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Perspective

Rnf12—A Jack of All Trades in X Inactivation?

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Placental mammals compensate the dosage imbalance of X-linked genes between males (XY) and females (XX) by silencing one randomly chosen X chromosome in females. This process is initiated during early embryonic development and can be recapitulated during differentiation of murine embryonic stem cells (mESCs). X chromosome inactivation (XCI) is initiated by up-regulation of a non-coding RNA on the future inactive X chromosome, named Xist, which lies within a large complex locus, called the X inactivation center (*Xic*). Subsequently, Xist RNA induces silencing of the entire chromosome in *cis* [1]. Although central to the XCI process, the molecular mechanisms underlying Xist's regulation still remain to be deciphered. In particular, it is unclear (1) how the up-regulation of Xist is triggered at the onset of differentiation, (2) why this is restricted to female cells, and (3) why one allele and not the other is affected? Although each aspect could in principle be controlled by distinct factors and sequence elements, one protein has recently been proposed to regulate Xist at all three levels: the E3 ubiquitin ligase Rnf12/Rlim [2]. The X-linked *Rnf12* gene acts as a dose-dependent activator of *Xist*, which is expressed at elevated levels in female relative to male cells and is up-regulated during differentiation. Two recent studies shed further light on the precise role of Rnf12 in XCI [3,4].

Developmental Regulation of *Xist*

Multiple stem cell–specific factors have been proposed to repress *Xist* in undifferentiated cells, and trigger its up-regulation when down-regulated during differentiation (Figure 1A and 1B, dark blue). The ability of some of these factors, e.g., Nanog, Oct3/4, and Sox2, to bind within the first intron of *Xist* led to the hypothesis that they could directly inhibit *Xist* expression [5]. However, the present study by Gribnau and colleagues reveals that deletion of this intronic site in mESCs is insufficient to activate *Xist* [3], thereby demonstrating the existence of additional mechanisms repressing *Xist* prior to differentiation. These could include repression by Tsix, *Xist's* repressive antisense transcript. Indeed, *Tsix* is also thought to be regulated by stem cell factors (Figure 1A) [6,7]. However, abrogating *Tsix* transcription does not lead to *Xist* derepression prior to differentiation [8]. As neither the sole deletion of *Tsix*, nor of *Xist* intron 1, result in *Xist* up-regulation, they might be targeted by two independent redundant pathways. Alternatively, stem cell factors might affect *Xist* by controlling another *Xist* regulator, such as Rnf12 itself. Gribnau and colleagues now provide evidence that Rnf12 can *trans*-activate the *Xist* promoter independently of *Tsix* [3]. As Rnf12 is itself up-regulated during early mESC differentiation (Figure 1B, red line), stem cell factors might control the correct developmental expression of *Xist* by repressing its activator Rnf12 (Figure 1A). Their strong binding within the *Rnf12* promoter would suggest a direct repression, but this remains to be explored [9].

Female-Specific Expression of *Xist*

The fact that XCI is only initiated in XX, but not XY cells, suggests that *Xist* up-regulation is controlled by an X-linked activator, which could be Rnf12. If a double dose of Rnf12 was the sole mechanism to ensure female-specific expression, then a cell heterozygous for an *Rnf12* deletion should fail to initiate XCI. However, heterozygous deletion of *Rnf12* delays, but does not prevent, random XCI in mESCs [2,4], which points to the existence of additional X-linked activators of *Xist* (Figure 1). Nevertheless, in mice as well as in differentiating mESCs, XCI is skewed towards the mutated *Rnf12* allele, suggesting either preferential up-regulation of *Xist* on the mutated allele, or a selective disadvantage of XX cells that have chosen to silence the wild-type allele, resulting in functional Rnf12 deficiency [2,4]. Importantly, $Rnf12^{-/Y}$ mice are fully viable and fertile, implying that the counter-selection mentioned above may be due to an initial inability to induce XCI. The capacity of complete null $Rnf12^{-/-}$ ES cells to initiate random XCI was also investigated in the two studies, though the conclusions diverged. Bach and colleagues report similarly delayed kinetics as for $Rnf12^{+/-}$ cells [4], whereas the Gribnau lab observes almost complete abrogation of XCI [3]. What could be the reasons for this discrepancy? First, Gribnau and co-workers generated the deletion in vitro in mESCs, while Bach and colleagues derived their ES cells from $Rnf12^{-/-}$ embryos. In the latter case, ES cells could have adapted to or have been selected for compensation of the *Rnf12* deletion. Second, ES cells can be subject to genetic or epigenetic differences, and in particular, female ES cells often lose one X chromosome and thus can survive differentiation without XCI. However, the ES cells in both studies were reported to have XX status in most cells. Alternatively, the two ES cell lines studied might carry polymorphisms in *Xist cis*-acting control elements, or differ in the levels or expression kinetics of *trans*-acting *Xist* regulators. For example, if an unknown X-linked activator (Figure 1C, dotted line) were to be down-regulated more quickly in one cell line (Figure 1C, left) than in the other (Figure 1C, right), XCI might occur only in the latter case. This raises the exciting possibility that comparison of the two lines might enable identification of these unknown activators.

Monoallelic Expression of *Xist*

Does Rnf12 participate in the mechanism that ensures that only one out of two X chromosomes up-regulates Xist? *Rnf12*

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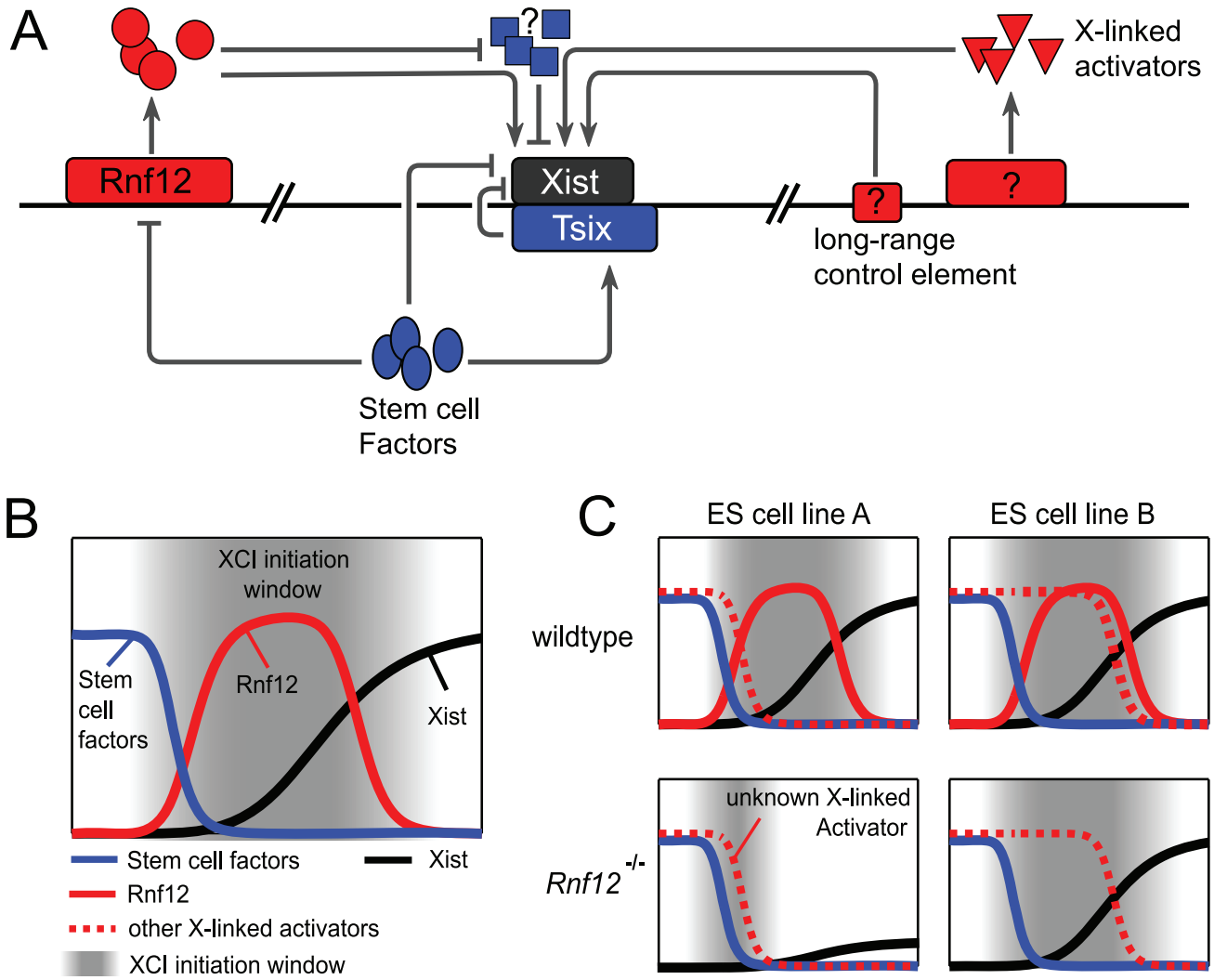

Figure 1. The X chromosome inactivation network. (A) *Xist* expression is controlled by counteracting activators (red) and repressors (blue). Stem cell factors (blue ovals) might repress *Xist* directly or indirectly via activating the repressive transcript Tsix or repressing the activator Rnf12. Rnf12 is the only known activator, and may function by targeting the *Xist* promoter directly and/or by inducing degradation of an unknown *Xist* repressor (blue squares). The existence of additional X-linked activators (red triangles) and long-range control elements such as *Xpr*, *Xce*, *Xite*, and others (red box) has been suggested [10]. (B) The time window when XCI can be initiated (grey) could be controlled by the down-regulation of *Xist* repressors such as stem cell factors (blue) and up-regulation of *Xist* activators like Rnf12 (red). (C) Different cell lines might require Rnf12 (ESC line B) or not (ESC line A), depending on the expression kinetics of other X-linked activators (dotted red line).
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has been suggested to act as a negative feedback regulator to ensure monoallelic *Xist* up-regulation: its silencing on the inactive X might prevent *Xist* up-regulation on the second X chromosome [2]. While this hypothesis still remains to be investigated, monoallelic *Xist* expression is much better understood in the other, imprinted form of XCI in the mouse. In female pre-implantation embryos, the paternal X is inactivated initially, while the maternal X is prevented from XCI by a repressive maternal imprint on *Xist* [1]. This situation is maintained in extra-embryonic tissues, but reversed in cells giving rise to the embryo, where it is followed by random XCI. The maternally transmitted deletion of *Rnf12*, which results in a loss of the maternal Rnf12 pool in the zygote, leads to female-restricted embryonic lethality [4]. The Rnf12 maternal pool thus seems to be essential for triggering paternal XCI during early development. It should be noted, however, that imprinted XCI differs from random XCI in that it initially occurs independently of the number of X chromosomes in the cell and is controlled by a smaller genomic region. Consequently, additional *cis*-regulators must be involved in random XCI [10], as well as additional *trans*-activators [2].

The characterization of Rnf12 in XCI is clearly an important step towards a better understanding of *Xist* regulation. However, important pieces of the puzzle are still missing. How does Rnf12 activate *Xist*? As a ubiquitin ligase, does it induce degradation of a repressor of *Xist*? Is the *Xist* promoter the direct target of Rnf12, or are other *Xic* sequences also involved, such as *Xce*, *Xpr*, or others [10]? What are the missing X-linked activators that compensate for a heterozygous *Rnf12* mutation in females? And more generally, at the very heart of X chromosome inactivation, why is only one and not both *Xist* alleles up-regulated during the random form of XCI? This could be due to stochastic activation of *Xist*, followed by *cis*-silencing of dose-dependent activators such as

Rnf12. But even if this was the case, it still remains to be understood why *Xist* is up-regulated with such a low probability that it is initially triggered in a mono-allelic fashion. Whatever the answers, the recent work on Rnf12 has provided us with exciting new insights into the regulatory network acting on *Xist*.

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