IMMUNE GENES ARE PRIMED FOR ROBUST TRANSCRIPTION BY PROXIMAL LONG NONCODING RNAs LOCATED IN NUCLEAR COMPARTMENTS

Aurora Mazzetti – Elena Masini – Martina D'Agostino

Regulation of gene expression by cis-acting lncRNAs

Long non-coding RNAs (lncRNAs) are defined arbitrarily as transcripts of more than 200 nucleotides that have been implicated in a wide array of cellular processes

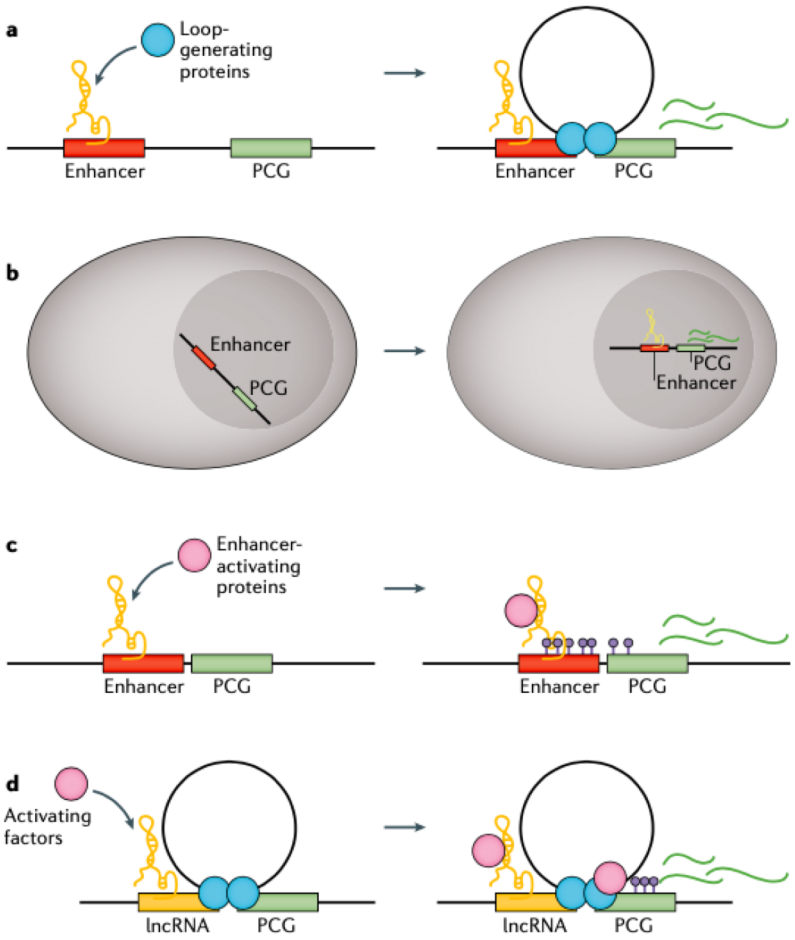
One type of lncRNA classification is based on the location at which the lncRNA functions relative to its transcription site

Trans-acting lncRNAs are transcribed from enhancers, then relocate to their sites of function to exert their influence elsewhere

- non-coding RNAs produced at enhancers, termed enhancer RNAs (eRNAs)

Cis-acting lncRNAs are those whose activity is based at and dependent on the loci from which they are transcribed. The largest group of cis-acting lncRNAs are those that function to augment the expression of target genes, akin to the function of enhancers

Cis-acting lncRNAs can activate target protein-coding genes (PCGs)



Recruitment of proteins that modulate chromatin loops

Potentiate the enhancer to activate target genes by affect its position

Potentiate the enhancer by the recruitment of proteins that enhance gene expression

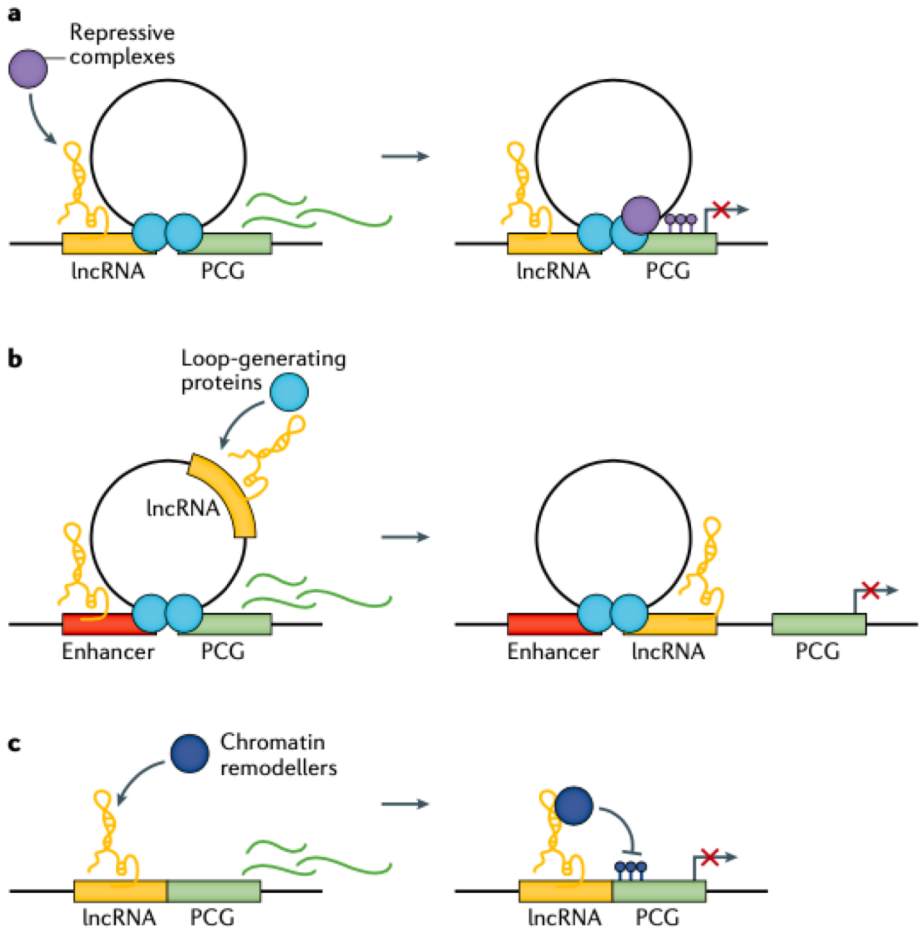
Pre-formed chromatin loops bring the lncRNA into the proximity of target genes

Cis-acting lncRNAs can act to repress the expression of target genes

Recruitment of proteins that repress gene expression

Competition over available enhancers in the vicinity

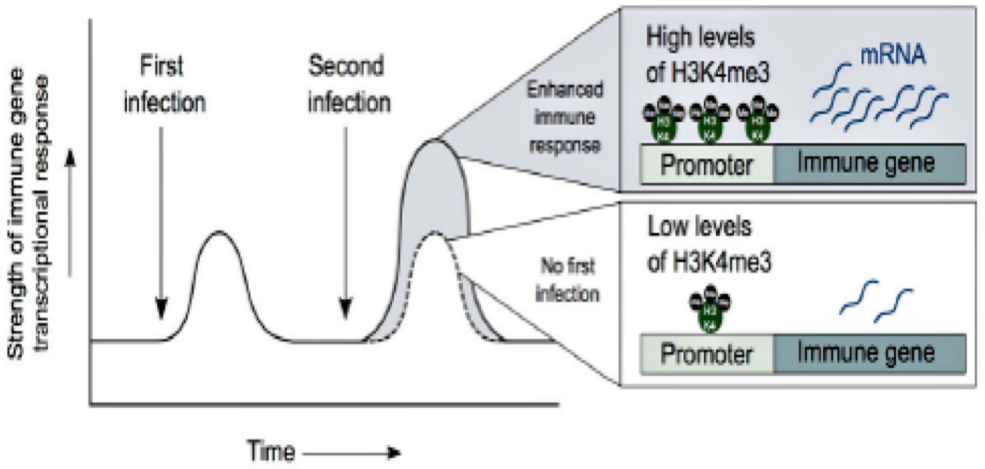
Transcriptional interference

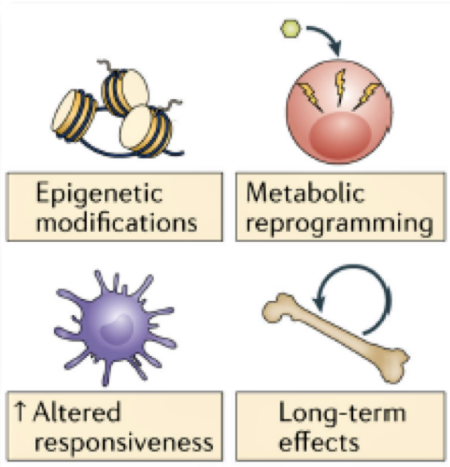


What is Trained Immunity?

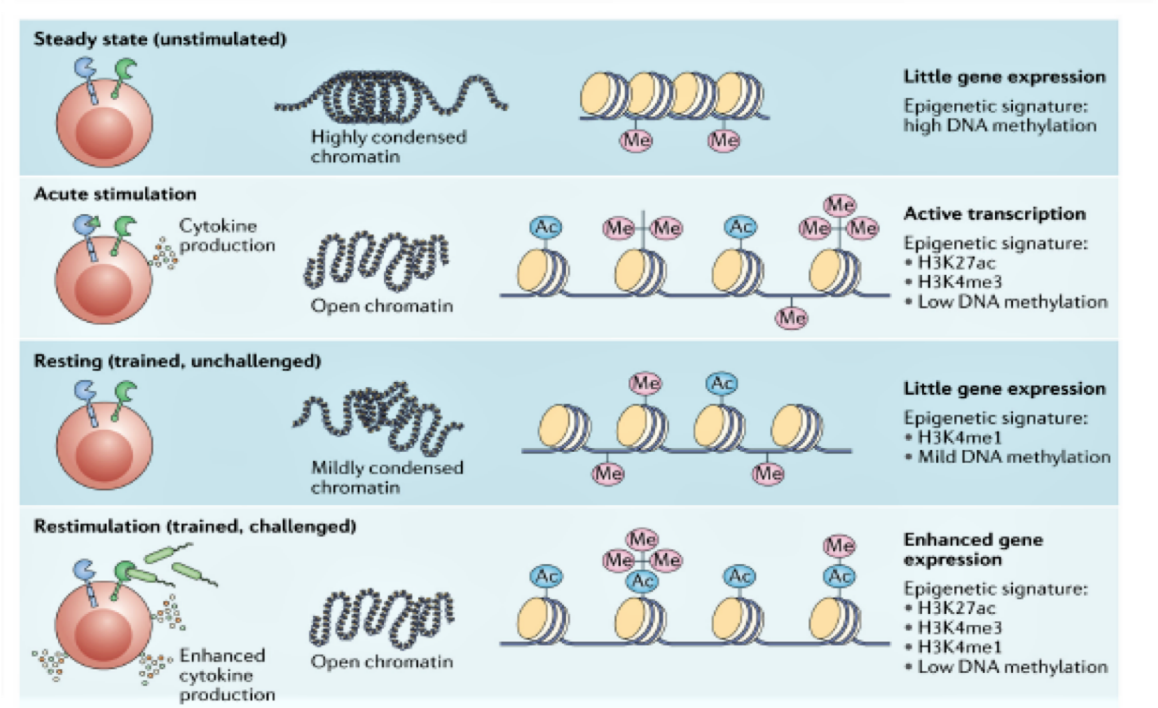
The ability of innate immune cells to form immunological memories of prior encounters with pathogens, resulting in **enhanced inflammatory response and an increased transcription** of innate immune genes

The hallmarks are the activation of various pathogen recognition signaling pathways, a shift in cellular metabolism towards a glycolytic state and **extensive epigenetic alterations throughout the genome** such as changes in chromatin organization at the level of TADs, transcription of lncRNAs, different DNA methylation pattern





“Epigenetic scar”



STIMULATION OF INNATE IMMUNE CELLS IS ACCOMPANIED BY THE DEPOSITION OF CHROMATIN MARKS, ALLOWING QUICKER AND ENHANCED RECRUITMENT OF TRANSCRIPTION FACTORS AFTER SECONDARY CHALLENGE

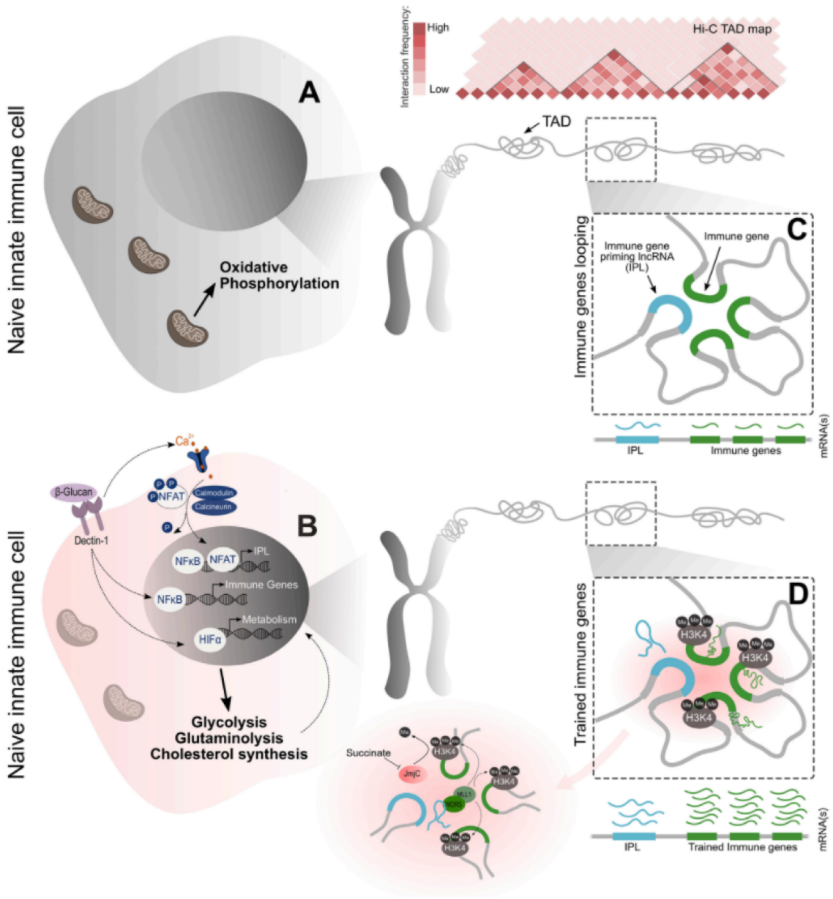
Nuclear architecture and lncRNAs influence epigenetic gene regulation

Many TNF-responsive innate immune genes are located within the same TAD, and engaged in preformed chromosomal contact with enhancers located within the same TAD

lncRNAs are emerging to be key modulators of gene activity by their influence upon epigenetic status regulating their trancription

the activity of several lncRNAs have been shown to be associated with a multitude of disease states, such as cancer and inflammation

HOTTIP and NeST are two lncRNAs that directly interact with WD repeat-containing protein 5 (WDR5) to direct mixed lineage leukemia protein 1 (MLL1) to target genes, to catalyse H3K4me3 at target gene promoters



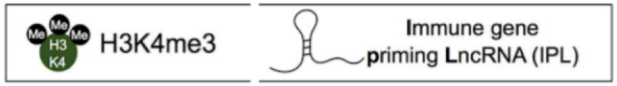
lncRNAs regulate the epigenetic reprogramming of innate immune genes

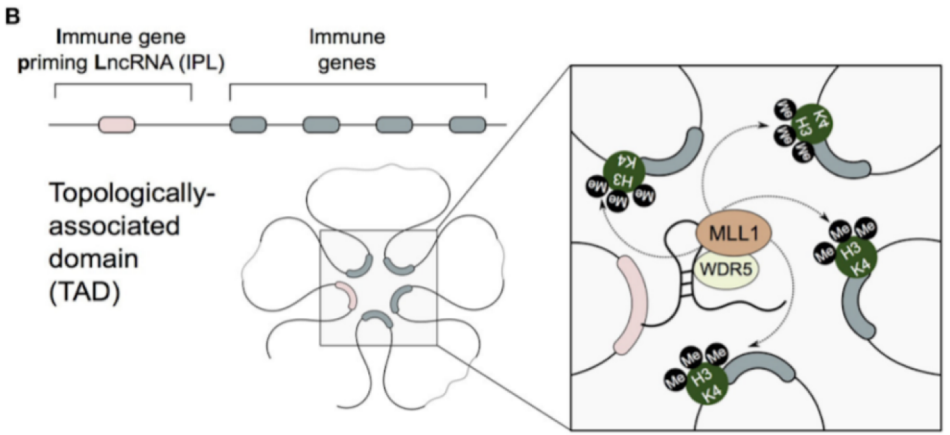
3D chromatin topology enables key trained immune genes (e.g., IL1β, IL-6, IL8) to engage in chromosomal contacts with a newly identified subset of lncRNAs: **IPLs** (immune-gene priming lncRNAs)

One formed chromosomal contacts with the ELR+ CXCL chemokines: **UMLILO** (Upstream Master LncRNA of the Inflammatory chemokine LOcus)

UMLILO acts in *cis* to direct the **WDR5/MLL1 complex** across the *CXCL* chemokine promoters enabling their **H3K4me3** epigenetic priming, prior to their transcriptional activation

Other trained immune genes (e.g., IL-6 and IL1β) are also regulated in a similar IPL-mediated manner



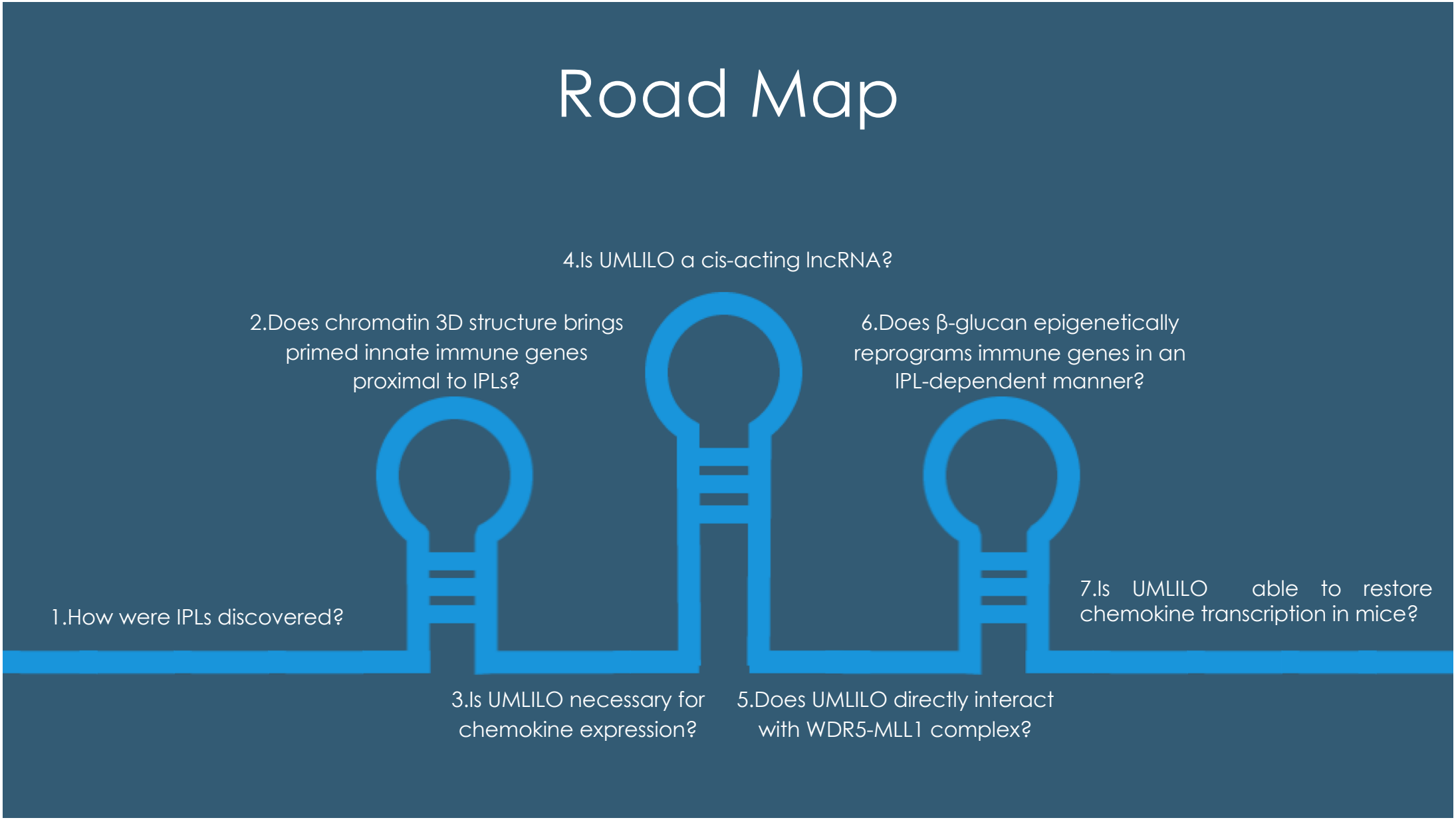


lncRNA-mediated regulation is central to the establishment of trained immunity

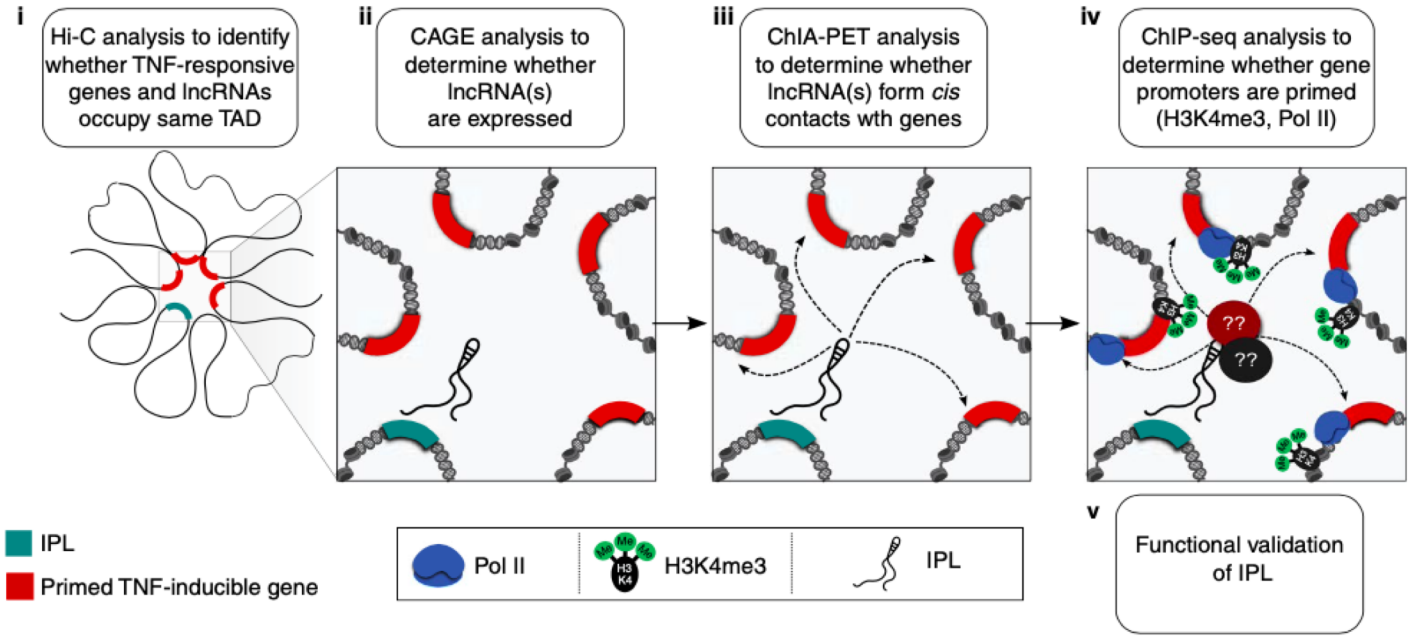
Take home messages

- **Cis-acting lncRNAs** activity is based at and dependent on the loci from which they are transcribed and they can activate or repress the expression of target genes
- Trained immune cells are **epigenetically reprogrammed** and as a result robustly express immune genes, enhancing their capability to resolve infection
- Accumulation of **H3K4me3** epigenetic marks on multiple immune gene promoters underlies robust transcriptional responses during trained immune responses
- Trained immune genes engage chromosomal contacts with a newly identified subset of lncRNAs: **IPLs**
- **UMLILO** is an IPL that acts in *cis* to direct the **WDR5/MLL1 complex** across the *CXCL* chemokine promoters enabling their training

Road Map


4.Is UMLILO a cis-acting lncRNA?
2.Does chromatin 3D structure brings primed innate immune genes proximal to IPLs?
6.Does β-glucan epigenetically reprograms immune genes in an IPL-dependent manner?
1.How were IPLs discovered?
7.Is UMLILO able to restore chemokine transcription in mice?
3.Is UMLILO necessary for chemokine expression?
5.Does UMLILO directly interact with WDR5-MLL1 complex?

How was UMLILO discovered?



Discovery pipeline to identify IPLs

ChIA-PET experimental protocol

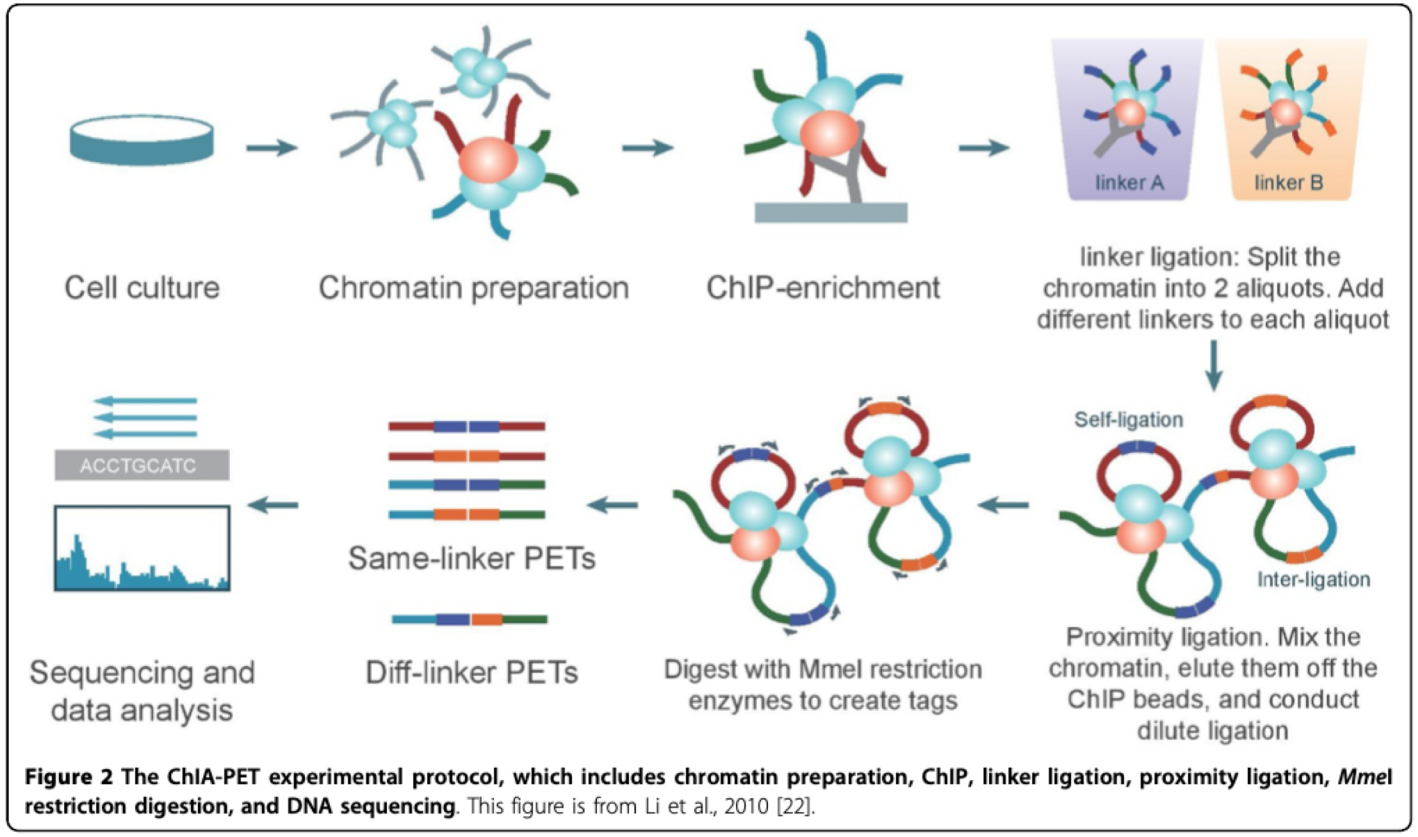
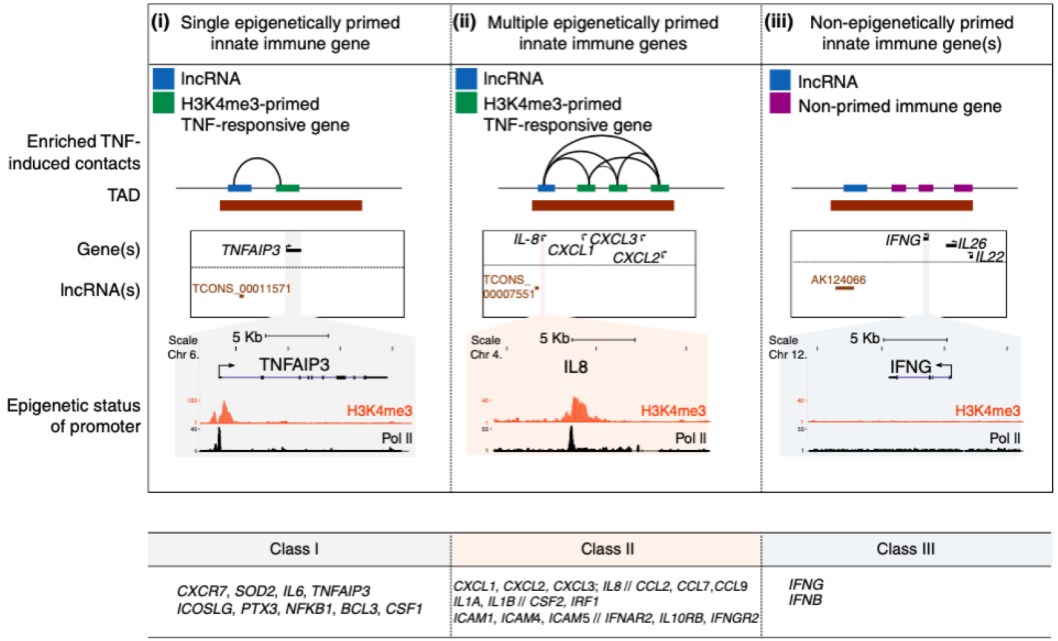


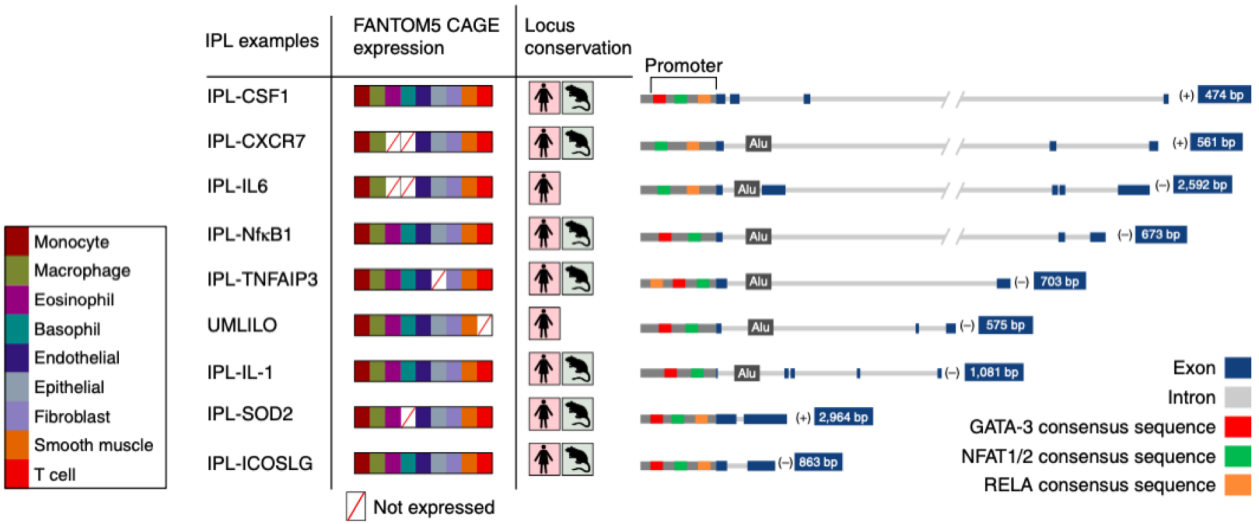
Figure 2 The ChIA-PET experimental protocol, which includes chromatin preparation, ChIP, linker ligation, proximity ligation, *Mme*I restriction digestion, and DNA sequencing. This figure is from Li et al., 2010 [22].



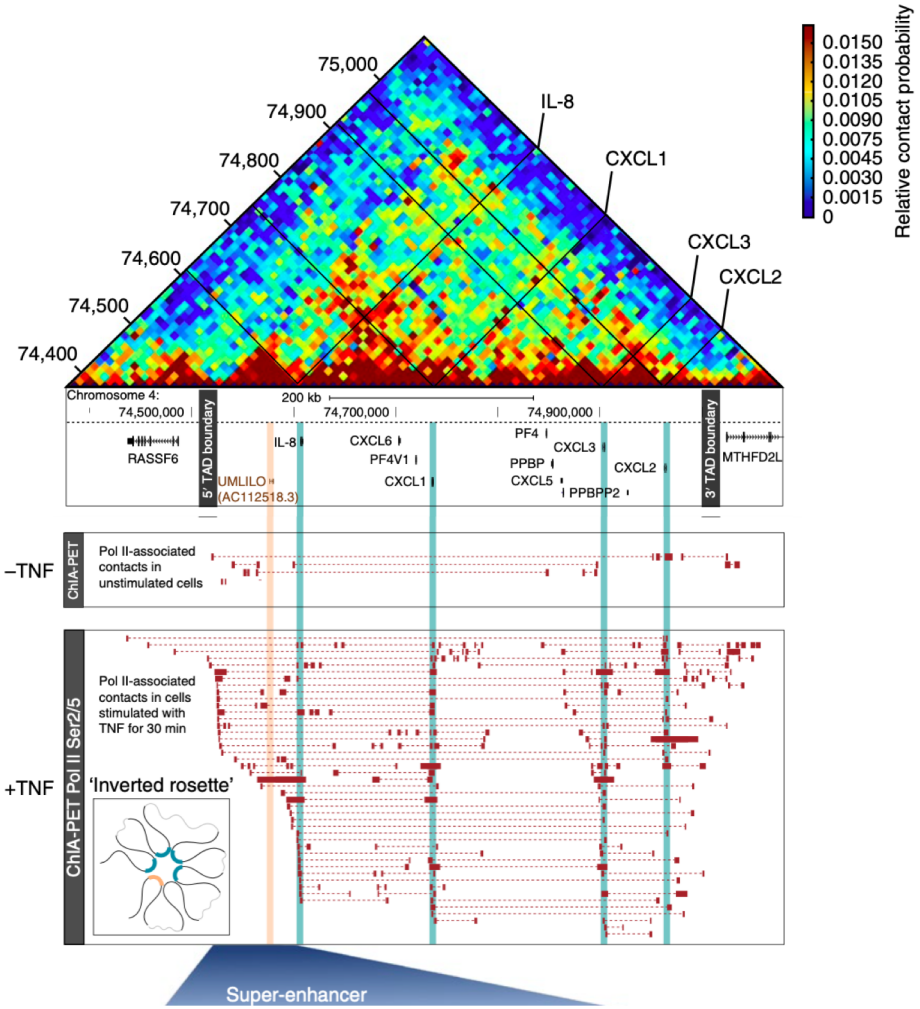
Does chromatin 3D structure brings primed innate immune genes proximal to IPLs?

Upon TNF stimulation, chromosomal contacts were enriched between TNF-responsive genes and IPLs for both class I and class II innate immune TADs

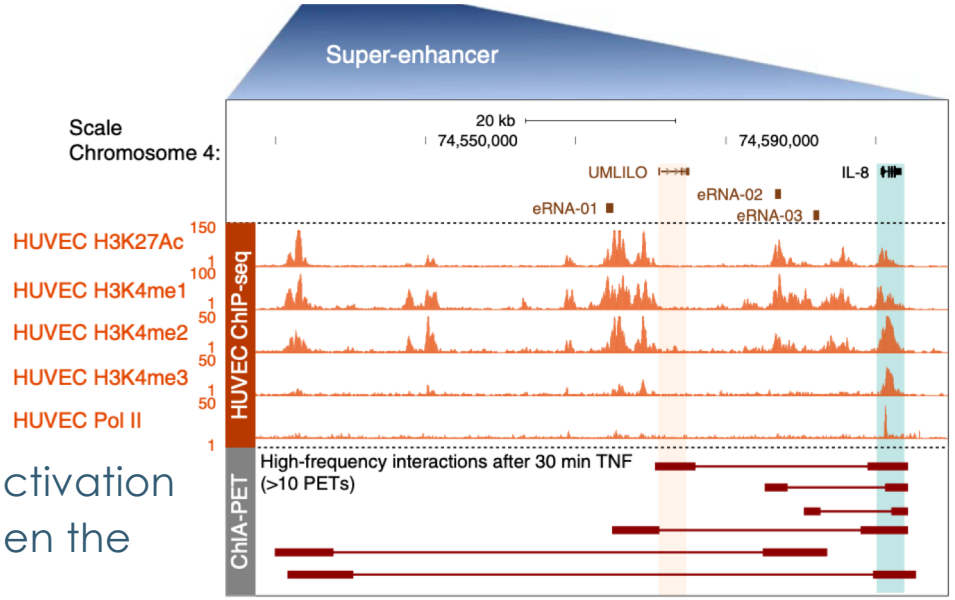
IPLs are multi-exonic, low-abundance lncRNAs expressed across a wide range of innate immune cell types



Is UMLILO transcribed within the CXCL chemokine TAD?

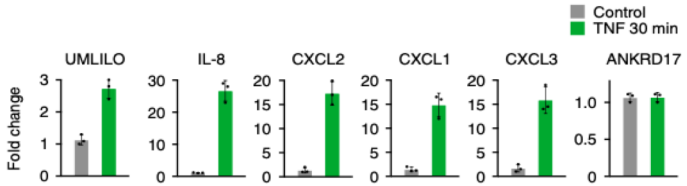


UMLILO does not possess the typical eRNA histone modifications, H3K4me1 and H3K27ac

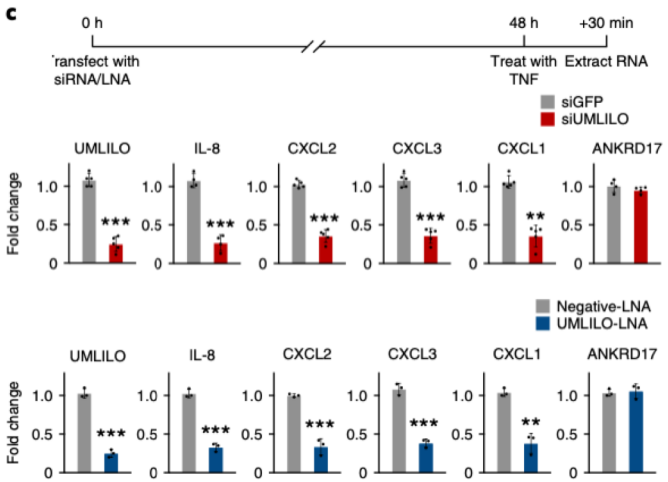


ChIA-PET analysis confirmed that upon gene activation with TNF there were **numerous contacts** between the super-enhancer region and the CXCL genes

Is UMLILO necessary for chemokine expression?



TNF strongly induces the chemokines concomitant with an increase in UMLILO expression from baseline levels



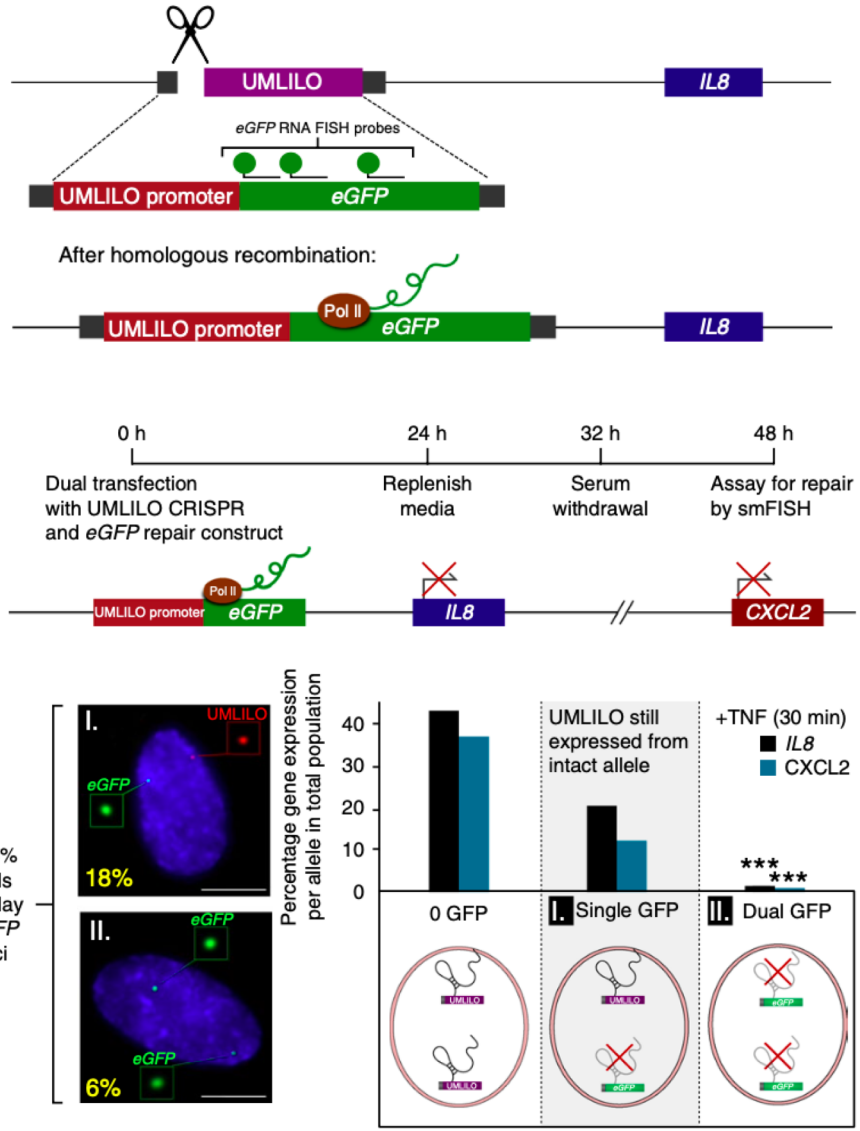
Knockdown of UMLILO with either siRNA or LNA GapmeRs, prior to chemokine gene induction using TNF, was sufficient to **significantly abrogate chemokine expression**

Is UMLILO necessary for chemokine expression?

The CRISPR–Cas9 system was used to delete the genomic sequence encoding UMLILO and replace it with an eGFP reporter sequence

There was a significant reduction in chemokine expression in cells displaying dual eGFP foci

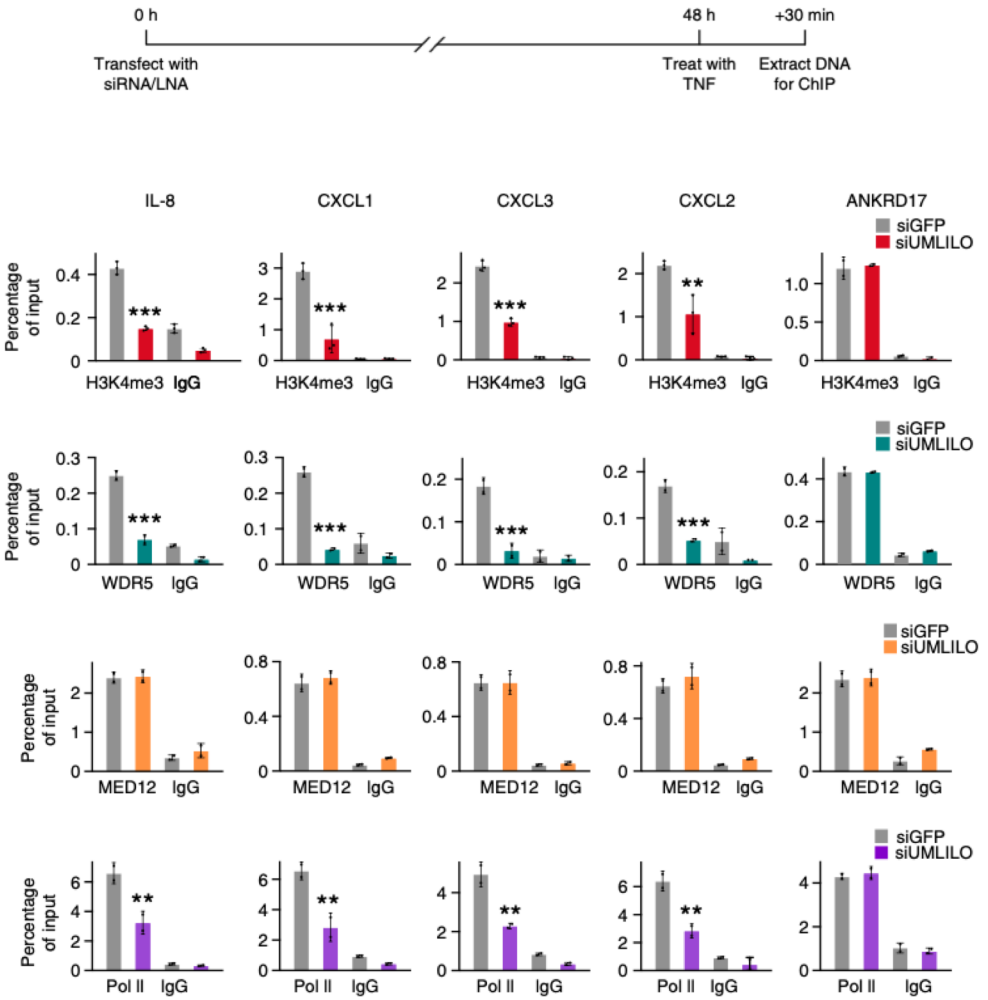
THESE EXPERIMENTS DEMONSTRATE THAT UMLILO INFLUENCE THE TRANSCRIPTION OF THE CXCL CHEMOKINES

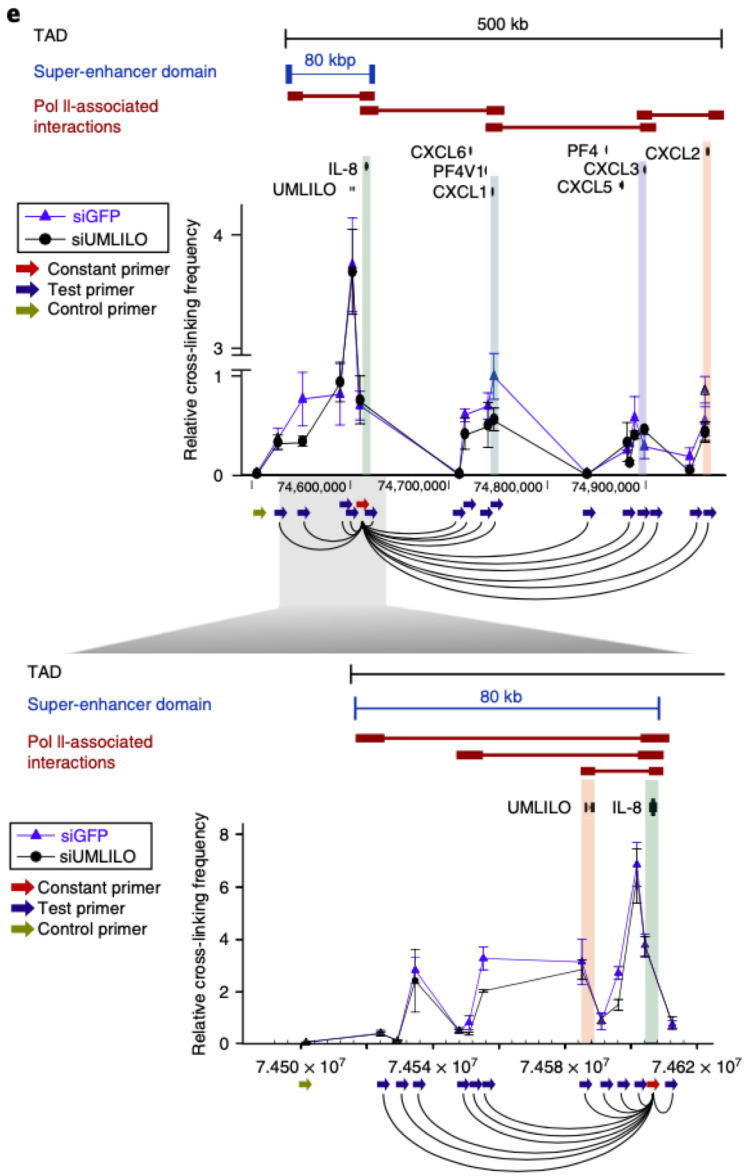


Does UMLILO regulate H3K4me3 across the CXCL chemokine promoters?

siRNA knockdown of UMLILO significantly reduced WDR5 binding and resulted in the loss of Pol II and H3K4me3 marks at chemokine promoters

THIS RESULTS INDICATE THAT UMLILO REGULATES H3K4ME3 ACROSS THE CXCL CHEMOKINE PROMOTERS





Does UMLILO instruct the chromosomal looping?

Silencing of UMLILO by siRNA followed by 3C analysis showed that **chromosomal contact** across the chemokine TAD **remained unaffected** by loss of the RNA

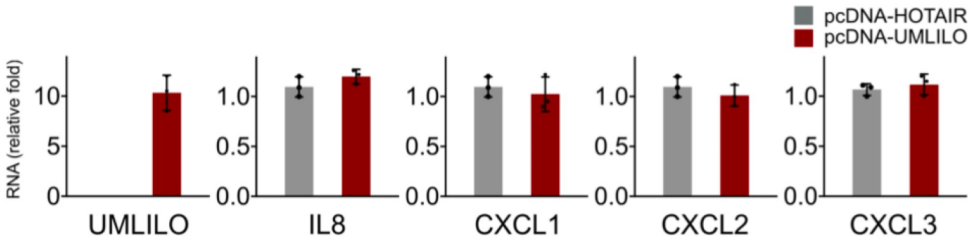
THIS DATA SUGGEST THAT THE UMLILO TRANSCRIPT DOES NOT INSTRUCT CHROMOSOMAL LOOPING

Take home messages

- Chromatin 3D structure brings H3K4me3-primed TNF-responsive genes proximal to IPLs
- UMLILO is a new super-enhancer-resident lncRNA that is transcribed within the ELR + CXC chemokine TAD
- Knockdown and knock-out of UMLILO abrogates chemokine transcription
- The UMLILO lncRNA regulates H3K4me3 across the CXCL chemokine promoters

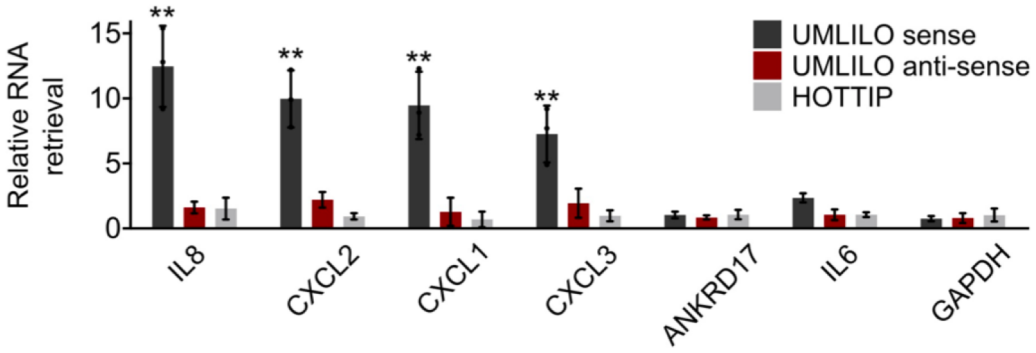
Is UMLILO a cis-acting lncRNA?

Overexpression of UMLILO did not alter chemokine transcription, suggesting that UMLILO may act in cis



ChIRP–qPCR showed that the UMLILO transcript is in close proximity to the CXCL promoters

THIS PROVIDED EVIDENCE THAT UMLILO IS A CIS-ACTING, CHROMATIN-ASSOCIATED TRANSCRIPT

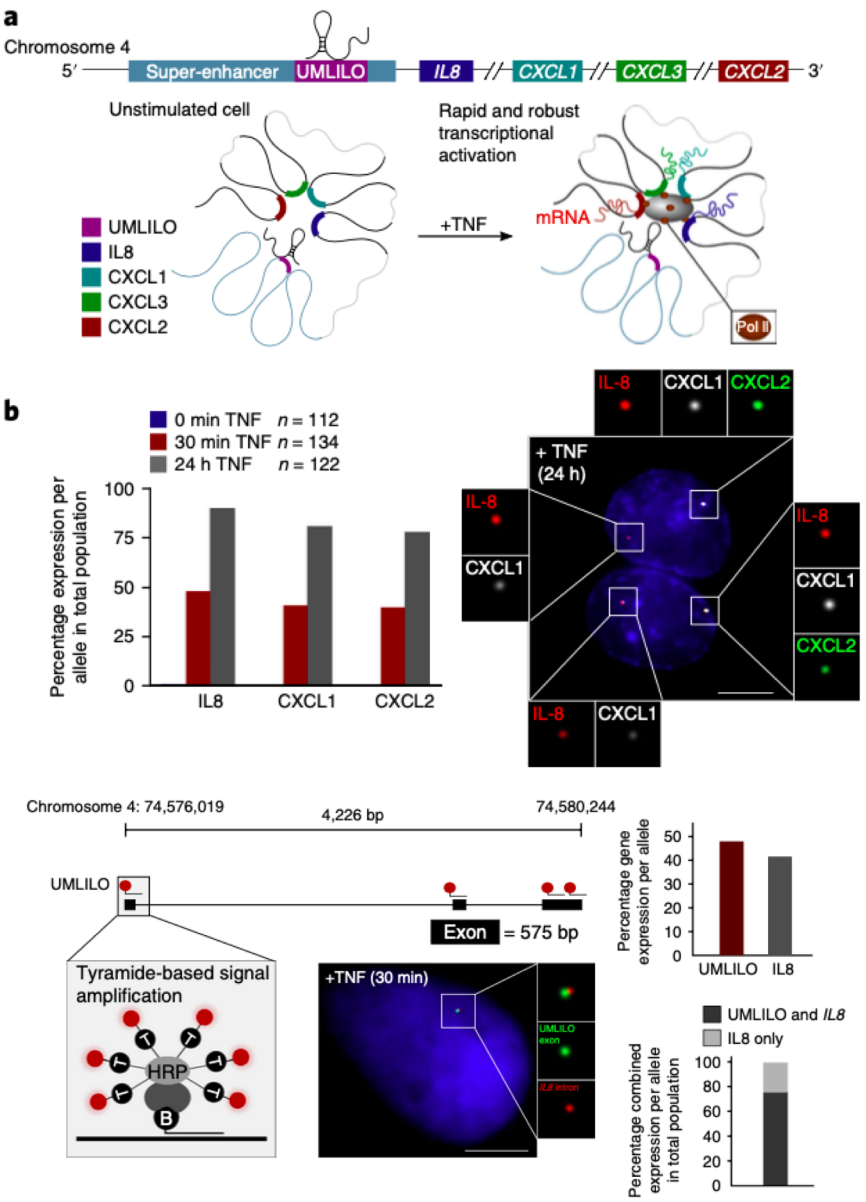


Is UMLILO a cis-acting lncRNA?

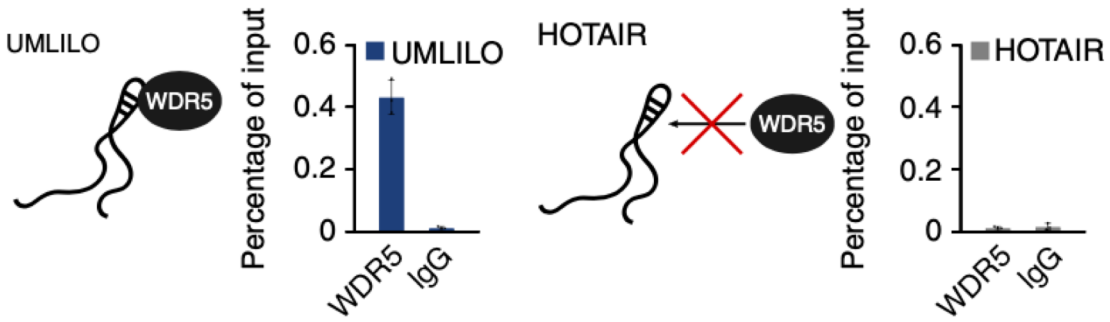
Intronic RNA FISH foci of co-expressed CXCL chemokine genes always co-localize

Simultaneous exonic TSA RNA FISH on UMLILO and IL8 showed that **co-localization** between exonic UMLILO and the intronic portion of IL8 was frequently observed

ADDITIONAL EVIDENCES THAT UMLILO IS A *CIS*-ACTING TRANSCRIPT



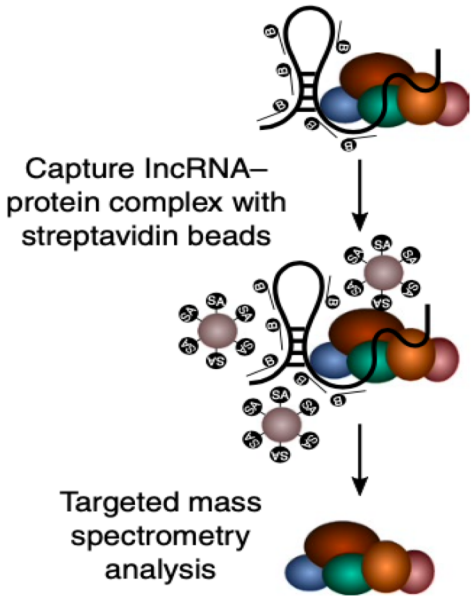
Does UMLILO directly interact with WDR5?

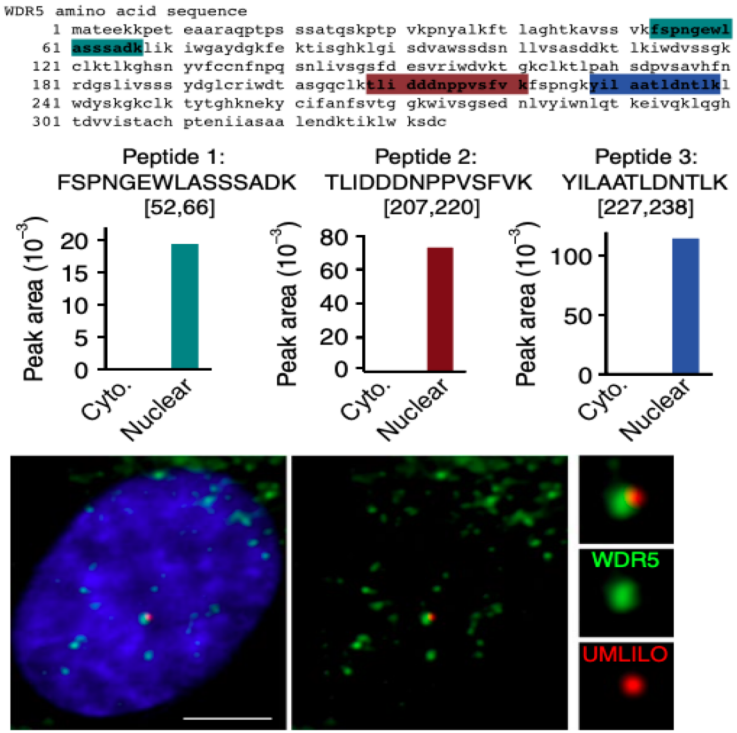


Immunoprecipitation of WDR5 retrieved UMLILO, but not HOTAIR

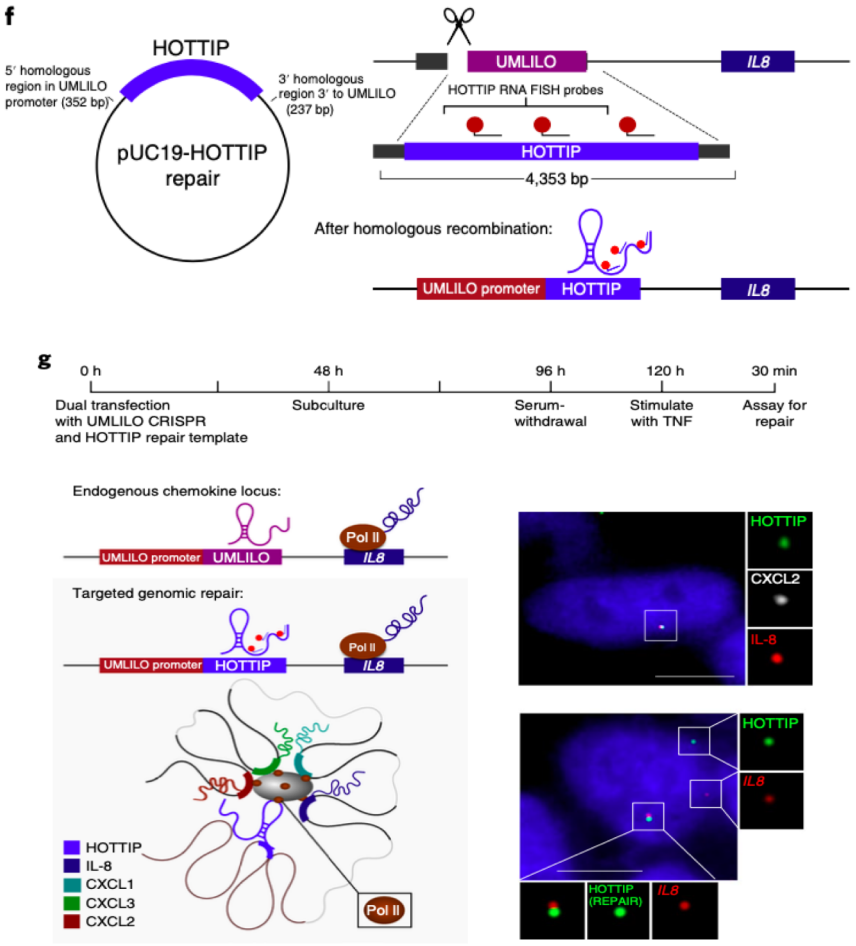
WDR5 has been identified in the nuclear fraction of the UMLILO pulldown

Immuno–RNA FISH showed that distinct foci of **WDR5 associate with UMLILO**





Is UMLILO activity strictly related with its capacity of binding WDR5?



HOTTIP is a lncRNA that binds to WDR5 to facilitate the H3K4me3-mediated activation of HoxA genes

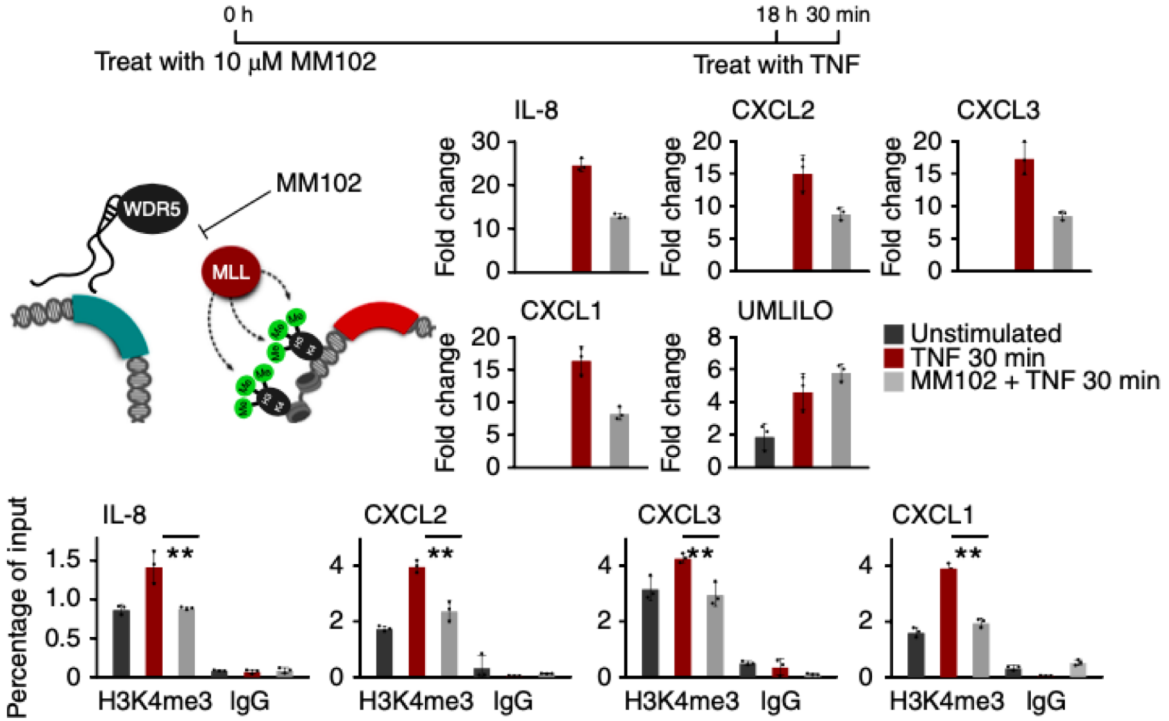
TheCRISPR–Cas9 system was used to delete the genomic sequence encoding UMLILO and replace it with the sequence of HOTTIP in HeLa cells. Replacing endogenous UMLILO with HOTTIP restores chemokine transcription

THIS DEMONSTRATES THAT SUBSTITUTING A WDR5-INTERACTING LNCRNA INTO THE UMLILO LOCUS RESTORES CHEMOKINE TRANSCRIPTION

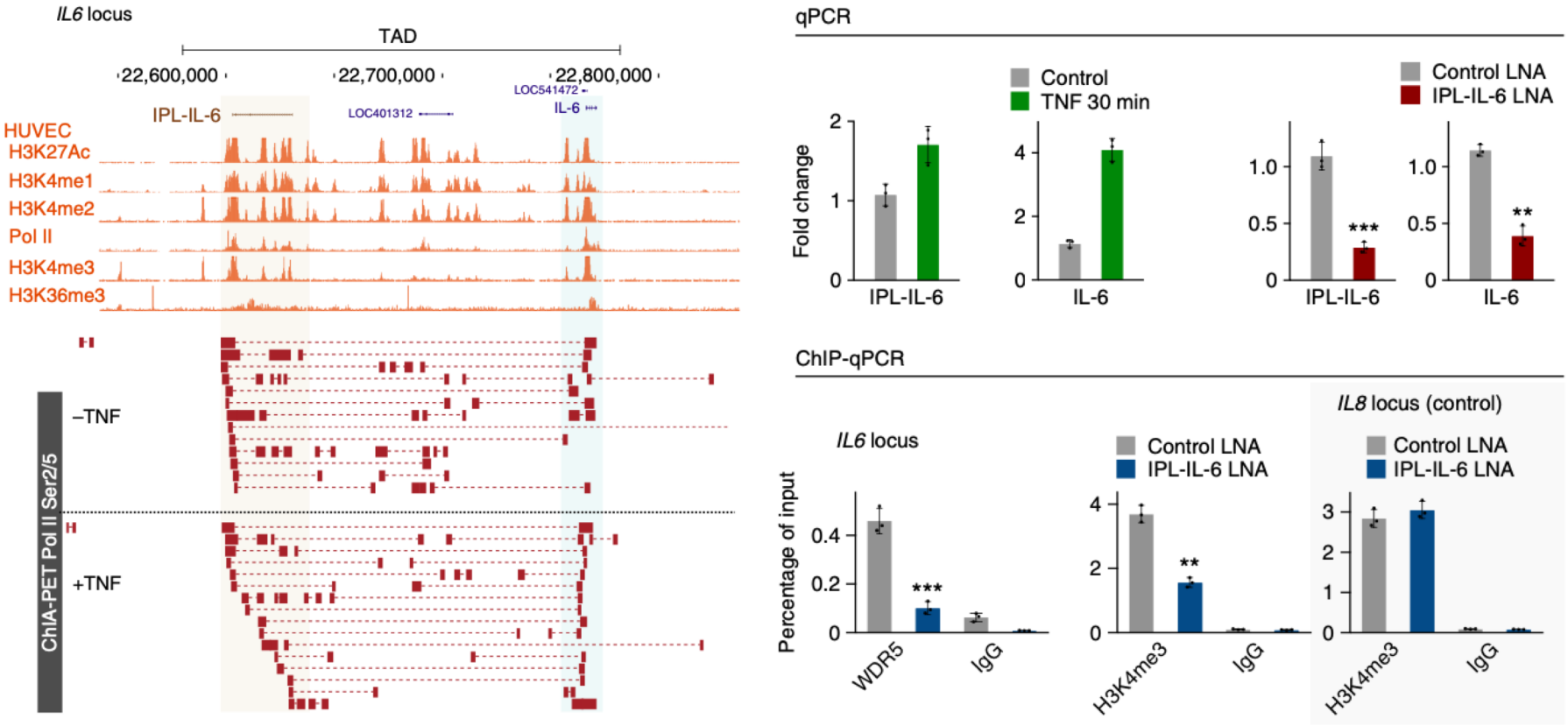
UMLILO-WDR5 complex interacts with MLL1

MM102, a small molecule inhibitor that prevents the WDR5–MLL1 interaction, led to a **significant reduction** in chemokine gene transcription and H3K4me3 epigenetic marks on the chemokine promoters

THESE DATA HIGHLIGHT THE NECESSITY OF MLL1-DEPENDENT H3K4ME3 DEPOSITION FOR IMMUNE GENE TRANSCRIPTION



IL-6 locus



WDR5–lncRNA regulation is a general mechanism of H3K4me3-primed TNF-responsive genes

Take home messages

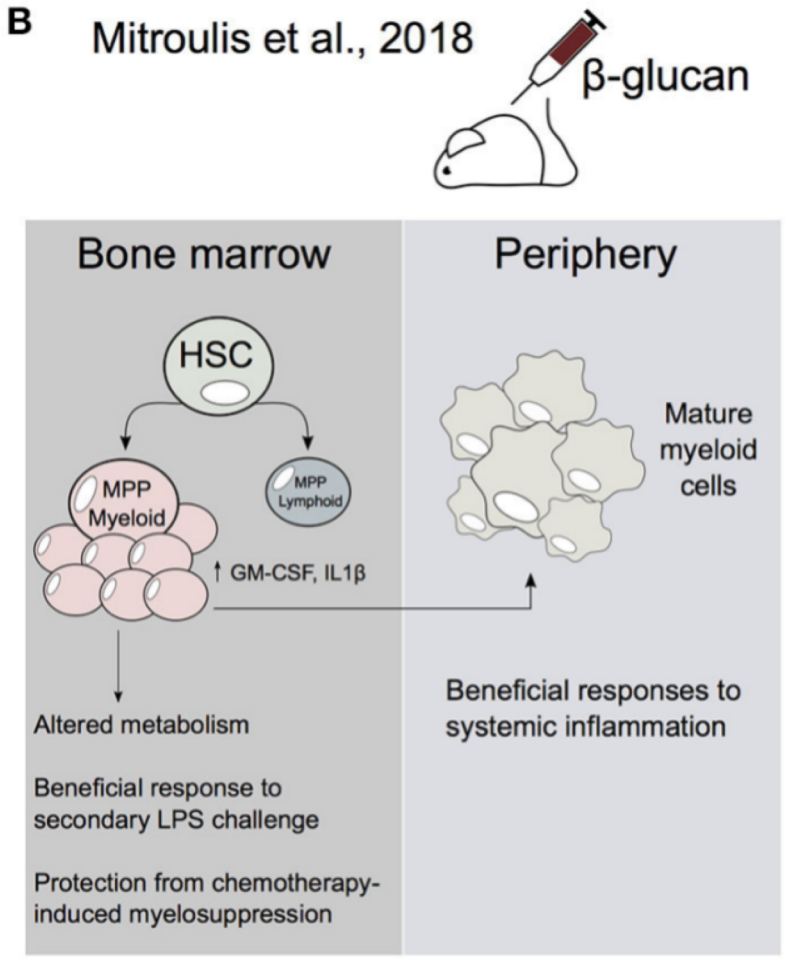
- UMLILO directly interacts with WDR5
- UMLILO is a cis-acting lncRNA which plays a key role in the expression of chemokine genes
- Replacing UMLILO with a well-characterized WDR5-interacting lncRNA restores CXCL transcription
- This IPL discovery pipeline represents a reliable approach to identify other IPLs

Is trained immunity regulated at the epigenetic level thanks to β-glucan?

β-glucan is:

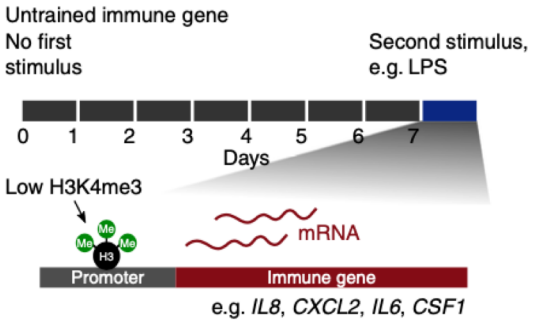
- Major component of the cell wall of Candida albicans
- Prototypical trained-immunity-inducing agonist

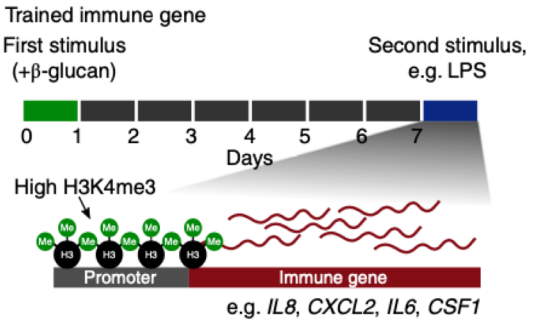
Administration of β-glucan resulted in the expansion and increased transcription of innate immune regulators (e.g., IL1β) in murine hematopoietic progenitors



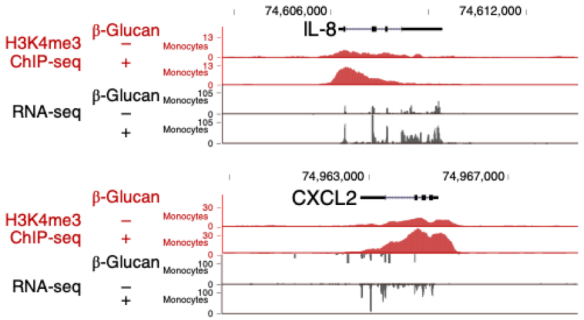
Does β-glucan epigenetically reprograms immune genes in an IPL-dependent manner?

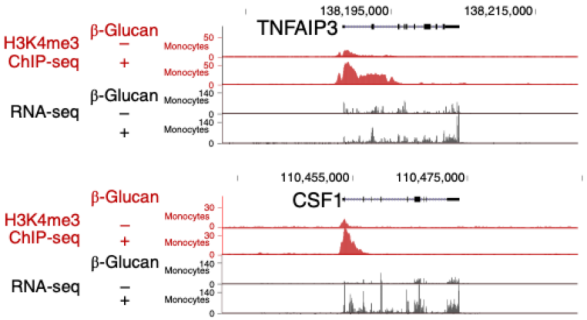
The increased responsiveness of trained monocytes is driven by epigenetic reprogramming





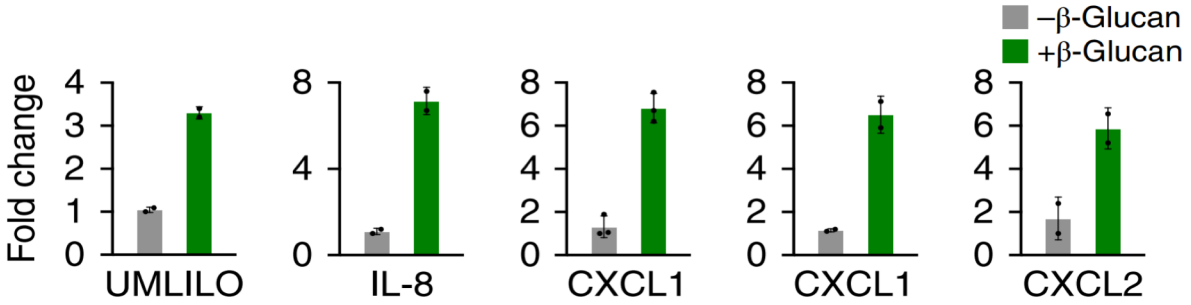
H3K4me3 ChIP-seq and RNA-seq data showed that **β-glucan**-mediated training of human monocytes increased transcription and H3K4me3 levels on IPL-regulated immune gene promoters





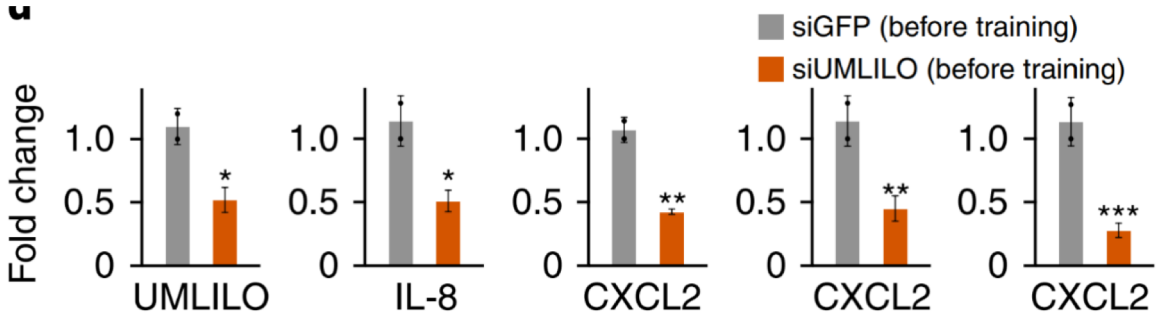
Does β-glucan epigenetically reprograms immune genes in an IPL-dependent manner?

β-Glucan-induced training increases UMLILO and *CXCL* transcription.



siRNAs targeting UMLILO prior to β-glucan-induced training lead to a significant reduction in expression of UMLILO

siUMLILO significantly decreased chemokine transcription



Does β-glucan epigenetically reprograms immune genes in an IPL-dependent manner?

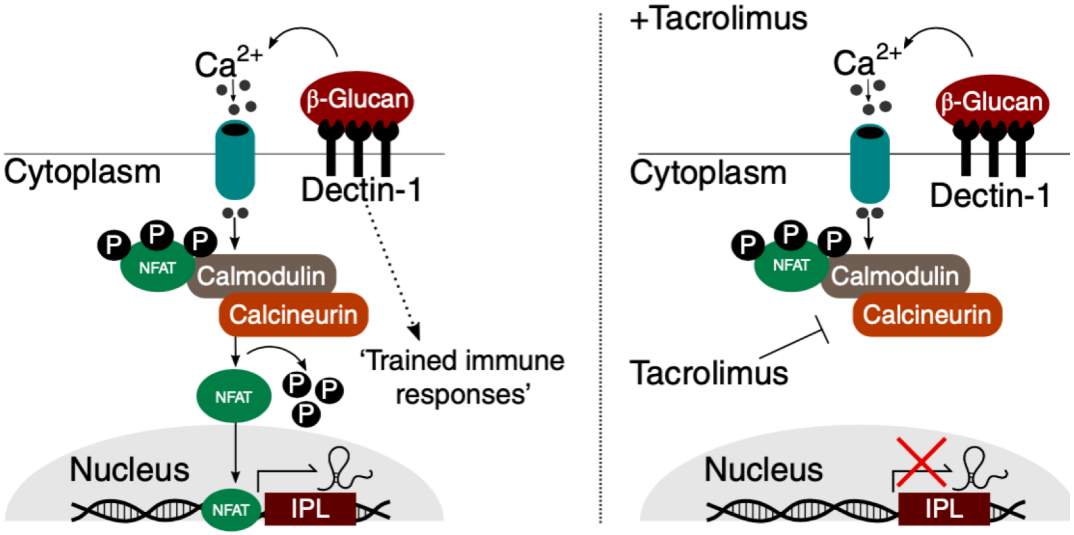
Training of monocytes is mediated by the β-glucan receptor, **dectin-1**.

β-Glucan/dectin-1 signaling triggers activation of calcium-dependent **NFAT**.

All IPL promoters contain NFAT motifs so:

IPL upregulation in trained monocytes is triggered via NFAT?

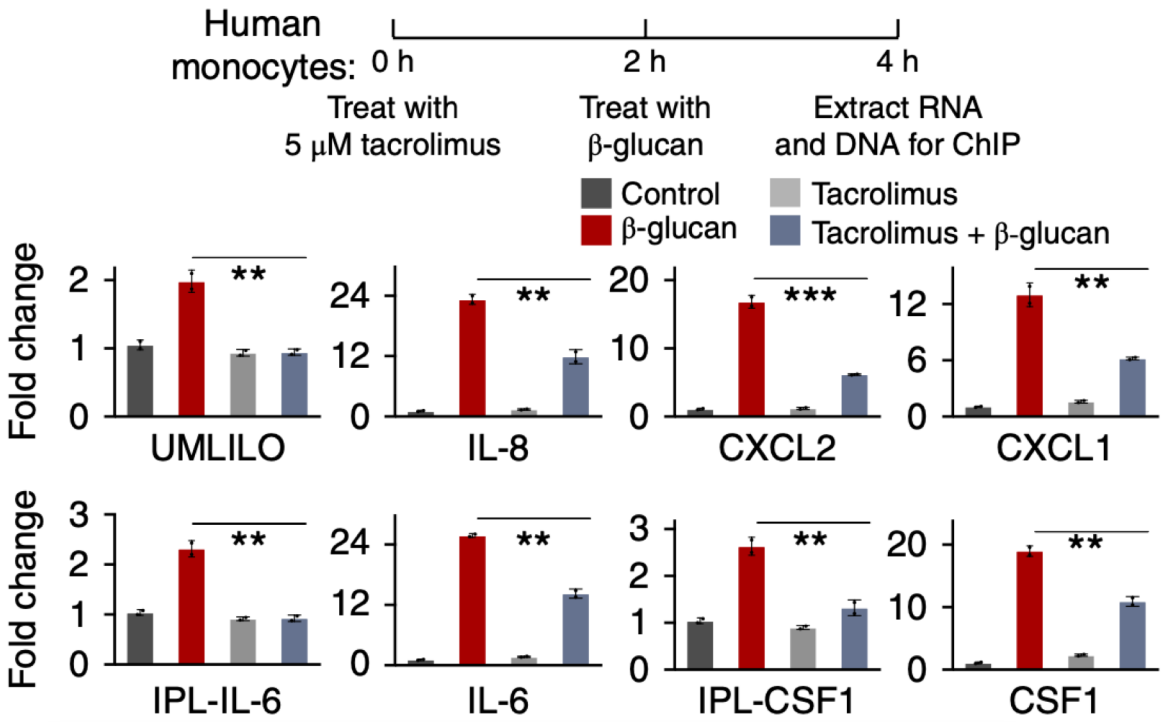
Tacrolimus inhibits calcineurin activation and, as a consequence, NFAT nuclear translocation



IPL upregulation in trained monocytes is triggered via NFAT?

Exposing monocytes to β-glucan increased expression of UMLILO and other IPLs

Pre-treatment with tacrolimus prevented the β-glucan-induced upregulation of IPLs from baseline levels, as well as target gene transcription

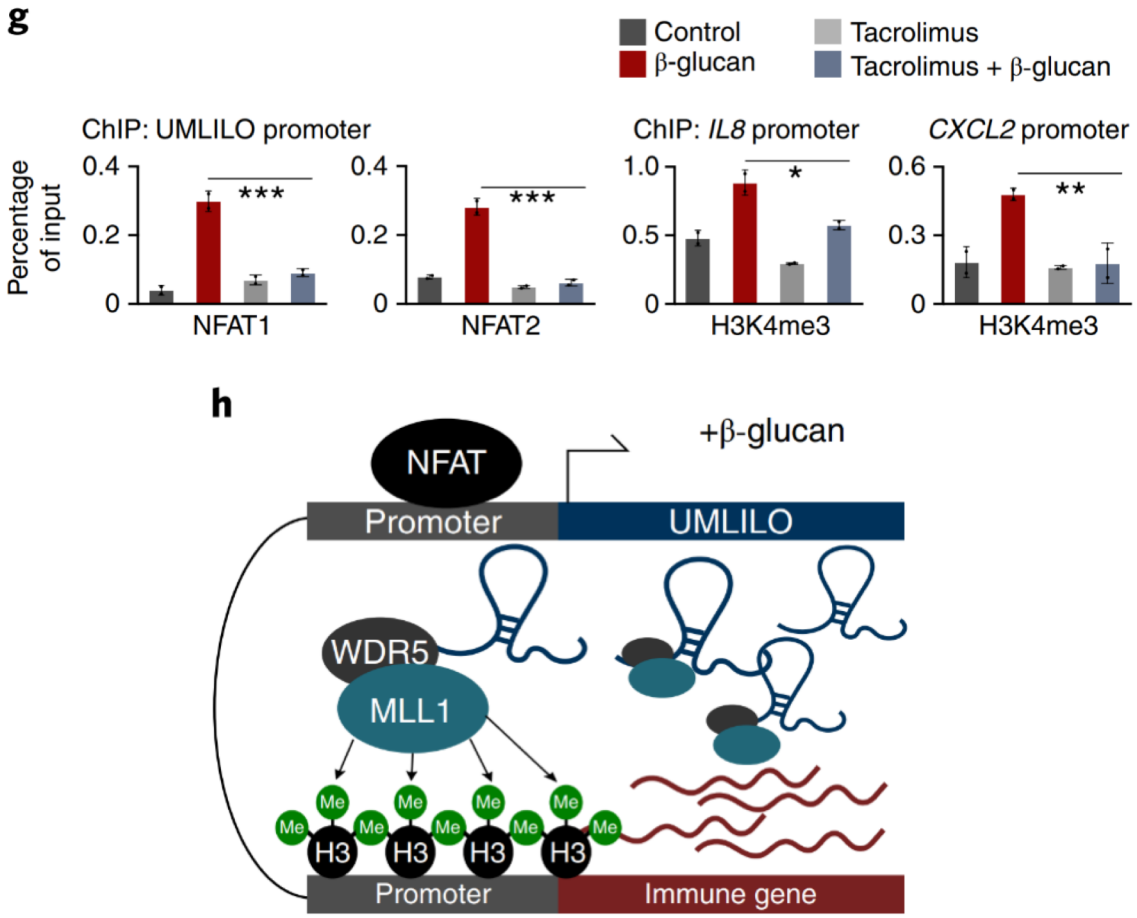


IPL upregulation in trained monocytes is triggered via NFAT?

β-glucan induced an increase in NFAT levels on the UMLILO promoter, while pre-treatment with tacrolimus abrogated this increase

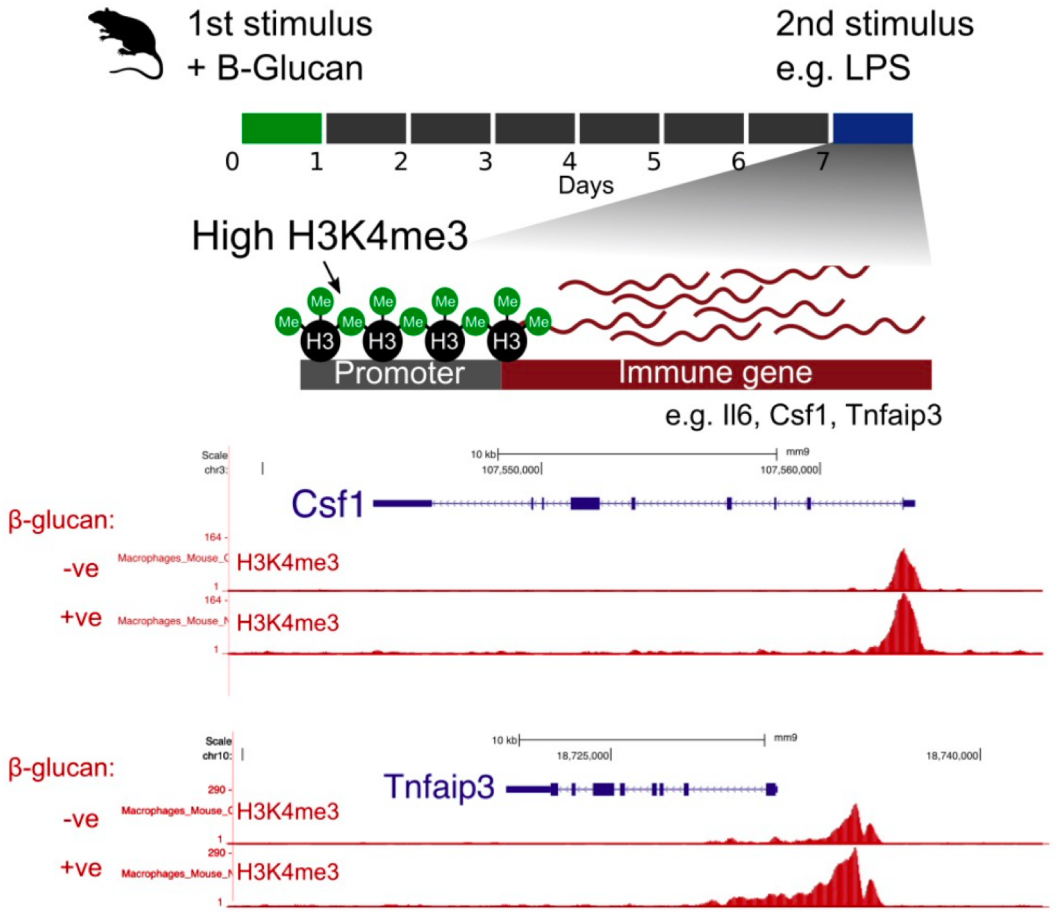
Upon exposure to β-glucan, NFAT signaling leads to an upregulation of IPL transcription

This increases the concentration of the WDR5–MLL1 complex proximal to the innate immune gene promoters, facilitating the specific H3K4me3 priming of the innate immune response genes.



The effects of β-glucan in mouse immune genes

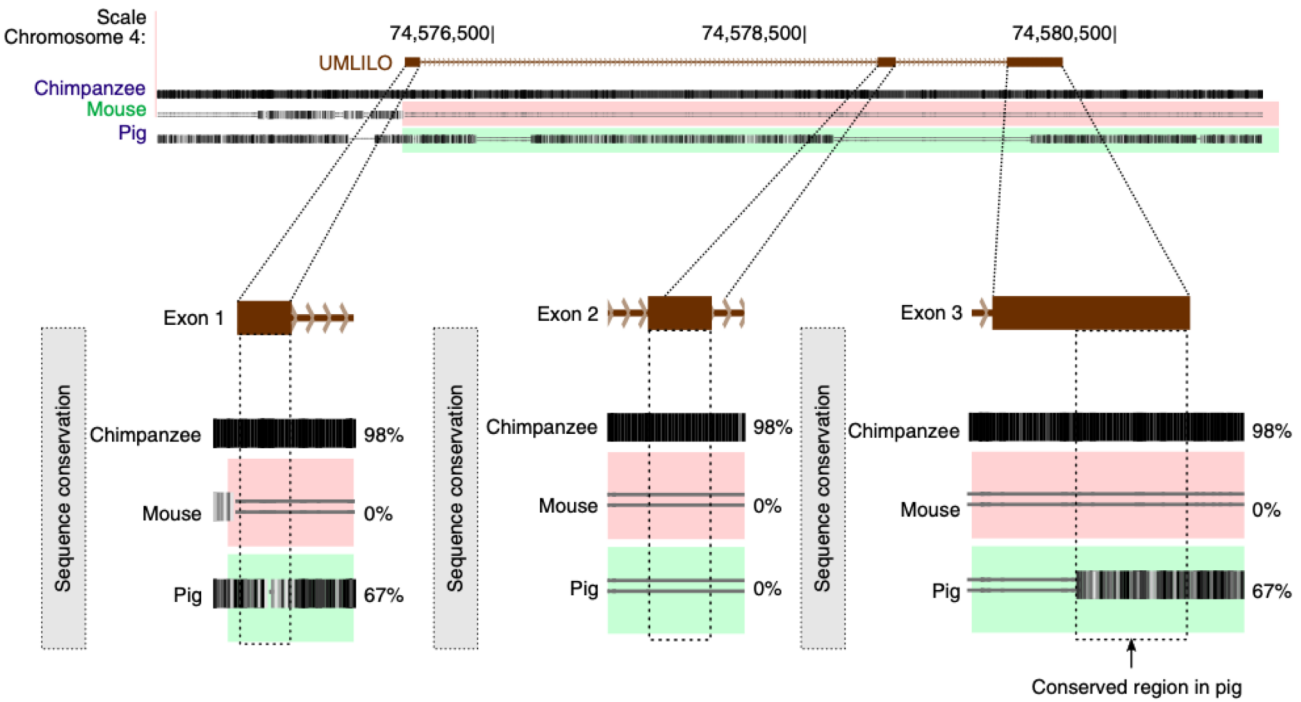
β-Glucan-induced training increases H3K4me3 on several mouse immune gene promoters



There's an homolog of UMLILO in mouse?

BUT no homolog of UMLILO exists in mice

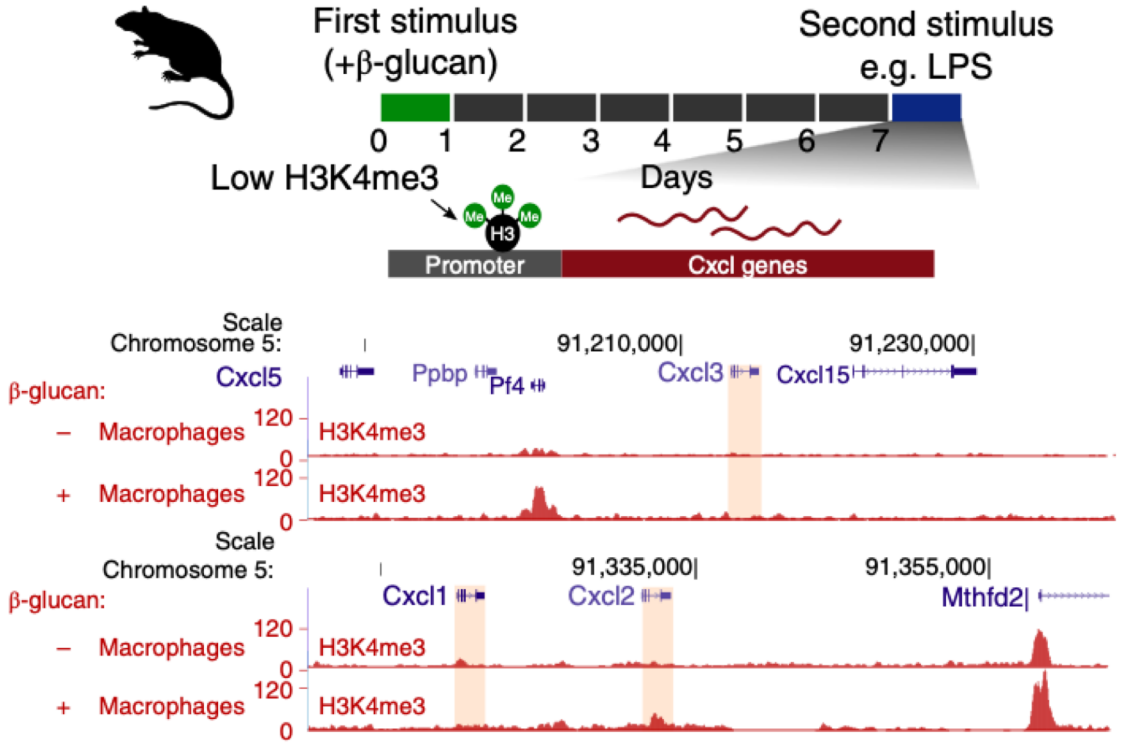
Murine *Cxcl* TAD lacks UMLILO



What happens in the *Cxcl* TAD if we treat with β-glucan?

What happens in the *Cxcl* TAD if we treat with β-glucan?

No increase in H3K4me3 at the *Cxcl1*, *Cxcl2* or *Cxcl3* promoters is observed after β-glucan-induced training

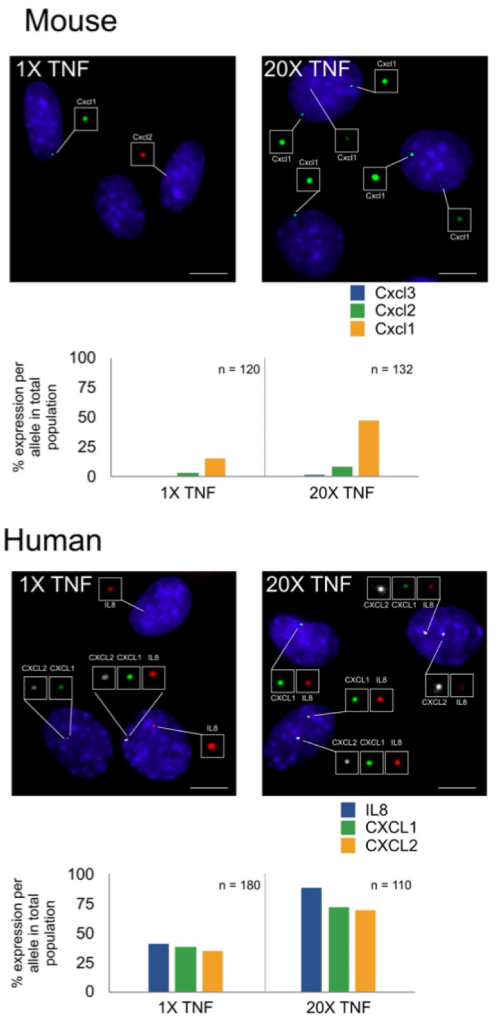


What happens in the *Cxcl* TAD if we treat with β-glucan?

Mouse chemokines were weakly transcribed upon treatment with physiologically relevant levels of TNF in humans (1X). The mouse chemokines were more strongly expressed post treatment with 20X higher levels of TNF

Robust transcriptional response in human cells treated with physiologically relevant levels of TNF (1X)

Is this due to the lack of IPL-mediated regulation in the mouse *Cxcl* TAD?



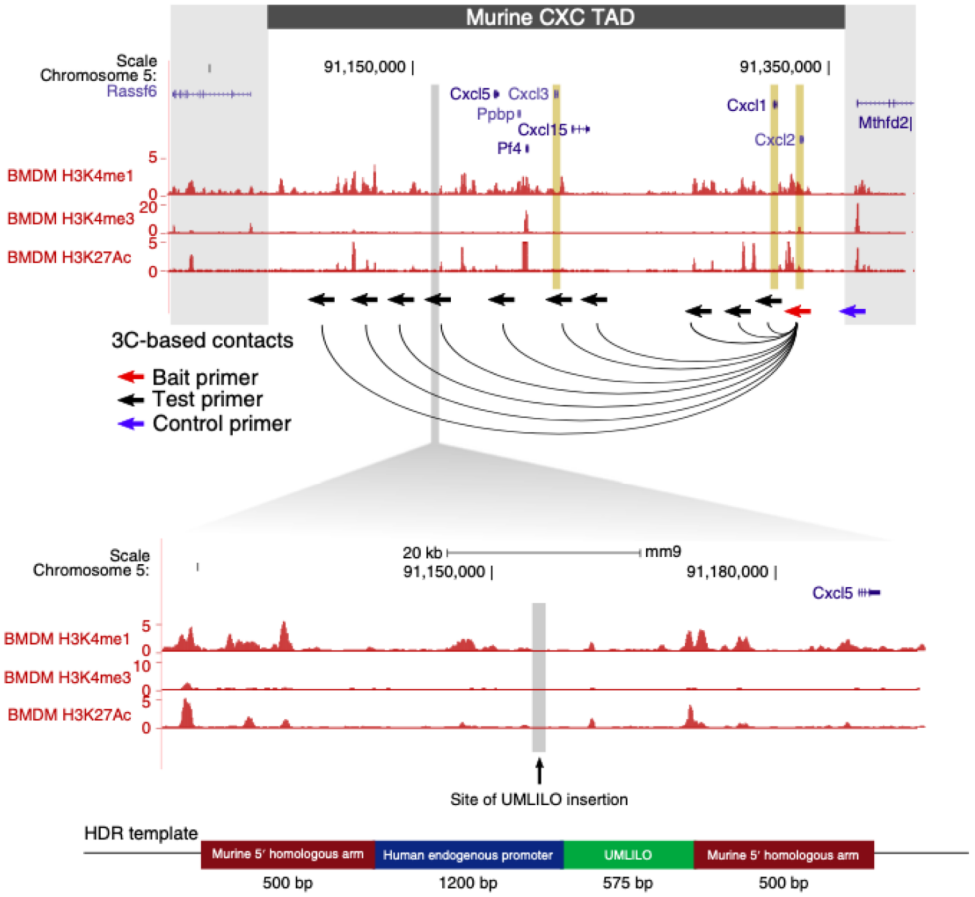
Is UMLILO able to restore chemokine transcription?

Knock-in experiment in the mouse RAW 264.7 macrophage-like cell line

Designed a repair template that included UMLILO with its human endogenous promoter to insert via HDR mediated by CRISPR–Cas9

Using 3C analysis in murine cells, an insert region was selected within the mouse super-enhancer that engages in chromosomal contact with *Cxcl2*

UMLILO was successfully edited into the mouse *Cxcl* chemokine TAD in the cell line to generate a knock-in clone

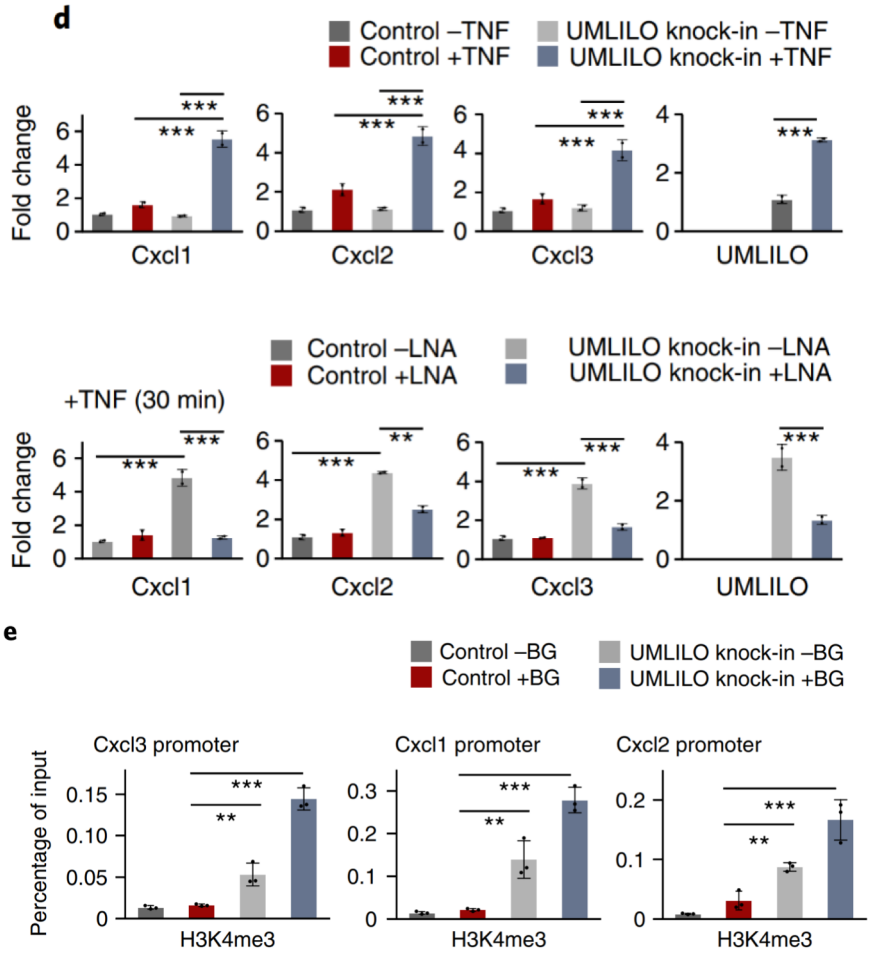


Is UMLILO able to restore chemokine transcription?

Performing ChIP and qPCR in UMLILO knock-in cells they observed a significant increase in chemokine transcription

LNA knockdown (knockdown of lncRNAs by Locked Nucleic Acid) of UMLILO in knock-in cells confirmed that this was due to UMLILO RNA and not to UMLILO genomic DNA.

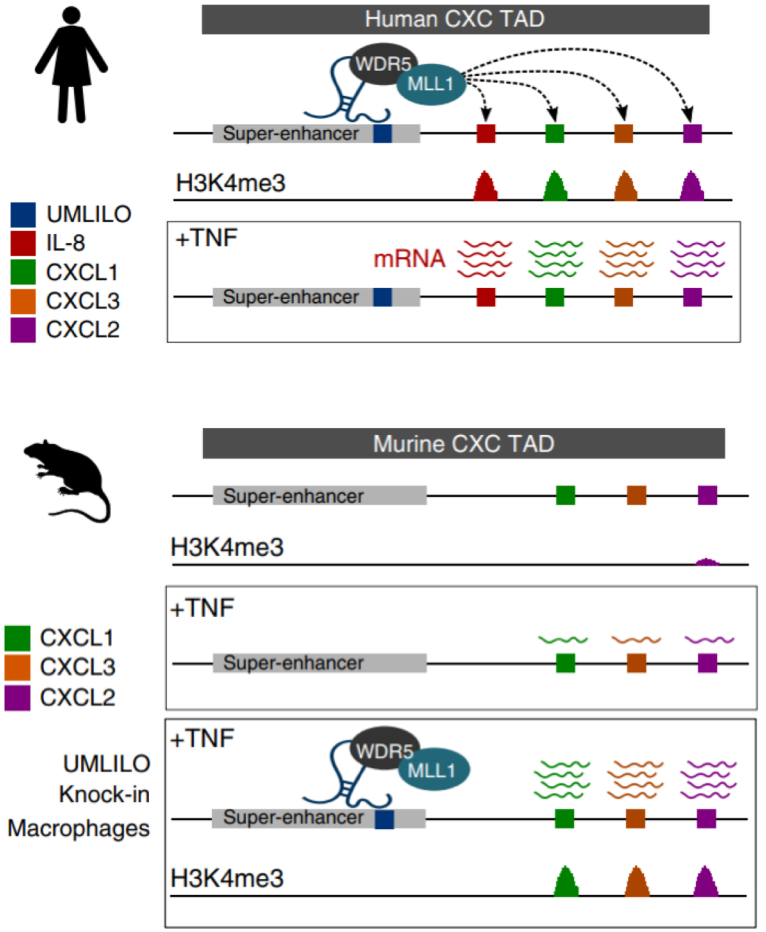
Subsequent β-glucan-induced training of the UMLILO knock-in cells resulted in a significant accumulation of H3K4me3 at *Cxcl* promoters



Is UMLILO able to restore chemokine transcription?

These data provided strong evidence that:

- UMLILO is central to the deposition of H3K4me3 on chemokine promoters during trained immune responses
- IPLs are the bona fide mechanism by which essential trained immune genes acquire enrichment of the H3K4me3 promoter mark.



Take home messages

- **β-glucan** epigenetically reprograms immune genes by upregulating IPLs in an NFAT-dependent manner
- The addition of **UMLILO** to the chemokine TAD **in mouse macrophages** significantly increased chemokine expression and resulted in training of the chemokine genes
- Though highly elevated, these levels did not fully recapitulate those observed at human CXCL genes. It could be that IPLs are **not strictly necessary**, but increase H3K4me3 levels on target genes aiding robust immune gene transcription
- Genes displaying **non-stochastic gene expression** may be assisted in transcriptional regulation by 'IPL-like' lncRNAs, such as UMLILO

Future Perspectives

- Monocytes exposed to lipopolysaccharide (LPS) exhibit a tolerized phenotype, which is characterized by a reduction of epigenetic marks at immune gene promoters and express lower levels of cytokines upon exposure to a secondary, unrelated infection
- It was shown that β-glucan can partly reverse this phenotype and reactivate unresponsive genes, by reprogramming distal histone modifications. Thanks to β-glucan/ NFAT signaling that upregulates IPLs and, as a result, H3K4me3 on target immune gene promoters
- This suggests that assaying IPL transcription levels may be a **useful biomarker** for assessing effective innate immune activation by various methods, including vaccination
- As aberrant expression of innate immune genes underlies many diseases, adjustment of immune gene levels by directly altering the activity of IPLs may represent a valuable **therapeutic strategy** to achieve tailored **immunomodulation**

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THANK YOU
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ATTENTION