

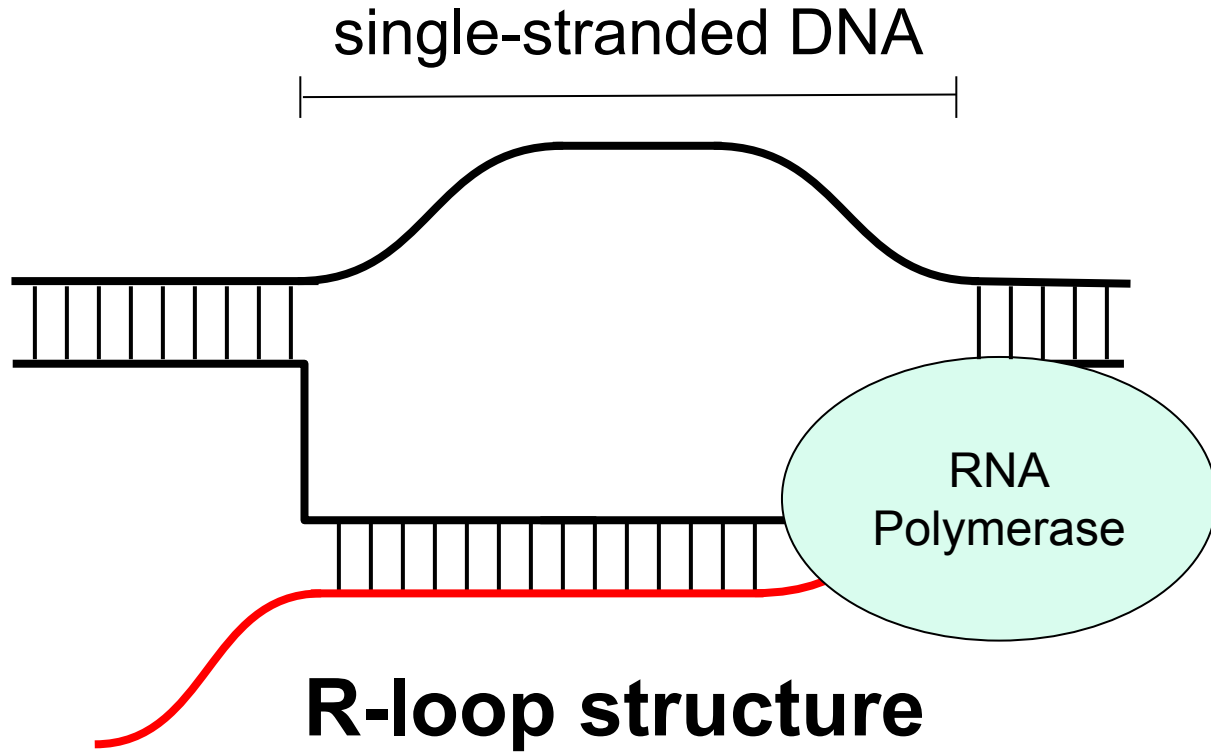


# R-loops

## GENE EXPRESSION CHROMATIN STRUCTURE



PRESENTED BY:  
Violina POTLOG  
Margot LADISLAS  
Isabella CONCINA



Rare and accidental  
byproducts of transcription ?

Rare and accidental  
byproducts of transcription ?

**NO**

# WITHOUT / WITH R-loops ?

## Preventing factors

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RNA-binding proteins  
Splicing factors  
mRNA export factors  
Topoisomerases  
Cleavage and polyadenylation factors  
Transcription elongation factors



## Promoting factors

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G-rich nascent RNA  
Negative supercoiling

## Nascent Connections: R-Loops and Chromatin Patterning

Frédéric Chédi

Basic Determinants of Co-Transcriptional R-Loop Formation

## 2 Methods for R-loops Mapping

- By using Bisulfite treatment
- By using immunoprecipitation

## 2 Methods for R-loops Mapping

- By using Bisulfite treatment



## 2 Methods for R-loops Mapping

- By using Bisulfite treatment





## 2 Methods for R-loops Mapping

- By using Bisulfite treatment



## 2 Methods for R-loops Mapping

- By using Bisulfite treatment

ATAC**C**GAC**C**CG      Bisulfite      ATAC**C**GAC**C**CG  
TATG**C**CTGG**C**            TATG**C**CTGG**C**

**No Denaturation**

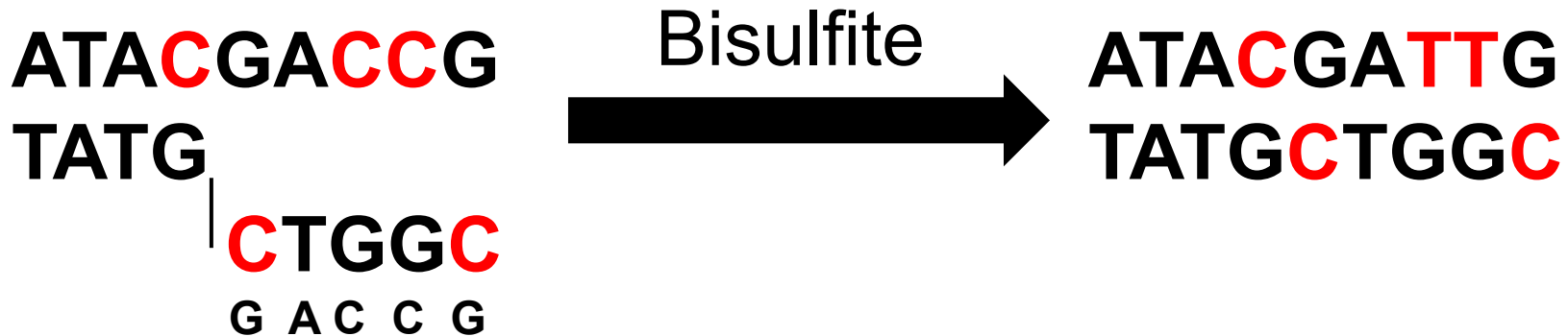
## 2 Methods for R-loops Mapping

- By using Bisulfite treatment



## 2 Methods for R-loops Mapping

- By using Bisulfite treatment



## 2 Methods for R-loops Mapping

- By using Bisulfite treatment



## 2 Methods for R-loops Mapping

- By using Bisulfite treatment

**= low-throughput  
single-molecule approach**

## 2 Methods for R-loops Mapping

- By using immunoprecipitation

~~ChIP-seq~~

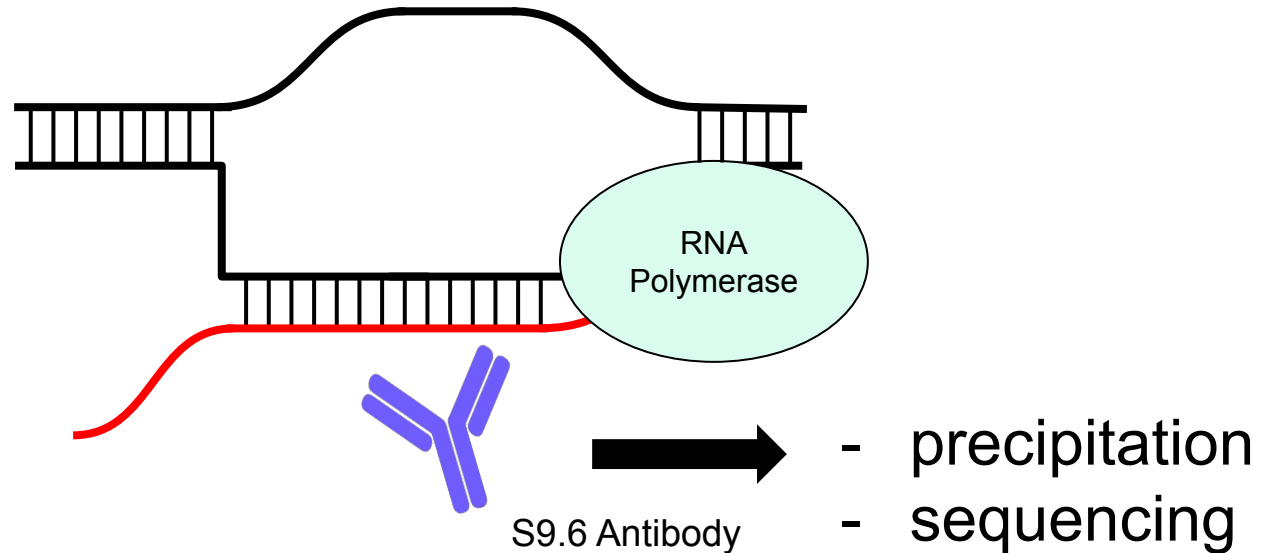


**DRIP-seq**

= DNA-RNA immunoprecipitation

## 2 Methods for R-loops Mapping

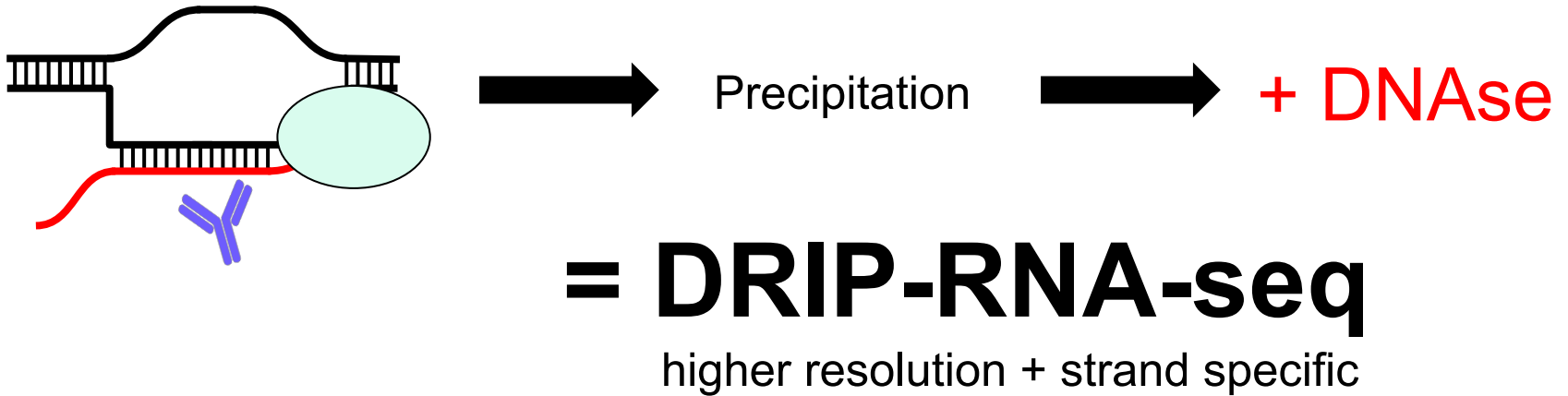
- By using immunoprecipitation





## 2 Methods for R-loops Mapping

- By using immunoprecipitation



## 2 Methods for R-loops Mapping

- By using immunoprecipitation  
**= high-throughput  
population average  
technique**

R-loops are more prevalent  
than we thought:

**10.000 loci**

**= 150 Mb**

**= 5% of the genome**

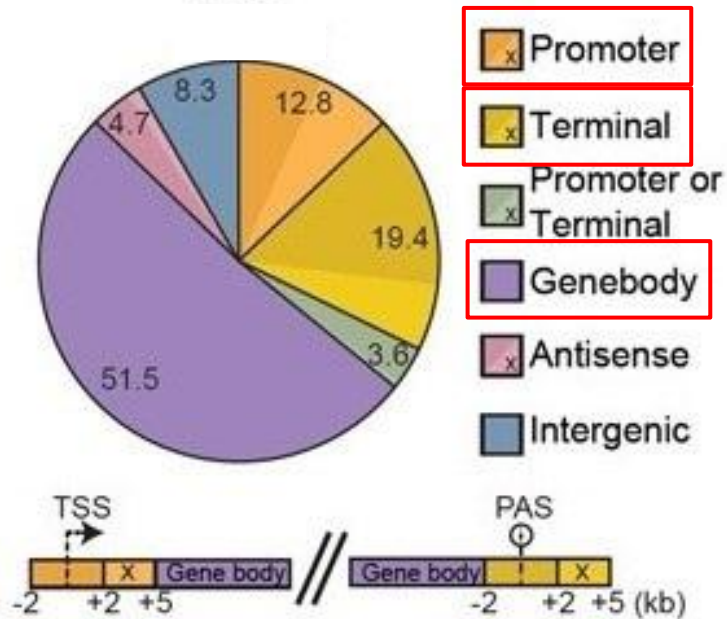


Function ?

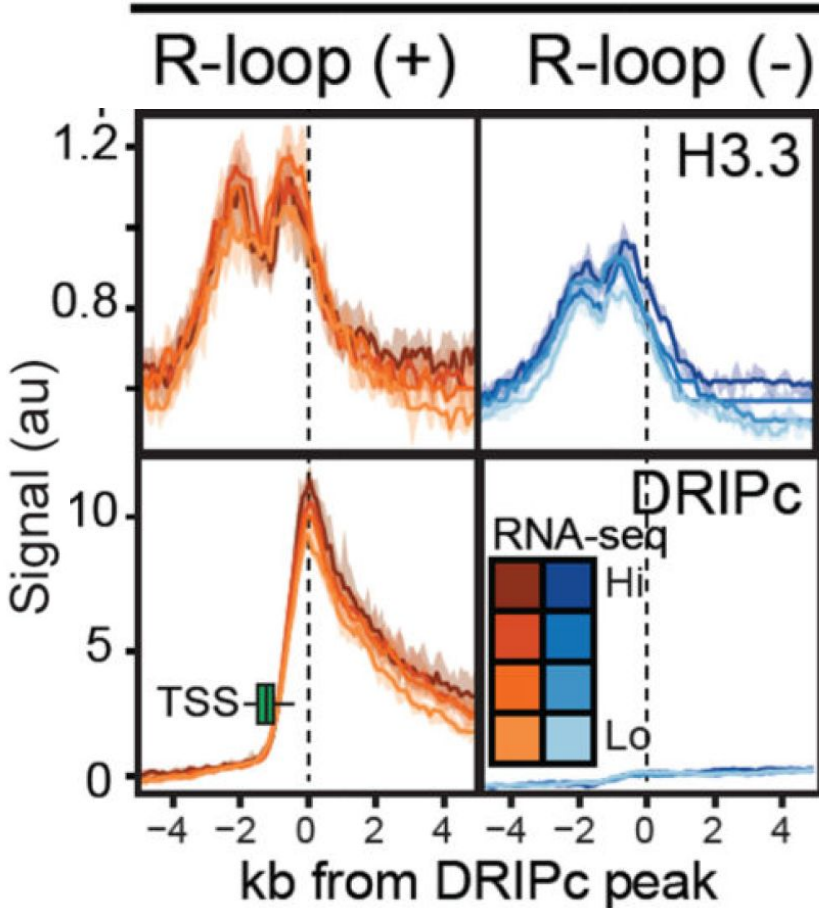


# Where's R-loops?

- in promoter regions
- in terminator regions
- in gene bodies



# Promoter



Hp: R-loops interfere with nucleosome repositioning behind the advancing RNAP

H3.3 = histone variant that dynamically replaces H3 lost owing to nucleosome disruption

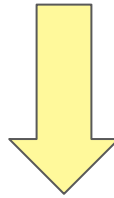


R-loops locally open the chromatin structure by regulating nucleosome:

- occupancy,
- positioning
- turn-over

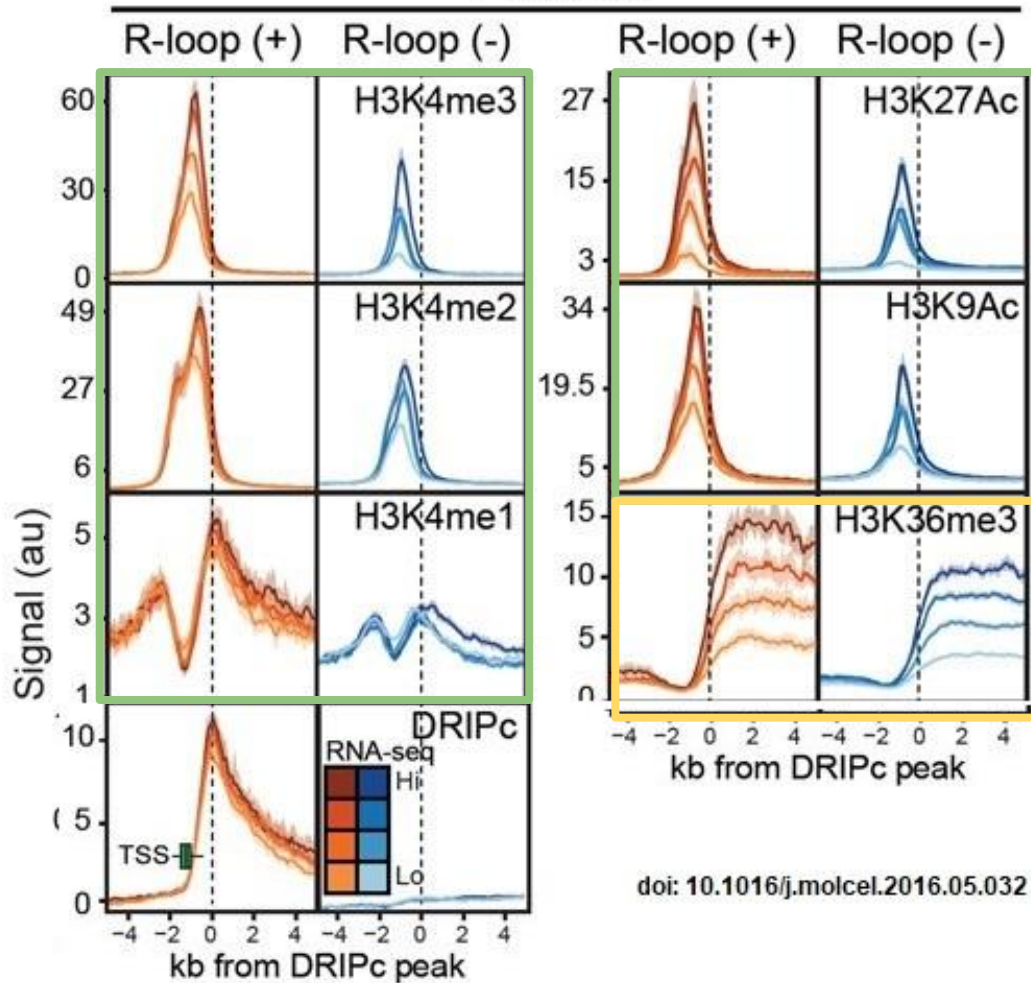
# Histone modification signatures of R-loop chromatin

Identification of a set of histone modifications associated with R-loop chromatin under normal conditions



R-loops = additional layer of epigenetic information!

## Promoter



## Promoter regions markers

Active transcription:

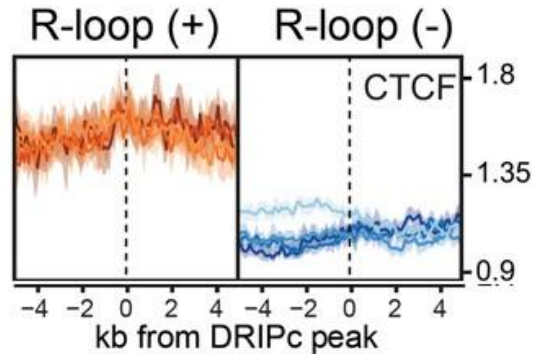
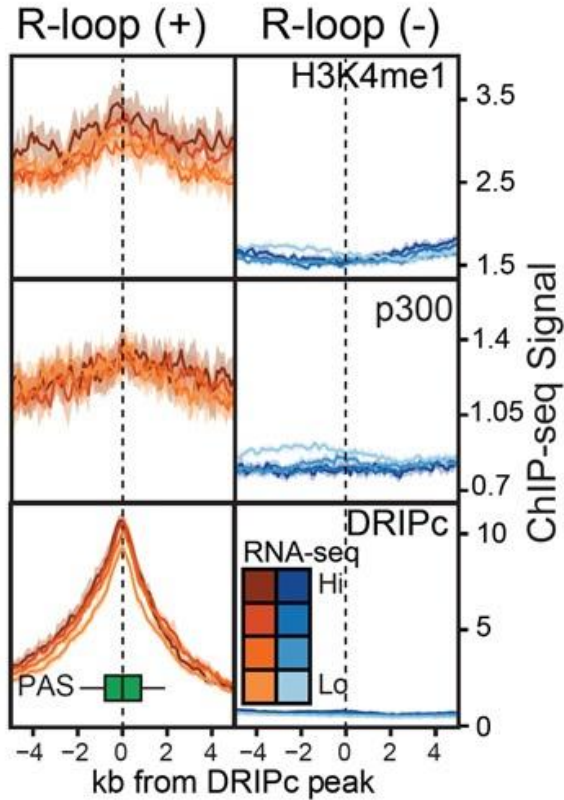
- H3K4me1
- H3K4me3
- H3 acetylation

Transcription elongation:

- H3K36me3

doi: 10.1016/j.molcel.2016.05.032

# Terminal R-loops markers



- H3K4me1 → common mark of R-loop regions
- p300 acetyltransferase
- CTCF-cohesin complex

R-loop regions =  
enhancer-like  
chromatin state



What is the mechanism of R-loop mediated patterning ?

R- loops favor the recruitment of chromatin - modifying complexes

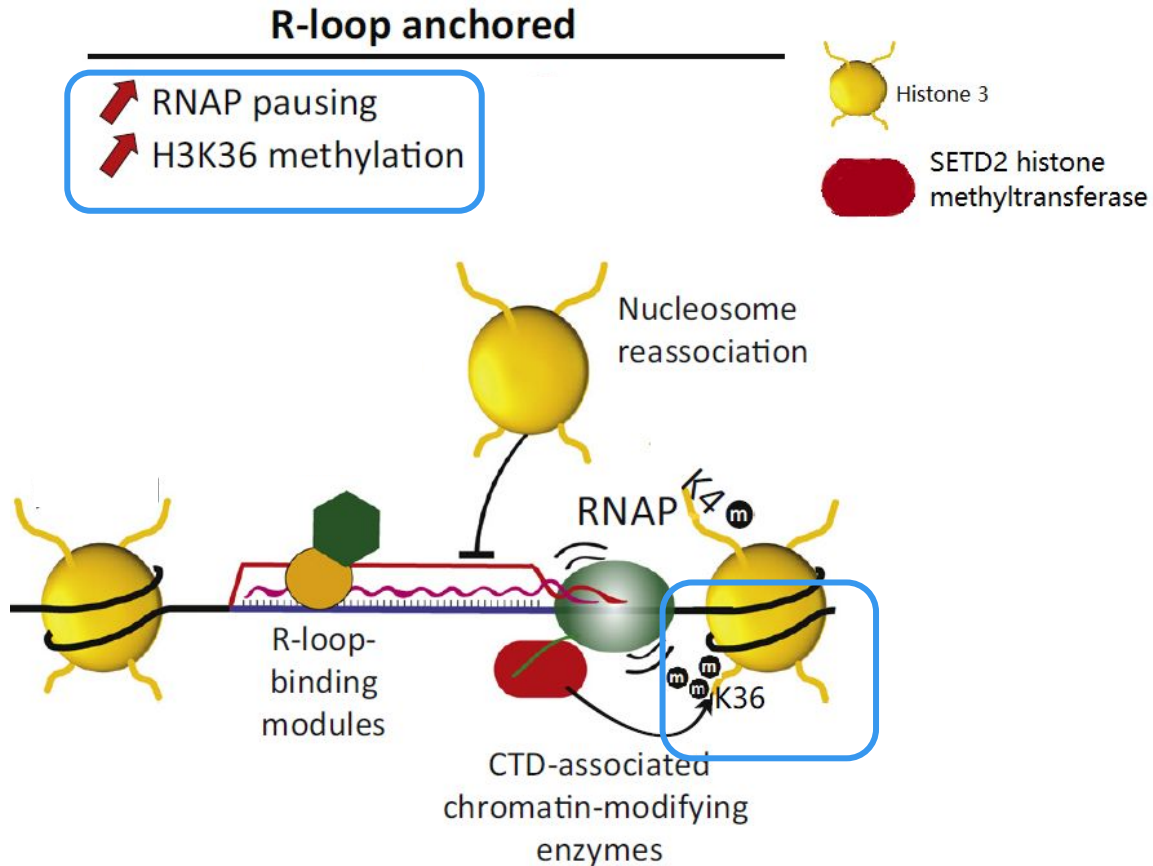
# H3K36me3

Observations:

- SETD2 histone methyltransferase interact with CTD of RNAP
- $\uparrow$  levels of H3K36me3 +  $\uparrow$  RNAP density at R-loop(+) promoters



R-loops facilitate recruitment of SETD2 to RNAP complexes by transiently stalling the transcription machinery



# H3K4me1/3

TSS anchored

H3K4me2 / me3

R-loop anchored

RNAP pausing

H3K36 methylation

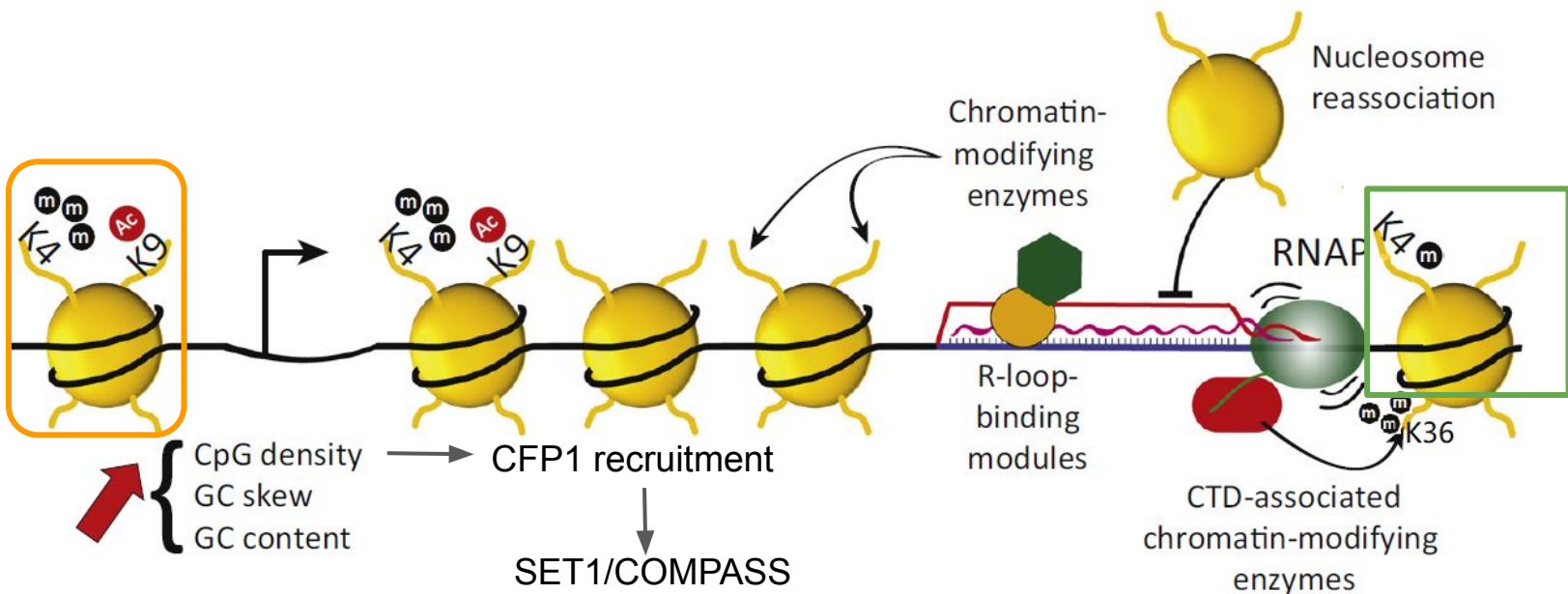
H3K4me1



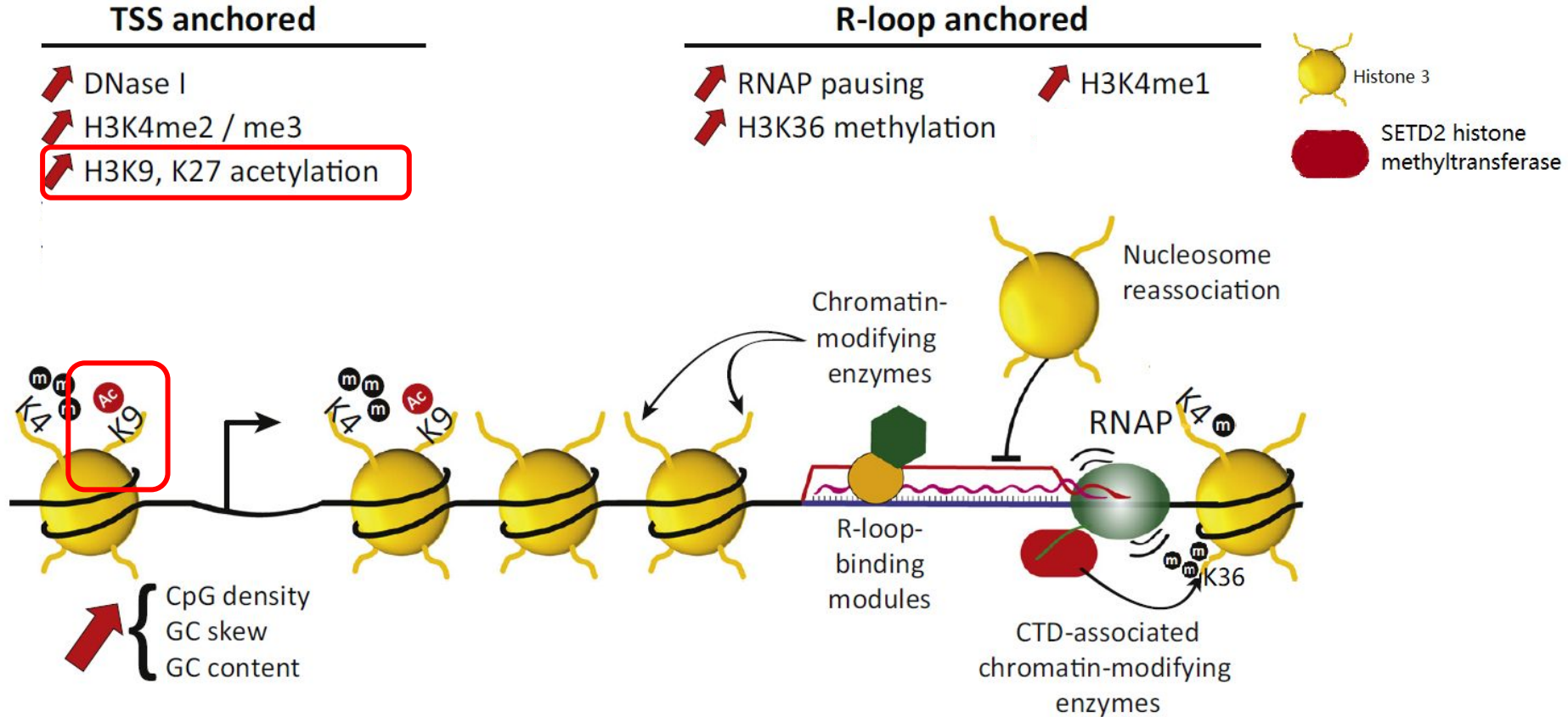
Histone 3



SETD2 histone methyltransferase



# Histone acetylation



## R-loops recruit Tip60-p400

### Observation in mESCs:

↓ Tip60-p400 chromatin remodelling and acetylase complex in mESCs  
expressing RNaseH1



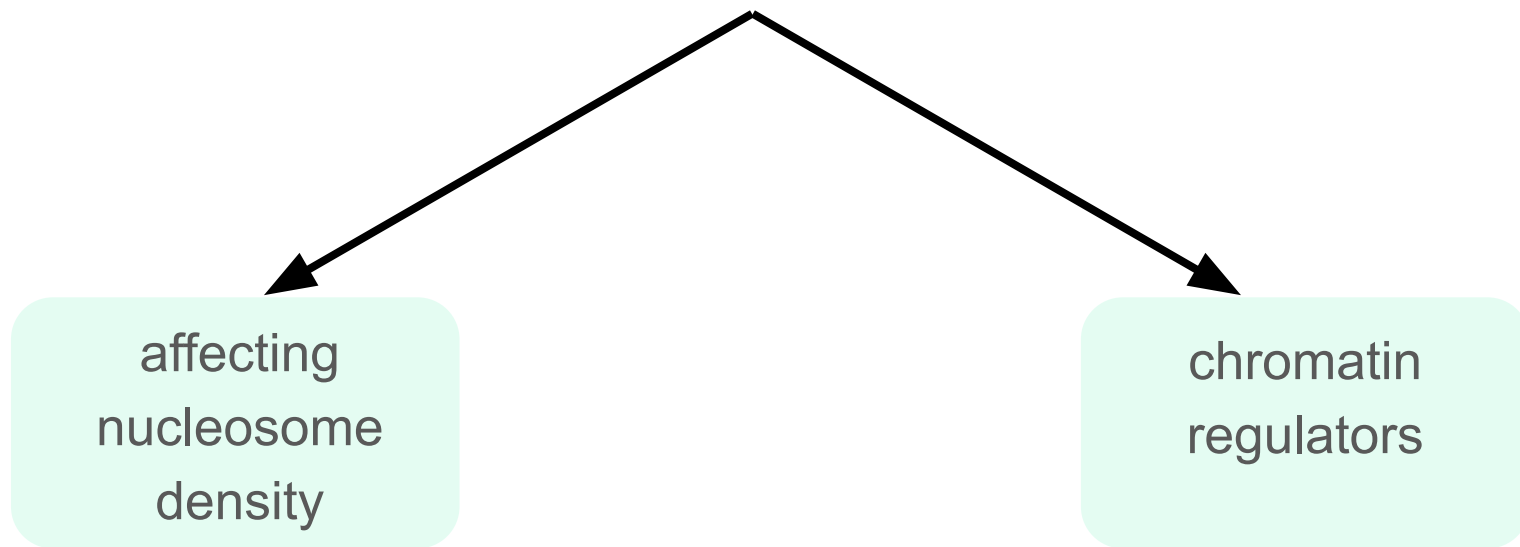
↑ Tip60-p400 targets form R-loops



R-loops contribute to recruiting the Tip60-p400 complex to chromatin.

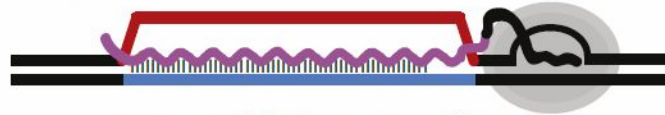
## Summarising...

R-loops influence chromatin modification states by:



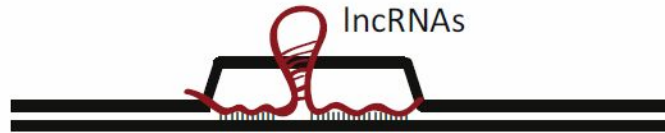
# CIS or TRANS R-loops

Formation in *cis*



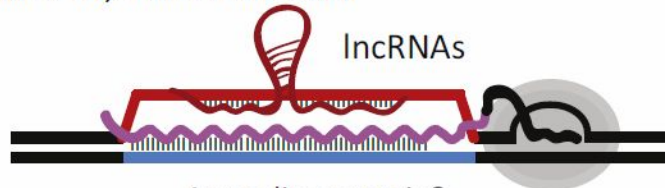
Transcription/DNA sequence/topology

Formation in *trans*



IncRNAs  
Homology search?  
DNA strand exchange?

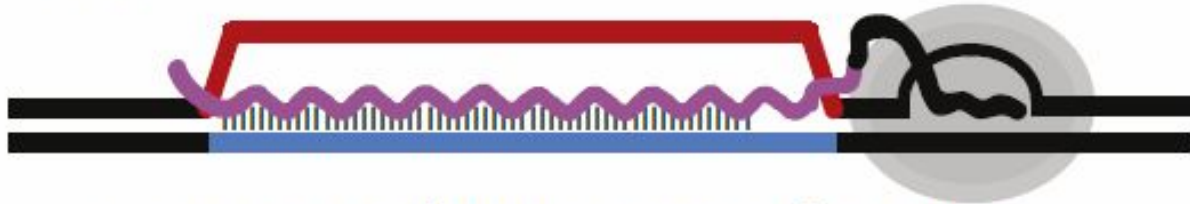
Mixed *cis/trans* formation



Annealing protein?

# CIS or TRANS R-loops

Formation in *cis*



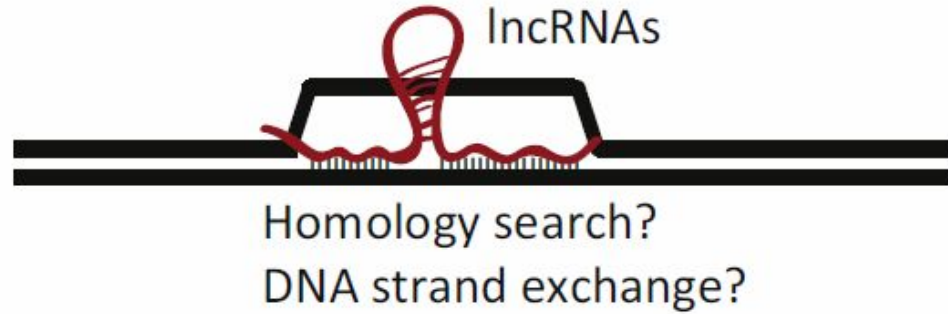
Transcription/DNA sequence/topology

- 1) In 90% of cases R-loops are formed co-directionally with transcription;
- 2) R-loops levels correlate with expression levels and respond to transcriptional changes;
- 3) DNA based and RNA based R-loops maps agree well with each other;
- 4) Genes showing allele-specific expression show allele specific R-loops.



# CIS or TRANS R-loops

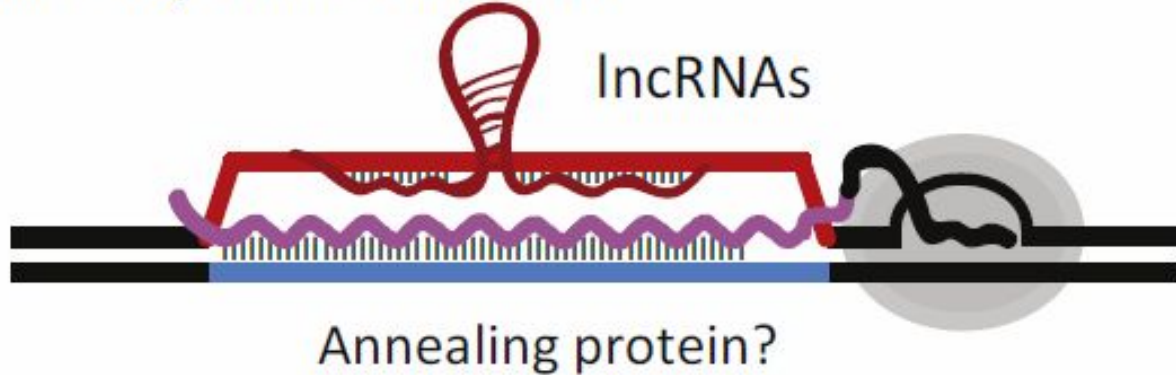
Formation in *trans*



- 1) RNA strand transcribed from a locus can hybridize onto a distant locus through regions of RNA-DNA complementary;
- 2) This mechanism likely requires proteins to catalyse strand invasion and assist in homology searching;
- 3) Evidence obtained in different models and provides an attractive mechanism for directing lncRNA to potential targets.

# CIS or TRANS R-loops

Mixed *cis/trans* formation



- 1) R-loops might provide a ready-made ssDNA landing pad on the non-template strand for lncRNA to interact with in trans;
- 2) RNA-DNA hybrid formation might be easier to achieve mechanistically and only requires the activity of a protein with strand annealing capacity (Rad52).

# Aberrant R-loops and chromatin condensation

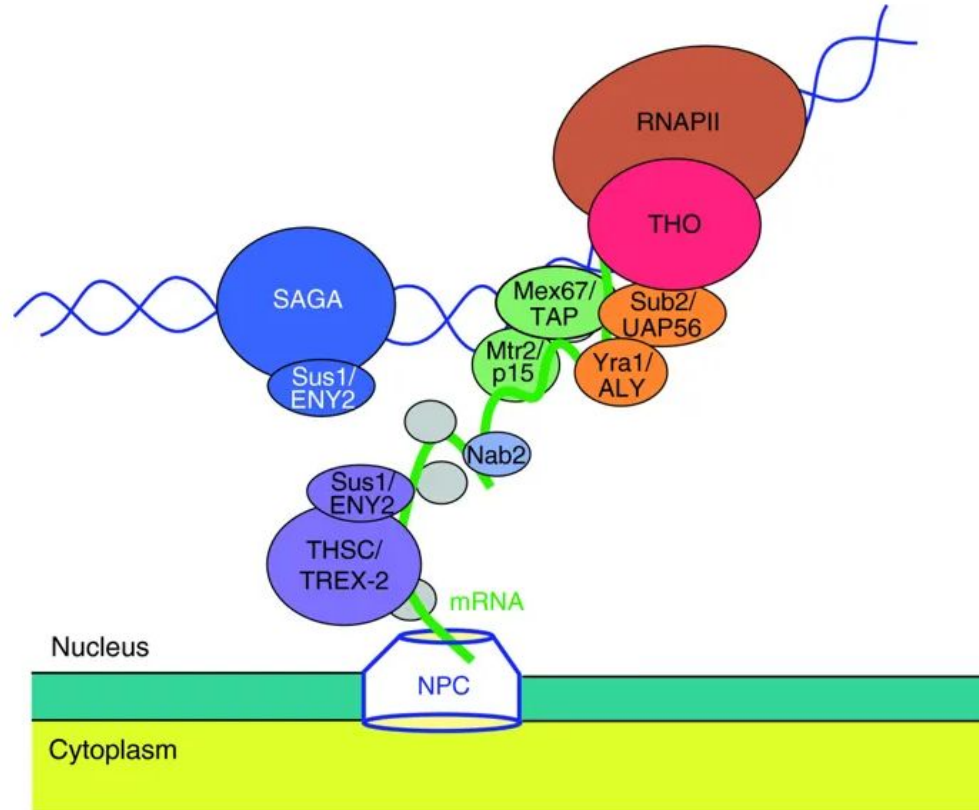
I.

**Defects** in the **THO mRNA export** complex in yeast trigger marked genomic **instability** and increased **R-loops formation**.

II.

**Increase of R-loops** is accompanied by **higher levels** of H3S10P which is a marker of **condensed chromosomes** during mitosis.

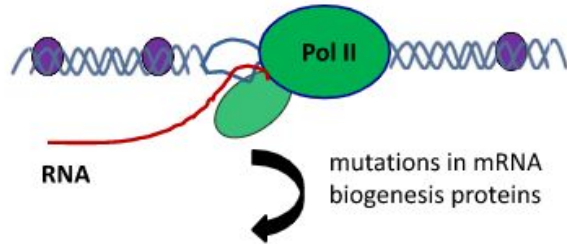
# Aberrant R-loops and chromatin condensation



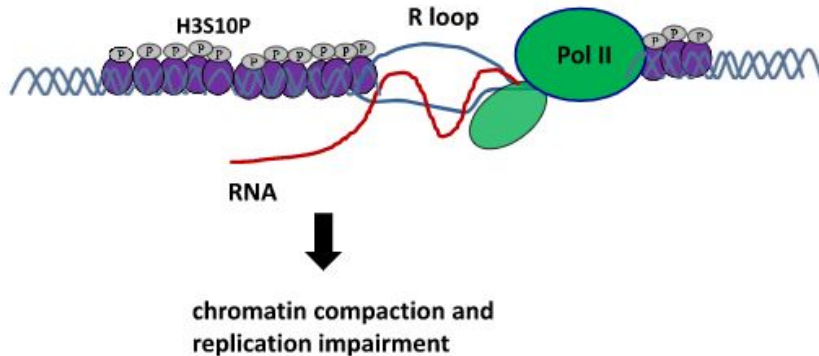
- I. Defects in the THO mRNA export complex in yeast triggers marked genomic instability and increased R-loops formation.

# Aberrant R-loops and chromatin condensation

a. WT cells



b. R loop accumulating cells



II. Increase of R-loops is accompanied by higher levels of H3S10P which is a marker of condensed chromosomes during mitosis.

Castellano-Pozo, M. et al. (2013) R loops are linked to histone H3S10 phosphorylation and chromatin condensation. *Mol. Cell* 52, 583–590

# Defining aberrant R-loops formation under pathological conditions

R-loops association with genomic instability is clear in the context of defects in a variety of factors:

- 1) involved in co-transcriptional processes (splicing mRNA, export, 3' RNA processing, transcription elongation);
- 2) which are thought to prevent R-loops formation (topoisomerase);
- 3) which are thought to mediate R-loops resolution (ribonucleaseH, RNA/DNA elicase).

# Defining aberrant R-loops formation under pathological conditions

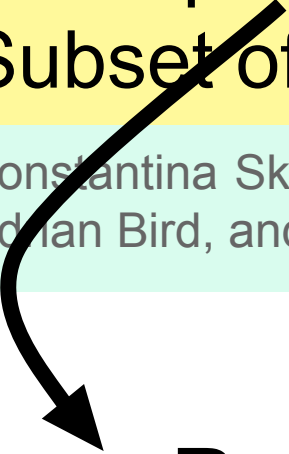
Assumptions about increased genic R-loops LEVELS in pathological situation may NOT be correct



Alterations of R-loops TURNOVER RATES may lead to persistent R-loops and threaten genome stability.

# R-Loops Enhance Polycomb Repression at a Subset of Developmental Regulator Genes

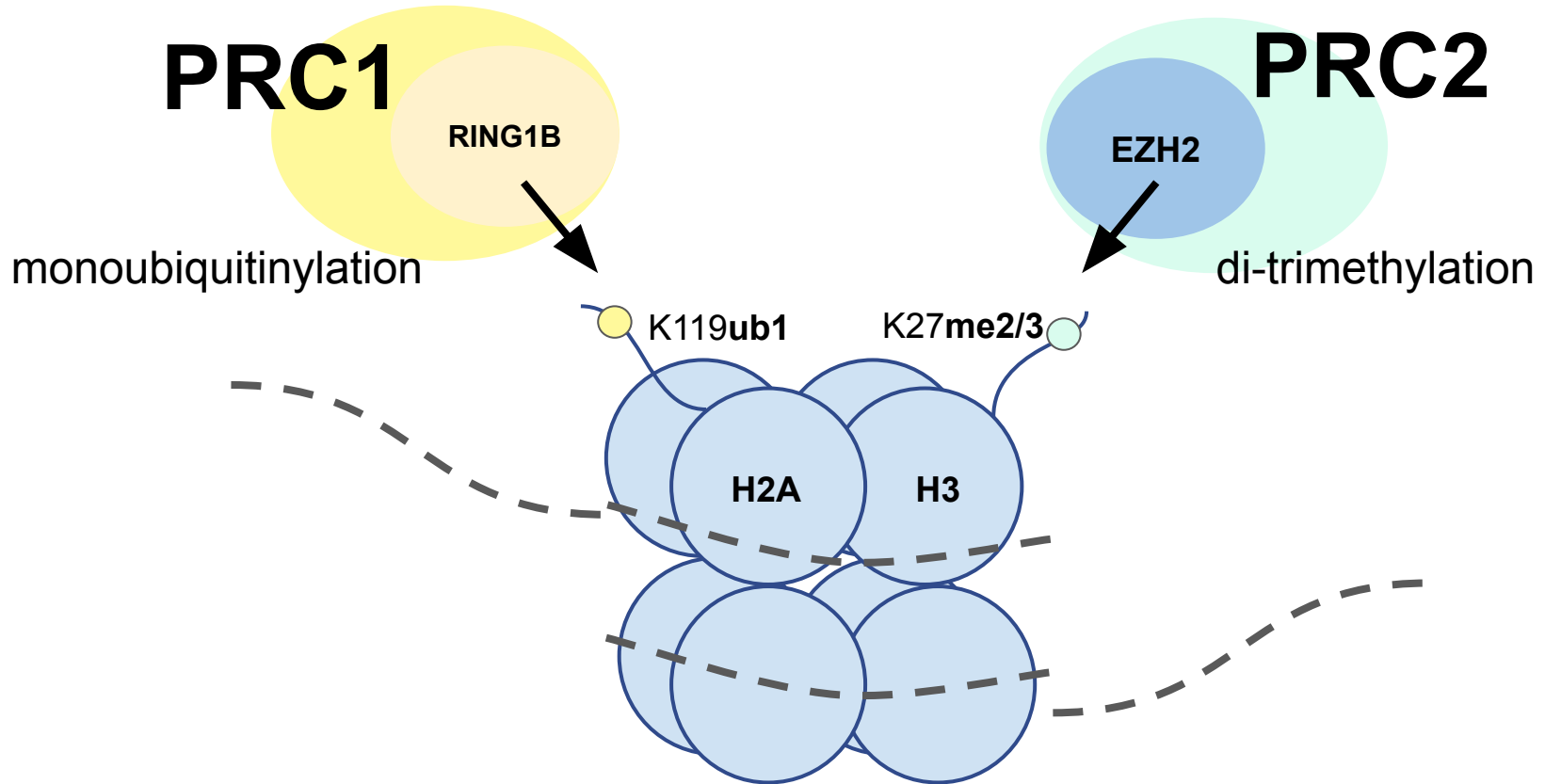
Konstantina Skourti-Stathaki, Elena Torlai Triglia, Marie Warburton, Philipp Voigt, Adrian Bird, and Ana Pombo



Polycomb group proteins (PcG)  
= Epigenetic regulators  
= Transcription repressor

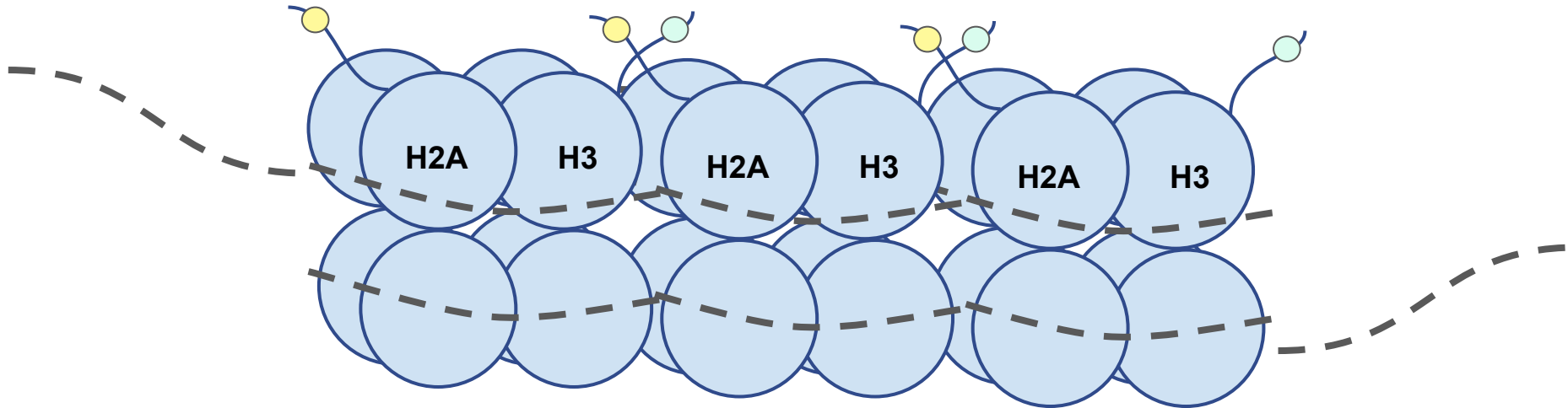


# Polycomb repressive complex:



- silence CpG-rich developmental regulator genes in mESCs
- maintain patterns of gene expression established during cell commitment

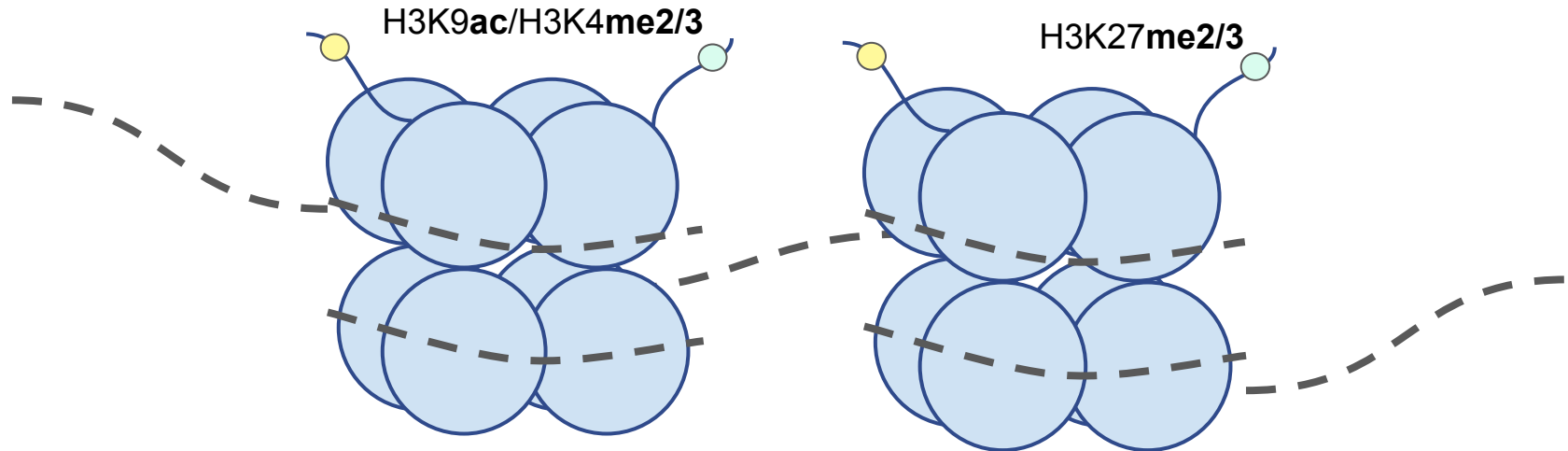
**= Repressed chromatin**



## In mESC cells :

PcG prevent the inappropriate expression of specific differentiated cell genes

**BUT** Those genes also have active marks on the chromatin

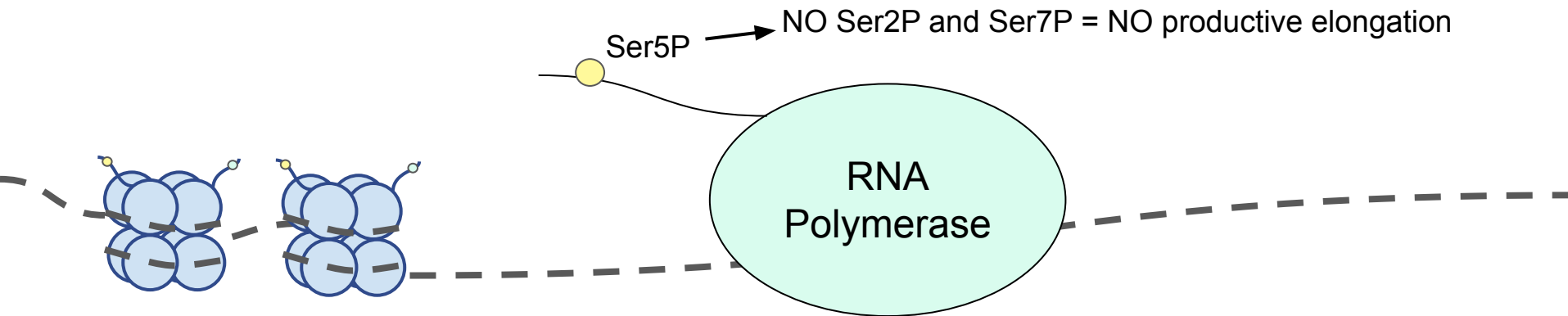


## In mESC cells :

PcG prevent the inappropriate expression of specific differentiated cell genes

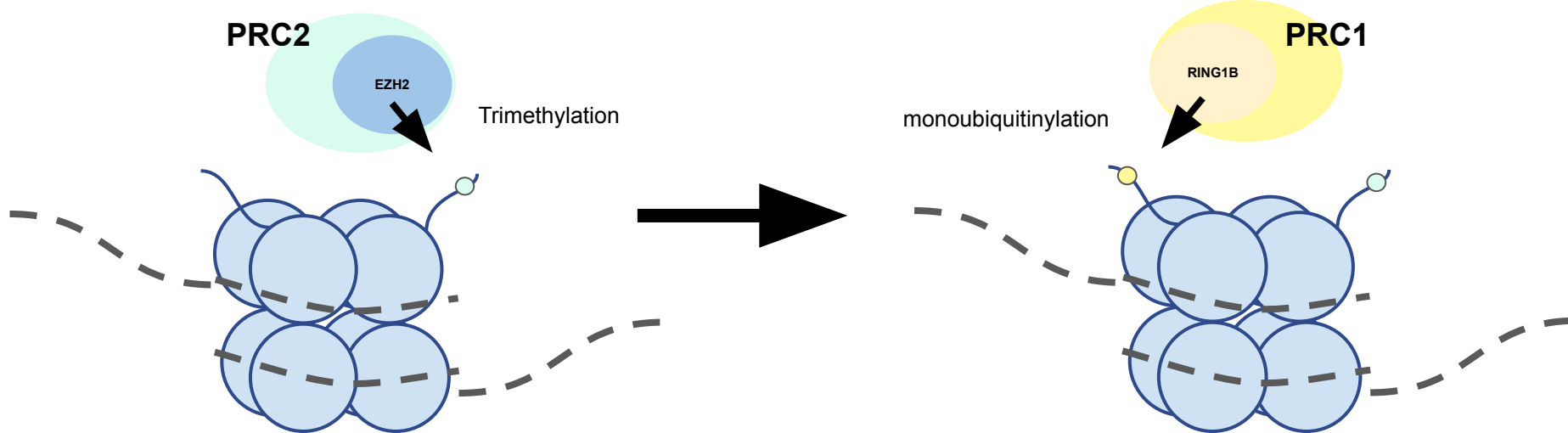
**BUT** Those genes also have active marks on the chromatin

**Though** Poised polymerase = NO mRNA



# PcG recruitment:

- Gene silencing = recruitment of PRC2 to CGI promoters
- PRC1 occur **after** trimethylation of H3K27 by PRC2



## R-loops x PcG ? → Correlations

 human ESCs

 mouse fibroblast

How does R-loops  
regulate PcG  
repression?

I) Impact of R-loops on  
PcG recruitment

II) Impact of R-loops on  
PcG-repressed genes

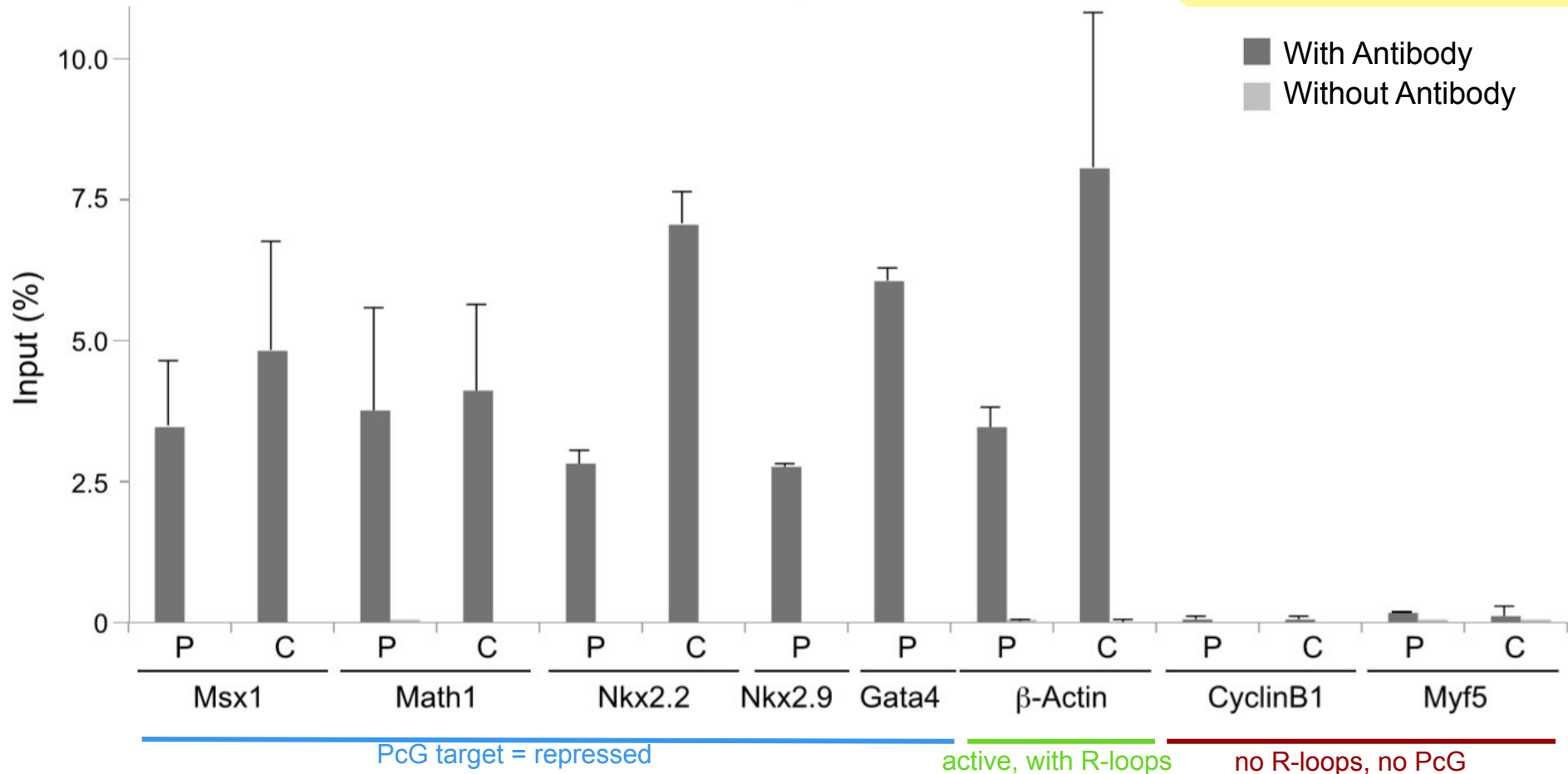
III) Impact of PcG on  
R-loops and PcG-target  
genes

## Sidenote : The genes mentioned in the article

- Msx1, Math1, Nkx2.2,  
Nkx2.9, Gata4 PcG target = repressed
- B-actin active, with R-loops
- CyclinB1 active, no R-Loops
- Myf5 inactive, no PcG

# R-loops DRIP : R-loops form over PcG-repressed genes

## Results

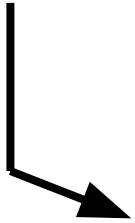




There are R-loops at PcG repressed genes



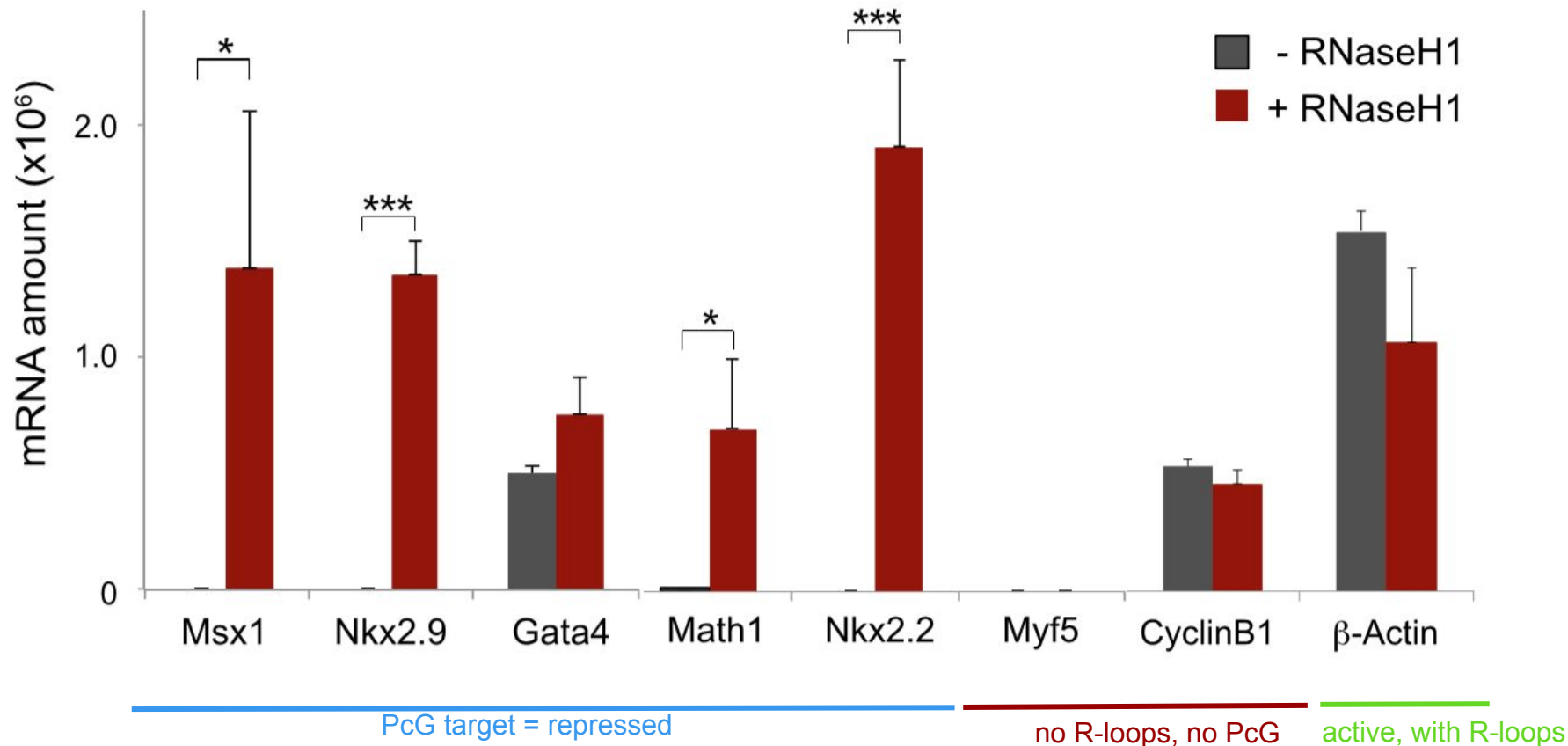
**But do they have a role in the repression?**



Do we have derepression when we remove R-loops?  
→ quantify RNA after **RNase H1** treatment

# RT-PCR of mature RNA :

# Results



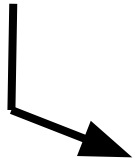
There are R-loops at PcG repressed genes



They have a role in the repression



**This role is linked to PcG?**

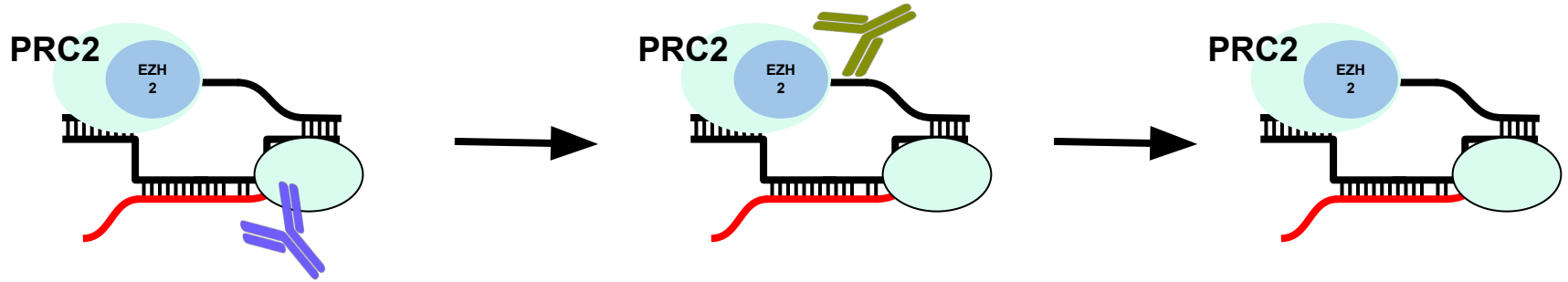


Does R-Loops Co-occupy Chromatin with PcG Enzymes ?

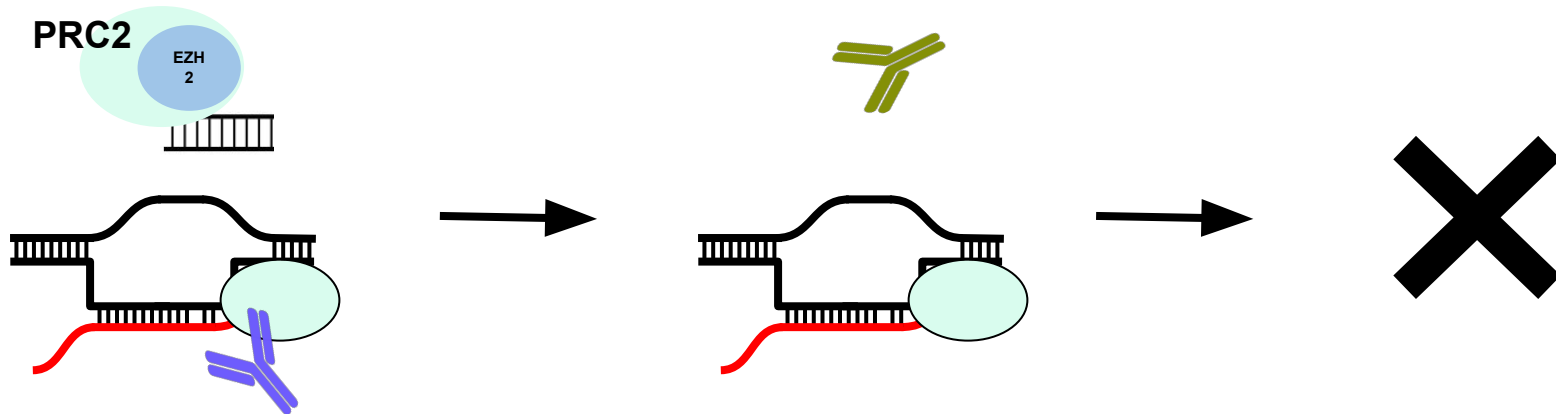
# Sequential Native Chip : R-loops $\rightarrow$ EZH2

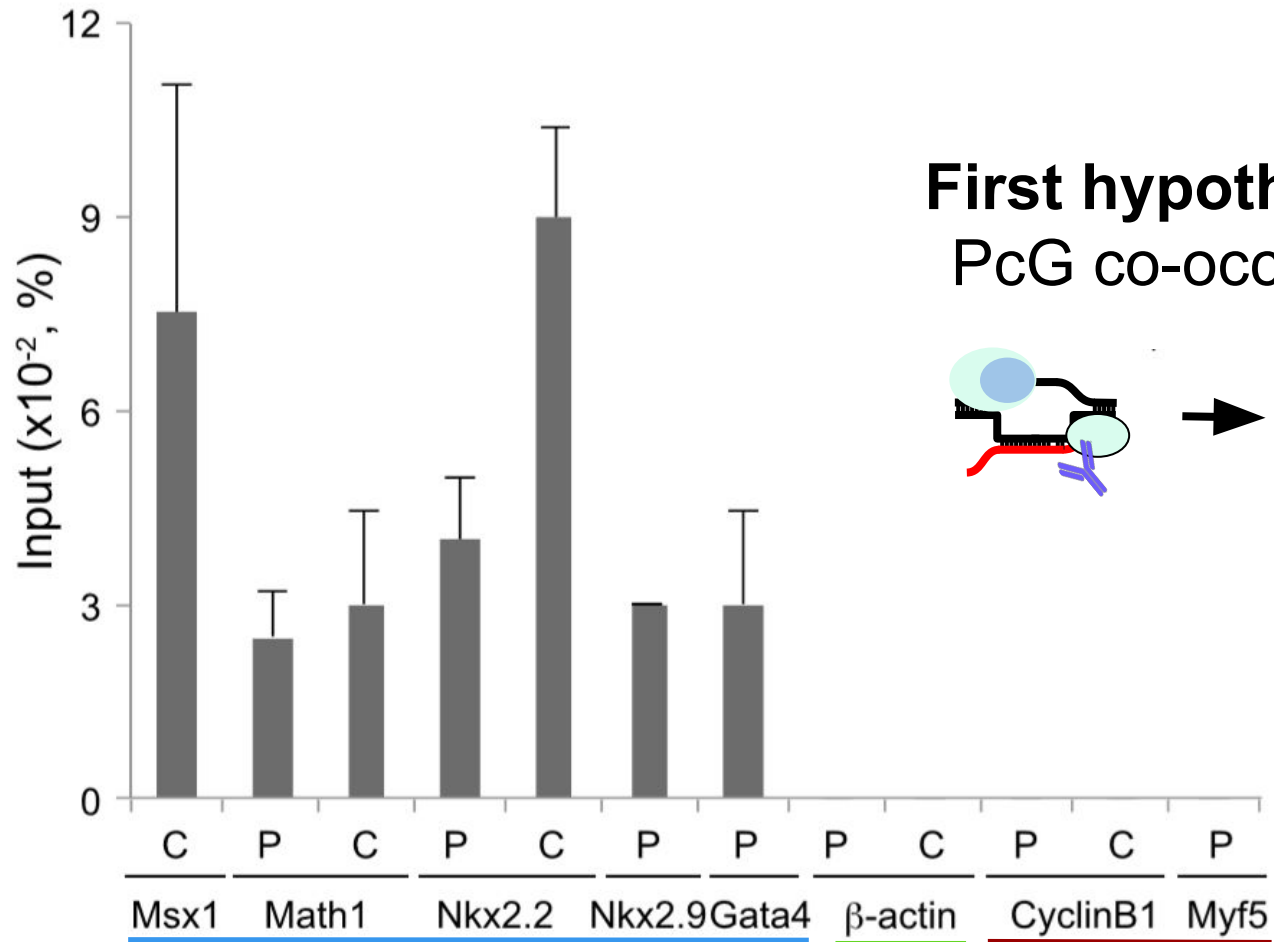
# Results

**First hypothesis** : R-loops and PcG co-occupy the chromatin

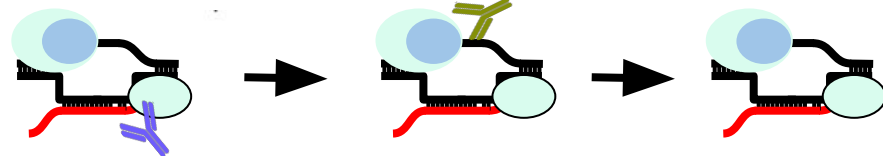


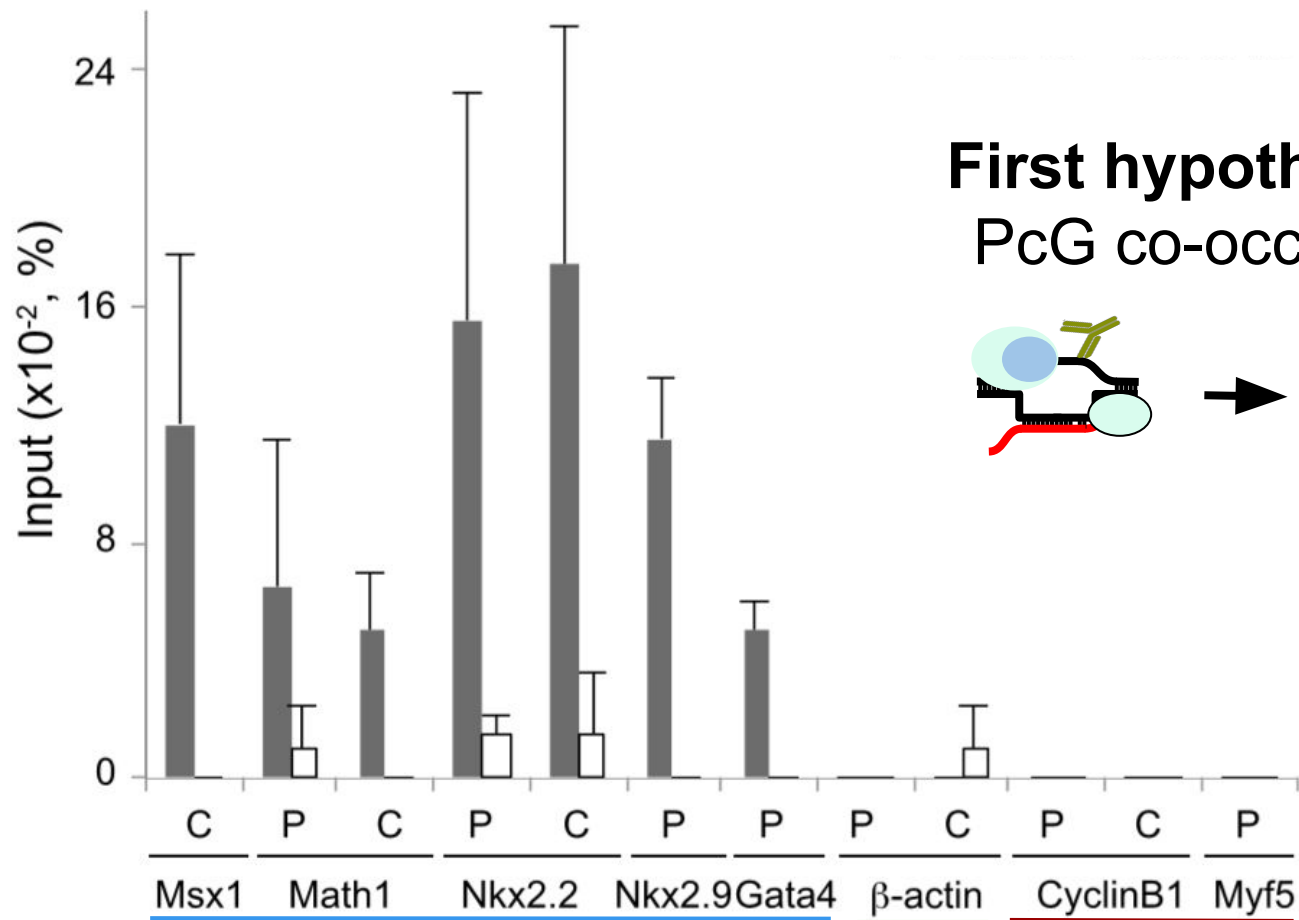
**Second hypothesis** : R-loops and PcG don't co-occupy the chromatin



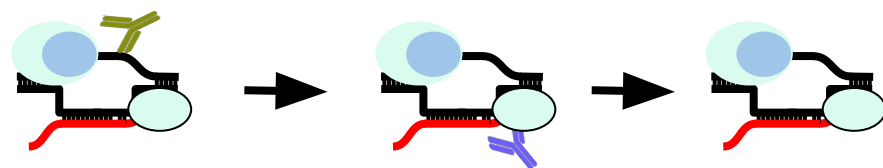


**First hypothesis : R-loops and PcG co-occupy the chromatin**





**First hypothesis : R-loops and PcG co-occupy the chromatin**



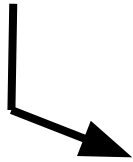
There are R-loops at PcG repressed genes



They have a role in the repression



**This role is linked to PcG?**



R-Loops Co-occupy Chromatin with PcG Enzymes



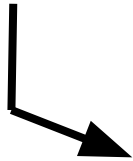
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**This role is linked to PcG?**



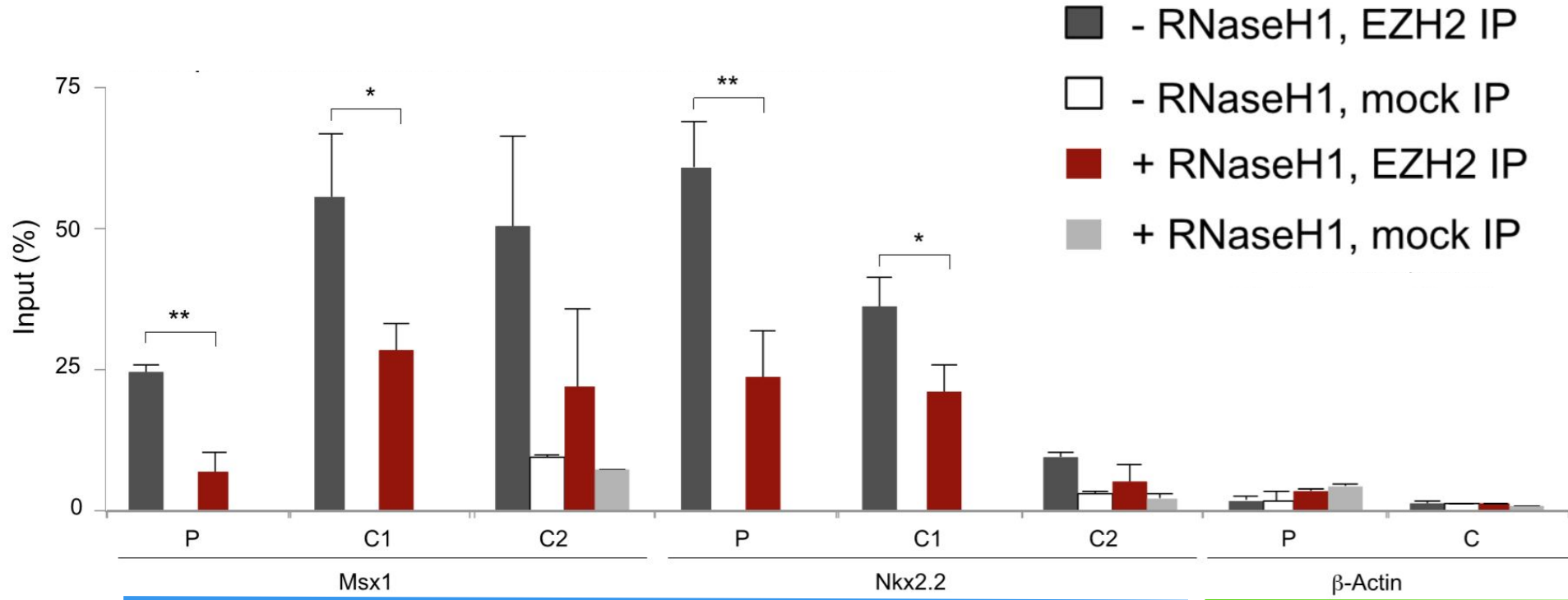
Does R-loops help recruitment of PcG?



# RNase H1 treatment + CHIP: EZH2 (PRC2)

# Results

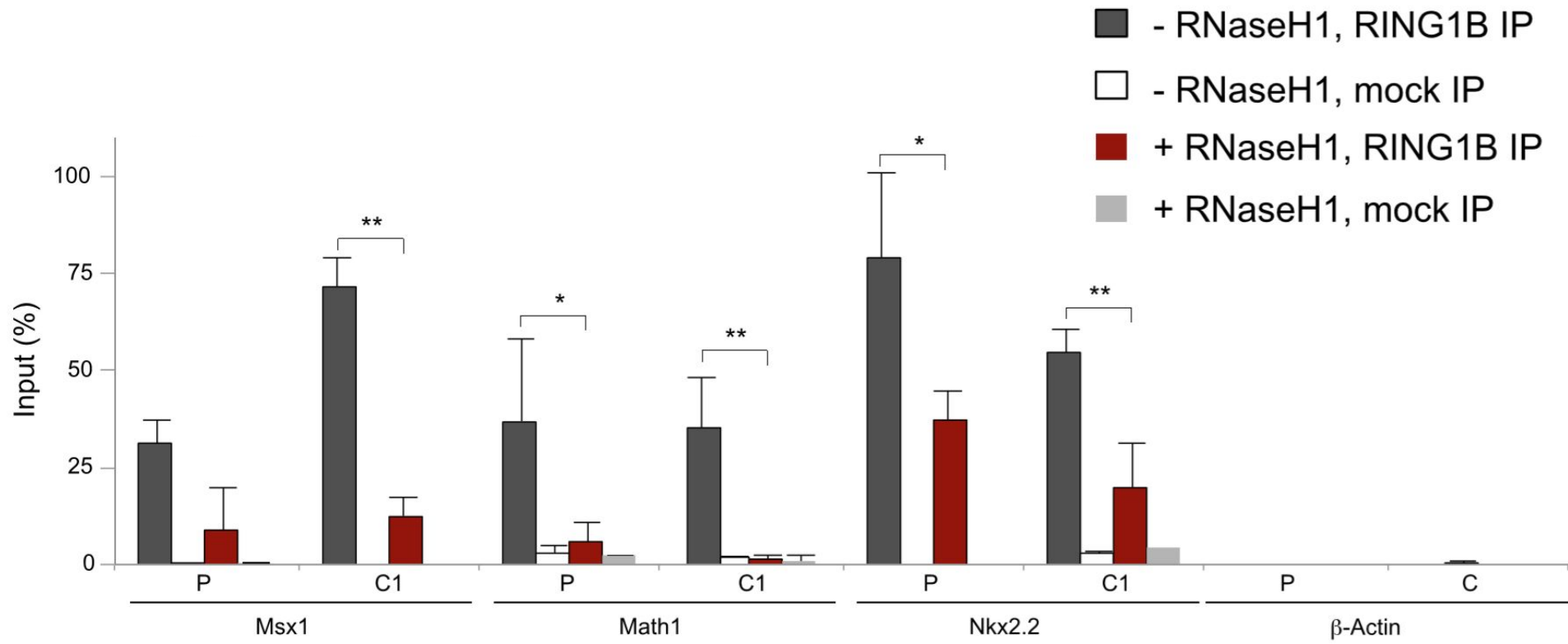
→ If we remove R-loops, will PcG still be recruited ?



# RNase H1 treatment + CHIP: RING1B (PRC1)

# Results

→ If we remove R-loops, will PcG still be recruited ?



There are R-loops at PcG repressed genes



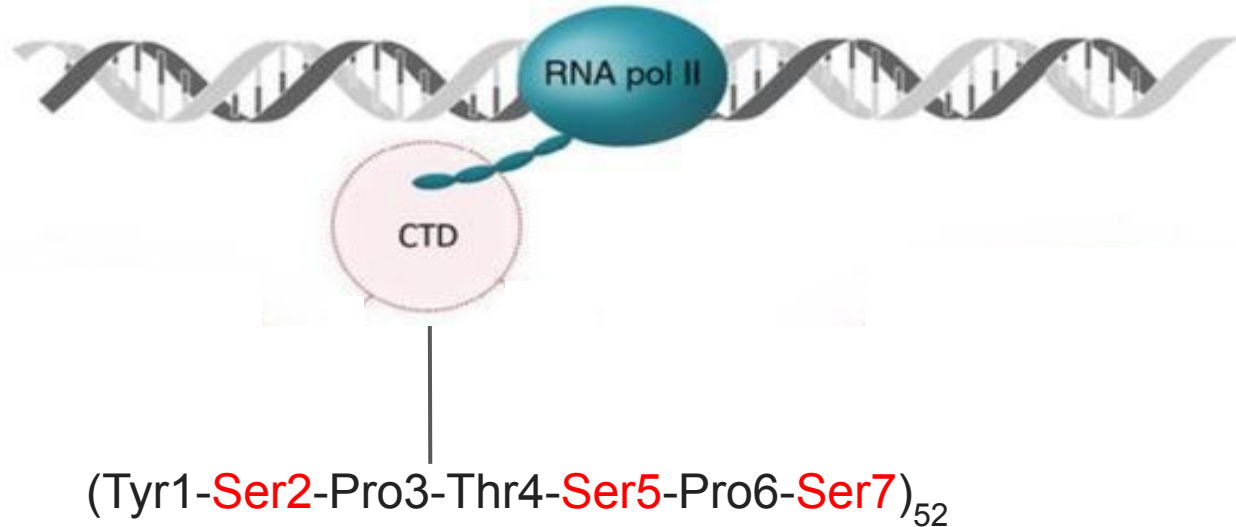
They have a role in the repression



R-loops facilitate binding of PcG



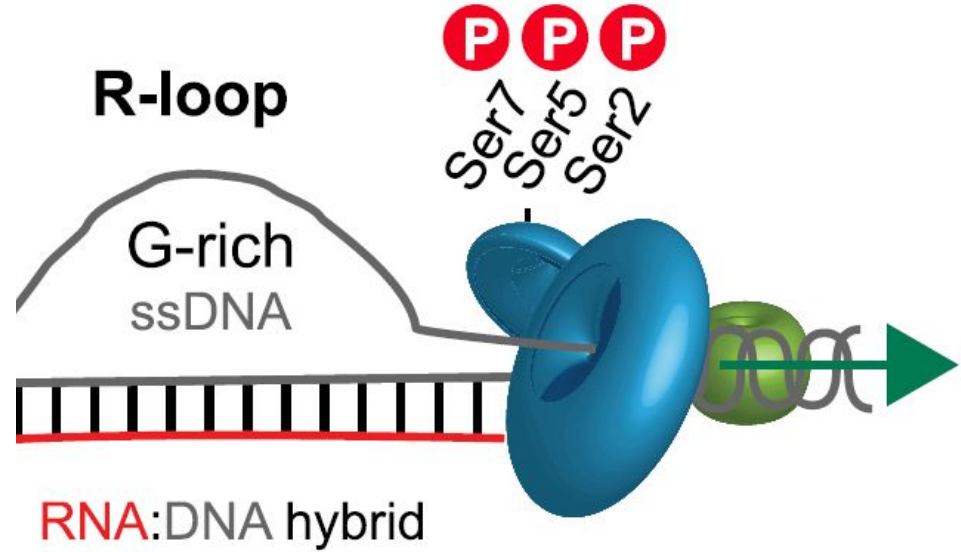
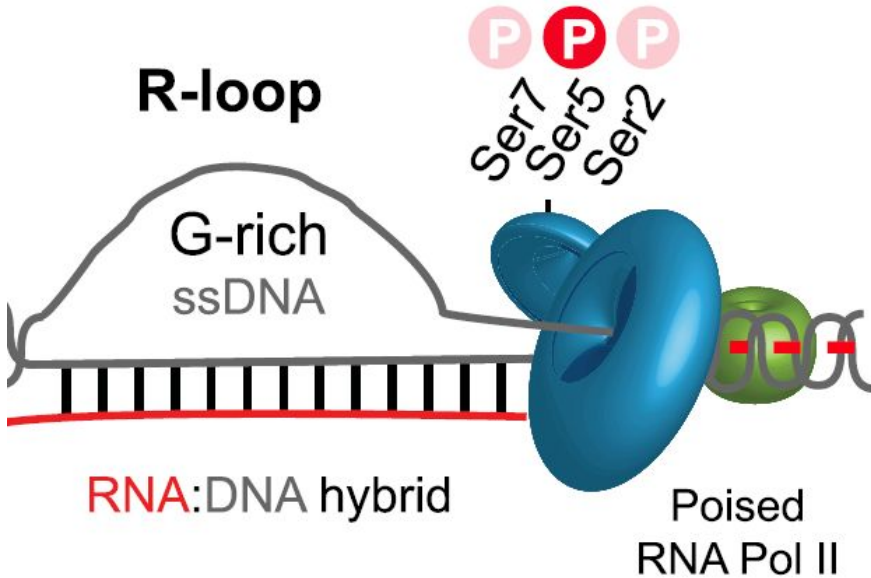
# The effect of R-loop (loss) on RNA Pol II activation



Basic concepts

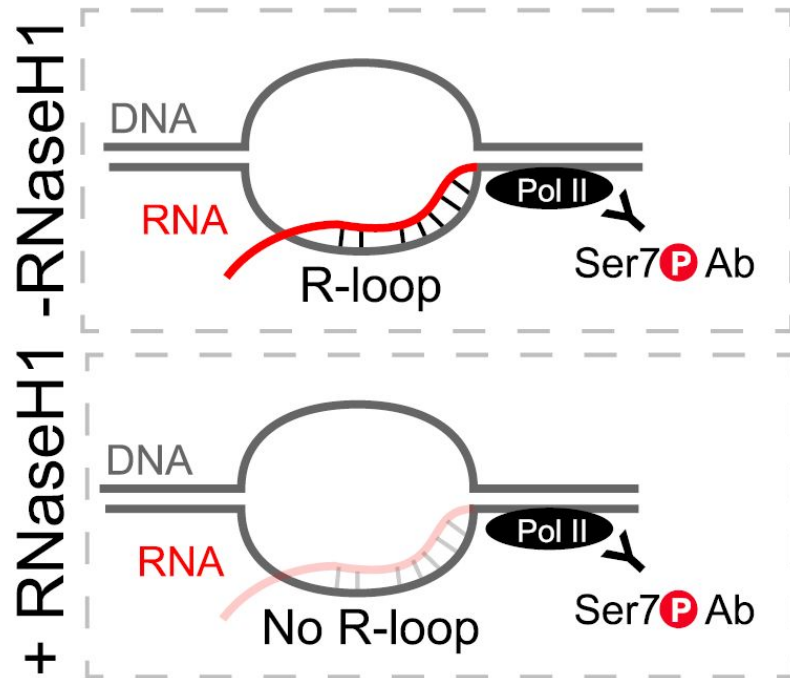
PcG repressed genes

Active genes



## Goal

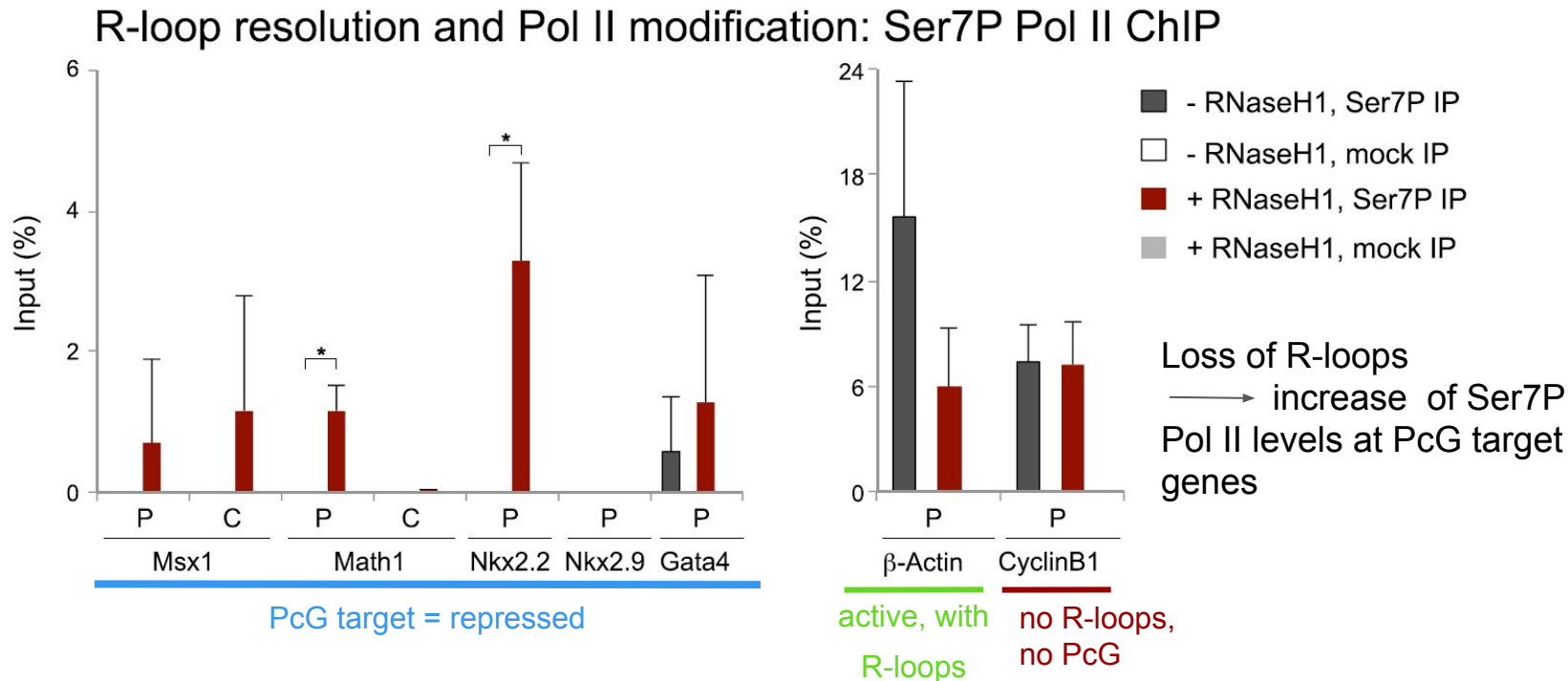
Determine whether the transcriptional activation of PcG target genes observed on R-loop removal is linked to changes in CTD modification.



ChIP with Ser7P Ab

Remember: Ser7P = active genes!

PcG target genes exhibit an increase in Ser7P Pol II levels after R-loops resolution



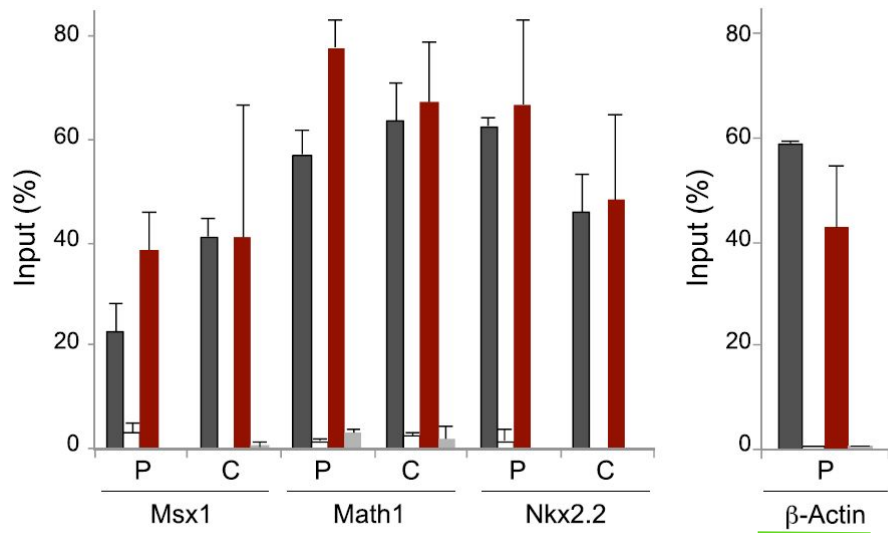
Removal of R-loops at PcG target genes leads to a **specific** change in Pol II CTD modification



# R-loop depletion has NO detectable effect on Ser5P occupancy levels over PcG target genes



## R-loop resolution and Pol II modification: Ser5P Pol II ChIP



Ser5P is not affected by decreased occupancy of PcG enzymes upon R-loop depletion



Ser5P precedes R-loop formation



# The effect of R-loop (loss) on RNA Pol II activation

Ser5P is NOT affected by the R-loops resolution



R-loops removal leads to an increase of Ser7P



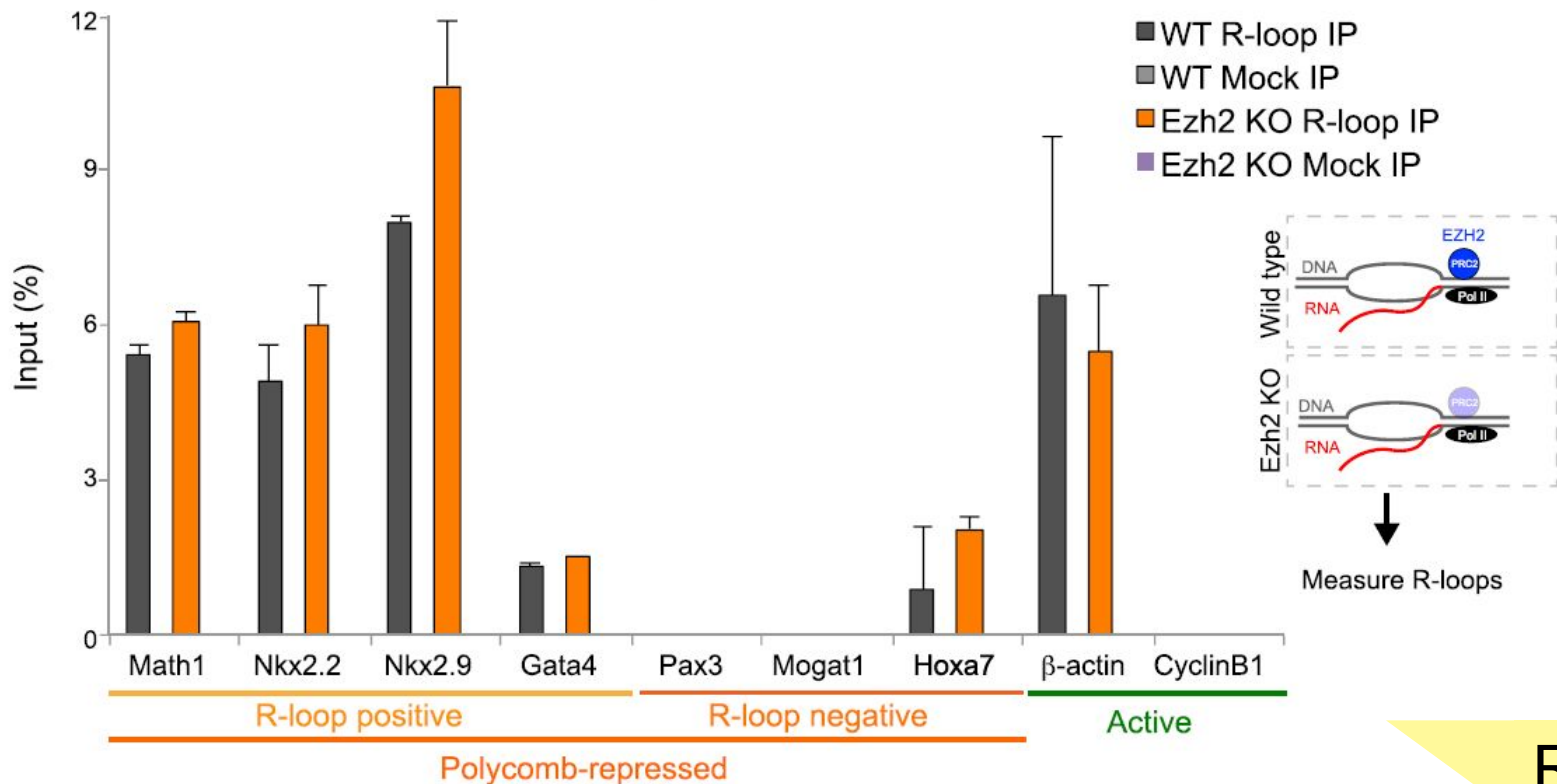
R-loops contribute to the transcriptional repression of PcG target genes via changes that affect not only PcG stability on chromatin but also Pol II activation

What is the role of PcG on R-loop formation and gene repression?

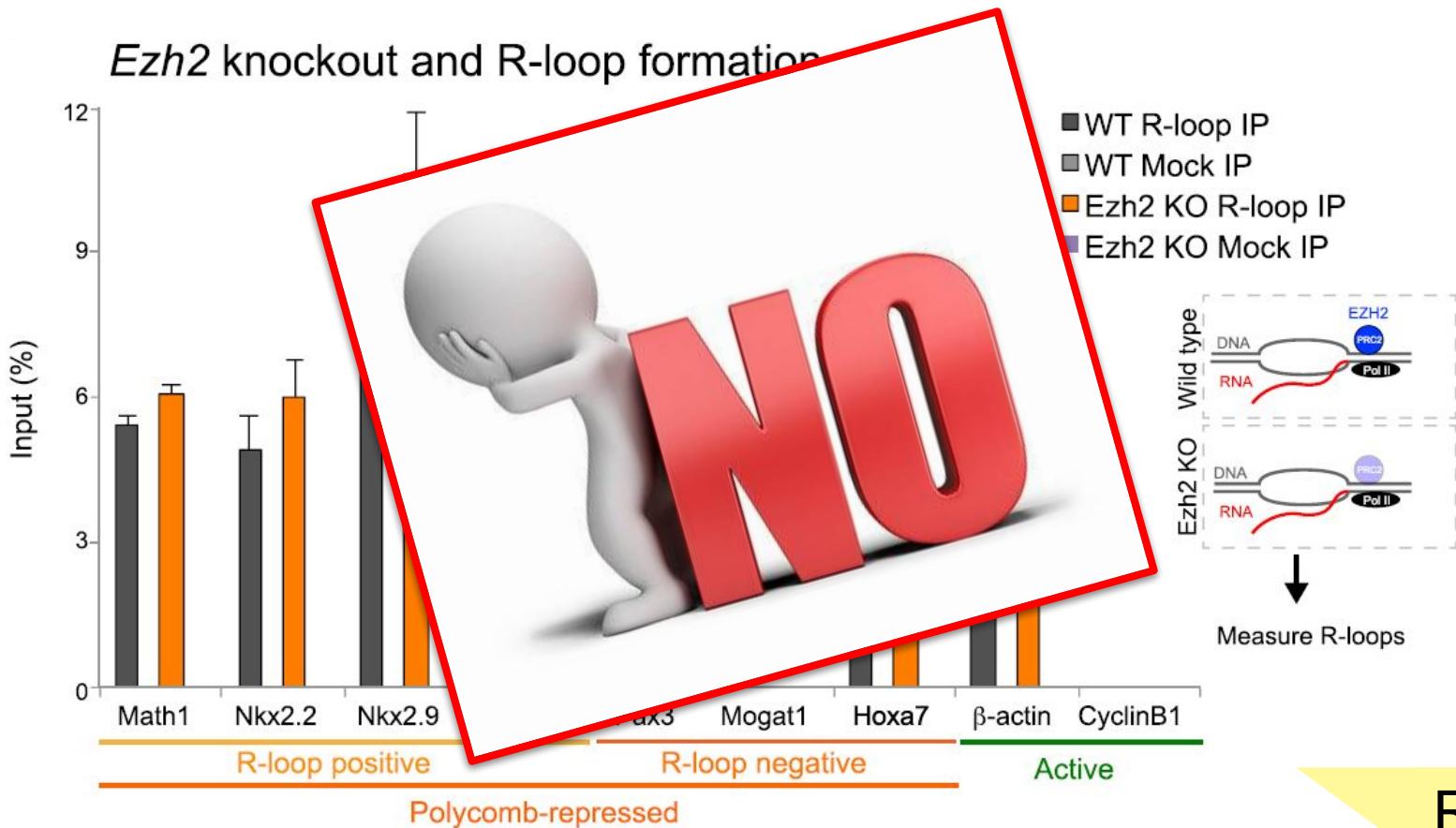


## Is R-loops formation affected upon PRC2 and H3K27me3 loss?

### *Ezh2* knockout and R-loop formation



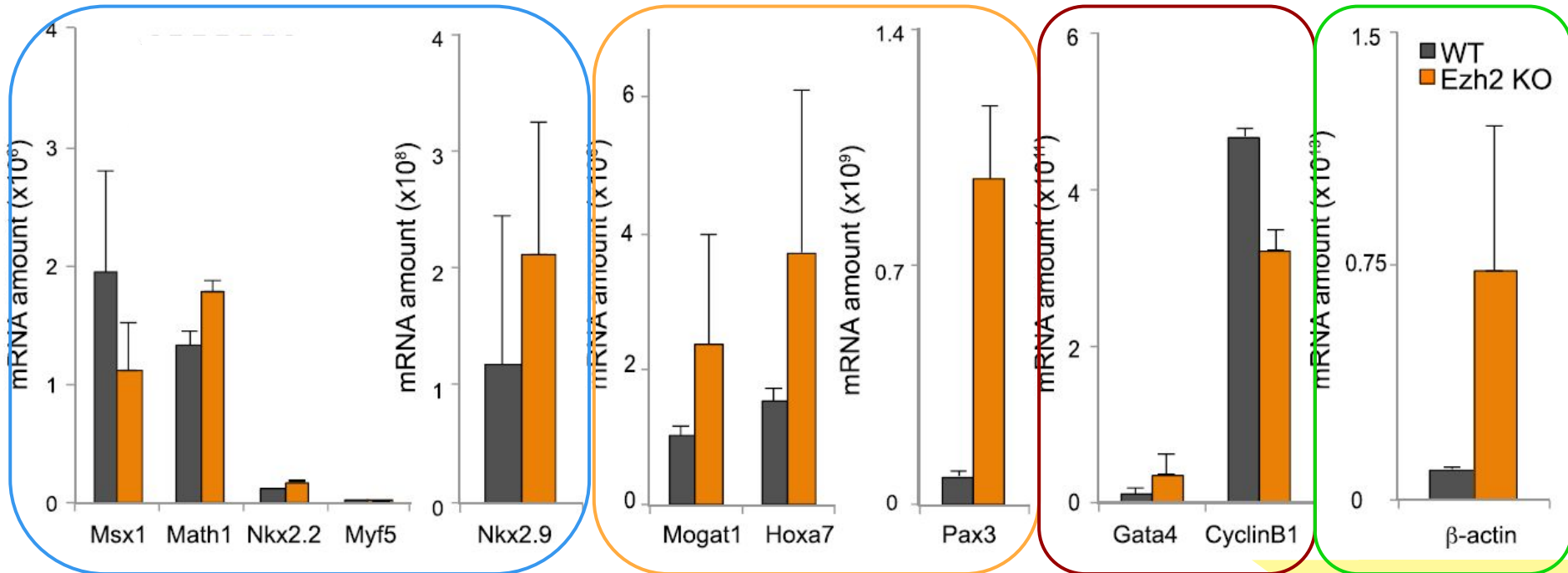
## Is R-loops formation affected upon PRC2 and H3K27me3 loss?



What is the role of PcG presence on R-loop formation and gene repression at PcG-repressed genes?

## Does EZH2 KO cause transcriptional derepression of R-loop (+) PcG-repressed genes?

### *Ezh2* knockout and spliced transcripts



Results

What is the role of PcG presence on R-loop formation and gene repression at PcG-repressed genes?

## Does EZH2 KO cause transcriptional derepression of R-loop (+) PcG-repressed genes?

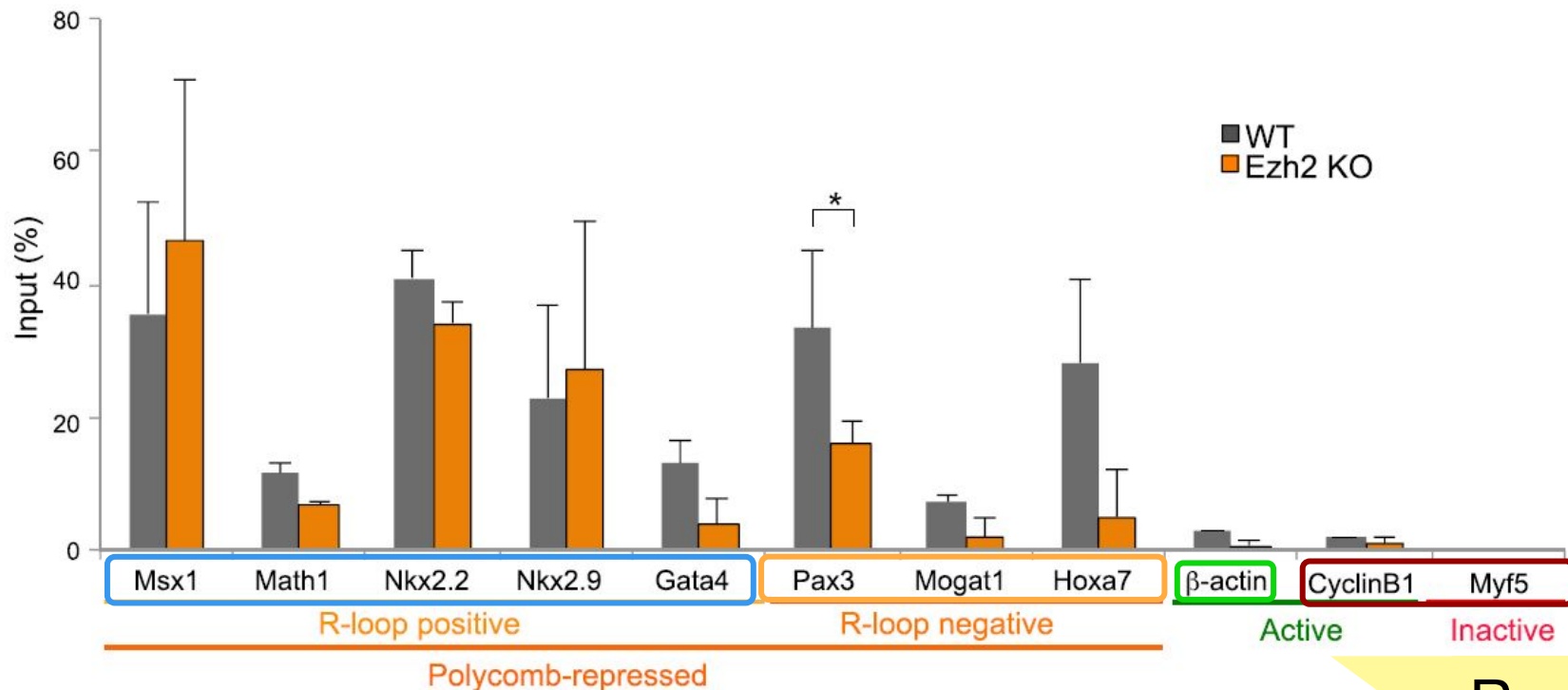
*Ezh2* knockout and spliced transcripts



Results

## Is PRC1 recruitment affected upon PRC2 KO?

### *Ezh2* knockout and PRC1: RING1B ChIP



## Is PRC1 recruitment affected upon PRC2 KO?





# What is the role of PcG on R-loop formation and gene repression?

- EZH2 KO does **NOT** affect R-loop formation
- EZH2 KO does **NOT** affect derepression of R-loop(+) PcG genes
- EZH2 KO does **NOT** affect RING1B recruitment on R-loop(+) PcG genes



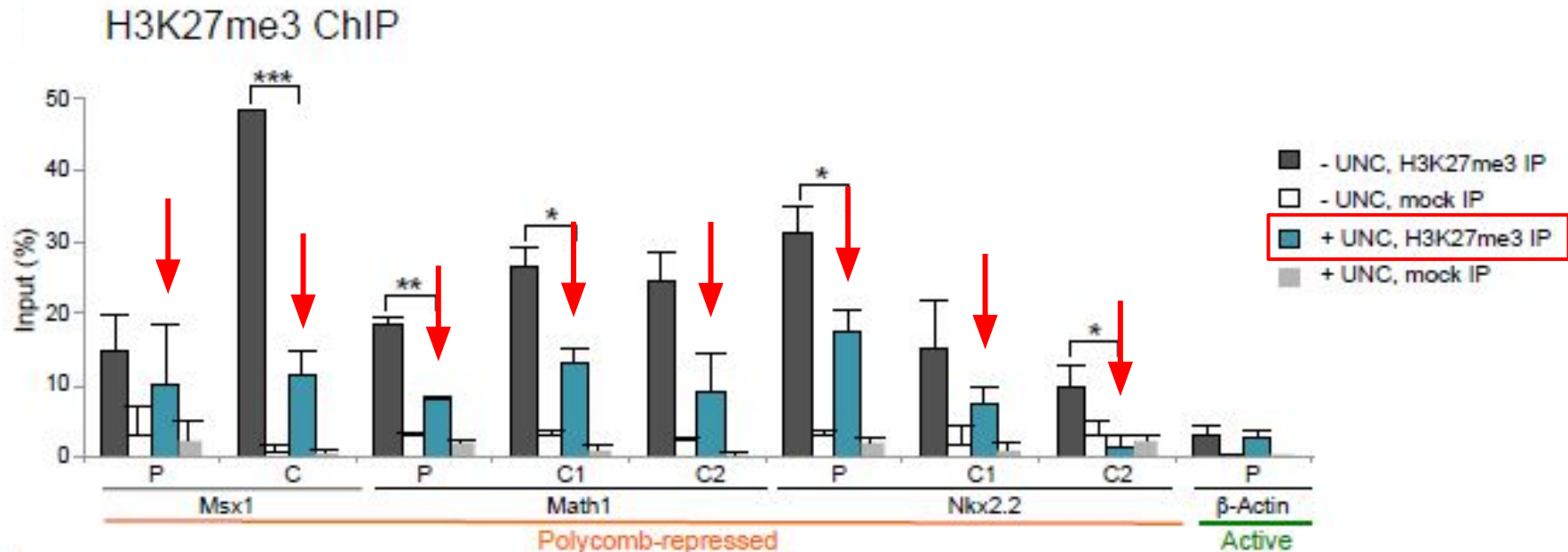
Presence of R-loops and RING1B explains the lack of derepression on R loop(+) PcG genes.

What if we chemically interfere with both EZH1 and EZH2 methyltransferase activity?



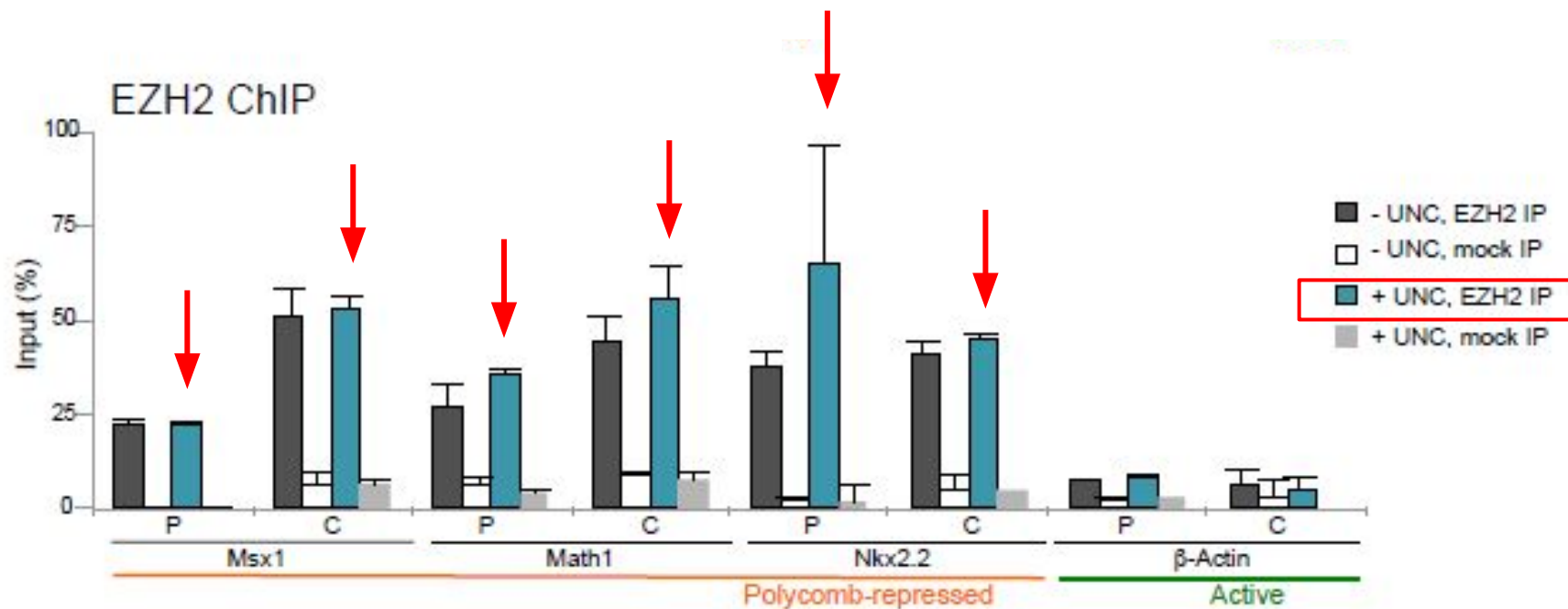
What if we chemically interact with both EZH1 and EZH2 methyltransferase activity?

## Levels of H3K27me3 upon EZH1-2 activity removal

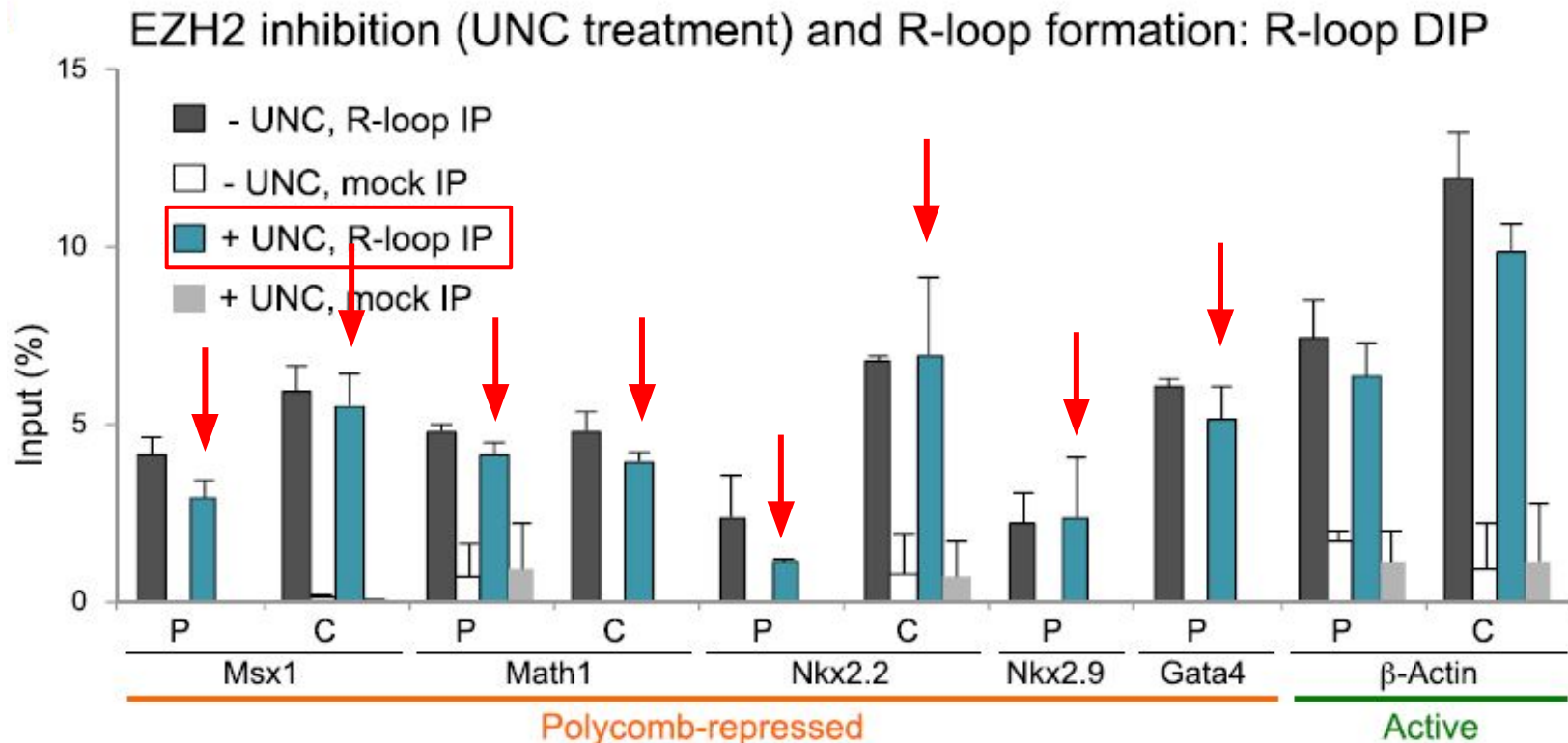


Results

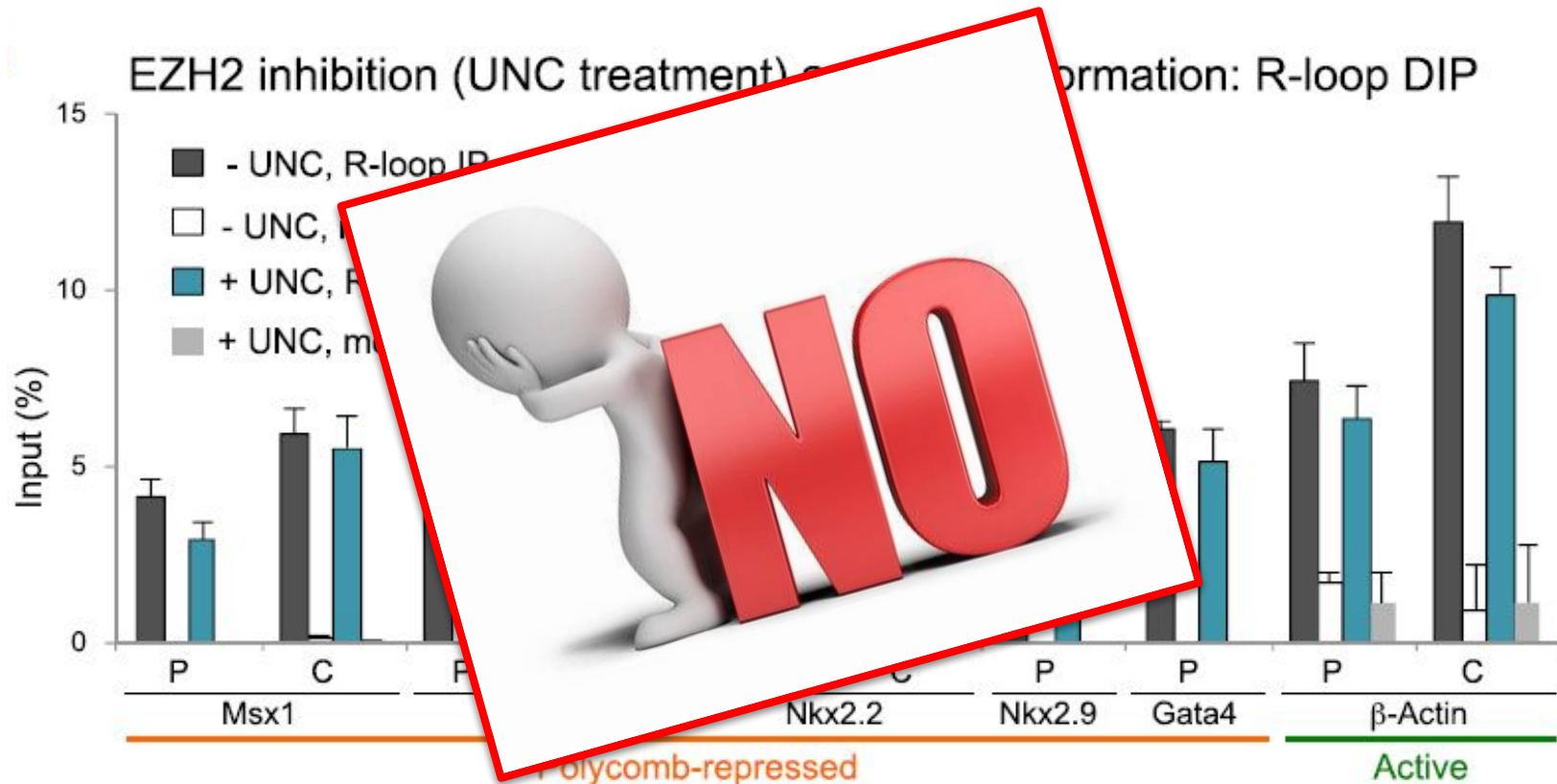
## Levels of EZH2 binding upon EZH1-2 activity removal



## Are R-loop levels altered upon EZH1-2 activity removal?

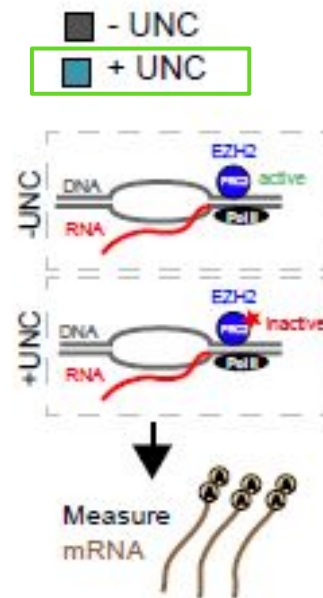
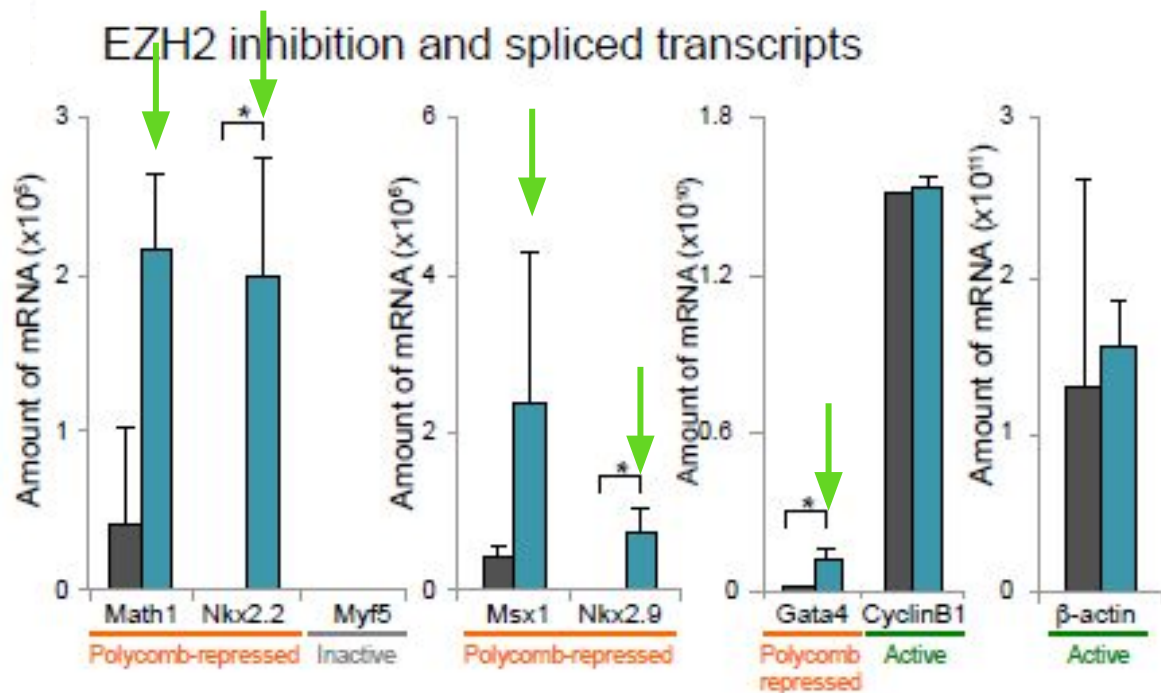


## Are R-loop levels altered upon EZH1-2 activity removal?



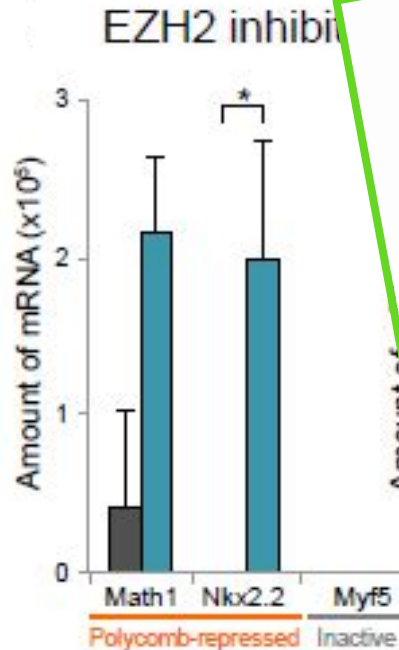
What if we chemically interact with both EZH1 and EZH2 methyltransferase activity?

## Are mRNA levels altered upon EZH1-2 activity removal?



Results

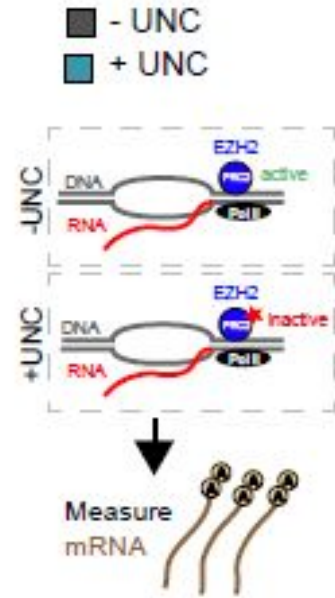
## Are mRNA levels altered upon EZH1-2 activity removal?



Amount of

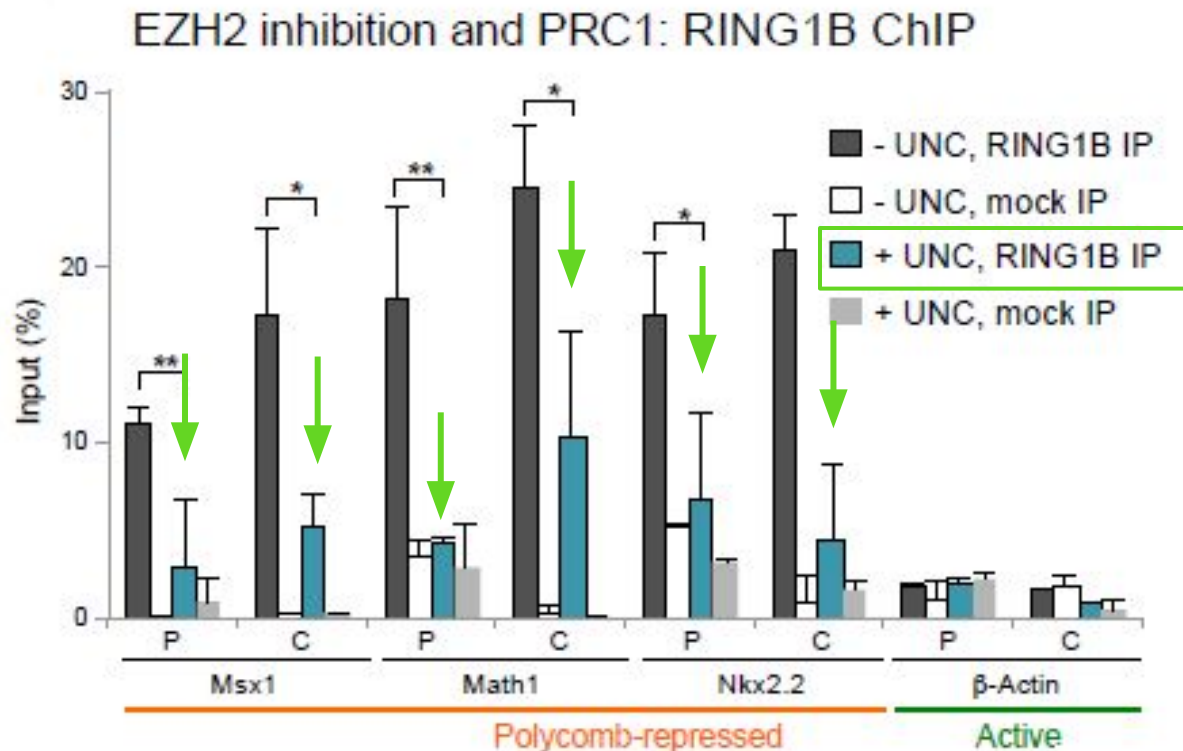


Active





## Is RING1B altered upon EZH1-2 activity removal?



What if we chemically interact with both EZH1 and EZH2 methyltransferase activity?

## Is RING1B altered upon EZH1-2 activity removal?



Results

# What if we chemically interfere with both EZH1 and EZH2 methyltransferase activity?

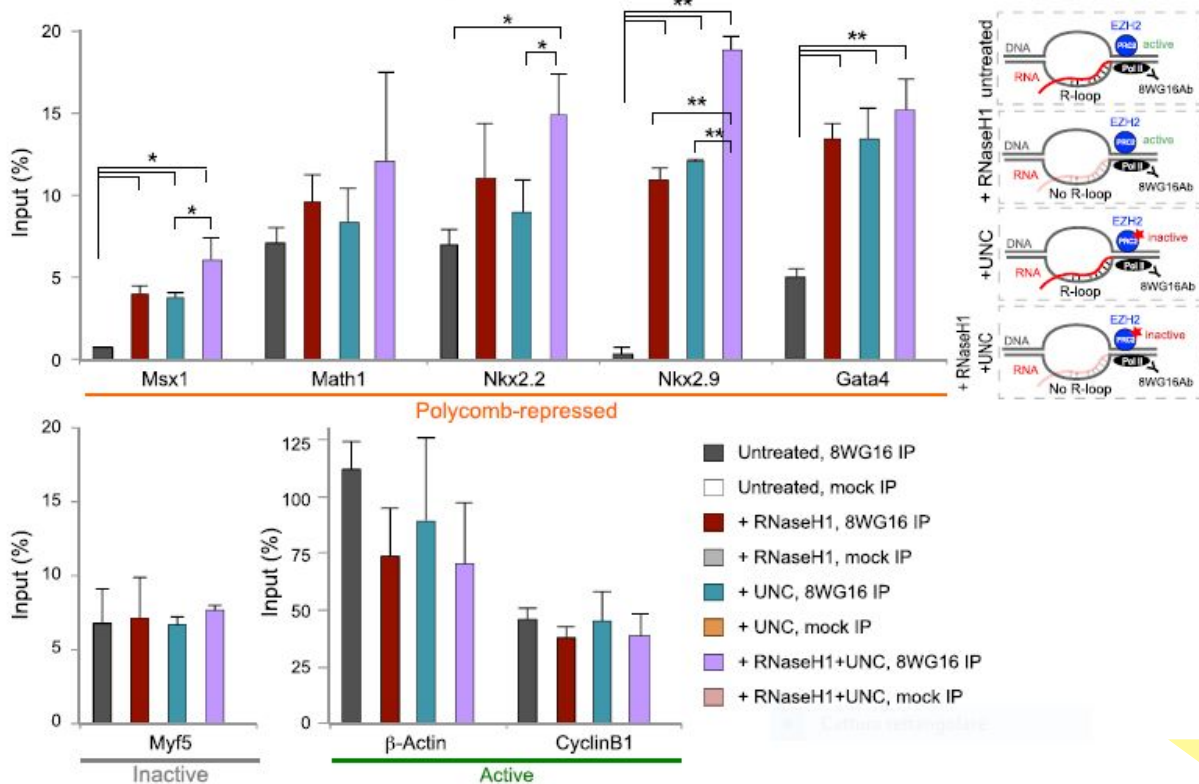
- **NO** loss of R-loops: R-loops form before EZH1-2 activity or presence
- R-loop(+) genes **derepression**
- **Reduced** recruitment of PRC1 on R-loop(+) genes

Do R-loops and EZH2 activity act through parallel pathways or do they have synergistic effects?



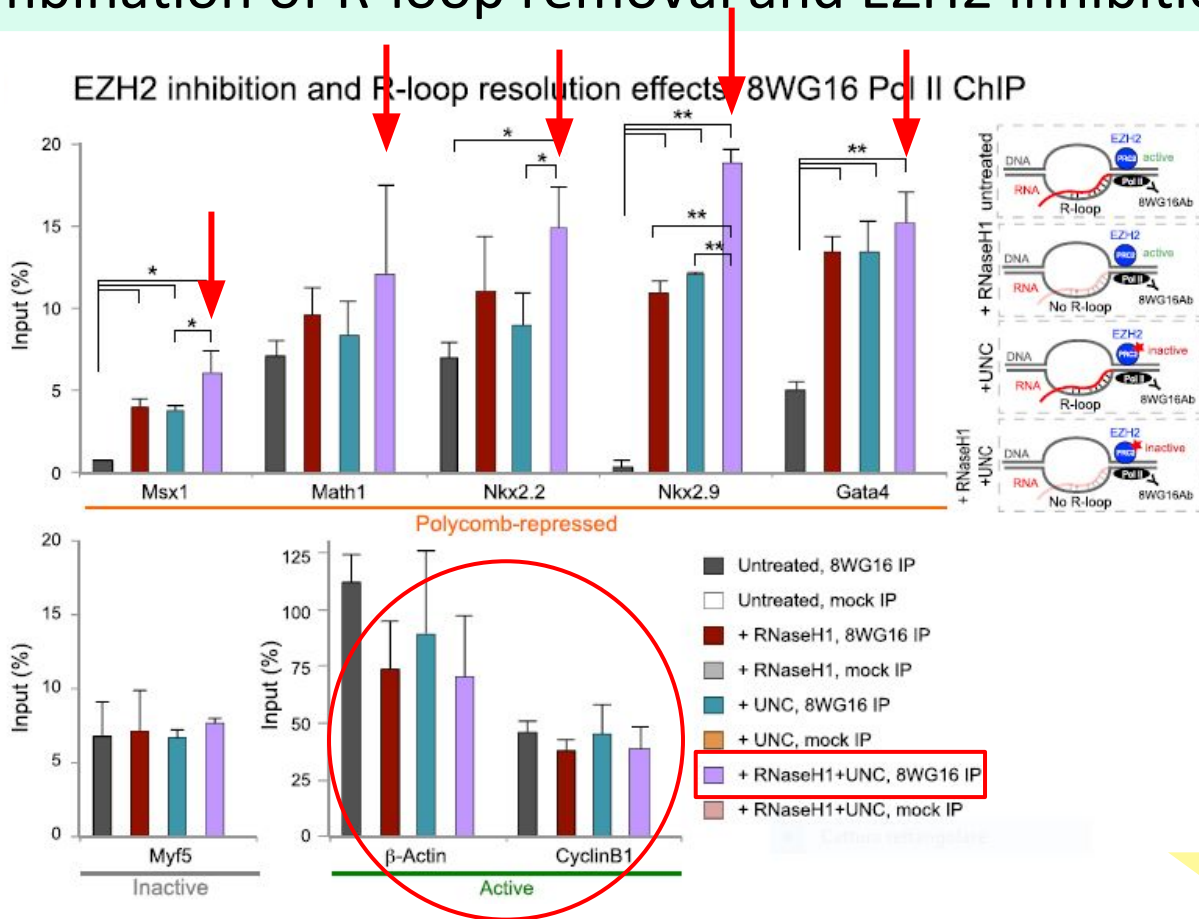
# Combination of R-loop removal and EZH2 inhibition

EZH2 inhibition and R-loop resolution effects: 8WG16 Pol II ChIP



Do R-loops and EZH2 activity act through parallel pathways or do they have synergistic effects?

## Combination of R-loop removal and EZH2 inhibition



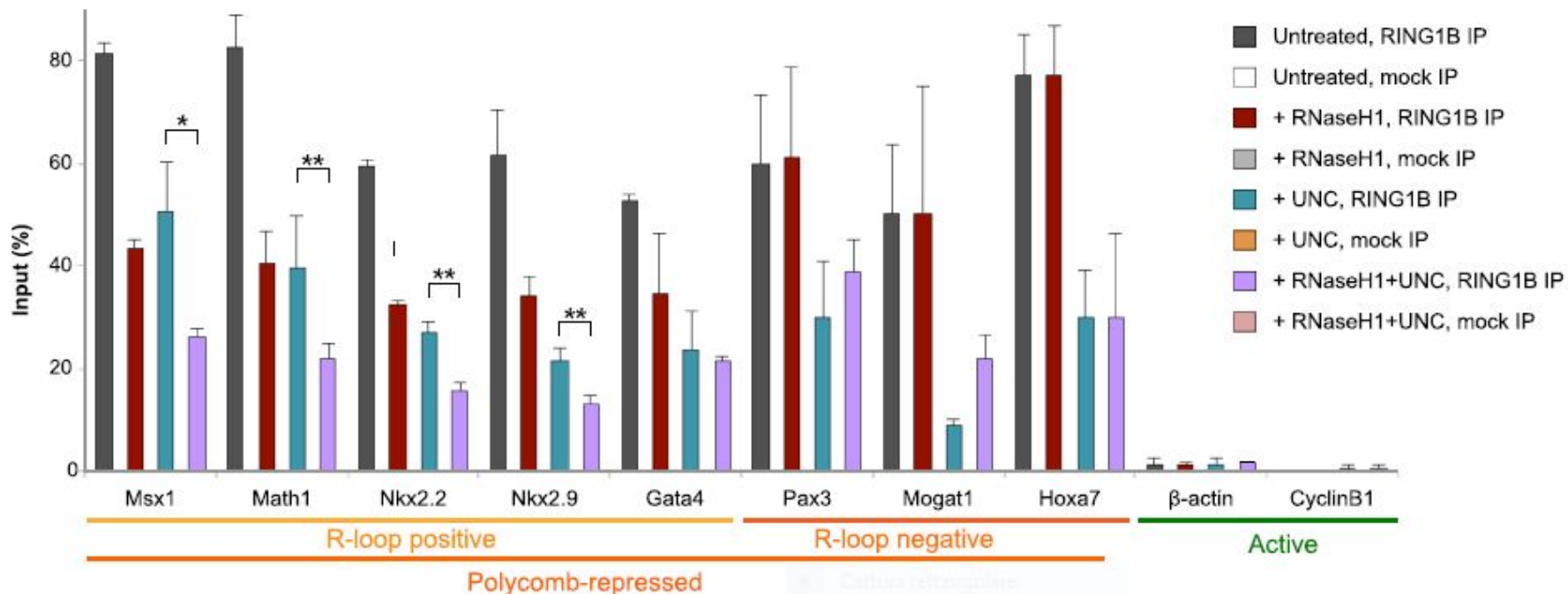
Results

Do R-loops and EZH2 activity act through parallel pathways or do they have synergistic effects?

- **Both** R-loop and EZH2 catalytic activity contribute to PcG repression

## What is the role of the reduced recruitment of RING1B?

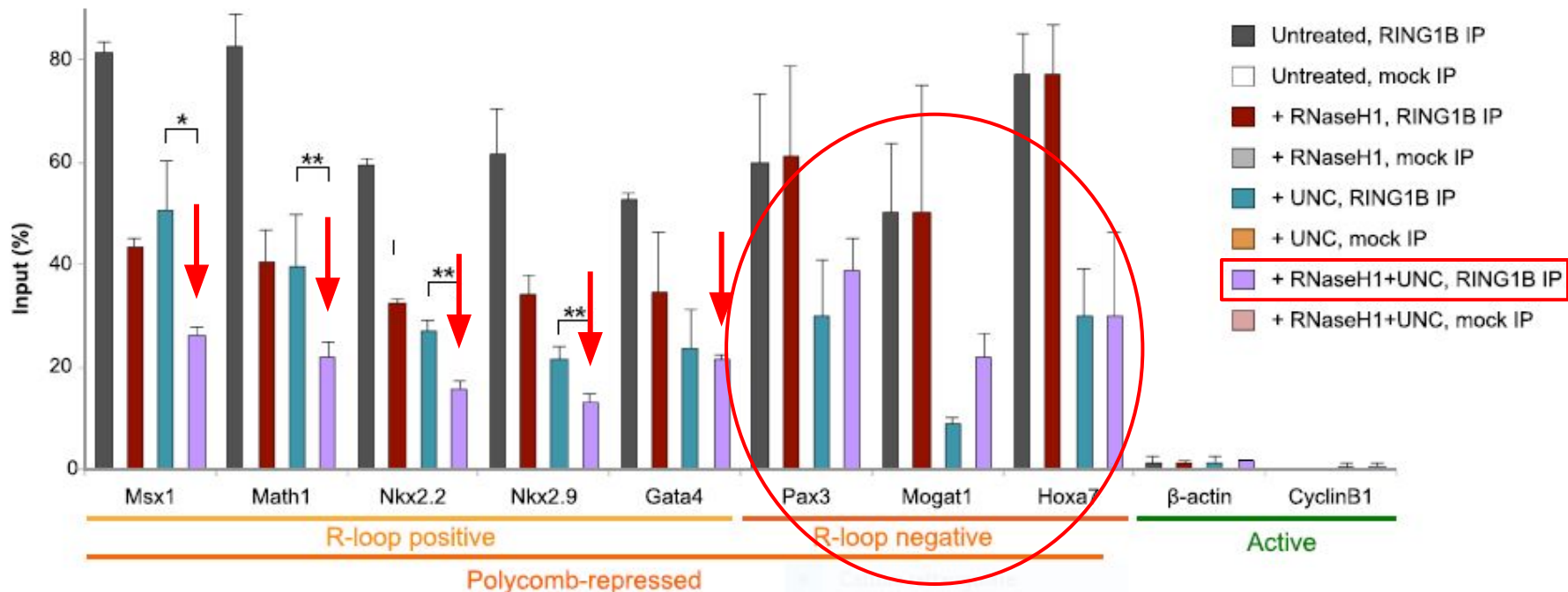
### EZH2 inhibition and R-loop resolution effects: RING1B ChIP





## What is the role of the reduced recruitment of RING1B?

### EZH2 inhibition and R-loop resolution effects: RING1B ChIP



# Do R-loops and EZH2 activity act through parallel pathways or do they have synergistic effects?

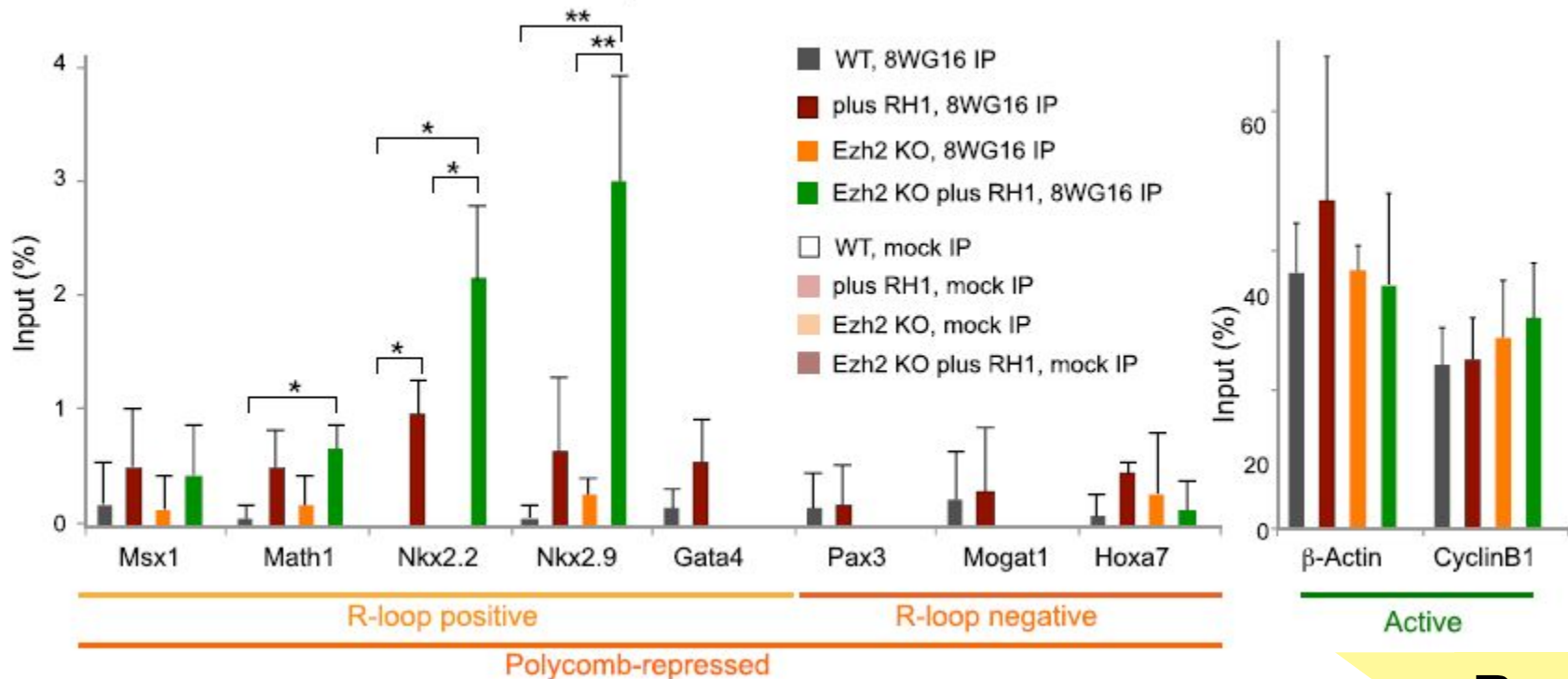
- **Both** R-loop and EZH2 catalytic activity contribute to PcG repression
- **Both** R-loop and RING1B recruitment on chromatin are important to repress R-loops(+) PcG targets genes
- RING1B recruitment can be regulated by R-loop formation in absence of PRC2 activity

Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?



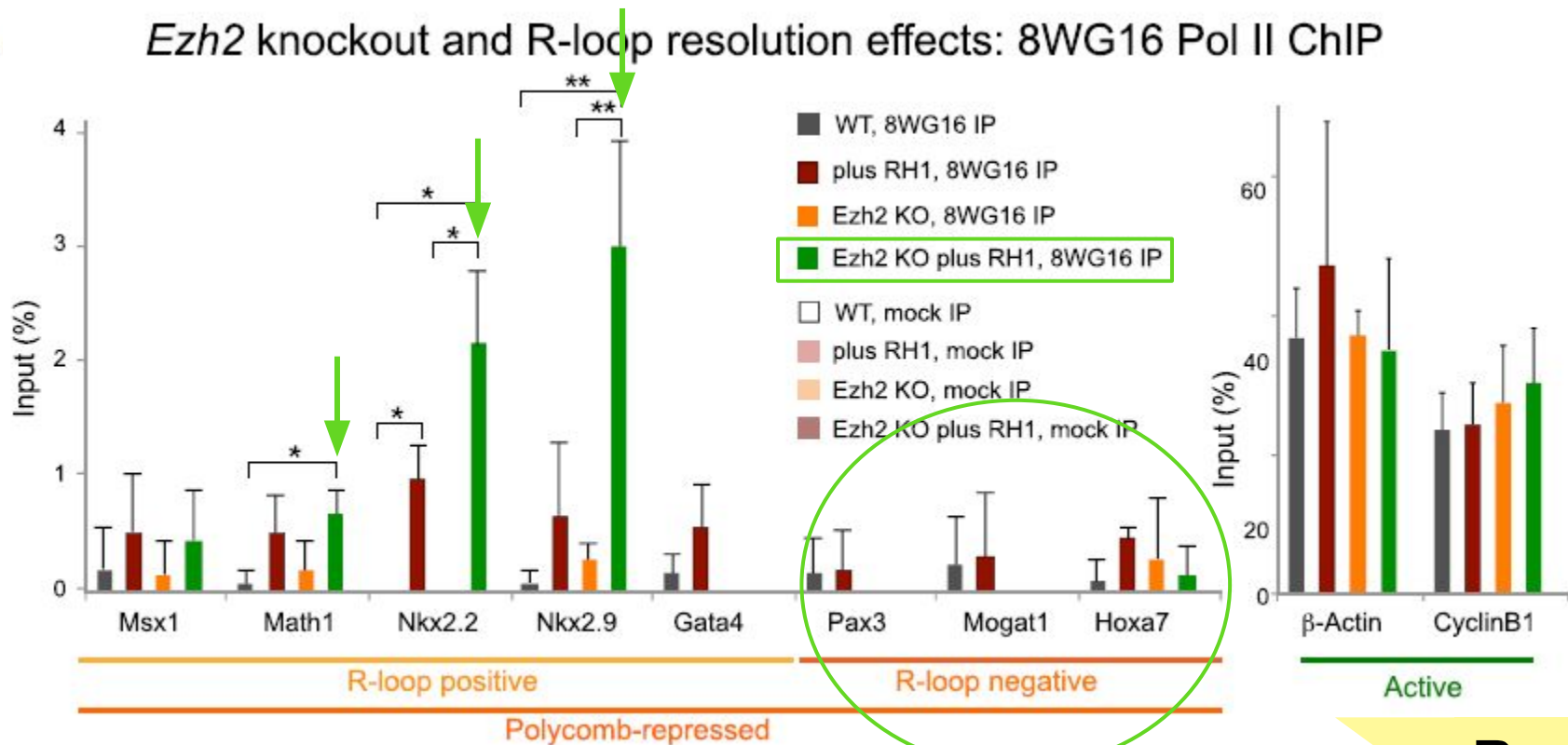
## Can R-loops removal induce activation in EZH2 absence?

*Ezh2* knockout and R-loop resolution effects: 8WG16 Pol II ChIP



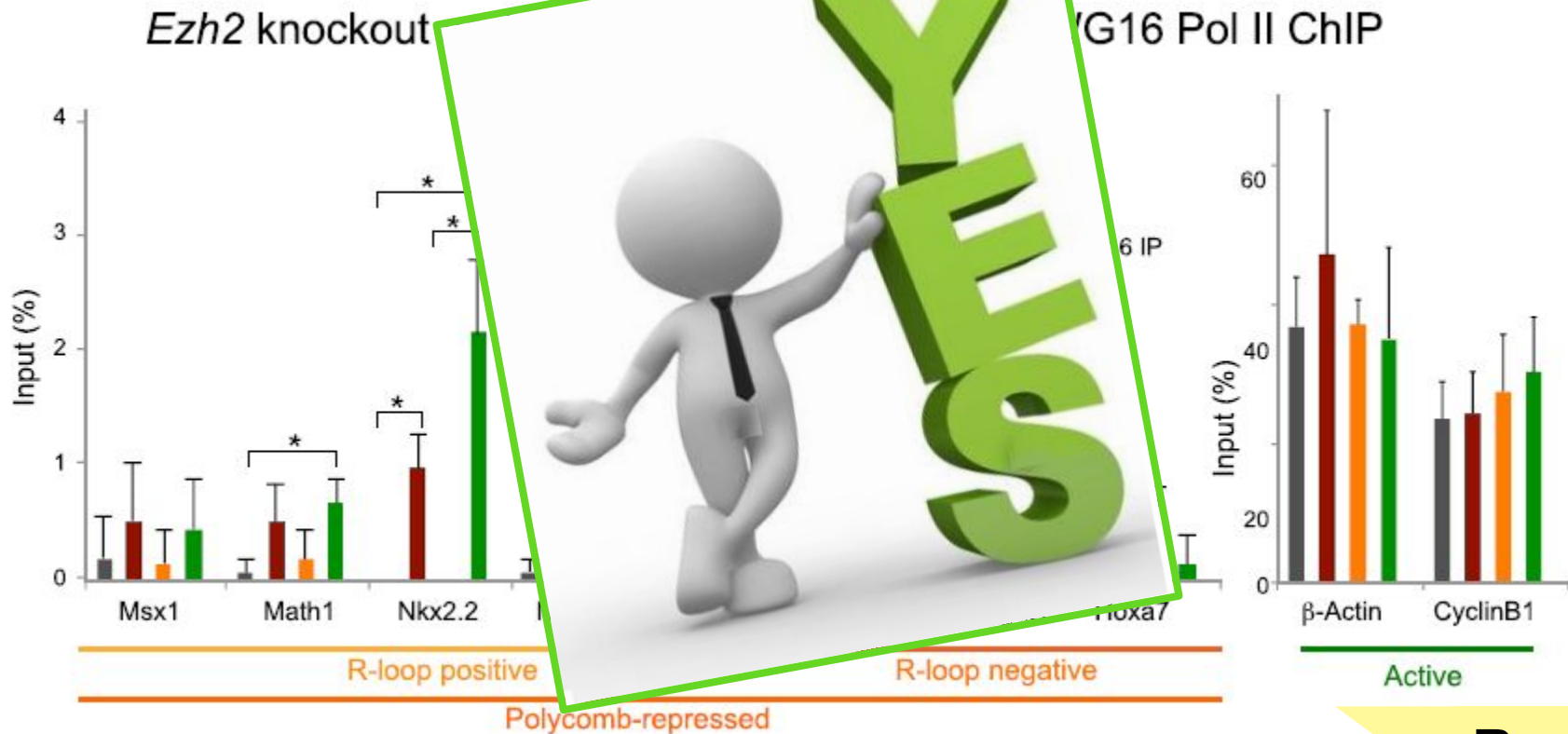
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## Can R-loops removal induce activation in EZH2 absence?



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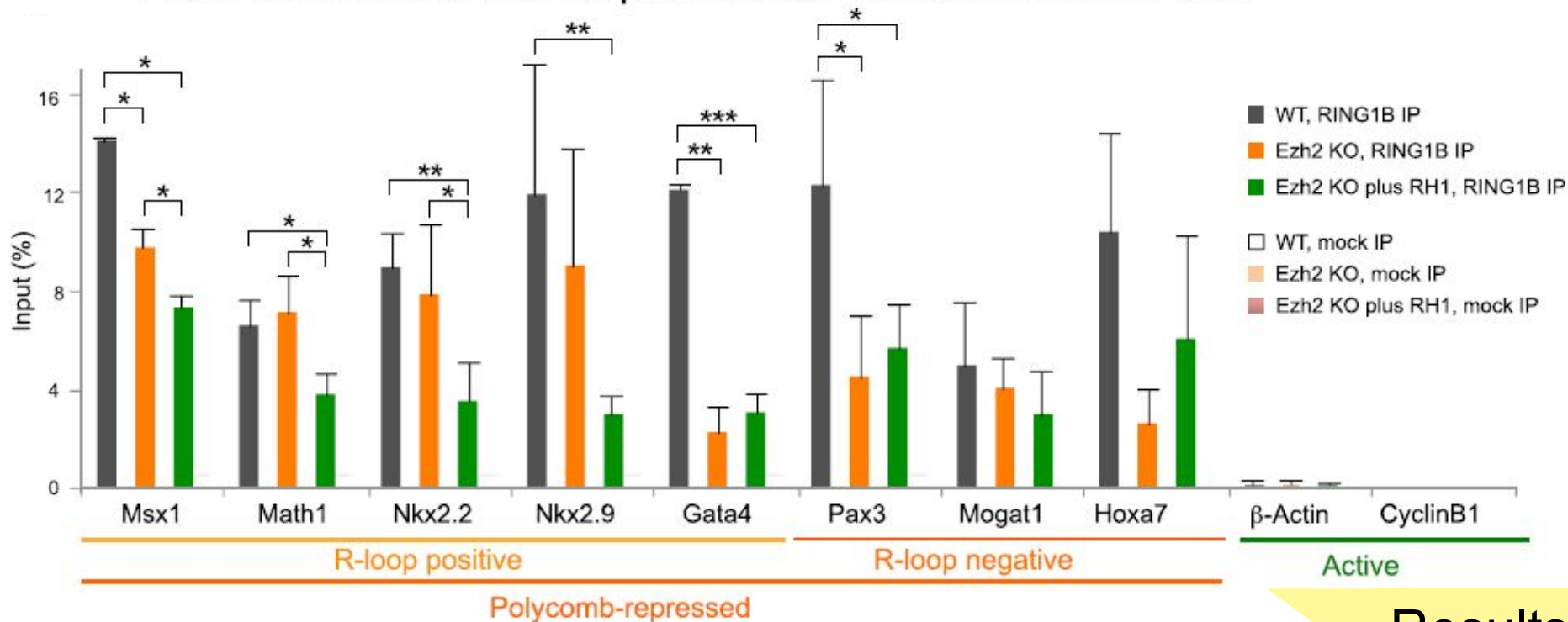
# Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?

- R-loops can act as transcriptional repressors in PcG system **independently** of EZH2 occupancy on chromatin

Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?

Is transcriptional change in EZH2 KO R-loops - due to RING changes?

### *Ezh2* knockout and R-loop resolution effects: RING1B ChIP



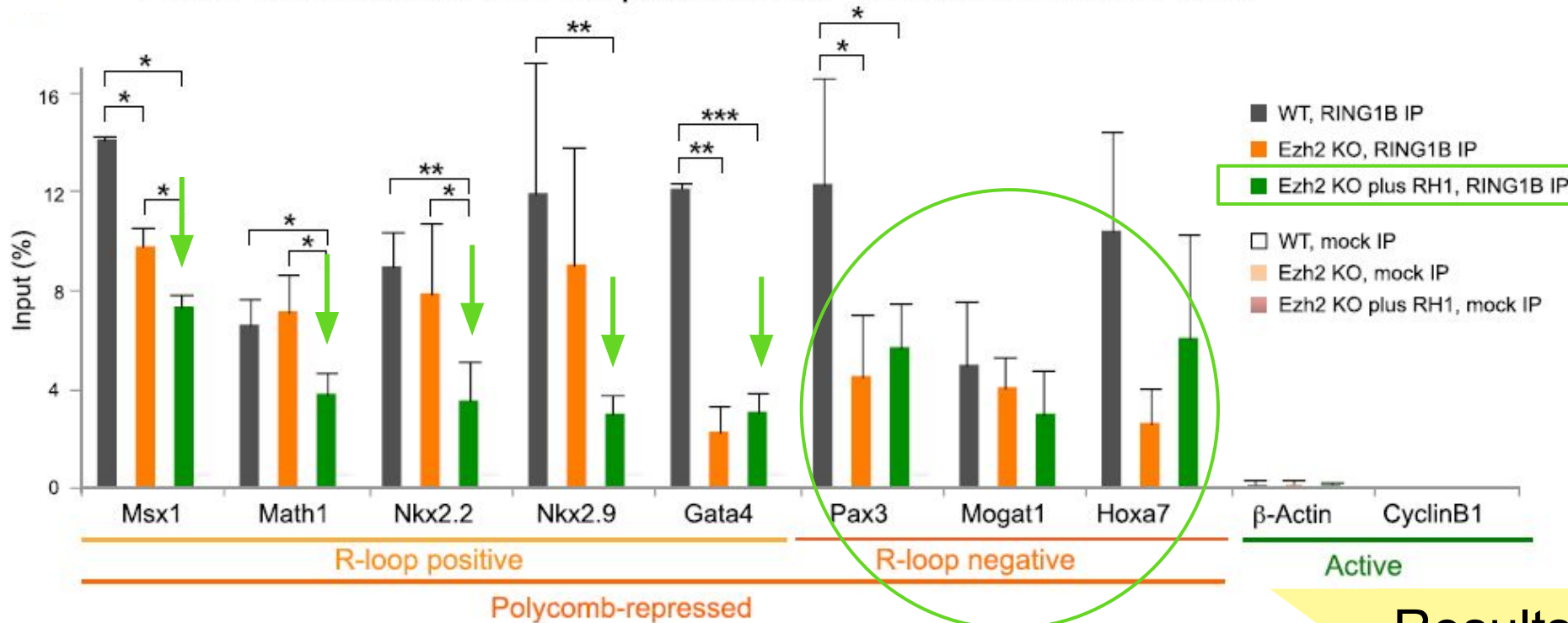
Results



Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?

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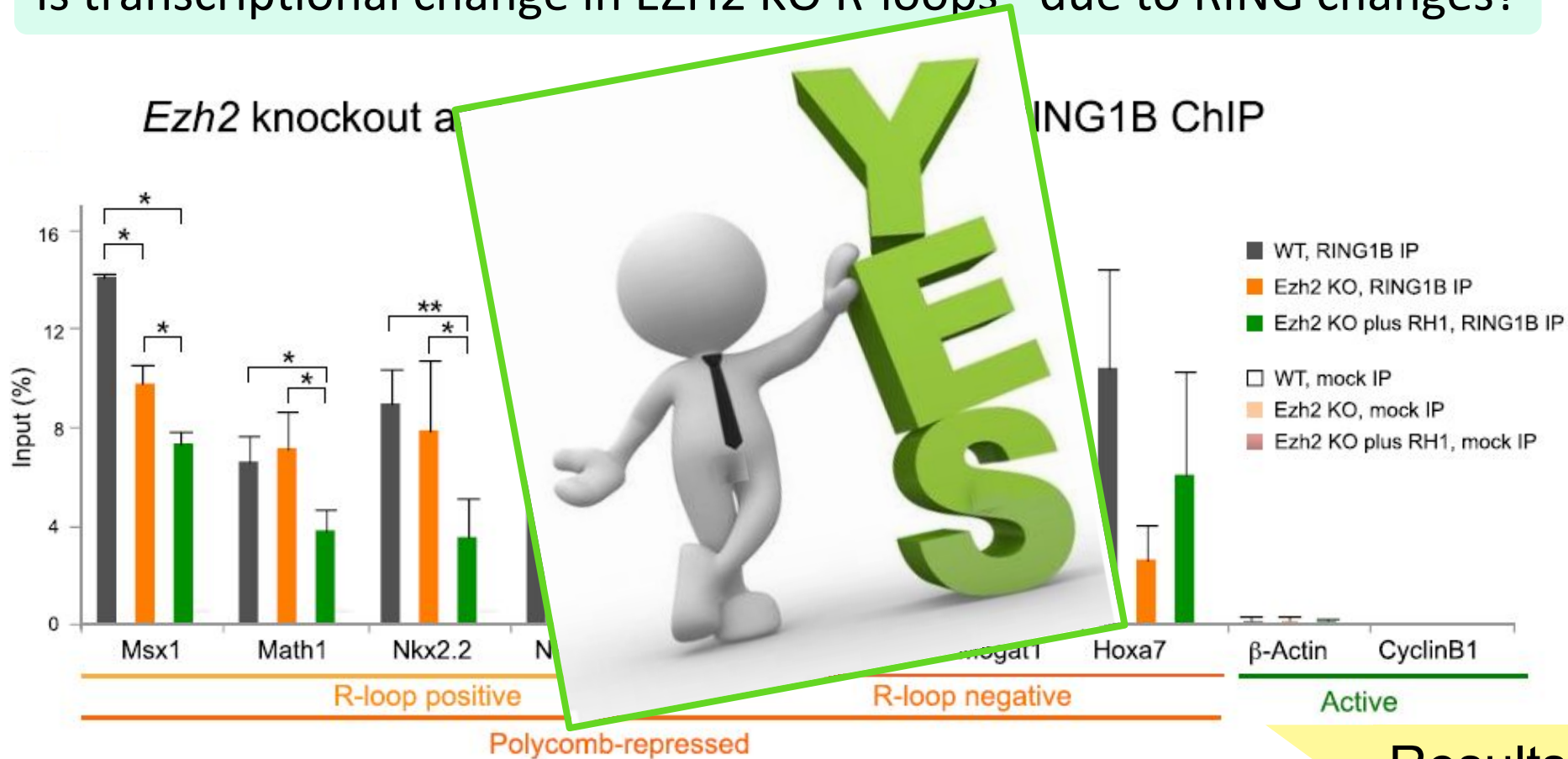
### *Ezh2* knockout and R-loop resolution effects: RING1B ChIP



Results

Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?

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Results

# Is the EZH2 occupancy on chromatin important for gene repression in R-loop forming PcG target genes?

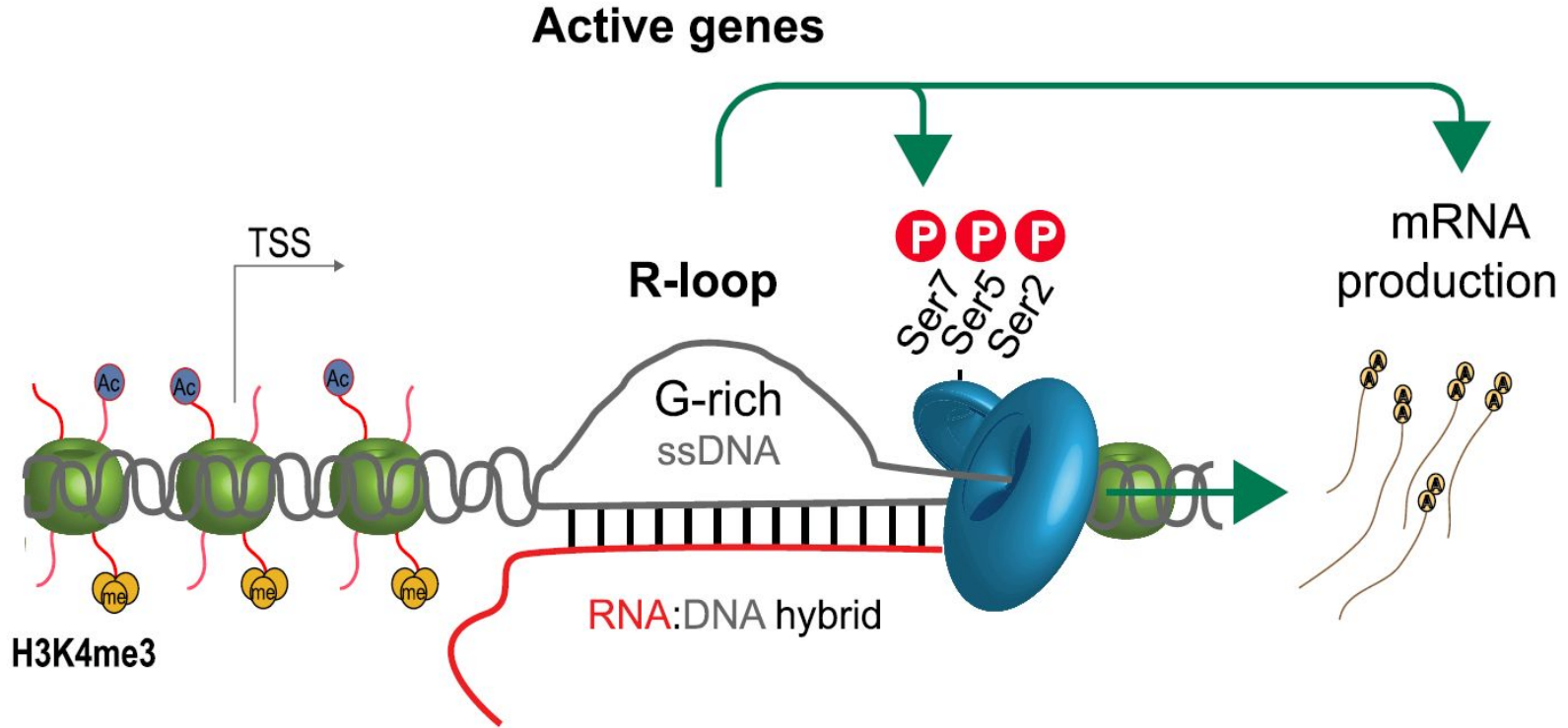
- R-loops can act as transcriptional repressors in PcG system independently of EZH2
- R-loops are important for RING1B recruitment specifically at the subset of PcG-repressed genes that form R-loops

# Let's sum it up

## REVIEW

- R-loops are important for both active and repressed genes
- In active genes: R-loops help the recruitment of chromatin modifying enzymes

# Let's sum it up



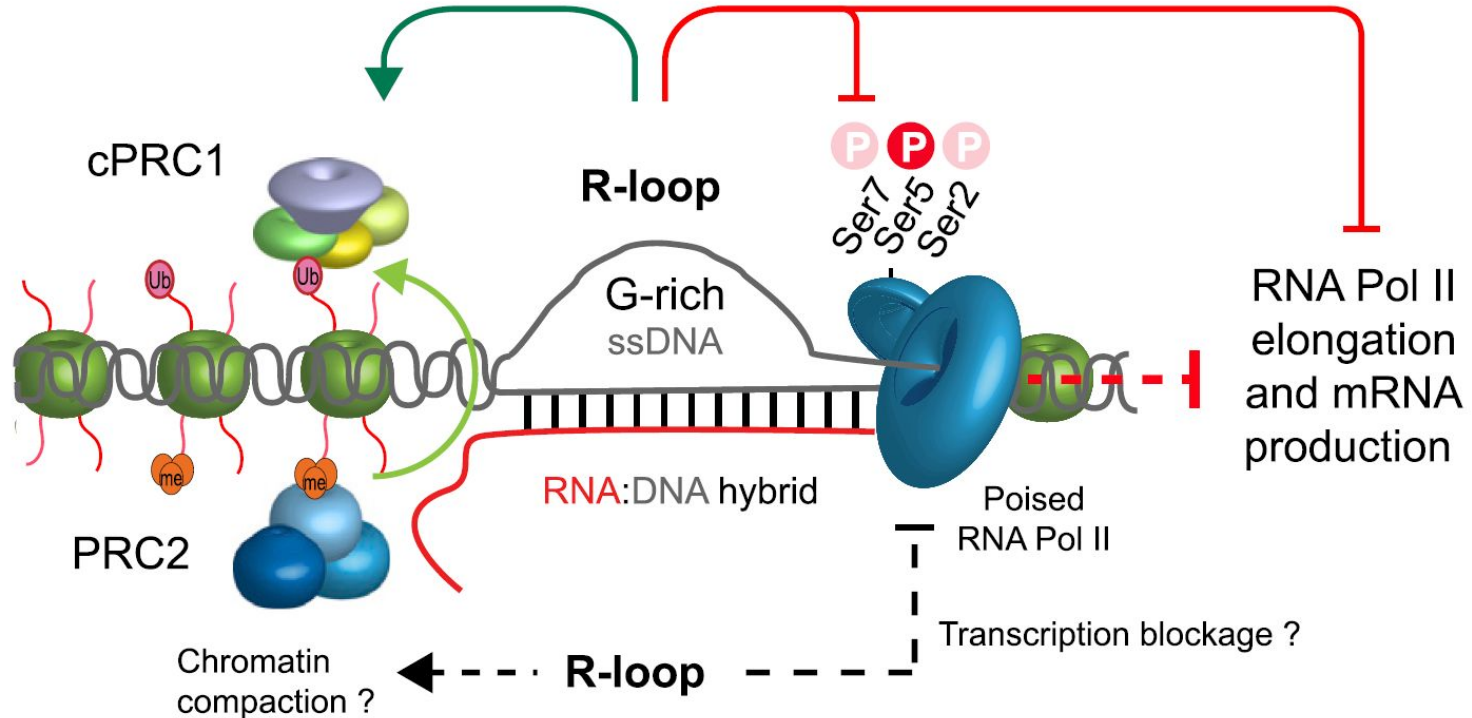
# Let's sum it up

## ARTICLE

- In mESC, R-loops correlate with PcG repressed genes
- R-loops collaborate with RING1B for the repression of the PcG repressed genes
- R-loops form independently of EZH1-2, but together they contribute to PcG repression
- R-loops can act as transcriptional repressors in PcG target genes independently of EZH2 occupancy on chr

# Let's sum it up

## Polycomb repressed genes: R-loop positive



**THANK YOU  
FOR YOUR  
ATTENTION!**

**Me and my friend after our  
powerpoint presentation**



**The teacher and the rest of the class**





# REFERENCES

Images without references on the slides are either made by the presenter, or are from the review and article studied.

## REVIEW:

Chédin, Frédéric. 2016. « Nascent Connections: R-Loops and Chromatin Patterning ». *Trends in Genetics* 32 (12): 828-38. <https://doi.org/10.1016/j.tig.2016.10.002>.

## ARTICLE:

Skourti-Stathaki, Konstantina, Elena Torlai Triglia, Marie Warburton, Philipp Voigt, Adrian Bird, et Ana Pombo. 2019. « R-Loops Enhance Polycomb Repression at a Subset of Developmental Regulator Genes ». *Molecular Cell* 73 (5): 930-945.e4. <https://doi.org/10.1016/j.molcel.2018.12.016>.