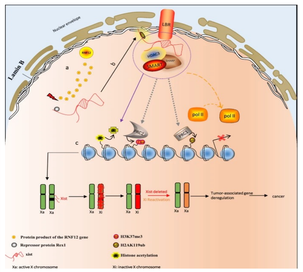
Generation of a Live Cell Imaging System to Investigate X-linked mRNA Expression Dynamics during Immune Responses

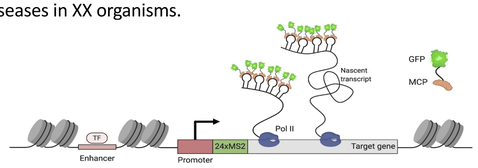
Powell, Alexis | SEAS 2025
Lim, Bomyi | Trudeau, Bobby
University of Pennsylvania School of Engineering and Applied Science | Chemical and Biomolecular Engineering | Penn Undergraduate Research Mentoring Program

Introduction

- Protein synthesis is regulated naturally or manipulated in lab. DNA is either accessible for transcription or inaccessible (heterochromatin); accessibility is modulated by DNA's interactions with histone proteins. Heterochromatin forms when histone tails are modified via methylation.
- X-chromosome inactivation (XCI) is an epigenetic process that prevents an XX organism from imbalances within its genome and regulates gene expression. Genomic imbalance may cause dysfunctionality or other immune implications.
- XCI occurs as a non-coding RNA, the X inactivation specific transcript (Xist), coats the X-chromosome to compact the chromosome into a barr body. DNA is inactivated by methylation.
- XCI maintenance was found to be diverse among single cells, cell types, and other species members. It functions differently in immune cells, as evidenced by varying patterns of Xist RNA localization.
- We are investigating this XCI and escape phenomenon as it relates to immunity. In XX organisms, instances of autoimmune diseases, such as rheumatoid arthritis, are much more prevalent than in their XY counterparts. The tendency of some X-chromosomes to escape inactivation is hypothesized to influence rates of autoimmune diseases in XX organisms.



Xist mechanism (Wang et al)

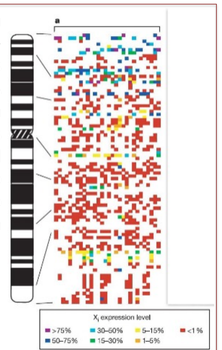


MS2-GFP system (Hoppe et al)

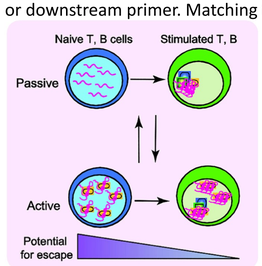
- Assay uses stem-loops formed by the coat protein of an MS2 (MCP) bacteriophage fused with a green fluorescent protein (GFP), and reporter mRNA consisting of stem-loops bound by the MCP-GFP system. MCP-GFP and PCP-RFP (PP7/red fluorescent protein) is highly specific to the RNA containing those stem-loops.

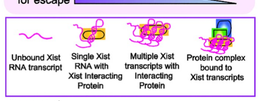
Abstract

The experiment was designed to investigate immune responses by editing stem cells to develop a mouse model. The X-linked genes–Rbm3, Was, and Pim2– were endogenously tagged with MS2 or PP7. We used CRISPR-mediated edits by which we matched the left and right homology arms of genomic DNA (gDNA). The donor vector was comprised of repeated MS2/PP7 stem-loops, an antibiotic resistance gene, neomycin (Neo), and fragments of DNA that serve as a template for repairing DNA breaks. Upon editing, we genotyped several colonies to confirm the correct insertion whereby a section of DNA specific to the correctly edited mouse cell is amplified. We used two pairs of primers to verify each side of the edit, one primer unique to the insert, and an upstream or downstream primer. Matching the correct distance between primers is confirmed using gel electrophoresis and verifying the existence of MS2, or PP7, and Neo between gDNA strands. If the insert is valid, cells from the colony with the correctly tagged X-linked gene are sent to Penn Vet to be grown. These cells are injected into a mouse blastocyst so that all cells become genetically identical to cells grown with edits. The blastocyst is implanted into a pseudo-pregnant female mouse for growth. Once mice with MS2 and PP7 tagged genes are developed, they will cross to produce offspring with both MS2 and PP7 tagged. The goal is to image the genes in the immune cells and bind their receptors to pathogenic infections to observe expression of the specific X-linked gene tagged. Additionally, we will examine the differences in expression between naïve and activated T and B cells by experimentally binding antibodies to CD28 and CD3 receptors.



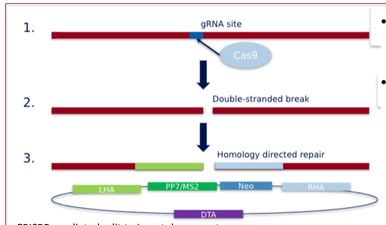
XCI Escape





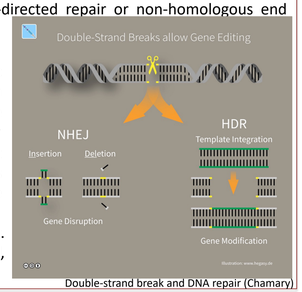
Sierra et al

Methodology



CRISPR-mediated edit to insert donor vector

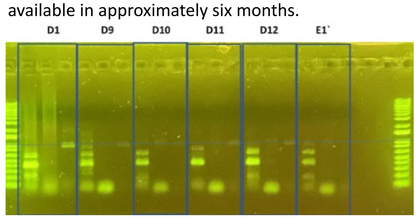
- Transcription dynamics and regulation are visualized with confocal microscopy, and quantitative imaging data are collected during live imaging.
- CRISPR-Cas9 is used to edit DNA—the mechanism in bacteria begins with spacer acquisition. crRNA is then processed as tracrRNA binds to the sequence. Cas9 cuts these fragments and integrates itself. The PAM sequence is recognized, and a double-strand break occurs at the site. Either a homology-directed repair or non-homologous end joining occurs.
- Confirm successful editing: gDNA samples from each gene-specific edited cell type are run in a polymerase chain reaction machine with water, GOTAQ (polymerase + NTPs), and forward (fw) + reverse (rv) primers. Product is loaded through 1% Agarose gel to verify whether the vector was inserted–determined by presence of bands at correct distance between primers on each homology arm (LHA/RHA). Bands were separated by polarity and compared to a DNA ladder with base pair distances labeled. DTA (toxin) and control were tested.
- Several genotyping trials were performed to validate 68 colonies. Various distances were attempted; multiple bands/smearing occurred, caused by non-specific binding of primers to other sequences.



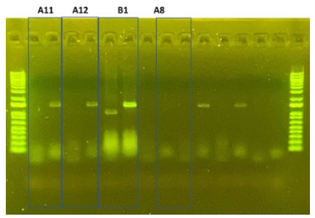
Double-strand break and DNA repair (Chamary)

Results

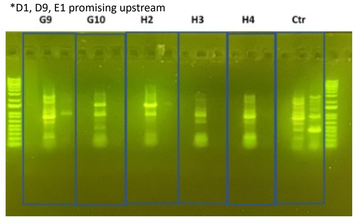
Research is still in progress, but the next step (input into mice cells) is underway. New data will be available in approximately six months.



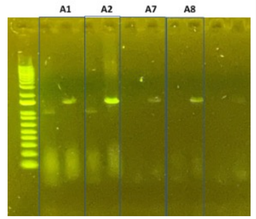
MS2-tagged Pim2 gDNA (1.0 kilobases (kb), upstream; 1.5 kb, downstream; 0.8 kb, DTA)
*D1, D9, E1 promising upstream



Rbm3 gDNA (1.5 kb, downstream, Neo to genome)

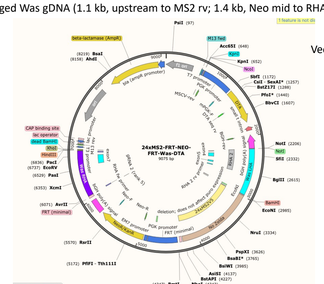


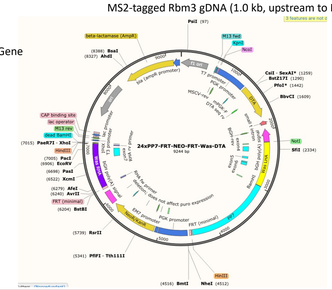
MS2-tagged Was gDNA (1.1 kb, upstream to MS2 rv; 1.4 kb, Neo mid to RHA rv)



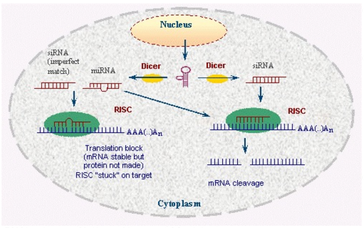
MS2-tagged Rbm3 gDNA (1.0 kb, upstream to MS2 LHA)

Vectors on SnapGene



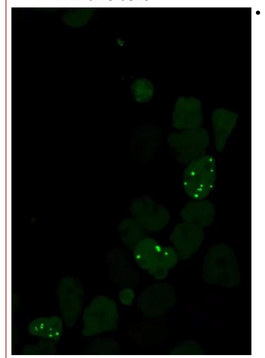


Future Work

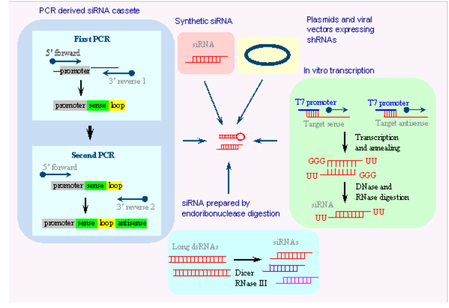


RNAi pathway (National Library of Medicine)

- Gene silencing can be conducted using RNA interference (RNAi) pathways.
- RNAi system occurs naturally to resist endogenous parasites and exogenous pathogenic nucleic acids; the mechanism uses both small interfering RNAs (siRNAs) and microRNAs.
- RNAi is triggered by foreign and viral DNA or double-stranded RNA, transposons, or pre-microRNAs.
- RNA transcript is spliced into mRNA and enters the cytoplasm. Double-stranded precursors bind to an RNase II enzyme, dividing the RNA. The guide strand binds to argonaut to form RNA-induced silencing complex (RISC). siRNAs lead RISC to mRNA strands according to their sequences; argonaut cleaves mRNA, causing it to degrade. microRNAs behave similarly, with imprecise targeting.
- RNAi is induced experimentally by introducing double-stranded RNAs or RNA loops. Experiments will use siRNAs in immune cells to target the transcription of specific proteins and induce XCI/escape.
- RNAi to unsilence an X-chromosome for both modeling escape and comparing the control (after introducing an immune challenge). The goal is to explore a new methodology to knock down Xist in immune cells.


Live cell imaging system of a reporter gene

- Future research on gene silencing will also use CRISPR interference. Cells controlled by RNA or CRISPR interference will be imaged.



RNAi future experiments (National Library of Medicine)

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- Lim Lab

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