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Epigenetic control of X chromosome reactivation: the when, where and how

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Layman's summary

Biologisch gezien is er een duidelijk verschil tussen mannen en vrouwen: naast 22 andere chromosomen hebben mannen over het algemeen een X en een Y chromosoom en vrouwen twee X chromosomen. Chromosomen zijn dragers van genetische informatie in de vorm van genen; op ieder chromosoom liggen verschillende genen, dus verschillende informatie. Chromosomen bestaan echter uit paren van twee, dus ieder gen komt twee keer voor in een cel van zoogdieren. Althans, dit zou het geval zijn zonder sekseverschillen, maar doordat mannen een X en een Y chromosoom hebben, zijn de genen op deze twee chromosomen maar één keer aanwezig. Vrouwen hebben wel twee keer een X chromosoom, wat betekent dat zij twee keer zoveel genetische informatie van de X zouden hebben. Door een compensatiemechanisme van de cel dat actief is tijdens de embryonale ontwikkeling is dit echter niet het geval, dit heet X chromosoom inactivatie (XCI). Tijdens dit proces wordt één van de twee X chromosomen uitgeschakeld, waardoor de genen op dat chromosoom niet meer actief zijn. Dit wordt strak gereguleerd door verschillende factoren, waaronder het lange RNA molecuul *Xist* wat uiteindelijk om het hele inactieve X chromosoom gewikkeld zit. De inactivatie die door deze regulatiemoleculen wordt gerealiseerd, zal door andere factoren omgezet worden in een permanente inactivatie zodat deze tijdens het leven van de vrouw in stand blijft in alle cellen. Dit houdt bijvoorbeeld in dat het chromosoom compacter opgevouwen wordt in de cel, waardoor genen niet beschikbaar voor aflezen zijn.

Er zijn ook cellen in het lichaam die wel twee X chromosomen geactiveerd willen hebben, bijvoorbeeld de voorlopers van de ei- en spermacellen. Deze ontstaan nadat XCI heeft plaatsgevonden in de embryo, wat betekent dat het geïnactiveerde X-chromosoom weer gereactiveerd moet worden in een proces genaamd X chromosoom reactivatie (XCR). Dit gebeurt in meerdere stappen: de omwikkeling van *Xist* wordt verbroken, de onderhoudsfactoren worden uitgeschakeld en de algehele staat van het X chromosoom wordt weer actief in plaats van inactief. Ook dit proces wordt streng gereguleerd, deels door dezelfde factoren als bij XCI maar ook door factoren met andere functies. Buiten de biologische systemen van een lichaam kan XCR ook plaatsvinden tijdens het kunstmatig reprogrammeren van een gespecificeerde cel, zoals een levercel, naar een niet-gespecificeerde cel, zoals een stamcel. Dit proces verloopt grotendeels hetzelfde.

Op het X-chromosoom komen veel verschillende genen voor, die als ze gemuteerd zijn ook ziektes kunnen veroorzaken. Eén van deze ziektes is het Rett syndroom, wat alleen voorkomt bij vrouwen doordat in sommige cellen het gemuteerde gen actief is en in anderen het gezonde gen. Desalniettemin is het gezonde gen in alle cellen van het lichaam aanwezig, maar in sommigen dus inactief. Door de mogelijkheid om XCR kunstmatig te laten gebeuren, zou dit kunnen worden ingezet tegen de ziekte. bij Het reactiveren van het aanwezige gezonde gen zou als therapie gebruikt kunnen worden om symptomen van Rett syndroom te verlichten. Dit lijkt alleen minder effectief dan gedacht, waardoor andere therapieën mogelijk meer kans van slagen hebben.

Abstract
X-chromosome inactivation (XCI) is a process necessary in mammals for gene dosage compensation between the sex chromosomes in males and females. This process is dependent on a key player, *Xist*. This factor is regulated tightly throughout the process of XCI, and the inactive state is maintained by repressive histone marks such as H3K37me3, DNA methylation and an inaccessible chromatin conformation. Despite the tight maintenance of XCI, reactivation of the X chromosome is possible and happens during development in the ICM of mice and in the formation of germ cells. In this review, we describe the process and mechanisms of X chromosome reactivation (XCR). This includes the role of *Xist*, key characteristics of the process *in vivo* and the differences between endogenous biological XCR and during *in vitro* reprogramming. XCR's potential as a therapeutic aid is discussed in the context of Rett syndrome.

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Introduction

In 1961, Mary Lyon suggested that, during development, one X-chromosome in female mammals is inactivated (Lyon, 1961). This is now known as X-chromosome inactivation (XCI), a means of gene dosage compensation between male and female individuals of X-linked genes (Lyon, 1961, 1962). A classic model for studying XCI is the mouse, *Mus musculus*, in which the phenomenon was first described. However, there is some variation in the process of XCI between mammalian species (Okamoto et al., 2011; Vasques et al., 2002). One of the main differences is the mode of inactivation, i.e. imprinted (iXCI) (Okamoto et al., 2005) or random (rXCI) (Okamoto et al., 2011). While marsupials show only paternal iXCI (Xi^p), meaning the X-chromosome originating from the father is inactivated (Mahadevaiah et al., 2009; Sharman, 1971), humans only show rXCI (Moreira de Mello et al., 2010). In contrast, mice show both iXCI and rXCI. In practice this means that at the 2-4 cell stage, the paternal X-chromosome (X^p) is inactivated, and will stay inactive in the extraembryonic lineages, while in the inner cell mass (ICM) the Xi^p is reactivated and upon implantation into the uterine lining random XCI takes place (Borensztein, Okamoto, et al., 2017; Okamoto et al., 2004, 2005; Rebuzzini et al., 2020). This reactivation is not exclusive to the mouse, as it also happens naturally in humans (Chitiashvili et al., 2020). However, the mechanics of this process known as X chromosome reactivation (XCI) are not as well-researched as XCI, despite its biological importance.

Initiation of X-chromosome inactivation is dependent on many positive and negative regulators

As stated previously, humans, mice and marsupials use different paths to inactivate an X-chromosome. While these species seem quite divergent in their way of XCI, the mechanisms of epigenetic regulation and silencing by long non-coding RNAs (lncRNAs) are in reality quite conserved between species (Froberg et al., 2013; Mahadevaiah et al., 2009).

Main actors in the XCI process

One lncRNA is considered the master-regulator of XCI: X inactive-specific transcript or *Xist* (Penny et al., 1996). In mice it is only expressed from the future inactivated X (Xi), meaning it acts on the same chromosome it originates from i.e. *cis*-acting (Borsani et al., 1991; Brockdorff et al., 1991). Once expressed from the Xi, it coats this chromosome starting at gene-rich places on the X, later covering the intermediate parts (Clemson et al., 1996; Simon et al., 2013). For humans, XCI is preceded by a state called X-chromosome dampening (XCD) in which both X-chromosomes express *XIST* but in small amounts, until one Xi is established later (Petropoulos et al., 2016). Upon upregulation, *Xist* recruits repressors such as the Polycomb Repressor Complex (PRC) proteins 1 and 2, responsible for the deposition of repressive histone marks H2AK119ub and H3K27me3 respectively (De Napoles et al., 2004; Plath et al., 2003). Expression of *Xist* itself is controlled by other factors, one of which is its primate-specific repressor *XACT* which coats the Xa and exhibits overlapping expression patterns with *XIST* prior to XCI. However, their localization pattern on the X chromosome differs (Vallot et al., 2013, 2017). GATA factors, essential for several developmental processes (reviewed in Tremblay et al., 2018), have recently been implicated in initial activation of *Xist* during iXCI just after fertilization through binding to *Xist* enhancers (Ravid Lustig et al., 2023). These GATA factors do not have an effect on rXCI during differentiation. In addition, the chromatin remodeler CHD8 has an effect on *Xist* expression in rXCI (Cerase et al., 2021). The authors reveal that CHD8 resides on the *Xist* promoter in both differentiated and undifferentiated states, where CHD8 acts as a chromatin remodeler at the *Xist* promoter, to regulate the expression of *Xist* during differentiation. Without CHD8, *Xist* expression is upregulated by other activators such as YY1 (Cerase et al., 2021).

The *Xist* gene lies within the X inactivation center or Xic, a specific region of the X-chromosome which contains genes involved in the process of XCI in both human and mouse (Brown et al., 1991; Lee et al., 1996; Figure 1). This locus is specific for eutherian animals, since the Xic arose after the split of marsupials and eutherians (Romito & Rougeulle, 2011). The Xic harbors many more genes involved in regulating XCI, both coding and non-coding (Figure 1), which makes it essential for the process although *Xist* is also regulated by distal structures (Gjaltema et al., 2022; Rebuzzini et al., 2020; Yin et al., 2021). The most prominent ones are *Tsix* (Lee et al., 1999; T. Sado et al., 2001)*, Jpx* (Tian et al., 2010)*, Ftx* (Chureau et al., 2011) and *Rnf12* (Jonkers et al., 2009). Of these, only *Rnf12* is protein-coding, indicating it is a *trans*-acting regulator (Barakat et al., 2011). This E3 ubiquitin ligase works through negative regulation of REX1, a pluripotency-associated factor and *Xist* inhibitor, by ubiquitinating it for degradation by the proteasome (Gontan et al., 2012a, 2018), indicating that RNF12 is an indirect activator of *Xist*.

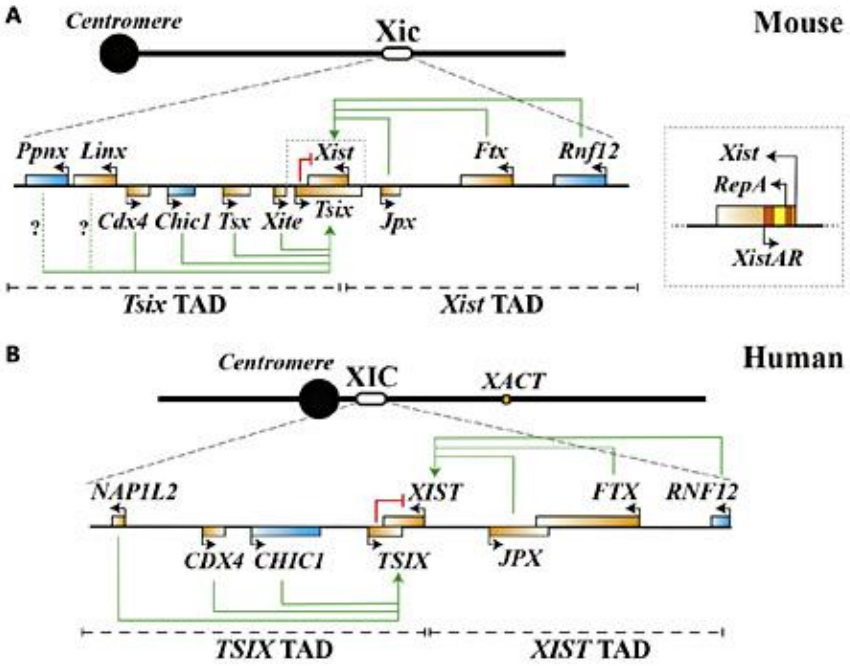

Figure 1: The X inactivation center encompasses *Xist* as well as its many regulators, both positive and negative. A. Location of the Xic on the murine X-chromosome. B. The XIC on the human X-chromosome can be seen, as well as the location of the *XACT* gene. For both A and B the zoom-in displays all different XCI-related factors, divided into the *Xist* and *Tsix* topologically associating domains (TADs). Genes depicted in blue are protein-coding, while the orange genes are not. The figure is adapted from Rebuzzini et al (2020).

The *Tsix* gene encodes for an lncRNA that is anti-sense to *Xist* (Figure 1) and is expressed in three stages: bi-allelic, mono-allelic on the Xa and repressed(Lee et al., 1999). In this manner, it negatively regulates the early expression of *Xist* by hypermethylating its promoter, allowing *Xist* upregulation when *Tsix* disappears. Conversely, *Jpx* and *Ftx* are activators of *Xist*. *Jpx's* function is to eliminate CTCF from *Xist* on the future Xi, making the promoter available for other regulators (S. Sun et al., 2013). The positive regulation on *Xist* by *Ftx* comes from its effect on the *Xist* promoter, where the non-coding *Ftx* transcript withholds DNA methylation of the CpG island (Chureau et al., 2011). This activates the transcription of *Xist*, making *Ftx* an activator of XCI. This effect on CpG methylation is also opposite of *Tsix* action, since *Tsix* hypermethylates the *Xist* promoter (Navarro et al., 2006).

Topologically associated domains in XCI initiation

As mentioned before, Xist is regulated by *cis* and *trans* mechanisms. Another way of explaining *cis*-regulation is through topologically associated domains or TADs. These domains

are characterized by an increased amount of interactions within itself and are established by cohesin and CTCF (Dixon et al., 2012; Fudenberg et al., 2016; Nora et al., 2012). As mentioned before, CTCF is a repressor of *Xist* expression, undone by *Jpx* action (S. Sun et al., 2013). However, CTCF is most commonly known as a barrier protein between TADs (Davidson et al., 2023). TADs exist within the Xic as well (Figure 1), segregating the regulatory domains of *Xist* and *Tsix* in a spatial manner with a conserved genetic region called *RS14* separating the two domains. *RS14* is bound by CTCF (Nora et al., 2012; Spencer et al., 2011). This plays an important role in the timing of XCI, as demonstrated in the paper of van Bemmel et al. (2019). Here the authors inverted the *Xist* and *Tsix* loci, placing them in each others' TAD leading them to be expressed by each other's promoters. This resulted in premature *Xist* expression, while *Tsix* expression remains active longer than normal during differentiation(van Bemmel et al., 2019). Both of these effects can be explained by the influence of the (*cis*-acting) regulatory environment of the opposite gene, leading to a switch in expression dynamics. Apart from being regulated through TADs, *Xist* also has an effect on chromatin remodelers themselves. Where it acts as an attractive agent to for example PRC1 and 2, it has the complete opposite effect on cohesin. The active X chromosome contains many TADs that are replaced by two mega-domains separated by the tandem repeat *Dxz4* on the Xi(Cheng et al., 2021; Deng et al., 2015; Gdula et al., 2019; Giorgetti et al., 2016). When knocking-out *Xist* specifically from the Xi, the amount of cohesin-bound sites on the Xi increased to similar levels as the Xa (Minajigi et al., 2015). This indicates that normally *Xist* would block the ability of cohesins to bind the Xi, decreasing TAD numbers in the process.

A key-player in the establishment of the chromatin conformation of the Xi is SmcHD1, a member of the SMC family of proteins important for chromatin structure (Gdula et al., 2019). In XCI, SmcHD1 is responsible for the repression of genes on the Xi through its ability to change chromatin conformation, which renders the entire Xa to behave more like Xi. Loss of SmcHD1 leads to increased TAD formation on the Xi, resulting in subsequent changes in transcription among other characteristics (Gdula et al., 2019). Since SmcHD1 acts late in the XCI process, it is suggested that the transcriptional repression it ensures is mainly needed for maintenance of XCI (Gdula et al., 2019; Gendrel et al., 2012). The transcriptional repression of Xi is linked to the loss of TADs during XCI, since the transcription machinery cannot bind X-linked genes anymore, presumably because of the compaction of the chromatin on the Xi (Collombet et al., 2023; Rego et al., 2008).

Maintenance of XCI

However important *Xist* seems to be in initiating XCI, its role in maintenance of the Xi is disputed. Some studies suggest that maintenance is completely independent of Xist (Wutz & Jaenisch, 2000), while more recently others have found that XCI deteriorates in the absence of the lncRNA (Mira-Bontenbal et al., 2022; Yang et al., 2022), leading to an increased incidence of escapee genes. These are genes on the X-chromosome which escape XCI and are thus expressed biallelically in female cells. In mice, the cell-type specific behavior of some escape genes has been characterized (Berletch et al., 2015). However, Tukiainen et al. (2017) found that escapees are cell-type specific in humans as well, with escape from XCI of some genes happening specifically in e.g. skin or long tissue. This evading of XCI is never complete; escape genes show, on average, one third of the amount of transcription as their counterparts on the Xa (Tukiainen et al., 2017). The TADs discussed above can have an effect on escaping XCI as well, since several facultative escapees were found to have CTCF binding sites at the borders of their gene only in cells where they escape XCI (Fang et al., 2023). This goes together with depletion of the repressive H3K27me3 mark on all described escapees. Apart from depletion of histone methylation, a decrease in DNA methylation is also linked to a higher incidence of escape genes (Sharp et al., 2011).

As said previously, the role of *Xist* in XCI maintenance is unclear. It has been reported that, upon deletion of *Xist* after XCI, the maintenance is affected in terms of repressive characteristics like DNA methylation. Strikingly, the absence of *Xist* does not have an effect on the function of the tissue researched (Adrianse et al., 2018). *Xist*'s effect on histone marks has been described by others as well, with Yu et al. (2021) noting that *XIST* maintains XCI in B cells through continuous deacetylation of the activating histone mark H3K27ac. However, they also report that the *XIST* cofactor TRIM28 acts as a cell-type specific transcriptional repressor. From this they speculate that regulation of XCI maintenance may vary from cell to cell, leading to differing effects of *XIST* deletions in these cells. Apart from *Xist* itself affecting maintenance, the *Xist*-recruited PRC1 and 2 have recently been implicated in this process. When inhibiting PRC1/2 after XCI, certain X-linked genes escape inactivation only in extra-embryonic lineages (Masui et al., 2023). This effect is independent of the PRCs effect on each other, although there is some overlap in the genes that escape XCI between the two Polyclomb complexes.

In summary, *Xist* is involved in the initiation of XCI in several different ways, from influencing the chromatin conformation of the Xi to recruitment of repressive protein complexes. It does this with aid of both *cis*- and *trans*-acting regulatory elements originating mainly from the Xic, which dictate timing and intensity of *Xist* expression and thereby XCI. To maintain inactivation of X-linked genes, chromatin state and histone marks seem to be important while the role of *Xist* is not completely clear.

In vivo reactivation of the X-chromosome

It has now been established that XCI takes place in every cell of the post-implantation embryo. However, XCI can be undone through a process called X-chromosome reactivation (XCR). As the name suggests, during this process the X chromosome is reactivated.

Reactivation in mouse and human

In mice, iXCI takes place at the 2-4 cell stage (Borensztein, Syx, et al., 2017). The maternal X is thus active in the entire embryo up to the blastocyst stage. There, the paternal X is reactivated in the ICM, leading to biallelic X-linked expression, while cells belonging to the extraembryonic lineages keep the paternal X silent (Borensztein, Syx, et al., 2017; Okamoto et al., 2004). This reactivation happens relatively fast, between E3.5 and E5.5 (Kobayashi et al., 2016; Figure 2). Reactivation of the Xi^p is detectable through a loss of H3K27me3 from Xi in cells of the ICM in fully matured blastocysts before reappearing again during rXCI post-implantation (Borensztein, Okamoto, et al., 2017; Mak et al., 2004). H3K27me3 methylation occurs to a greater extent during rXCI, leading the embryonic lineages to have a more stably inactivated Xi than extra-embryonic lineages (Csankovszki et al., 2001; Takashi Sado et al., 2000).

Inactivation is reversed in another cell type as well: primordial germ cells (PGCs) (Figure 2). PGCs, the precursors of oocytes and sperm, originate from the epiblast and migrate to the genital ridges that will develop into the adult gonads (McLaren, 2003). Oocytes possess two active X-chromosomes (Epstein, 1969), indicating that the Xi is reactivated during development of these cells. In mice, this process of XCR in female PGCs starts at E7.0, shortly after rXCI in the epiblast has taken place at E6.5 (Mak et al., 2004; Sugimoto & Abe, 2007). Monoallelic expression of some genes prevails until the PGCs are well into their first meiosis at E14.5, suggesting that XCR happens gradually over a longer period. During this time, *Xist* is switched off at inconsistent times between PGCs, with some cells displaying absence of *Xist* accumulation as early as E7.5 (Sugimoto & Abe, 2007).

Human primordial germ cells (hPGCs) develop between the third week post-fertilization up until the tenth week, when they start meiosis (Figure 2) (Tang et al., 2016). XCR in hPGCs

differs from mice since hPGCs display XCD, the same as human pre-implantation embryos as discussed before (Chitiashvili et al., 2020; Petropoulos et al., 2016). XCD is not a permanent state in the pre-implantation embryo, since complete XCI will take place upon implantation. This is also true for hPGCs, although XCD functions as a rather stable state which holds up until the first meiosis in the primitive germ cells (Chitiashvili et al., 2020). Dampening is linked to *XIST* in that its expression is seen from both X chromosomes only for hPGCs, while a larger extent of cells that no longer express X*IST* is observed once hPGCs advance in their differentiation. The fact that XCD is found in the process of attaining both XCI and XCR suggests that it is a vital intermediate phase between XaXa and XaXi in humans.

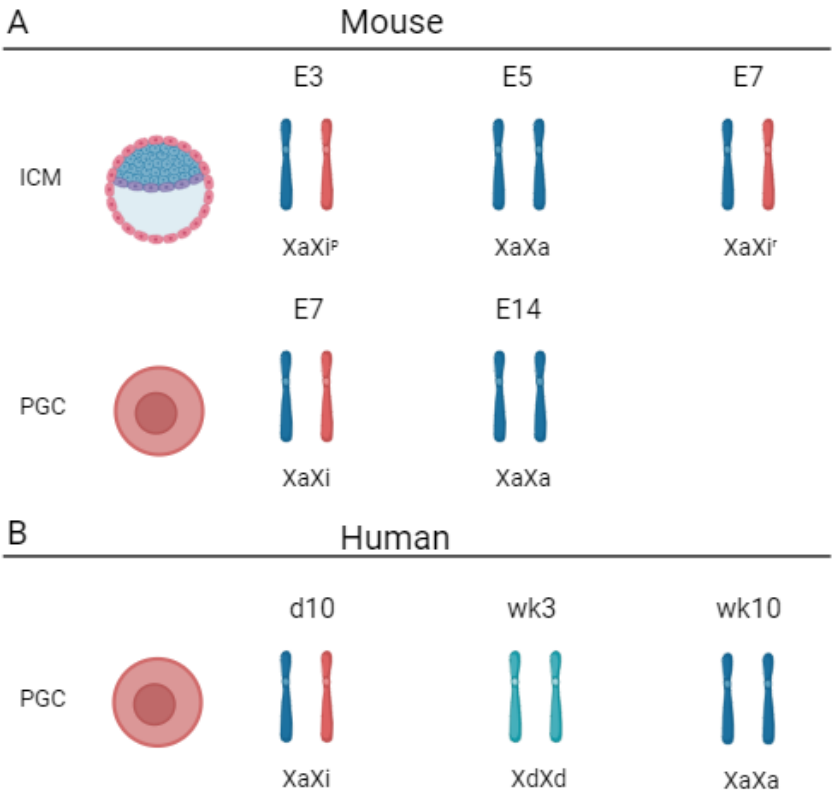

Figure 2: Overview of the X chromosome state during embryonic development in mouse and human. A shows the X state in E (embryonic days) of the developing mouse for both the ICM and PGCs. For the ICM, all cells start out with iXCI of the paternal X before going through XCR which allows cells of the ICM to go through rXCI. PGC development starts with the reactivation seen in the ICM before going through rXCI at post-implantation and finally XCR in their PGC fate. B displays the human development of PGCs both in d (days post fertilization) and wk (weeks post fertilization), thereby showing the process of initial XCI in the blastocyst up until d10 and reactivation in PGCs afterwards. ICM: inner cell mass, PGC: primordial germ cell, Xa: active X chromosome, Xi: inactive X chromosome, Xi^{P}: paternally inactived X chromosome, Xi^{r}: randomly inactivated X chromosome Xd: dampened X chromosome.

Proteins involved in reactivation

Despite XCR happening in different cell types during development, these share several key aspects of the process. One key aspect of XCR is the pluripotency state. PGCs have to return to the most undifferentiated state possible considering that they will give rise to totipotent germ cells. This return to pluripotency is seen as a hallmark of XCR (Pasque & Plath, 2015). It is seen in the epiblast cells of the ICM as well, where the pluripotency marker *Nanog* is expressed specifically (Plusa et al., 2008). Well-known pluripotency genes are also expressed during germline development in humans, where *OCT4* and *KLF4* expression are both regulated by TFAP2C which functions through naïve-state specific enhancers and is necessary for hPGC formation (Chen et al., 2018). It has also been established that, in mouse pluripotent embryonic stem cells derived from the ICM, OCT4 and SOX2 bind *Tsix* (Donohoe et al., 2009).

Both pluripotency factors and *Tsix* become downregulated concomitantly with *Xist*'s upregulation, illustrating the fact that pluripotency and *Xist* expression are mutually exclusive in the mouse.

During XCI, factors that promote pluripotency are suppressed. The clearest example of this is the inhibiting action of RNF12 on REX1 (Gontan et al., 2012b). REX1 has been established as a pluripotency factor in both mice and humans, where in the mouse it is mostly involved in properly establishing the preimplantation embryo (Climent et al., 2013). However, in humans its function also extends to deterring differentiation and heightening glycolytic metabolism needed for the energy expenditure of pluripotent stem cells (Son et al., 2013). If REX1 decreases during XCI it could be implicated in XCR as well. Gontan et al. (2018) show the opposite however: absence of REX1 does not affect the rate of XCR, both in the ICM and PGCs (Gontan et al., 2018).

This link between Xist-related factors and pluripotency is not unique to REX1/RNF12. Both *Xist* and *Tsix* are regulated by the important pluripotency factors NANOG, OCT4 and SOX2 (Navarro et al., 2008, 2010). Especially for *Xist*, this effect seems to contribute to the reactivation of the Xi^{p} in the murine ICM, while the effect of *Tsix* lies more in the subsequent differentiation and rXCI. The binding sites of NANOG, OCT3/4 and SOX2 lie in the first intron of the *Xist* gene and seem to magnify each other's' effect of inhibiting *Xist* expression (Navarro et al., 2008). The primate-specific *XIST* regulator *XACT* is also involved in the context of pluripotency, with its expression decreasing during differentiation probably due to an enhancer for *XACT* that is regulated by OCT4, SOX2 and NANOG among others (Casanova et al., 2019). Interestingly, in mouse, when *Tsix* expression is ectopically stimulated in extraembryonic lineages at E6.5 the Xi becomes reactivated through repression of *Xist* (Ohhata et al., 2011).

Tsix does have another role in the XCR process, in collaboration with another pluripotency factor called PRDM14 (Payer et al., 2013). The role of PRDM14 has been characterized in development of PGCs in mice (Yamaji et al., 2008). There it is both involved in re-establishing a pluripotent state through activation of genes involved in the pluripotency network such as SOX2, and reprogramming of the epigenetic marks starting with inhibition of the histone methyltransferase responsible for the H3K9me2 repressive mark. Through these mechanisms PRDM14 is essential for establishing the PGC fate (Yamaji et al., 2008). Knock-outs of *Prdm14*, *Tsix* or their double knock-out compromises the erasure of H3K27me3 marks needed to reactivate Xi^{p}, suggesting a shared regulatory pathway (Payer et al., 2013). *Tsix* and PRDM14 turned out to converge on the first intron of *Xist*, where *Tsix* was responsible for efficient binding of *Prdm14* to the intron to facilitate *Xist* repression. This effect of PRDM14 on H3K27me3 was also found in PGCs, where the mark is removed during migration of the PGCs through the genital ridge (Mallol et al., 2019).

Chromatin signatures involved in XCR

Since the Xi is completely reorganized during XCR, proteins responsible for remodeling need to be expressed. *In vivo*, epigenetic modifiers are more expressed during XCR in the ICM than at the same time in non-ICM cells (Borensztein, Okamoto, et al., 2017). This correlates with the erasure of H3K27me3 discussed before being a characteristic of XCR in the blastocyst (Mak et al., 2004). This epigenetic mark is linked to the timing of genes that are reactivated on the X. Late-reactivating genes have a higher density of H3K27me3 on their genes than genes that reactivate early during XCR (Borensztein, Okamoto, et al., 2017). Conversely, early-reactivating genes are enriched for Myc-interaction sites, indicating a heightened sensitivity to TFs earlier on in XCR (Borensztein, Okamoto, et al., 2017). Myc is a transcription factor used to reprogram somatic cells into pluripotent stem cells (Takahashi & Yamanaka, 2016). The existence of early- and late-reactivating genes is exemplified during *in vivo* XCR in PGCs,

where for the *Pgk1* locus most PGCs reactivate between E12.5 and E13.5, while the *Hprt* locus does not begin XCR until E11.5 (Haramoto et al., 2021).

In summary, reactivation of the Xi happens biologically in the ICM of mice before implantation and in PGCs of both mouse and human. Three key characteristics define XCR *in vivo*: return to pluripotent state, repression of *Xist* and repressive histone mark erasure. There is a large interplay between the factors that characterize XCR, with *Xist*-related factors influencing pluripotency and vice versa. The same is true between epigenetic marks and the pluripotency network. This leads to an intricate process that is necessary for correct further development of specific tissues.

Therapeutic promise of XCR

XCR is a naturally occurring process that reactivates X-linked genes, characterized by 1) a return to pluripotent state, 2) *Xist* repression, and 3) erasing repressive epigenetic marks. These characteristics could be used *in vitro* to reactivate specific genes in X-linked diseases as means of a therapy.

XCR during reprogramming

In the last decade, XCR research has focused on the mechanics of reactivation during reprogramming. Pasque et al (2014) provide insight into the progression of cells undergoing a reprogramming treatment with OCT4, SOX2, c-MYC and KLF4 (Takahashi & Yamanaka, 2006). Several stages of reprogramming are defined through this, starting with the mesenchymal-epithelial-transition (MET) and followed by CDH1 expression. Part of the CDH1+ cells will then activate NANOG. Only when NANOG is expressed, the H3K27me3 marks are erased from the Xi together with the disappearance of macroH2A1 (Pasque et al., 2014). Subsequent to the reactivation of NANOG the Xist clouds that were still present in the first NANOG+ cells dissipate, while *Tsix* becomes active. Other pluripotency genes such as REX1 become reactivated around the same time. Biallelic expression of X-linked genes only starts to appear after this, indicating it is a late event. A live cell imaging paradigm, employed to visualize the process of XCR, showed the same repression of *Xist* at the same time as pluripotency factors reactivated (Tran et al., 2018).

Similar to reactivation *in vivo*, early- and late-reactivating genes are found during reprogramming (Janiszewski et al., 2019). The authors found that some X-linked genes even start becoming reactivated before complete *Xist* disappearance, contrary to the results of Pasque et al (2014). Furthermore, in contrast to the association of H3K27me3 density and late-reactivating genes *in vivo* there does not seem to be such an effect during reprogramming, where pluripotency TFs such as SOX2 and OCT4 are suggested to bind early and intermediate genes (Borensztein, Okamoto, et al., 2017; Janiszewski et al., 2019). The distance from escape genes has also been correlated to reactivation of genes, with genes that are closer to escapees having a higher incidence of being reactivated (Mira-Bontenbal et al., 2022). Other reports implicate the relative distance between genes and the centromere as a factor in whether a gene reactivates early or late (Aizawa et al., 2022). Genes that belong to a region closer to the centromere express earlier during the reprogramming process, even when *Xist* was still active. It seems like this region has a less condensed chromatin structure than the rest of the Xi, leading to higher accessibility (Aizawa et al., 2022). Significantly, the chromatin structure of the Xi and its decompaction are of great importance to the process of XCR as a whole (Generoso et al., 2023). This works through the cohesin subunit SMC1A, responsible for the open structure of the Xa and for establishing this open structure on the Xi as well during XCR. Intriguingly, *Xist* repels cohesin during XCI which suggests that *Xist,* by denying SMC1A action, causes the TAD-poor chromatin conformation of the Xi. The reformation of TADs is

therefore also included in XCR and starts in *Xist*-poor compartments of the Xi and surprisingly precedes chromatin decompaction and gene reactivation (Bauer et al., 2021).

XCR for therapeutic goals

Evidently, during reprogramming X-linked genes are reactivated. Some genes on the X chromosome have been linked to disease (reviewed in Z. Sun et al., 2022), including for example *GLA*, causing Fabry disease associated with lysosomal defficiency, *SMC1A* truncations that cause Cornelia de Lange syndrome, and *MECP2.* Heterozygous mutations in this last gene, which codes for methyl-CpG binding protein 2, cause a neurodevelopmental disorder named Rett syndrome (RTT) (Amir et al., 1999; Lyst & Bird, 2015). Interestingly, RTT is almost exclusively found in females as the hemizygous mutation in XY males is mostly lethal (Amir et al., 1999; Ben Zeev et al., 2002; Meloni et al., 2000). In female cells affected by the mutation (*MECP2*$^{\Delta}$), the X-chromosome with the wildtype *MECP2* copy is inactivated through XCI. Since in humans XCI is a random process, not all cells will express the mutated gene. This is called mosaicism and explains the different degrees to which RTT patients with the same *MECP2* mutation can be affected (Ishii et al., 2001). However, since the silenced wild type copy of *MECP2* is present in all cells of the patients, the possibility of targeted reactivation as a cure has been proposed and researched in recent years.

The earliest phenotypic signs of RTT show at 6-18 months of age, since neuronal maturation is affected by MECP2 (Guy et al., 2007; Kishi & Macklis, 2004). It has been shown that re-expressing *Mecp2* postnatally can alleviate phenotypic symptoms in mice (Guy et al., 2007). The authors here used a Cre-lox system implemented into a mouse model of RTT to reactivate *Mecp2* in adult mice. More recent studies tried to mimic this reactivation through disturbing the XCI process alone (Przanowski et al., 2018). Here the authors used a small-molecule inhibitor that affects the XCI promoting factors ACVR1 and PDPK1 (Bhatnagar et al., 2014). When the ACVR1 inhibitor was added to cells with monoallelic *Mecp2* expression, the amount of cells harboring biallelic expression increased to at least 60% of the population (Przanowski et al., 2018). This activation could be reversed when the inhibitors were removed from the system. For PDPK1 the return to biallelic expression was seen in around half the cells in the population and this effect could also be reversed. When tested in living adult mice (4 weeks old), the Xi reactivated in a third of the cells of the treated brain hemisphere after three weeks (Przanowski et al., 2018). Another small-molecule inhibitor that affects DNA methylation has been studied as well, in combination with an antisense oligonucleotide that targets Xist, and functions to restore some *Mecp2* expression from the Xi (Carrette et al., 2018; Mira-Bontenbal et al., 2022). *In vitro*, this combination faithfully restores *Mecp2* expression from the Xi at the level of 2% of that of the Xa, which is 12000 times more expression than without treatment. Although 2% does not seem like a lot, it was found that restoration of 5% non-mutated *Mecp2* can have great phenotypic effects (Guy et al., 2007). However, both studies do not show whether reactivation of a healthy allele of *Mecp2* could solve the problems that arise because of the mutated *Mecp2.*

In summary, reactivation of *MECP2* is possible *in vivo*, but is not complete and it does not completely reverse the phenotype of RTT. Furthermore, *in vitro* studies focused on *Mecp2* reactivation also do not show full reactivation. However, full reactivation may not be necessary to achieve the phenotypic reversal and relief for the affected patients.

Discussion

Every eutherian mammal with two X chromosomes is a mosaic of X-linked gene expression due to the rXCI that happens during development. This process is initiated by expression of the lncRNA *Xist* from the future Xi. *Xist* is encoded from the Xic locus on the X-chromosome containing several other genes important for the regulation of XCI. Their regulatory mechanisms are either *cis*-acting or *trans*-acting. *Cis*-acting regulators can also be said to reside within the same TAD, of which there are two in the Xic: one for *Xist* and one for *Tsix* (Figure 1). Their respective TADS are important for ensuring correct expression patterns and thus correct XCI. Upon inactivation, *Xist* eliminates the TADs on the entire X-chromosome by repelling cohesin. This creates a characteristic chromatin conformation on the Xi, consisting of two mega-domains separated by *Dxz4* (Figure 3). Changes to the chromatin are a key factor in stably maintaining XCI. Especially histone methylation was found to be important. If the methylation falls away, XCR will be the consequence.

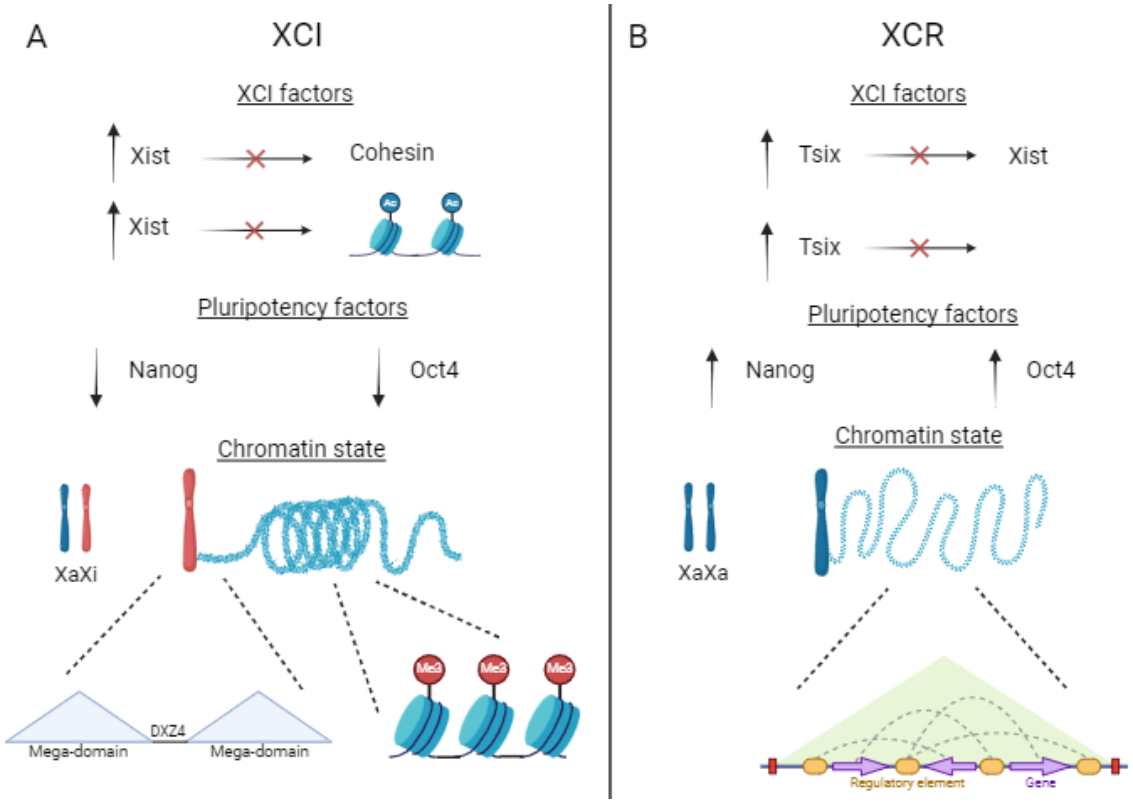

Figure 3: Comparison of the processes happening in XCI and XCR. For both XCI (A) and XCR (B) the condition of XCI factors, pluripotency factors and chromatin state are shown for the respective processes. For XCI, all these factors lean more towards a differentiated state as exemplified by the closed chromatin, low expression of *Nanog* and *Oct4*, and the absence of TADs. These repressive characteristics lead to the XaXi configuration. The reverse is true for XCR, with two active X chromosomes (XaXa), where *Xist* expression is repressed, which causes pluripotency markers to increase and facilitates an open chromatin state.

XCR naturally occurs in the ICM of mice to transition between iXCI and rXCI, as well as in PGCs of both mice and humans (Figure 2). The reactivation in PGCs is a gradual process, spanning almost a week in mice, while in the ICM it is completed within two days. These differing contexts of biological XCR could realistically play a role in the course of reactivation. The environments are inherently distinct, if not only because of the difference in developmental timing (blastocyst vs early differentiated tissues). An added factor for PGCs is the fact that their development happens during cellular migration. This means several different cell types could have an effect on migrating PGCs, while also giving rise to the possibility that the energy expenditure in a migrating cell allows less resources to go to achieving XCR. Cell-dependent

differences in XCR would not be unrealistic, seeing as escapees during XCI are also cell-type dependent (Tukiainen et al., 2017). Research into this might provide insight into contextual differences during development as a whole.

In humans there is a vital intermediate phase between XCI and XCR in which the X-chromosome biallelically expresses *XIST* but at low levels (XCD; Figure 2). Three key steps happen in all biological XCRs: return to pluripotency, deactivation of *Xist* and erasure of repressive histone marks, as summarized in Figure 3. Factors that control these processes are not specific to one of these, but instead bind all three together. For example, *Tsix* has both a role in switching off *Xist* expression and in the return of pluripotency together with Prdm14, where it functions to remove H3K27me3 marks from the Xi. This histone mark is also important in determining the order in which genes are reactivated, with a higher density of H3K27me3 indicating a late reactivation. For both biological and artificial XCR there is a lack of data concerning other histone marks than H3K27me3. Although H3K27me3 is of course relevant because of the link to PRC2 and thus *Xist*, the same would be true for H2AK119ub as deposited by PRC1. Other than these repressive marks, more research into the effect of XCI factors on activating marks, as seen in Yu et al. (2021) where the authors found that *Xist* is responsible for deacetylation, might also prove interesting in the context of XCR.

During reprogramming, XCR also needs to take place for successful reprogramming to iPSCs. This follows roughly the same characteristics as biological XCR, although more information about the chromatin structure during reprogramming is known than during *in vivo*. This shows the importance of cohesin in the accessibility of the X chromosome (Generoso et al., 2023). Since cohesin has also been implicated as being affected by *Xist* (Minajigi et al., 2015), it would be worth investigating if cohesin interactions with the X chromosome also change during physiological XCR. Seeing as reactivation can happen fully in reprogrammed cells, this is an interesting target for X-linked diseases such as Rett syndrome. Patients with this syndrome are almost exclusively female and have a heterozygous mutation of *MECP2*, which is subject to rXCI and is thus expressed in a mosaic together with the wildtype gene. Because of the presence of the healthy *MECP2* in every cell, a therapy which reactivates this allele could prove successful. This has been demonstrated to work in cells when treated with small-molecule inhibitors that target XCI factors or DNA methylation.

However promising this sounds, therapies that use *MECP2* reactivation risk raising the MECP2 levels too high which causes *MECP2* duplication syndrome (Van Esch, 2020). At the same time, when the Xi is non-specifically reactivated other X-linked genes will reactivate as well, erasing XCI in all mature cells. The effect of losing XCI later in life is not known broadly, since little is known about XCI maintenance in adults (Jacobson et al., 2022). Other strategies to re-express *MECP2* have been developed as well, using AAV-based gene therapy (Powers et al., 2023). This therapy is based on a viral vector which transports a healthy *MECP2* construct including promoter into the affected cells. When tested, significant symptom reduction was found albeit no full reversal of the phenotype. The AAV-treatment is considered safe to use in both mice and the non-human primate *Macaca fasicularis,* which indicates it as an interesting candidate for human clinical trials.

Upcoming research into XCR will have to build on these knowledge gaps in order to paint a more complete picture of the process. This might prove helpful in developing new and specific therapeutics against Rett syndrome among other X-linked diseases. This substantial and paramount information can be unraveled if the processes concerning the (in)active X are as well.

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