activity, plays important roles in the Rnf12/RLIM-induced activation of ectopic XCI in male ESCs.

To identify roles of REX1 in XCI, day-3-differentiated male ESCs expressing lower REX1 levels (+/– for *Rex1*) or female ESCs overexpressing REX1 were examined for XCI as measured by the occurrence of *Xist* clouds coating an X chromosome. Indeed, whereas a significant number of the male *Rex1*$^{+/-}$ ESCs underwent ectopic XCI, REX1-overexpressing female ESCs experienced severe inhibition of XCI. Importantly, knockdown of *Rex1* in *Rnf12*$^{-/-}$ ESCs resulted in a significant rescue of XCI. These results reveal functions of REX1 downstream of *Rnf12,* directly linking the functions of both proteins during XCI and the formation of *Xist* clouds.

Using chromatin immunoprecipitation in undifferentiated ESCs, Gontan *et al*. [13] detected REX1 occupancy of promoter/regulatory elements of the *Xist* gene as well as those of *Tsix*, a gene that overlaps with the *Xist* locus on the X chromosome but is transcribed in the antisense direction, thereby inhibiting *Xist* transcription [2]. Indeed, consensus recognition sequences for REX1 binding were found on regulatory elements of both genes, suggesting that this transcription factor directly regulates the expression of these genes. To address this possibility, they performed transient cotranfections and detected a downregulation of *Xist* in cells overexpressing REX1, similar to cells that lack Rnf12/RLIM. These results indicate that REX1 functions as a repressor of *Xist*, thereby inhibiting XCI. To explain their findings that REX1 binds to regulatory sequences of *Tsix* and represses XCI in ESCs, the authors suggest that REX1 may function by activating *Tsix* expression. Based on these results, the scenario for female ESC differentiation and XCI would be as follows: Differentiation-induced downregulation of pluripotency factors trigger the upregulation of *Rnf12* transcription and an increase in cellular Rnf12/RLIM levels. Lower cellular levels of REX1 due to its increased proteasomal targeting by Rnf12/RLIM would then lead to downregulation of *Tsix* and activation of *Xist* transcription followed by initiation of XCI. Due to the fact that similar to *Xist*, *Rex1* is found only in placental mammals but not in marsupials that also lack *Xist*-mediated XCI, the authors propose that *Xist* and *Rex1* may have co-evolved with the appearance of an *Xist*-dependent XCI mechanism.

While this report identifies important mechanisms of Rnf12/RLIM functions during XCI activation, it also raises several questions. Going forward, one of the most pressing question concerns the relevance of the *Rnf12-Rex1* regulatory module for XCI *in vivo* as seemingly conflicting results have been reported for the requirement of Rnf12/RLIM for the initiation of rXCI. In an ES cell model Rnf12/RLIM is essential for initiation of rXCI [11] and the results obtained by Gontan *et al*. [13] are largely based on the same model. In contrast, evidence suggests that Rnf12/RLIM may be dispensable for rXCI in mice [8] and *Rex1*$^{-/-}$ mice are viable with no apparent phenotype [16]. Thus, it will be important to address the functional requirement of Rnf12/RLIM in conjunction with REX1 for rXCI *in vivo* and how they connect to the choice of which X chromosome to inactivate. Moreover, it will be interesting to examine whether the crucial functions of Rnf12/RLIM during iXCI are also connected to *Rex1* and whether targeted degradation of REX1 is the only role of Rnf12/RLIM in XCI. The further deciphering of the roles of *Rnf12* and *Rex1* in XCI promises to reveal new and exciting mechanisms of epigenetic regulation.

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