## In-class assignment: Fukuda et al. (2016) paper

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While the main claim of the paper is related to the maintenance of *Xist* imprinting, we will focus more on three aspects of the mechanism of this imprinting: chromatin ‘relaxation’, the effects of Kdm4b and TSA, and the real of Oct 4.

You are welcome to work on these questions individually or as part of a group. If you work in a group, your group will submit one set of answers for the whole group. Please ensure that all group members participate in the development of the answers.

The answers are to be submitted by email (as an attachment) by the end of class.

PLEASE FOCUS ON ANSWERING THE QUESTIONS IN PURPLE (2, 4, 6, 8, 9).

**Chromatin ‘relaxation’ and *Xist***

1.    The authors claim that the chromatin of and around the *Xist* locus is very relaxed on the paternal X of XX embryos, while it is very tight in mock-treated XmXm embryos. What evidence do they have to support this claim? How did they measure this ‘level of relaxation’ of the chromatin? Are you convinced by their evidence?

* Figure 1B shows significant differences in normalized distance (between Xist/Tsix region and Rnf12) between the DMSO (XmXm control) and the AE (XpY or XpXp)
* They did a DNA FISH measuring the distance of the Xist/Tsix region to the Rnf12 gene in bimaternal cells and biparental/XpY cells.
* Yeah sure.

2.    What can be said about the chromatin of and around the *Xist* locus in bimaternal, very early embryos that express Kdm4b and are treated with TSA? How do Kdm4b  and TSA affect the levels of chromatin ‘relaxation’? How does this relate to *Xist* expression and XCI?

  The chromatin around *Xist* locion the maternal chromosomes is highly condensed in early XmXm embryos. Embryos that express Kdm4b and are treated with TSA have a more relaxed chromatin structure around *Xist* locus than mock-treated XmXm embryos. These embryos have a higher *Xist* expression and therefore are able to inactivate the chromosome. (The chromosome is more likely to be inactivated when the *Xist* locus is decondensed).

**Lethality of embryos without paternal *Xist*.**

3.    XmXp mouse embryos that have a non-functional paternal *Xist* cannot survive to term? What happens to them (include mechanisms)? Why is it that XmY embryos (who definitely don’t have a paternal *Xist*!) are perfectly viable?

In mice, all paternal X chromosomes are inactivated in the pre-implantation stage. This happens through *Xist* expression, that covers the chromosome and silences the genes. If paternal *Xist* is nonfunctional, the cells are unable to silence the paternal X and suffer from double dosage of X-linked genes. This is not an issue for XmY embryos, since they only have one X chromosome.

4.    How do you think Kdm4b and TSA can rescue the lethality of a missing paternal *Xist* in Xm Xp embryos?

A deletion in paternal Xist leads to lethality due to two active X chromosomes (defective Xp silencing).

Treatment with Kdm4b and TSA can rescue this lethality by decondesing the chromatin around maternal Xist, allowing maternal Xist expression and Xm inactivation. When Xm is inactivated, the biallelic expression if the X-linked genes is prevented, and the embryo is rescued from lethality of missing paternal *Xist.*

5.    Recall how we saw that bimaternal mouse embryos die early in development and show underdeveloped extra embryonic tissues, and said that this is due at least in part to the incorrect dosage of ~100 autosomal, imprinted genes.. In light of what you know now about XCI, how could this lack of extra embryonic tissues be connected to the X chromosome in bimaternal embryos?

In mice, the initial paternal XCI is reversed in the embryo proper, but not in the extra embryonic tissues. The lack of extra embryonic tissue in bimaternal mouse embryos could stem from the lack of XCI (since these tissues presumably don’t have a mechanism for inactivating the maternal chromosome). Two active X’s in the extra embryonic tissues could lead to this impaired development.

**Roles of the pluripotency factor Oct 4**

6.    What can you directly conclude from Figure 5a?

siRNA against Oct4 is sufficient to decrease the expression of Xist and Tsix in XmXm morulae.

Unaltered Oct4 levels are necessary for ‘normal’ levels of Xist and Tsix in the XmXm morulae (i.e. the levels observed in controls).

7.    *Xist* and *Tsix* have antagonistic functions.Are the results in 5a consistent with this? Why or why not? Explain.

The results in Fig 5a indicate that *Xist* and *Tsix* are regulated similarly by Oct4. When Oct4 is knocked out, both transcript levels drop, suggesting that Oct4 is a positive regulator of expression of both genes. It is surprising for two genes with antagonistic functions to have the same positive regulator. Usually, the cell will express two antagonistic genes differentially, so the results are not particularly consistent with this. However, Oct4 is a pluripotency factor, and there are several examples of pluripotency factors having opposing effects on pathways. In this way, the results don’t contradict the common understanding of the field either.

8.    Consider Figure 6: The authors claim that Oct 4 elicits decondensation of the chromatin on and around the *Xist/Tsix* locus in male, female, and bimaternal embryos. What pieces of evidence do they present in this figure that supports this claim? If you had to select one single, most impactful/convincing piece of evidence form that figure, which one would you select and why?

Evidence:

Fig6a and 6b show that siRNA mediated knock-down of Oct4 and Rnf12 has an impact on the levels of H3K9me3 and H3K27me3 histone modifications in five regions around the Xist gene and promotor.

Fig6c shows that an Oct4 knock-down leads to more condensed chromatin (less distance between Xist and Rnf12) in XmXm embryos. A Rnf12 knockdown has no effect on chromatin condensation.

Fig6d shows that the Oct4 knockdown in biparental embryos has a similar condensing effect (as in XmXm) on Xm, but no effect on Xp.

Fig6e shows the same knockdown condensed chromatin on Xm in XmY embryos.

Fig6F shows that the ratio of the cells that express Tsix in an XmXp genotype have a statistically

       significant difference in cells that are controlled and have an OCT4 KD.

The most impactful evidence is found in Fig6d. This evidence suggests that the decondensation mediated by Oct4 is specific to the maternal X chromosome in XmXp embryos. This is interesting, because it reveals a possible mechanism for maternal X inactivation mediated by Oct4 activity.

**Mega-model**

9.    Now, try to fit everything you have learned about XCI into a sensible model that explains how XCI occurs in mouse at the pre-implantation and post-implantation stages. Your model should include at least *Xist, Tsix, Jpx*, CTCF, Oct 4, Nanog, Cdx2, H3K27me3 and H3K(various)ac. If you feel adventurous, make a note of which part of the model corresponds to which hypothetical mechanism seen at the start of the unit (i.e. blocking factor, symmetry-breaking factor, alternate states of the two Xs, *etc*).

Early pre-implantation stages:

Paternal XCI in all tissues. (relaxed chromatin around Xist/Tsix region).

Maternal X chromosome is active. The chromatin around Xist/tsix region is highly condensed (contains many H3K9me3 heterochromatin marks), possibly due to the prescence of the paternal genome during pronucleus formation.

Late pre-implantation stages:

Pluripotency factors (Oct4 and Nanog) induce Xist activation directly and Xite, which leads to Tsix activation which in turn represses Xist; thus, mediating opposing effects. This effect of the pluripotency factors prepare the cell for the subsequent random XCI, preparing both chromosomes available for inactivation.

Oct4 acts as a chromatin decondenser, relaxing the chromatin around maternal Xist/Tsix locus through chromatin alterations. This leaves the Xist/Tsix locus transcriptionally permissive (Xm-Xist imprint erasure?).

Physiological levels of Rnf12 are essential - an overdose leads to Xm inactivation by Xist expression.

Post-implantation stages:

CTCF is biallelically expressed.

Jpx is expressed from the X chromosome.

CTCF binds to the Xist locus, silencing the gene. Jpx binds to CTCF, and inhibits its activity, mediating Xist expression.

In females (XX), the Jpx:CTCF ratio is 1:1 (equal amounts of expression). This seems to be enough for Jpx to bind CTCF and inhibits its activity on one X chromosome, leading to XCI of a random chromosome.

In males (and Turner syndrome), there is twice as much CTCF expressed as Jpx (only one X), which results in no XCI.

The Cdx2 binds to the intron region of Xist and promotes the expression of Xist and is mostly found on the parental X,