

Title: Functional dissection of conserved sequences of Xist lncRNA during X-inactivation

Synopsis:

X-inactivation is an epigenetic process in mammals whereby one of the two X-chromosomes in females is shutdown to establish gene dosage equity between XX females and XY males. The master regulator of this process is a long non-coding RNA (lncRNA) called Xist (X-inactive-specific transcript). Xist lncRNA is essential for female survival and represents one of the best studied cases of a lncRNA with a clear regulatory and important function. Xist is monoallelically upregulated from the X-chromosome chosen for inactivation, 'coats' the entire chromosome *in cis*, establishes gene silencing and recruits several chromatin repressive complexes thought to be important for the maintenance of the repressive heterochromatic state of the inactive X-chromosome (Xi) [1]. The molecular mechanisms through which Xist guarantees these three functions and their interdependency are still not completely clear, but are crucial for the maintenance of the inactive state of the Xi, inert to re-activation.

Xist lncRNA is capped, spliced and polyadenylated, just like a common mRNA, however lacks a significant open reading frame. It is retained in the nucleus and remains attached to the future Xi. It is a remarkably long RNA, being 15-17 Kb in size and is poorly conserved at the sequence level. An exception to that are six regions of tandem arranged repeats, named A to F, that are somehow more conserved in sequence. However, their specific sequence, size and number of motifs can also vary greatly between species. The A-repeat which lies on the 5' end of Xist is the most conserved piece of this RNA and genetic analysis has revealed its crucial role on X-linked gene silencing [2]. Interestingly, the multiple chromatin transforming functions seems to be independent of the A-repeat [3]. Interestingly, the ability of coating seems to be guaranteed with some degree of redundancy by different parts of the transcript [2]. This shows the multi-tasking ability of Xist lncRNA, with several portions of the transcript performing different tasks. However, the specific role of each conserved tandem repeat region and a possible interplay between them has not been systematically addressed. This knowledge will be crucial to dissect the molecular mechanisms through which this unusual lncRNA acts to ensure a stable inactive state of the full Xi.

Objectives

This master project proposal aims at understanding the biological role of the different conserved tandem repeats of Xist in the process of X-inactivation. It has two specific aims:

- Aim 1 – Generation of inducible Xist mutants in mouse embryonic stem cells (mESCs) using CRISPR/Cas9 genome editing tools.
- Aim 2 – Characterization of the defects in X-inactivation caused by the different Xist mutants

Plan and Methods

Aim 1

In order to generate inducible Xist mutants, we will take advantage of a male ESC line harboring an inducible promoter at the Xist endogenous locus. In this cell line, administration of doxycycline to the medium causes Xist expression. Like the normal endogenous Xist in

females, inducible *Xist* in this system is capable to coat the X-chromosome, silence X-linked genes and induces typical chromatin changes of the Xi [2]. Prolonged *Xist* expression in this system causes cell lethality due to loss of expression from the sole X-chromosome. *Xist* deletions will be performed using the powerful CRISPR/Cas9 system. Briefly, two guide RNAs (gRNAs) flanking the repeat region to delete will be selected and cloned into standard CRISPR plasmid for genome editing. The plasmids are then electroporated into the cells and subjected to a short puromycin selection and single colonies are then picked up and screen for the deletion by PCR. As a proof of principle, *Xist* deletions have been efficiently generated through this strategy in this cell line (da Rocha et al., unpublished). After the initial PCR screen, the deleted region will be sequenced and RNA analysis will be performed under inducible conditions to verify that the mutant version is expressed and splice pattern is not altered. With this approach, we will efficiently generate 5 *Xist* mutants (for repeats B to F, as A is already available).

Techniques involved:

- *Tissue culture*: culture of mouse ESCs; generation of new ESC clones.
- *Molecular biology*: CRISPR/Cas9 genome editing; DNA/RNA extractions; genetic screening by PCR; expression analysis (RT-qPCR).

Aim 2

After generation and validation of the different *Xist* induced mutants, we will evaluate their capacity to coat, to silence X-linked genes and establish the heterochromatin status of the Xi. First, we will analyse the capacity of *Xist* coating of these mutants by performing RNA FISH for *Xist* upon inducible conditions. The capacity of *Xist* to silence X-linked genes will be assessed by both RT-qPCR and RNA FISH for several genes across the Xi, comparing inducible with non-inducible conditions. Finally, coupling *Xist* RNA FISH with immunofluorescence (IF) for chromatin factors (such as, Polycomb repressive complexes 1 and 2) and marks of heterochromatin (namely, H3K27m3 and H2Aub), we will monitor the chromatin transforming properties of the *Xist* mutants. This analysis will give us insights of the functional tasks played by the different *Xist* tandem repeats.

Techniques involved:

- *Tissue culture*: culture of mouse ESCs.
- *Molecular biology*: RNA extraction; expression analysis (RT-qPCR).
- *Cell biology & microscopy*: RNA FISH; IF/RNA FISH.

Expected outcomes and perspectives

As a result of this work, a new series of inducible *Xist* mutants will be generated, which are a valuable set of biological tools for the field of X-inactivation. Our initial characterization will give the first hints on the functional role of the different conserved repeats of *Xist* during XCI. Our future directions will be to use these *Xist* mutants to fish the protein interactors of the different repeats by ChIRP-MS, a method for comprehensive identification of RNA binding proteins by mass spectrometry [4]. Furthermore, we would like to extend our study the human *Xist* RNA using induced pluripotent stem cells (iPSCs) as model systems to understand how evolutionary conserved are the functions of each repetitive domain across species.

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