UNIVERSITÀ DEGLI STUDI DI TRIESTE
Dipartimento di Scienze della Vita

PNAS
RESEARCH ARTICLE - IMMUNOLOGY AND INFLAMMATION

From Mechanisms to Function: lncRNA Gene Regulation, CRISPR Screening, and LOUP as a Key Regulator

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Outline

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Introduction

02

CRISPRi Screen Setup

03

LOUP Genomic Context and Expression

04

LOUP Functions in NFκB and SPI1 Regulation

05

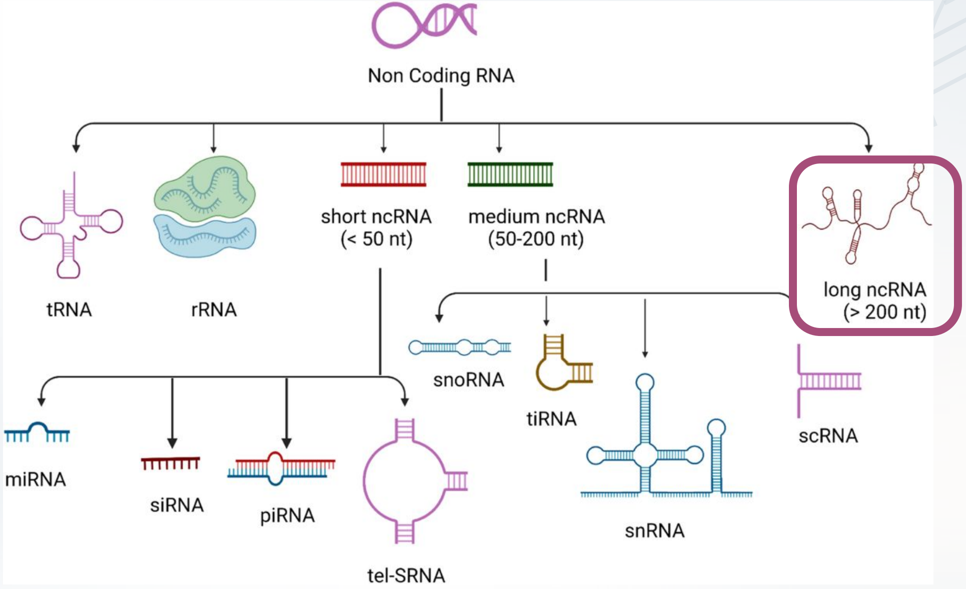
Conclusion

Long non-coding RNAs: overview

lncRNAs:

- Transcripts >200 nt
- General functions: gene regulation, chromatin modulation, splicing, translation, signaling pathways

20,000 annotated lncRNAs (GENCODE)

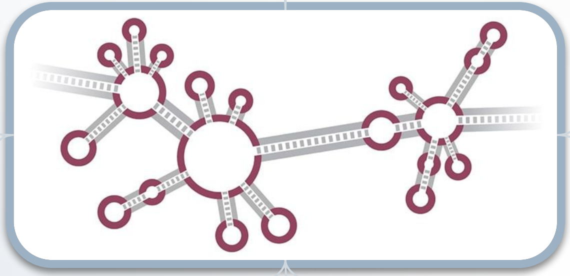


lncRNAs and gene expression control

Interference with Transcription Machinery

Gene Silencing & Epigenetic Memory

Interact with DNA, RNA and proteins



Genomic Position Effects

Chromatin Architecture Control

Direct DNA Interactions

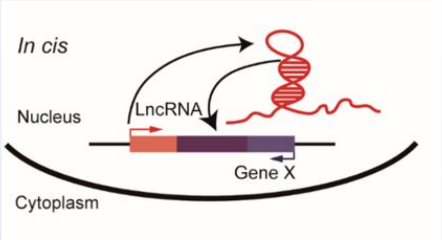
Decoy Activity

Recruitment or Blocking of Protein Complexes

cis and trans Actions

Cis- acting lncRNAs

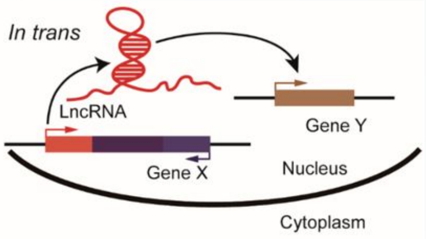
- Regulate genes located in the same chromosome
- Chromatin remodeling, recruitment of epigenetic complex, transcription-dependent effect



XIST acts in cis by coating its own chromosome and triggering X-chromosome inactivation

Trans- acting lncRNAs

- Act on distant genes
- Scaffolding, RNA- protein interactions, modulation of signaling pathways

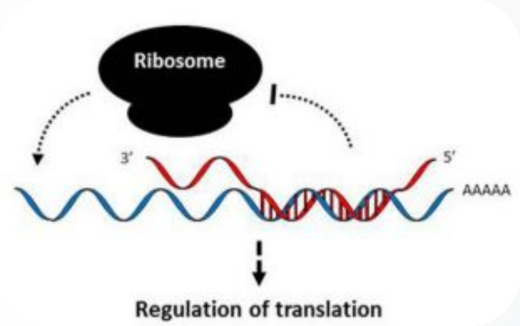


HOTAIR — trans-repressive regulator that recruits PRC2 to HOXD locus on another chromosome

Post-transcriptional roles of lncRNAs

- Recruit / sequester regulatory proteins
- Influence splicing and transcript stability
- Scaffold or modulate protein complex formation

- Bind complementary mRNAs
- Promote degradation or stabilization
- Modulate translation efficiency
- Relocate dynamically in response to stimuli



Physiopathology and therapeutic potential

Biological roles

immune regulation, differentiation, tissue homeostasis

Central nervous system development, neuronal differentiation, and regeneration after injury

Immune gene activation and suppression

Pathological roles

cancer, immune disorders, neurodegeneration

BACE1-AS - *Alzheimer disease*

NRIR *favours hepatitis B virus replication*

Targeting lncRNAs in cancer

Many lncRNAs are associated with tumor proliferation, apoptosis, metabolism, chromatin architecture and interactions with the tumor microenvironment

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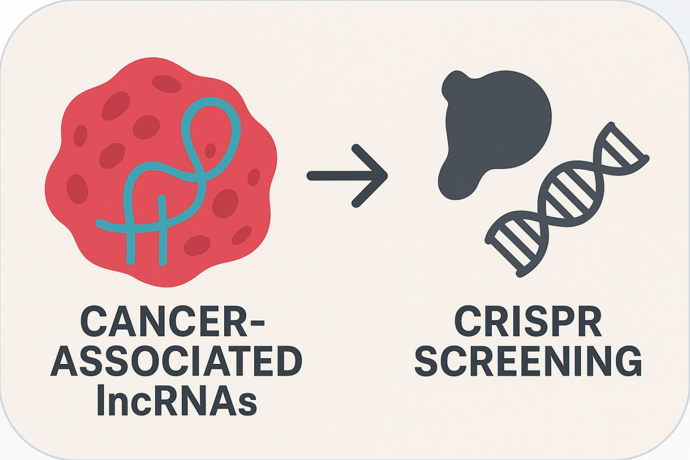
Need to identify **therapeutically actionable lncRNAs**



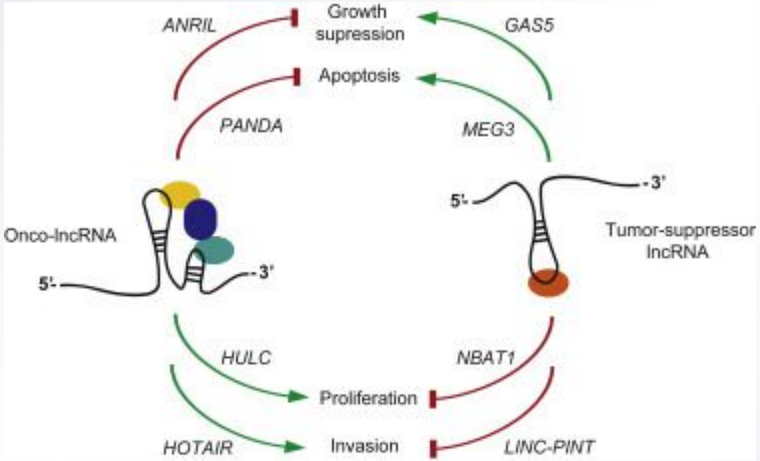
The Next Frontier of Cancer Targets

*Long non-coding RNAs can act either as **oncogenic drivers** or as **tumor-suppressor** molecules.*

Onco-lncRNAs

- HOTAIR
- NEAT1
- ANRIL
- MALAT1

Promote **proliferation**, **survival**, **invasion**



Tumor-suppressor lncRNAs

- GAS5
- MEG3
- NBAT1
- LINC-PINT

Limit growth and **support apoptosis** or **p53 signaling**

Nuclear oncogenic lncRNAs can be efficiently targeted using **antisense oligonucleotides (ASOs)**, currently the most advanced therapeutic approach for lncRNA inhibition.

Therapeutic Potential of lncRNAs in cancer

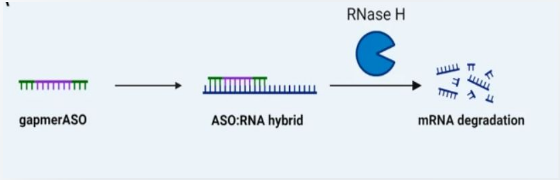
Classical Techniques

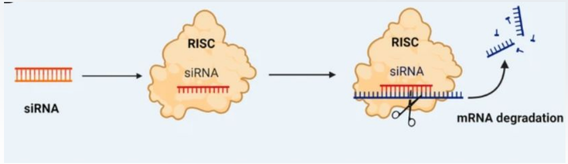
RNAi

- Functional in cytoplasm
- Temporary knockdown

ASO (Gampers)

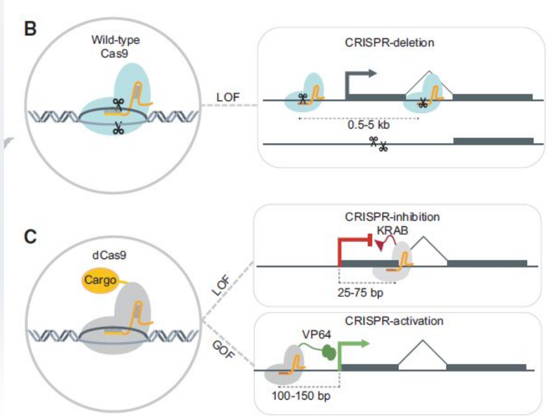
- Effective targeting also in the core
- Allows targeted KO of individual lncRNAs





CRISPR Screening

- enables high-scale of lncRNA
- **favor oncogenicity**
- promote **drug resistance**
- **"Druggable"** with ASO
- CRISPR act as a functional map of lncRNAs in tumors



Comparison:

ASO (Gampers)

- No high- scale screening: multiple lncRNA -> **cost problems and comparison difficulties**
- No information on the functional **role of lncRNAs** systemically

CRISPR

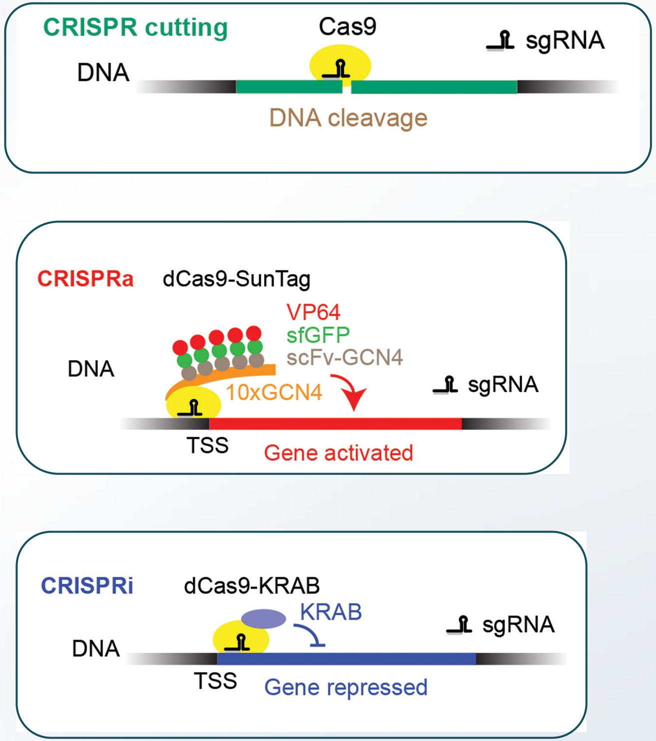
- **Translational approach**, from discovery to validation
- **Reduces the risk** of false positives

CRISPR technologies for the functional analysis of lncRNAs

- CRISPR exceeds the limits of RNAi
- Difference to approaches:

1. **CRISPR-del** ⇢ Cas9 cuts both sides of the lncRNAs locus to delete the promoter or gene region (loss-of-function)
2. **CRISPRa** ⇢ dCas9 fused to activator domains, upregulate silent or lowly expressed lncRNAs
3. **CRISPRi** ⇢ dCas9-KRAB binds the promoter and blocks transcription without cutting DNA

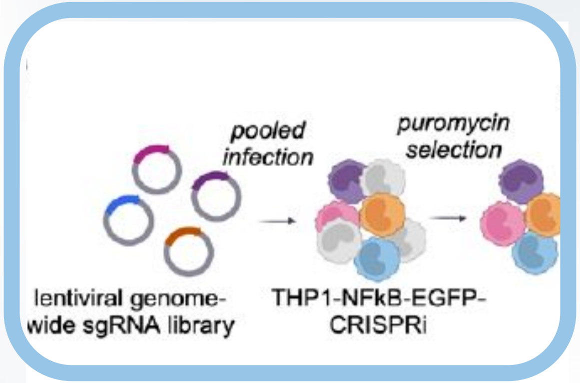
- **The functions of lncRNAs are much more Frequent than expected: 2 -20% of "functional hits" in screens**

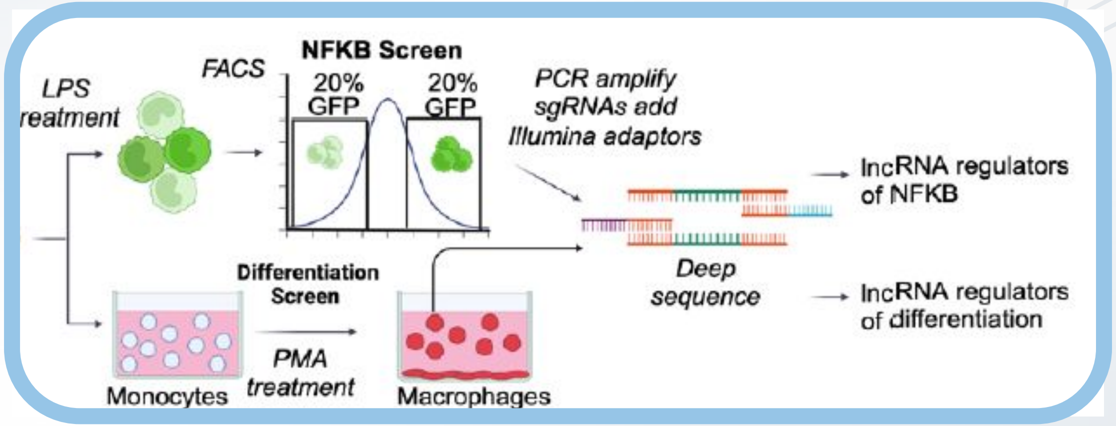


Strategies and Challenges: lncRNA CRISPR Screening

CRISPR screens use sgRNA libraries **to perturb hundreds of lncRNAs** at once in a single cell population, allowing rapid identification of those that drive a specific phenotype

Starting point..





1. **Cell Model** : THP1 NFkB-EGFP
2. **CRISPR perturbation**: dCas9-KRAB
3. **Custom sgRNA library** (2,342 lncRNAs)
4. **Infection and Antibiotic Selection**

5. **Treatment** to stimulation (LPS or PMA)
6. **Phenotypic Selection** (FACS Sorting -> NFKb -> GFP expression) Or Differentiation Screen
7. **Hit Identification** (DNA extractaction -> Hits)
8. **Calculate critical conditions**

Can CRISPR screens reveal functionally relevant lncRNAs in the regulation of differentiation programs and cellular responses?

PNAS RESEARCH ARTICLE | IMMUNOLOGY AND INFLAMMATION

CRISPRi screens identify the lncRNA, LOUP, as a multifunctional locus regulating macrophage differentiation and inflammatory signaling

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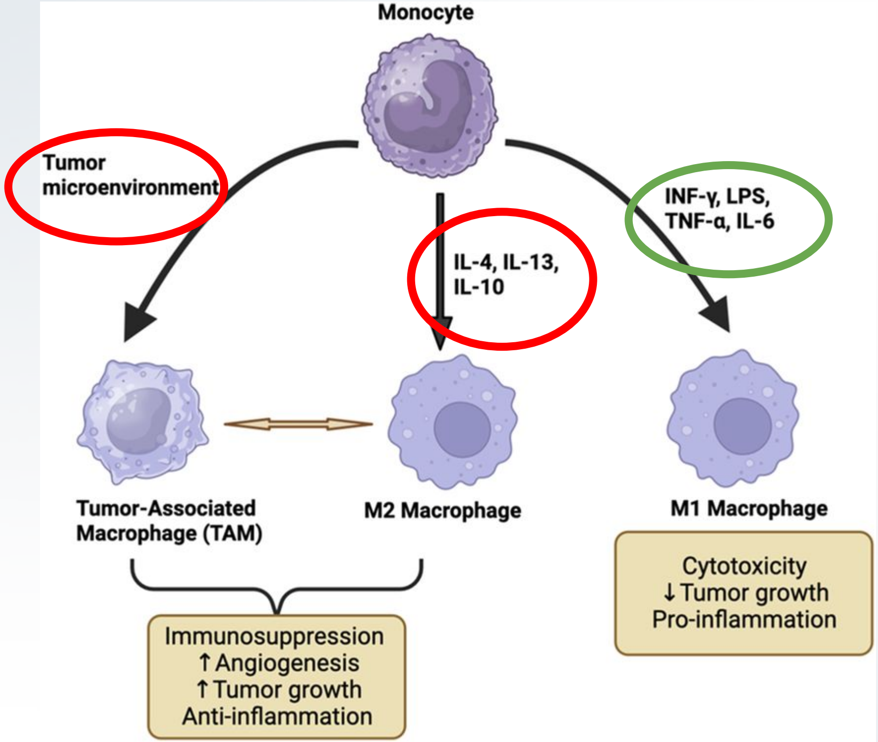
Tumor-associated macrophages are often M2-like

1. Promote tumor growth and angiogenesis
2. Suppress antitumor immune cells
3. Create immunosuppressive microenvironment



Monocyte line as Model

1. Detect and respond to pathogens
2. High plasticity
3. Inflammatory responses
4. Regulation by lncRNAs

- *lncRNAs can modulate macrophage differentiation, reshaping TAMs to reduce immunosuppression and limit tumor-supportive functions*

1. *Which lncRNAs regulate **NFkB** signaling and human **macrophage differentiation** ?*

THP1 : Human acute monocyte leukemia

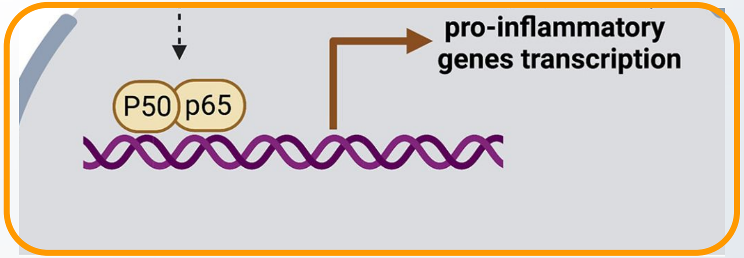
- Induces adherent, macrophage-like cells
- Expression of adhesion and pathogen recognitior
- Polarize cells toward M1 (pro-inflammation) or M2 (anti)

PMA

(ATCC TIB-202-AR)

Why NF -kB signaling?

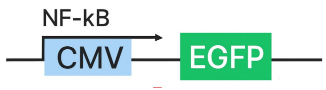
- Act as a **master regulator** of macrophage immunity
- Ideal **CRISPRi readout** to assess lncRNA effect on activation and inflammatory function



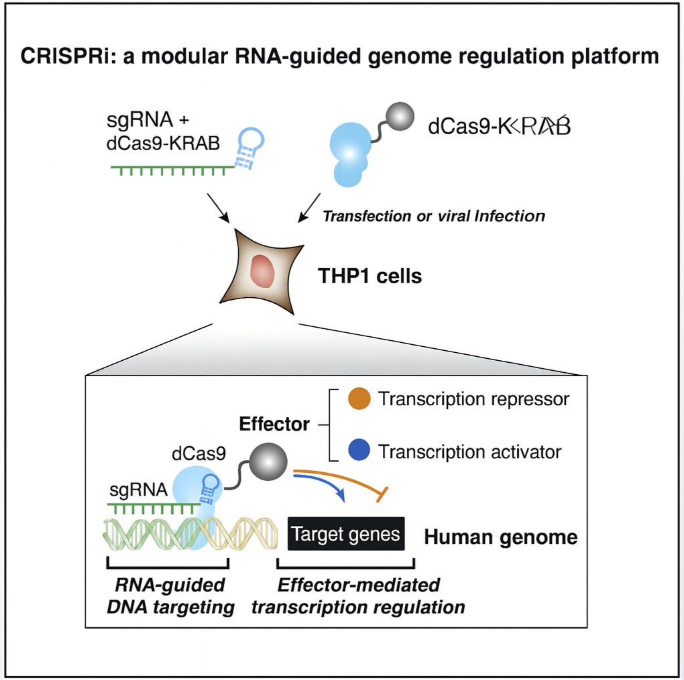
TNF-a
IL-1B
IL-6
CXCL8
CCL2
VCAM-1
ICAM-1

1. Which lncRNAs regulate *NFkB* signaling and human *macrophage differentiation* ?

- **THP1 as a cellular model**
  A human monocytic cell line used to study differentiation into macrophages and inflammatory signaling
- **NFkB Reporter**
  Measures the activity of this pathway: when NFkB is activated -> GFP is generating by the cells

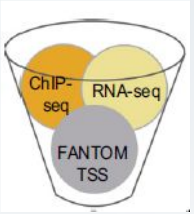


- **CRISPi (dCas9- KRAB)**
  To silence specific genes without cutting DNA.



1. *Which lncRNAs regulate **NFkB** signaling and human **macrophage differentiation** ?*

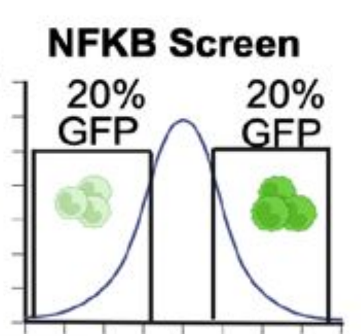
METHODS



1. **THP 1** cells were trasduced with a pooled **sgRNA library** to silence lncRNA expression through CRISPi

LPS

2. Cells were stimulated with **LPS** (24h) to activate NFkB pathway and induce **GFP** expression from the **NFkB** reporter



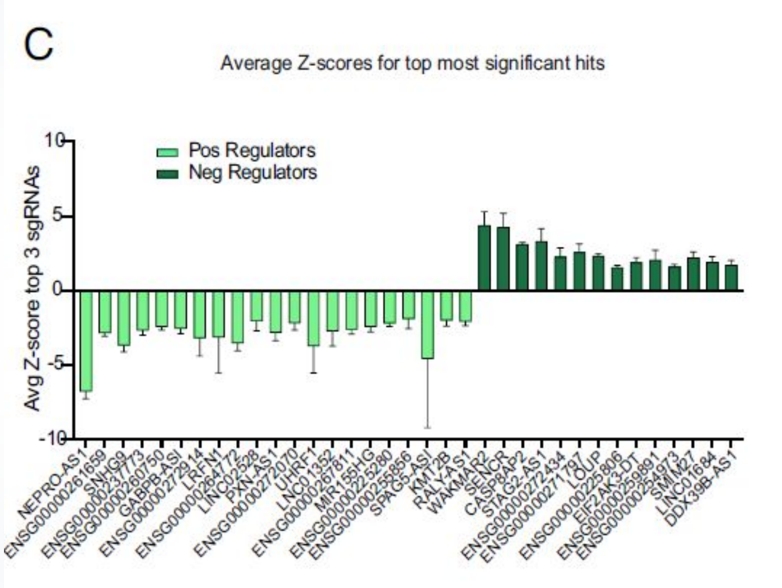
3. **FACS** to see the NFkB screen, that representing high and low NFkB activity as **regulators.** For each population, genomic DNA isolated, sgRNAs were PCR-amplified and sequenced.

1. *Which lncRNAs regulate **NFkB** signaling and human **macrophage differentiation** ?*

METHODS

4. NFkB Analysis

- sgRNA GFP-hig and GFP-low compared to unsorted population with **MAUDE**
- Each gene/lncRNA: top 3 sgRNA vs ctrl selected:
  Median Z-score calculated:
  Z < -3 act as positive regulators
  Z < 3 act as negative regulators
- Genes exceeding threshold = top hits



1. *Which lncRNAs regulate **NFkB** signaling and human **macrophage differentiation** ?*

METHODS

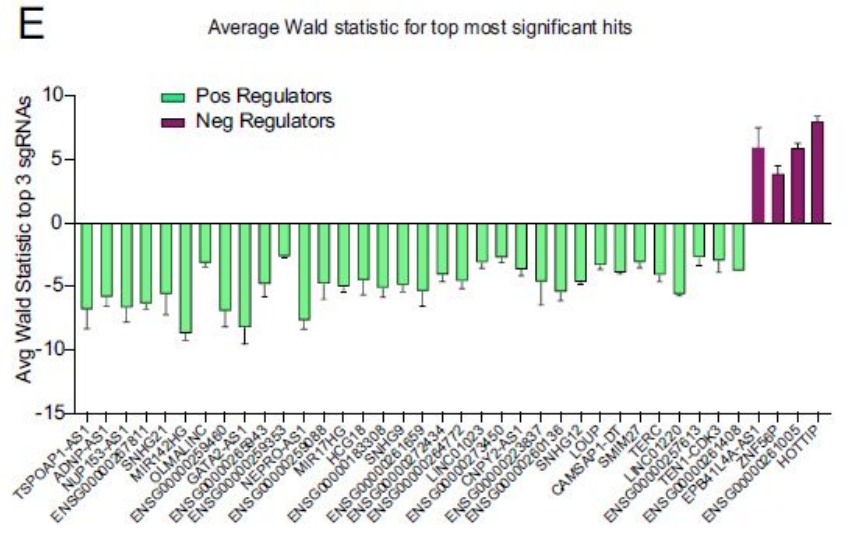
5. Macrophage Differentiation Screening

- THP1 cells treated with PMA three times over 11 days post-library selection
- 50% of cells differentiated

↓

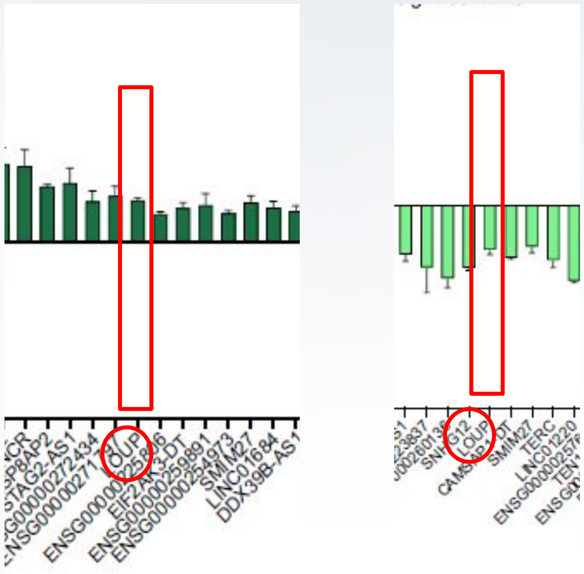
6. Data Analysis

- Log 2fold change of sgRNAs (untretaed vs. PMA-treated) calculated with DESeq2.
- Gene- level significance: Mann- Whitney U test (MWU)
  MWU ≥ 3 or ≤ −3 → significant.



Conclusion?...

1. *Which lncRNAs regulate **NFkB** signaling and human **macrophage differentiation** ?*



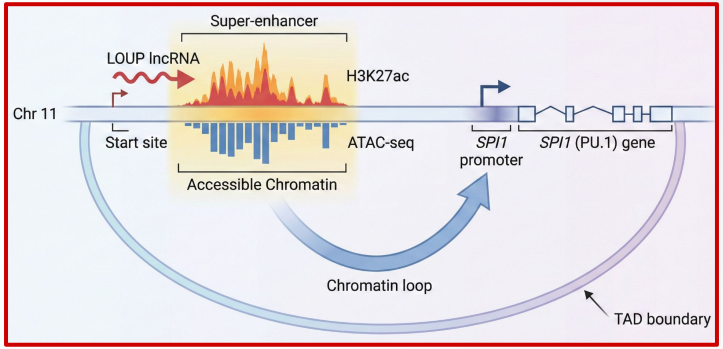
LOUP significant lncRNA hits in both of analysis

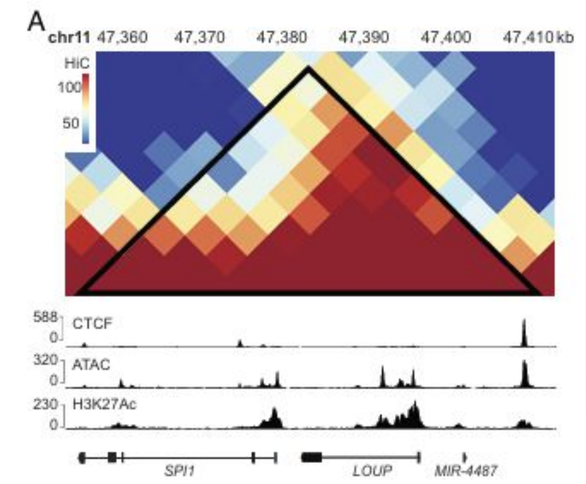
- The differentiation screen identified **38 lncRNAs** that significantly regulate the differentiation. **More than 75%** -> positive regulators
- **7 lncRNAs** were identified as hits in both the NFkB reporter screen and the differentiation screen, dual roles: <u>inflammatory signaling & macrophage maturation</u>
- ***LOUP*** was the only intergenic lncRNA near ***SPI1***, a key myeloid differentiation gene, highlighting it as a top candidate for follow-up

2. How does the LOUP locus regulate SPI1?

Background

- *LOUP = enhancer-derived lncRNA*
- *Super-enhancer features*
- *Myeloid differentiation involves 3D activation hubs and enhancer–gene looping*



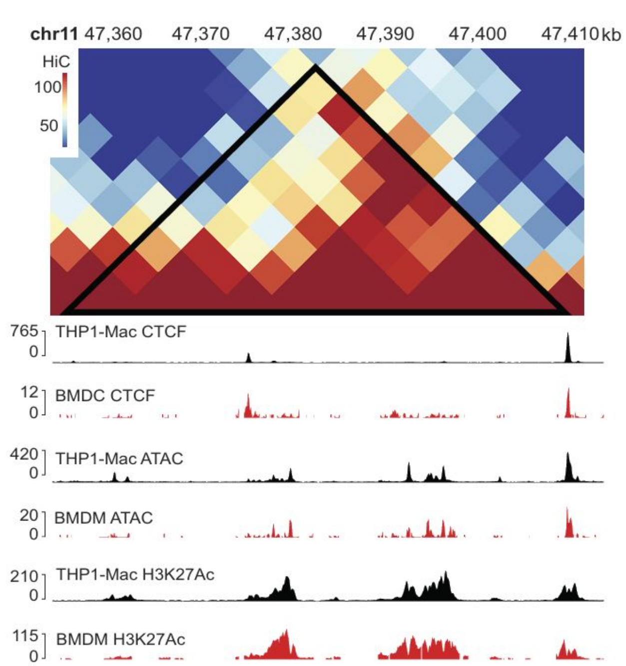


Experimental Evidence

- In THP1 → ATAC-seq and H3K27ac ChIP-seq show SE-like chromatin at the LOUP locus
- Hi-C and CTCF ChIP-seq show that **LOUP and SPI1 occupy the same TAD**

2. How does the LOUP locus regulate SPI1?

LOUP was identified as a hit in the PMA-induced THP1 differentiation screen, prompting investigation of whether LOUP or SPI1 expression changes were driven by local chromatin alterations



Hi-C, ATAC-seq and H3K27ac patterns **remain unchanged after PMA treatment** in THP1 cells

Suggests differences in LOUP/SPI1 expression during differentiation are not due to major epigenetic remodeling

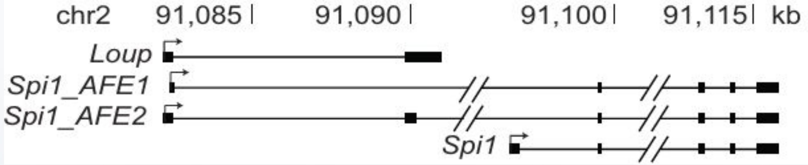
Epigenetic features are **conserved across species** (mouse BMDMs/BMDCs vs human macrophages).

2. How does the LOUP locus regulate SPI1?

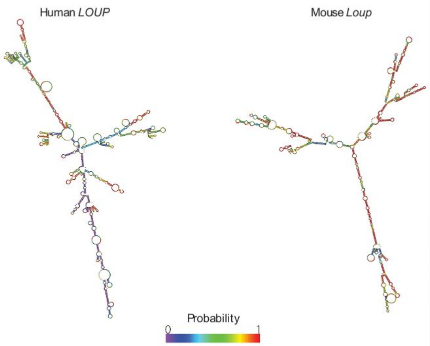
Human and mouse LOUP transcripts are structurally different

→ Human LOUP: **independent lncRNA**

→ Mouse:
Two-exon Loup lncRNA *or*
Extended 5' UTR fused to Spi1 mRNA



Only **37%** sequence identity
Different predicted secondary structures



Sequence alignment of transcripts

Hypothesis

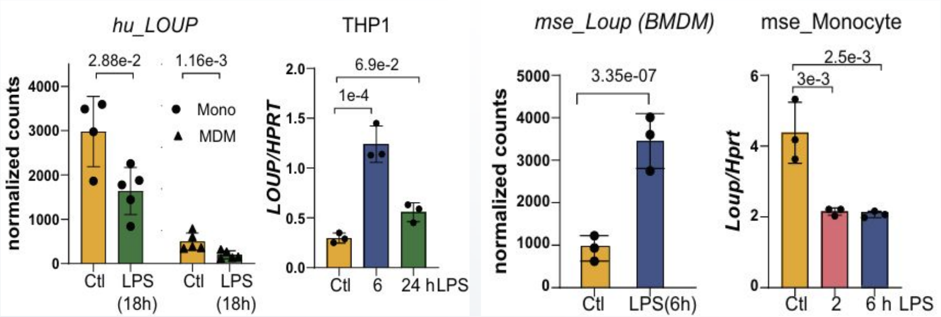
- Divergent RNA structure may cause species-specific functions

2. How does the LOUP locus regulate SPI1?

Does LOUP and SPI1 show comparable expression across species?

Method

- qPCR/RNA-seq in human and mouse monocytes + macrophages
- Baseline and LPS-treated cells



How LOUP and SPI1 respond?

- SPI1: induced by LPS in both species
- LOUP:
  - **Reduced** after LPS in primary monocytes
  - **Induced** after LPS in THP1 cells and mouse macrophages
- Indicates complex isoform regulation

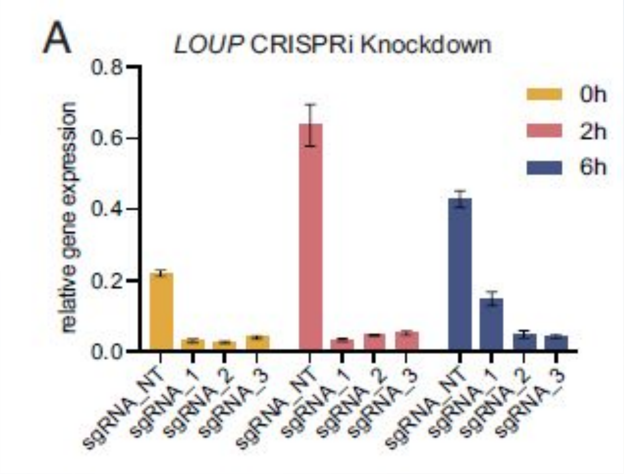
3. Does LOUP negatively regulate NFkB and inflammatory responses?

HYPOTHESIS

- LOUP making a candidate for further investigation; may act as a negative regulator of NFkB, restraining the expression of inflammatory genes and cytokines in macrophages

EXPERIMENTAL FOCUS - Effects of **LOUP-KD** on:

- RNA expression, transcription of inflammatory genes - 500
- Protein production, secretion of cytokines in response to stimulation

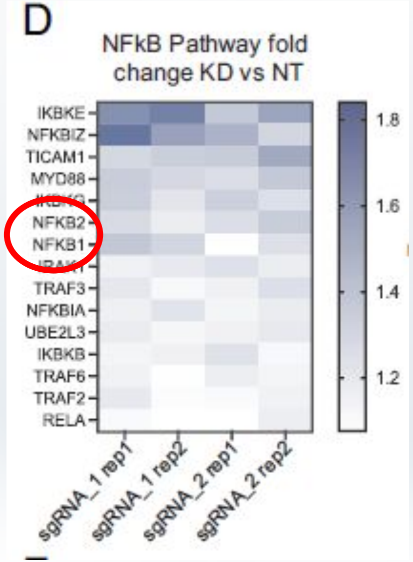


CRISPi KD of LOUP in THP 1. Three additional sgRNAs (1,2,3) and NT, in different times of stimulation

3. Does LOUP negatively regulate NFkB and inflammatory responses?

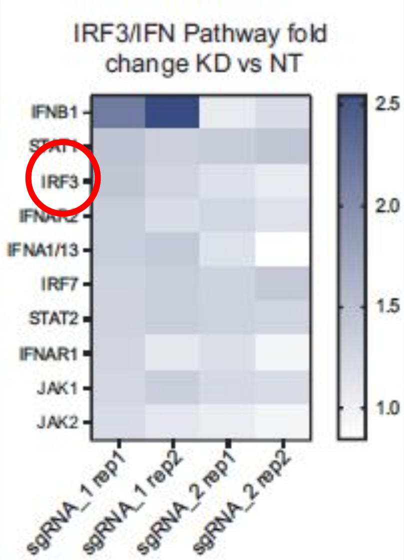
RESULTS

- LOUP-KD caused a broad upregulation of inflammatory genes even at baseline and after LPS stimulation .
- Two main pathways were affected:



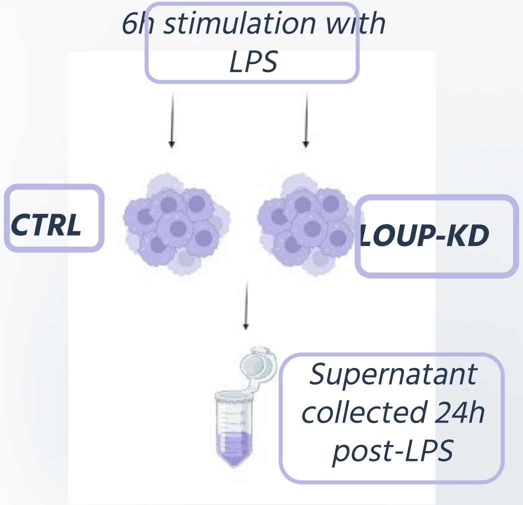
From HeatMap

1. ***TRL4/NFkB***: LPS activates TRL4, leading to NFkB mediated of inflammatory cytokines. LOUP normally restrain this activation.
2. ***IRF3/IFN***: LOUP-KD also enhanced transcription of these target genes.



3. Does LOUP negatively regulate NFkB and inflammatory responses?

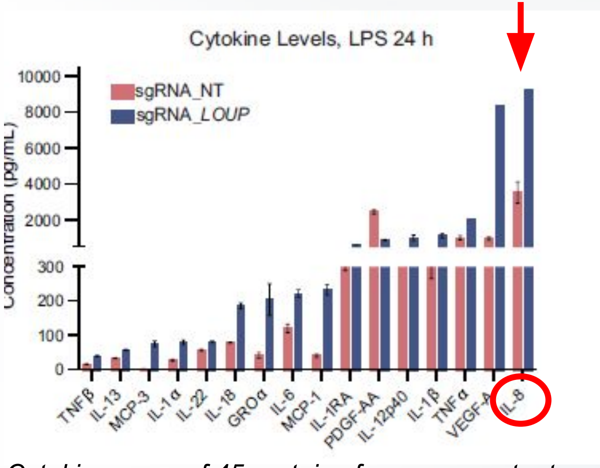
Effect of LOUP-KD on protein secretion



- A cytokine array measuring 45 different proteins was performed
- This allows simultaneous quantification of multiple cytokines to see which are regulated by LOUP

RESULTS

- **16** out of 45 were significantly **upregulated** in LOUP-KD compared to control.
- **IL8** showed the strongest, consistent the RNA-level data.

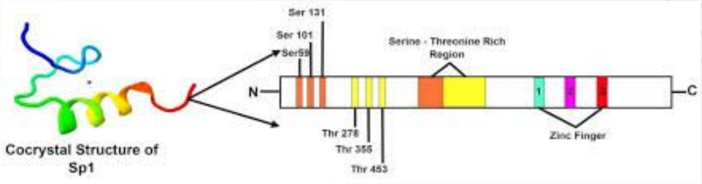


Cytokine array of 45 proteins from supernatants, analyzed by multiplex ELISA

3. Does LOUP negatively regulate SPI1 and inflammatory responses?

1. ***SPI1 in ctrl THP 1 cells:***

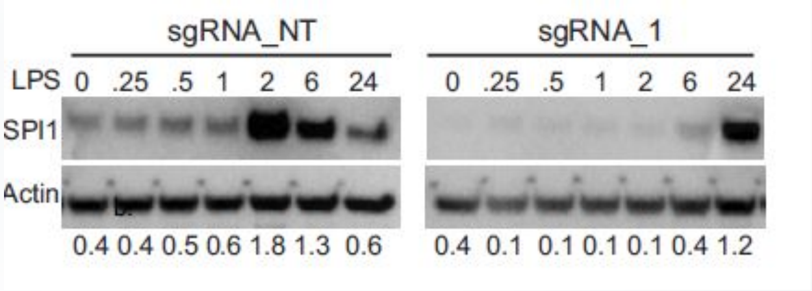
- Baseline: SPI1 is already detectable in WB even ***without stimulation***.
- After LPS (2-6h), early induction, SPI1 rapidly increase



SPI1 is a lineage- defining transcription factor for maintaining myeloid identity

2. SPI1 in LOUP-KD:

- Baseline: SPI1 protein is reduced.
- Induction is delayed, peaking only **24h** post-LPS



Representative LOUP-KD (sgRNA_1), similar for sgRNA_2,3

LOUP helps "prime" the inflammatory response without altering SPI1 mRNA

3. Does LOUP negatively regulate SPI1 and inflammatory responses?

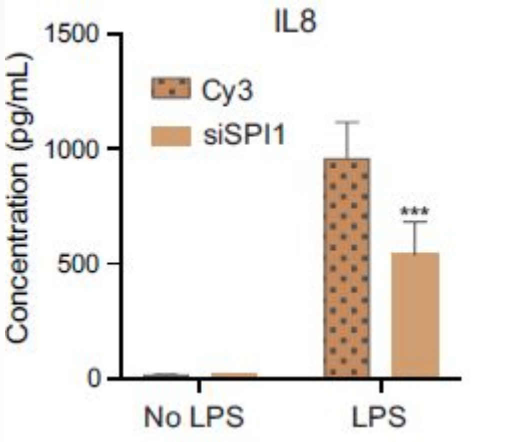
Experiment setup

1. WT THP 1 + **siRNA targeting SPI1** (siSPI 1) or **control siRNA** (siC y3)

1.Western Blot confirm the SPI1-KD

RESULTS:

- **IL8** measured by ELISA in supernatant after 24h-LPS.
- **SPI1-KD decreases IL8 levels**, opposite to LOUP-KD

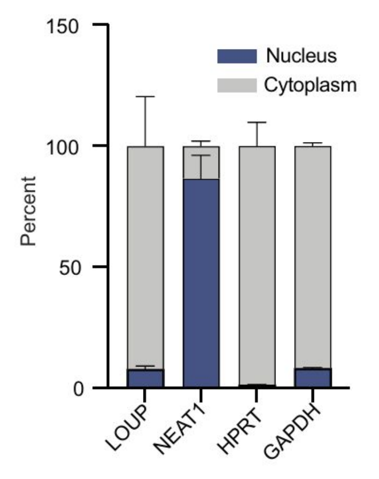


INTERPRETATION:

- SPI1, ***positive regulator*** of inflammation
- SPI1 loss: does not explain inflammatory gene upregulation in LOUP-KD
- LOUP: ***negative regulator of NF -kB***, independent of SPI1

4. What is the mechanistic contribution of LOUP-encoded small peptides?

LOUP RNA localizes to **both nucleus and cytoplasm**
Nuclear-cytoplasmic fractionation + qPCR



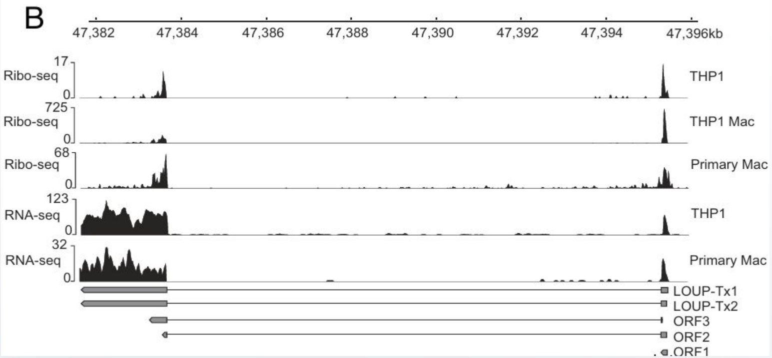
Results

- LOUP detected in *both* compartments
- Nuclear → cis regulation of SPI1
- Cytoplasmic → potential translation of sORFs

Hypothesis → **LOUP may encode functional SEPs that regulate immune genes**

4. What is the mechanistic contribution of LOUP-encoded small peptides?

Mining existing **Ribo-Seq data** from THP-1 cells, PMA-differentiated THP-1 macrophages and primary macrophages identified **three LOUP sORFs** with ribosome footprints



Results

3 sORFs actively translated

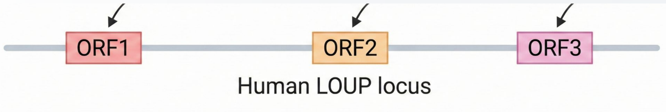
- *LOUP-Tx1*: contains **ORF1, ORF2, ORF3**
- *LOUP-Tx2*: contains only **ORF3**

Tx1- very short 5'UTR (14 nt), may allow **downstream initiation**, consistent with all ORFs being translated.

4. What is the mechanistic contribution of LOUP-encoded small peptides?

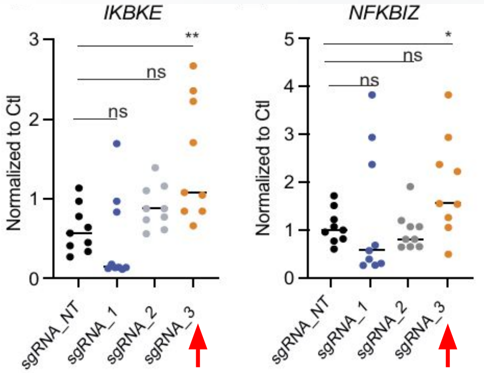
Does disrupting LOUP's sORFs with CRISPR/Cas9 increase basal inflammatory cytokine expression, consistent with the CRISPRi phenotype?

Individual LOUP sORFs were deleted using CRISPR/Cas9.



Researchers monitored two inflammation-related genes: **IKBKE** and **NFKBIZ**.

Results

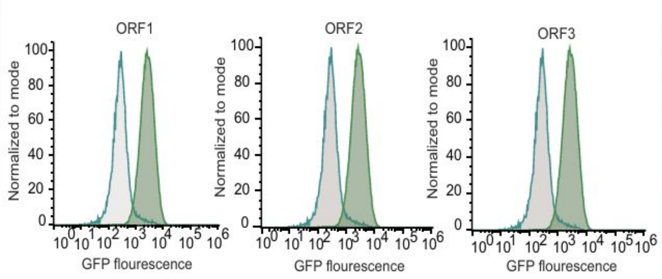


- **IKBKE** ↑ and **NFKBIZ** ↑ when ORF1 and ORF2 disrupted
- No effect with ORF2 or ORF2-ORF3
- LOUP & SPI1 RNA unchanged → post-transcriptional effect

4. What is the mechanistic contribution of LOUP-encoded small peptides?

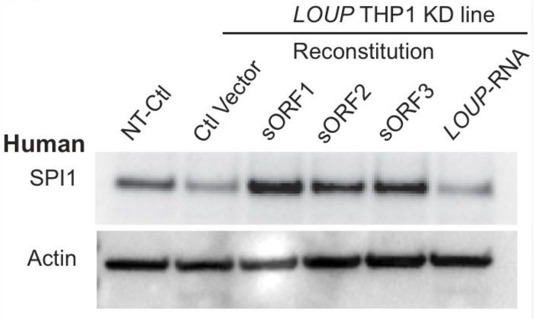
Method

1) sORF–GFP fusion expression - THP1- NFkB- CRISPRi cells
2) Rescue experiments in LOUP CRISPRi cells



Results

- All 3 sORFs produce GFP → translated
- Re-expression of SEPs → **SPI1 protein rescued**
- LOUP RNA without start codons → **no rescue**
- SPI1 RNA unchanged



4. What is the mechanistic contribution of LOUP-encoded small peptides?

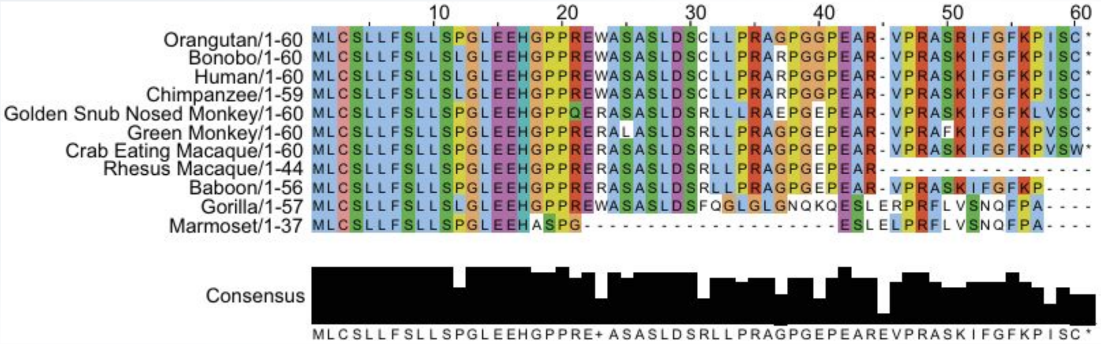
Are the small peptides encoded by LOUP conserved across species?

Results

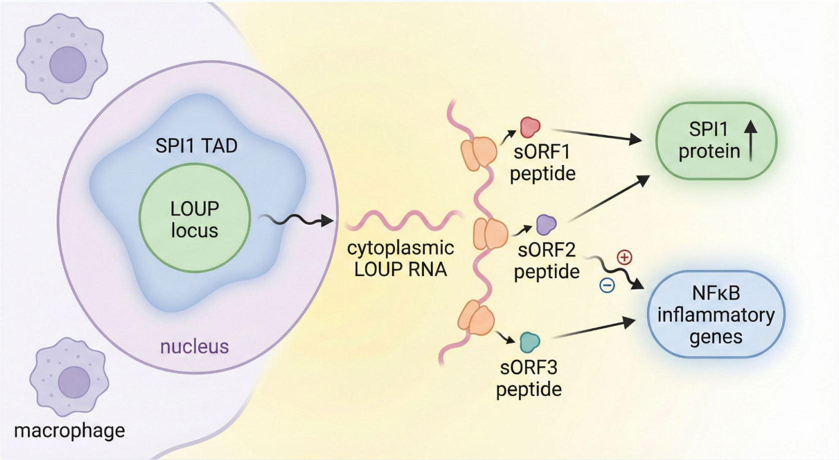
ORF1 and ORF2 are **highly conserved in primates**

ORF3 shows **no significant conservation**.

None of the ORFs are conserved in mouse genomes.



LOUP - A Multifunctional lncRNA Locus



- Identified in CRISPRi screens as a regulator of monocyte-to-macrophage differentiation and NF-κB signaling
- Cis-regulatory enhancer for SPI1
- SEPs from cytoplasmic LOUP RNA
- ORF1 and ORF2 are conserved in primates
- lncRNA bimodal regulators

What are the next steps to uncover LOUP's role?

Identify **targets and mechanisms** of LOUP-derived peptides

Study LOUP function in **varied inflammatory contexts**

Study **isoform-specific effects**

Thank you for your attention

Discussion and questions are welcome