UNIVERSITÀ DEGLI STUDI DI TRIESTE

DIPARTIMENTO DI SCIENZE DELLA VITA

Nascent RNA antagonizes the interaction of a set of regulatory proteins with chromatin

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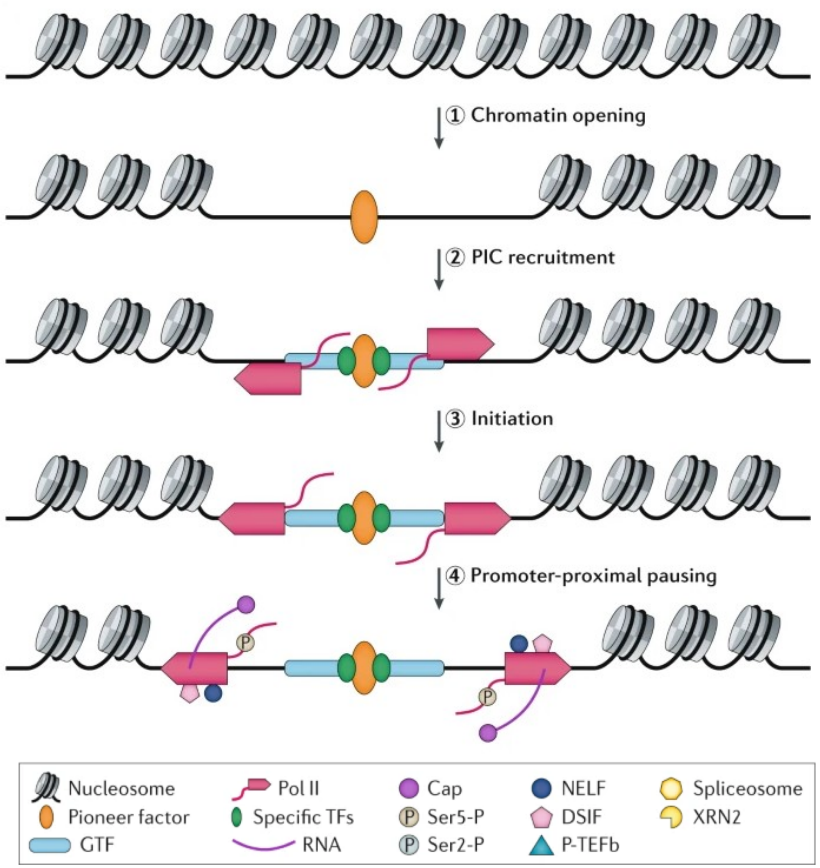
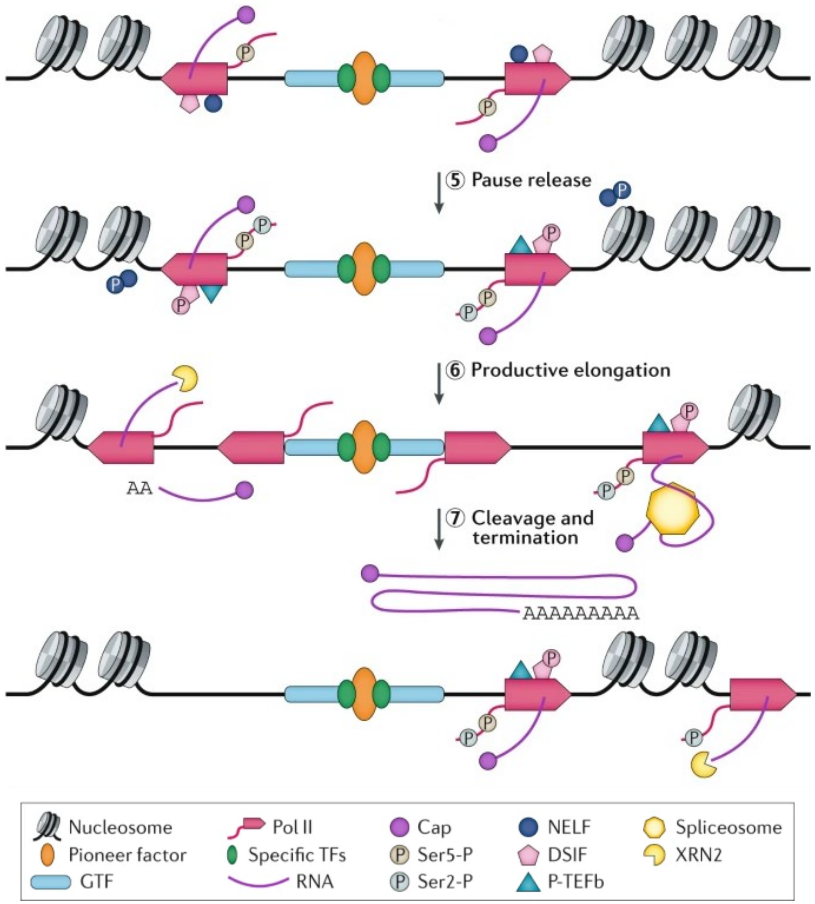
The transcription cycle

Transcription is the process by which RNA polymerases synthesize RNA molecules from a DNA template

1. A pioneer TF binds to a specific sequence motif and increases chromatin accessibility
2. Core promoters recruit additional TFs, such as general TFs (GTFs), and also RNA Pol II to form the pre-initiation complex (PIC)
3. A GTF unwinds DNA and Pol II initiates transcription
4. After transcribing 20-60 nucleotides, Pol II undergoes promoter-proximal pausing, that is stabilized by DSIF e NELF



The transcription cycle



5. Pol II escapes promoter-proximal pausing and enters productive elongation, largely thanks to P-TEFb

6. During productive elongation, multiple elongation factors (not shown) enhance the processivity of Pol. Co-transcriptional processes are going on including splicing, RNA methylation and RNA editing

7. The RNA is cleaved and polyadenylated; after termination, Pol II can initiate a new cycle

How is transcription regulated?

Promoters and enhancers
Proximal and distal regulatory elements

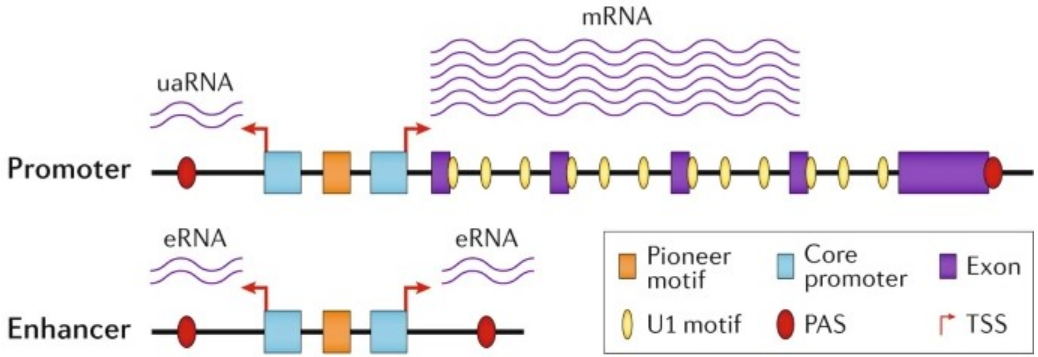
- Contain binding sites for specific transcription factors (TFs)
- Have very similar chromatin architecture
- Drive divergent transcription from their core initiation regions

C-terminal domain of Pol II
A signaling platform

- It consists of multiple repeats of a heptad consensus sequence
- It undergoes regulated post-translational modifications

RNA molecules

- Long non-coding RNA (lncRNA)
- TAR element: TAR binds the HIV transactivator protein TAT and they act together to release P-TEF-b from the inhibitory complex to activate HIV transcriptional elongation.
- RNA regulate chromatin state
- Nascent RNA antagonize the association of proteins with chromatin.
- miRNA, eRNA, lincRNA and others

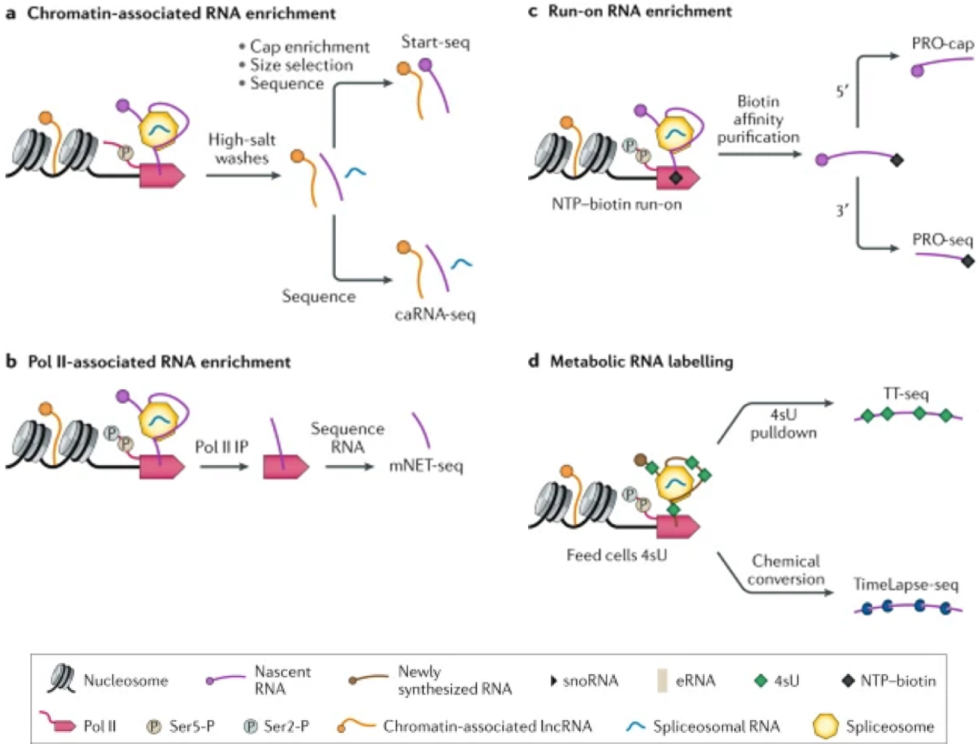


Nascent RNA analyses

Sequencing-based methods allow to perform genome-wide RNA analyses

- Investigation of RNA synthesis, detecting nascent or newly transcribed RNAs
- Density profiles of transcribing Pol II
- Transcription start sites (TSSs) or Pol II active sites at nucleotide resolution
- Measures of gene and enhancer transcription

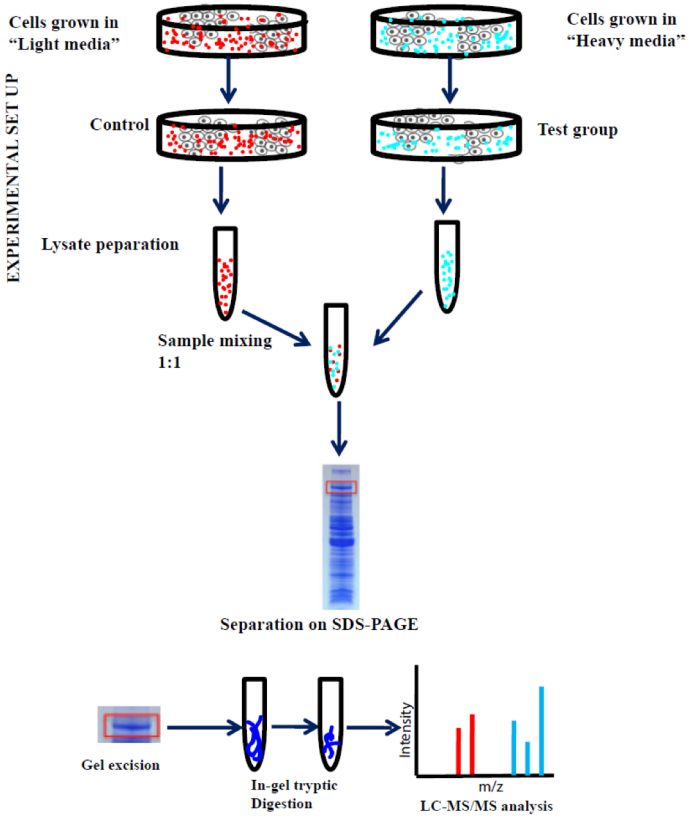
Different sensitivity and resolution!



Use of several methods together can provide an integrated view of active transcription, co-transcriptional processes and the half-lives of RNAs

Do nascent RNAs affect the interaction of proteins with chromatin?

SILAC-based quantitative proteomics approach in embryonic stem cells (ESCs)



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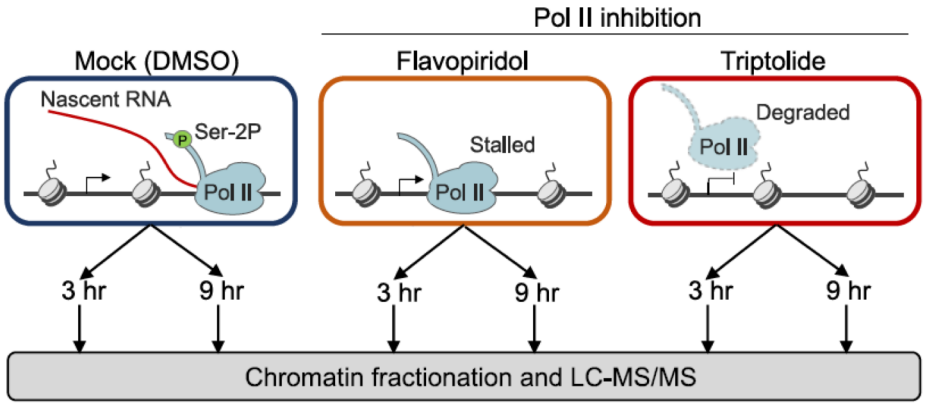

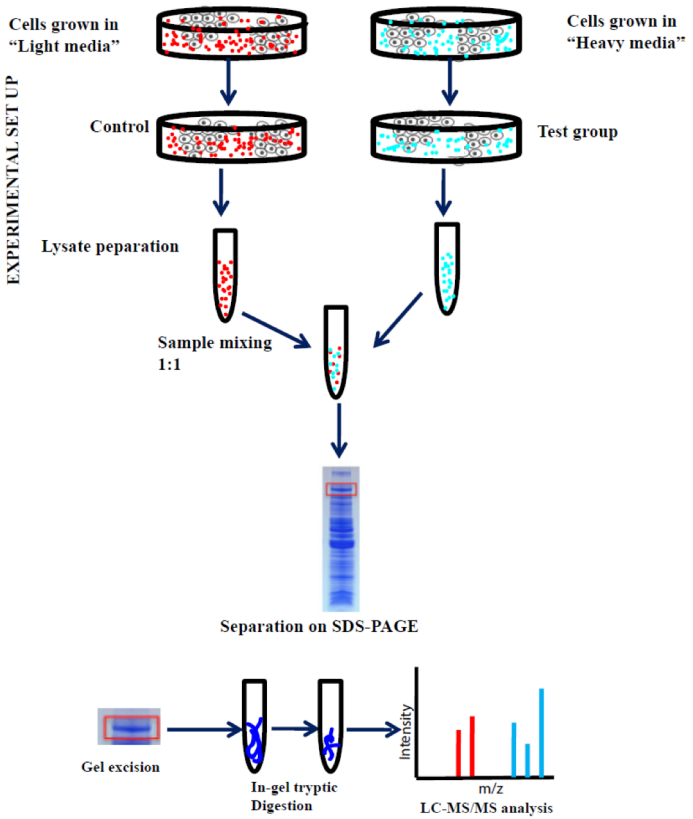

- Triptolide: TFIIH inhibitor which blocks transcriptional initiation and leads to RNA Pol II degradation
- Flavopiridol: CDK9 inhibitor (P-TEFb subunit) which blocks transcriptional elongation

Do nascent RNAs affect the interaction of proteins with chromatin?

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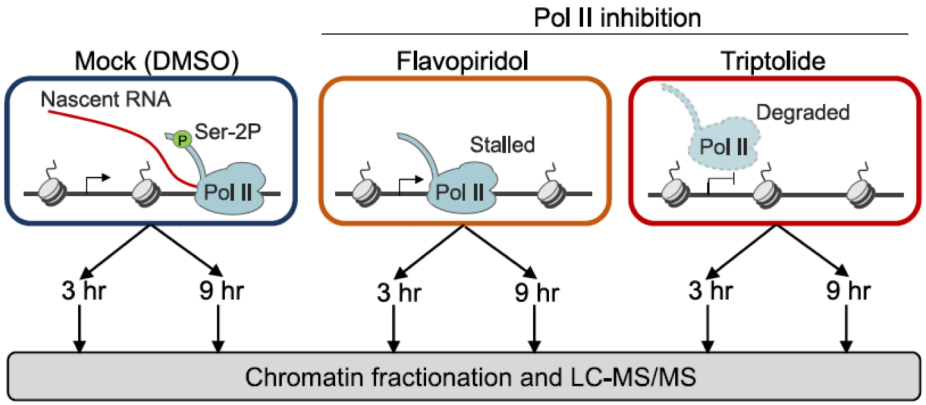


- **RNA-seq** revealed a great effect on chromatin-associated nascent RNA and that transcription had largely ceased
- Chromatin fractions were purified and verified by **silver stain and immunoblotting**
- Quantification by liquid chromatography-tandem mass spectrometry (**LC-MS/MS**)

- Triptolide: TFIIH inhibitor which blocks transcriptional initiation and leads to RNA Pol II degradation
- Flavopiridol: CDK9 inhibitor (P-TEFb subunit) which blocks transcriptional elongation

A set of proteins lost from chromatin and a set of proteins recruited to chromatin

Similar changes were evident at 3 and 9 h for both flavopiridol and triptolide

Do nascent RNAs affect the interaction of proteins with chromatin?

Set of proteins depleted from chromatin

Proteins with functions in RNA processing

Subunits of splicesome, exosome, polyA complex and RNA Pol II-bound factors

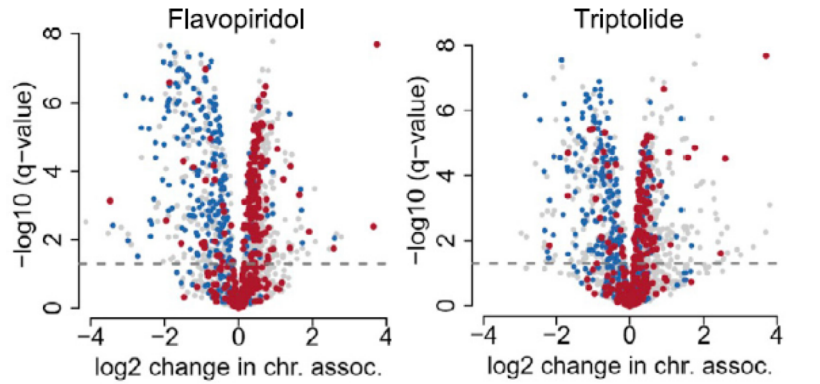
Set of proteins recruited to chromatin

Factors with roles in chromatin organization, gene expression and ESC pluripotency

PRC2 and other chromatin modifiers and nucleosome remodelers

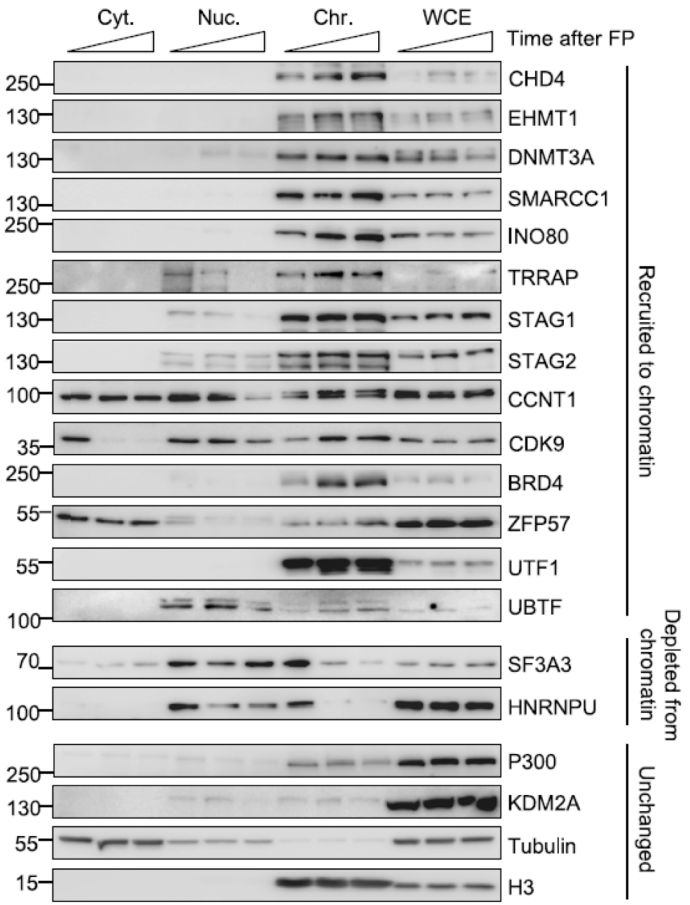
Regulators of RNA Pol II processivity, including P-TEFb

	Flavopiridol		Triptolide	
	Depleted	Recruited	Depleted	Recruited
Chromatin organisation	25	133	25	96
RNA processing	218	63	191	61
Other	143	251	124	245



Significance of changes in the association of proteins with chromatin upon treatment with flavopiridol (left) or triptolide (right) versus DMSO at 9 h. The GO term RNA processing (blue) was significantly enriched in the set of proteins depleted from chromatin in both treatments. The GO term Chromatin Organization (red) was significantly enriched in the set of proteins recruited to chromatin.

Do nascent RNAs affect the interaction of proteins with chromatin?



- **Immunoblotting** for 22 representative proteins that significantly increased their chromatin binding upon RNA Pol II inhibition

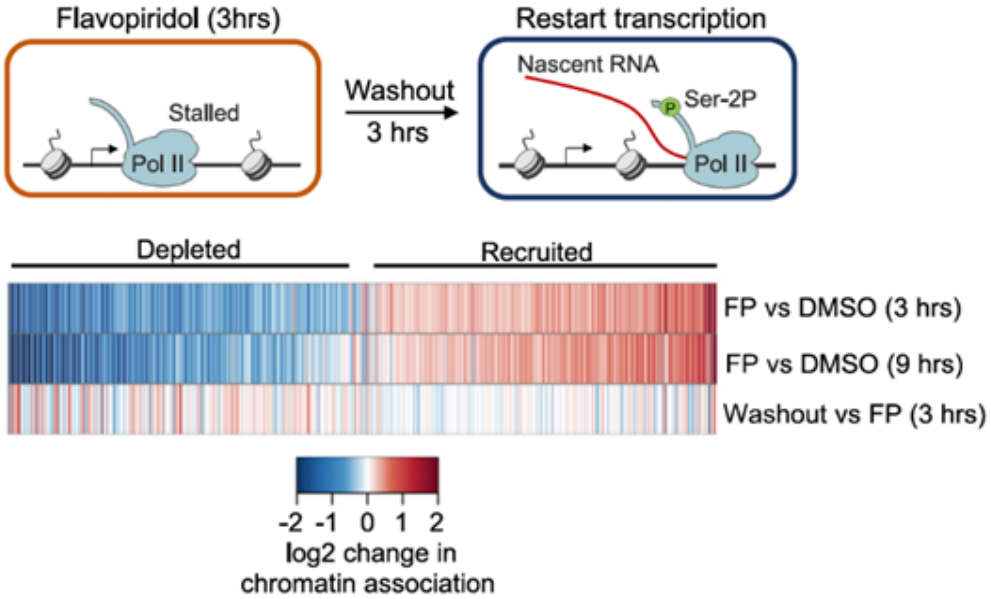
RNA pol inhibition induced changes in chromatin association for 16 of the 22 proteins tested (73%)

Immunoblots for representative proteins in the cytoplasmic, nucleoplasmic, and chromatin fractions and whole-cell extract (WCE) taken from ESCs after 0, 3, or 6 h of incubation with flavopiridol.

Does restarting transcription reverse the changes?

Washout of flavopiridol after 3h treatment

- Allowing transcriptional elongation to restart began to reverse the changes caused by flavopiridol treatment
- The magnitude of the effect was small relative to the initial treatment



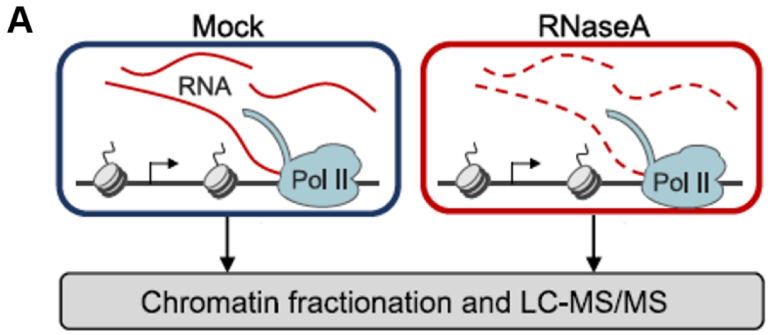
Transcription acts in a dynamic manner to regulate the association of a specific set of chromatin and transcriptional regulatory proteins with chromatin

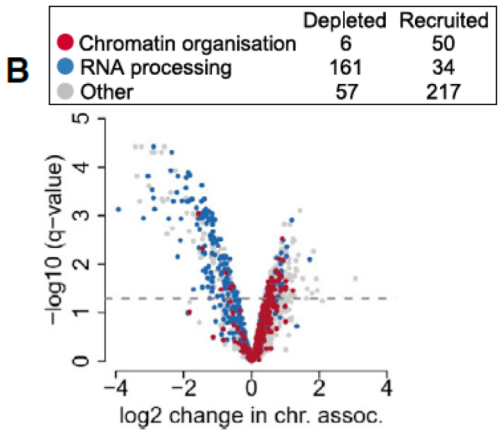
Do we observe the same effect upon RNAs degradation?

Quantification of proteins by LC-MS/MS after degradation of RNA in cells with RNaseA

- **RNA-seq** revealed a greater reduction of chromatin-associated intronic transcripts with respect to polyA+ exonic RNA

- Loss of factors involved in **RNA processing** from chromatin
- Recruitment of a set of transcriptional and **chromatin regulators**
- Significant correlation and overlap with the changes caused by RNA Pol II inhibition
- For a minority of factors RNA degradation had the opposite effect of RNA Pol II inhibition





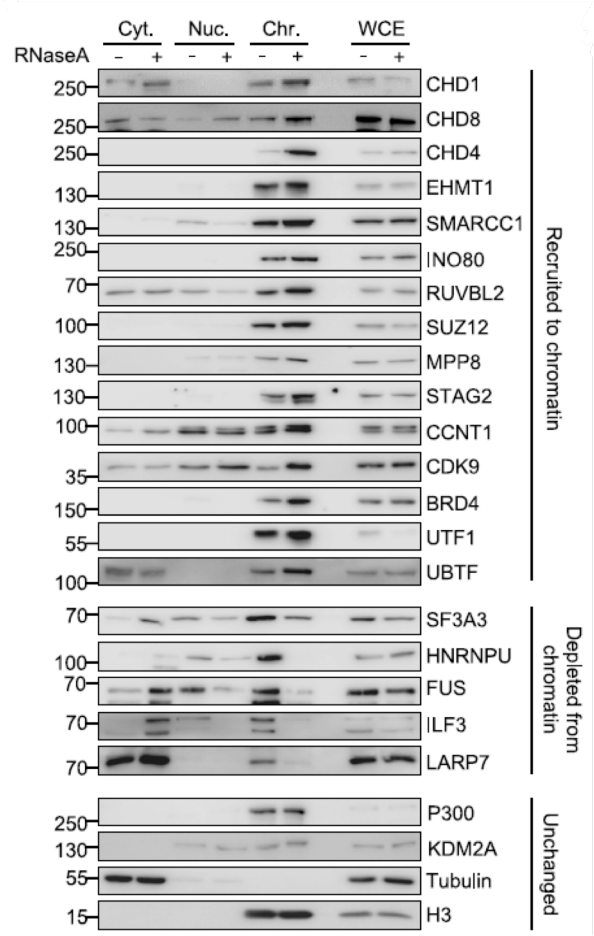
(A) Experimental strategy. ESCs were permeabilized, mock-treated or treated with RNaseA to degrade RNA, chromatin fractions purified, and proteins quantified by label-free LC-MS/MS.

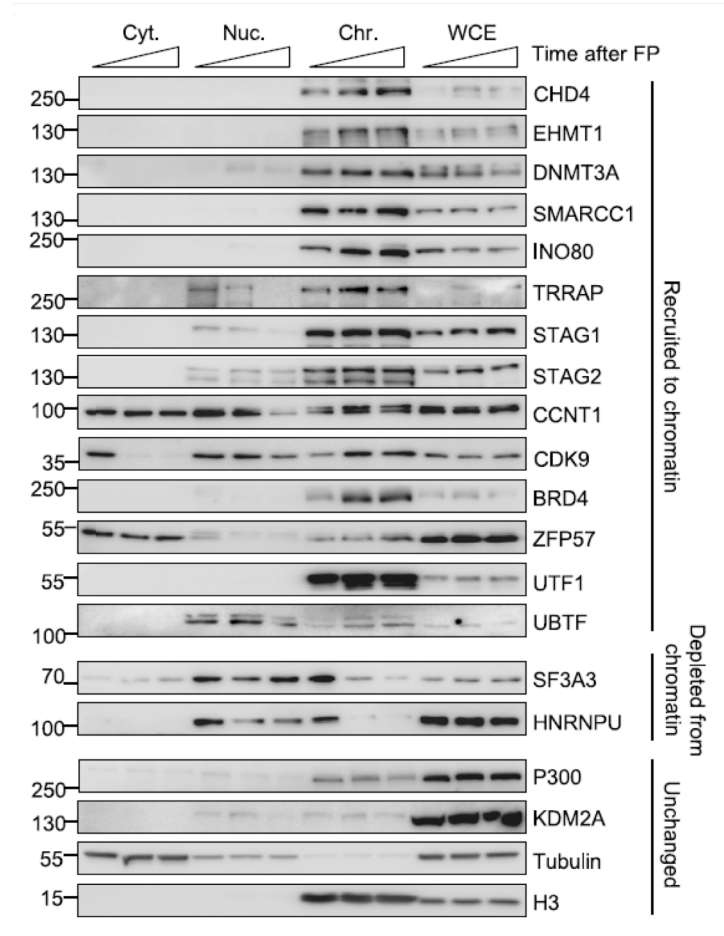
(B) Significance of changes in the association of proteins with chromatin upon RNaseA treatment versus mock-treated control. Proteins with functions in RNA processing and chromatin organization are highlighted in blue or red, respectively, and their frequencies in the sets of proteins recruited or depleted from chromatin (FDR < 0.05) shown above.

Do we observe the same effect upon RNAs degradation?

- **Immunoblotting** confirmed the same changes for regulatory proteins previously evaluated

RNA degradation recapitulates many of the changes in protein chromatin association caused by RNA Pol II inhibition





Left: Immunoblots for representative proteins in the cytoplasmic, nucleoplasmic, and chromatin fractions and WCE purified from ESC after mock treatment (-) or treatment with RNaseA (+).

Right: Immunoblots for representative proteins in the cytoplasmic, nucleoplasmic, and chromatin fractions and whole-cell extract (WCE) taken from ESCs after 0, 3, or 6 h of incubation with flavopiridol.

RNAs have a role in transcription regulation

Several methods for studying nascent RNAs

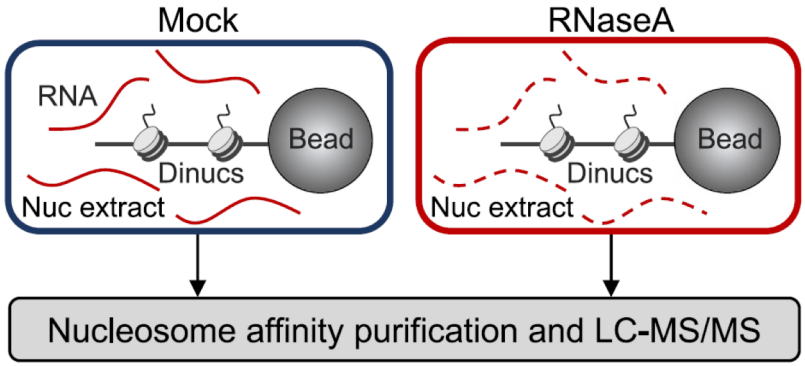
RNA pol II inhibition and RNA degradation cause similar changes in the association of proteins with chromatin

RNA Pol II inhibition and RNA degradation in cells are likely to have pleiotropic effects

Transcription acts in a dynamic manner to regulate the association of a specific set of chromatin and transcriptional regulatory proteins with chromatin

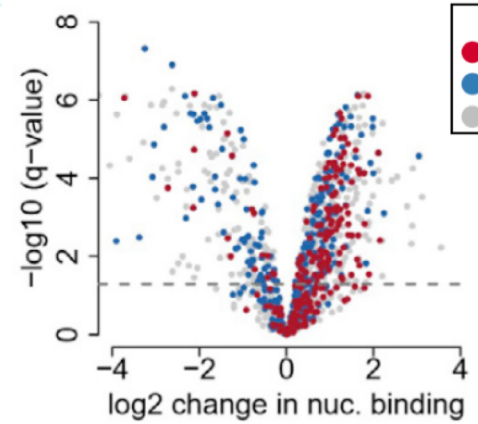
Does RNA antagonize nucleosome binding of chromatin regulators?

Proteins quantification by LC-MS/MS after RNaseA treatment and dinucleosomes purification



- Nascent RNA inhibits the association of PRC2 with chromatin
- RNA competes with nucleosomes for PRC2 binding and inhibits PRC2 catalytic activity

- **Proteins involved in chromatin organization exhibit increased nucleosome binding**
- **Significant overlap between proteins that exhibited increased association with chromatin upon RNA Pol II inhibition and those which show increased binding to nucleosomes after RNA degradation**



	Decreased	Increased
Chromatin organisation	16	129
RNA processing	85	135
Other	91	250

Significance of changes in the association of proteins with nucleosomes upon RNaseA treatment versus mock-treated control. Proteins with functions in RNA processing and chromatin organization are highlighted in blue or red, respectively.

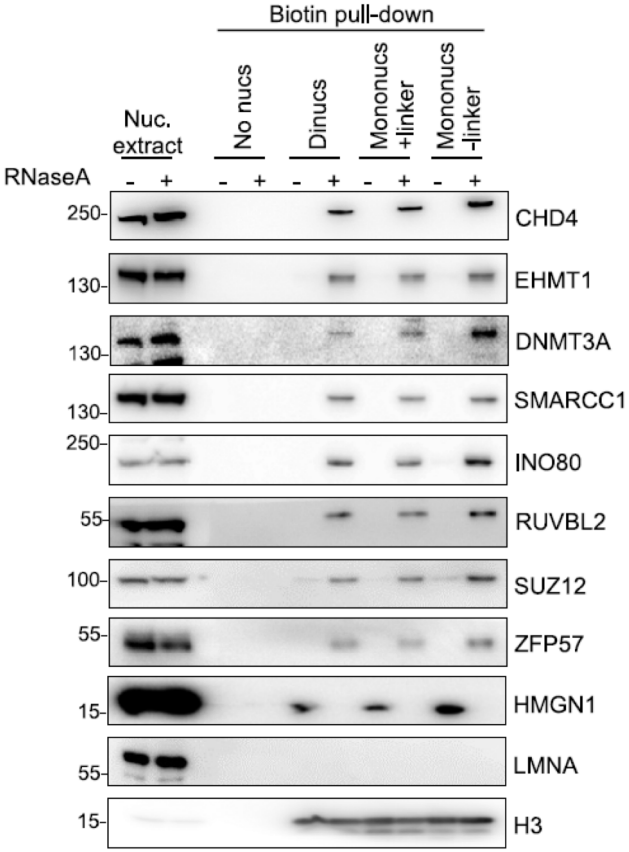
Does RNA antagonize nucleosome binding of chromatin regulators?

Repeating experiment with dinucleosomes and mononucleosomes incorporating or lacking linker DNA

- Of the 16 proteins tested, 13 were enriched by nucleosome affinity purification
- All of these exhibited increased nucleosome binding upon RNA degradation
- 6 factors also showed increased binding on chromatin upon RNA pol II inhibition and RNaseA treatment

Confirmation that RNA antagonized the interaction of PRC2 (SUZ12) with nucleosomes

Nuclear RNA inhibits the association of a set of regulatory proteins with nucleosomes

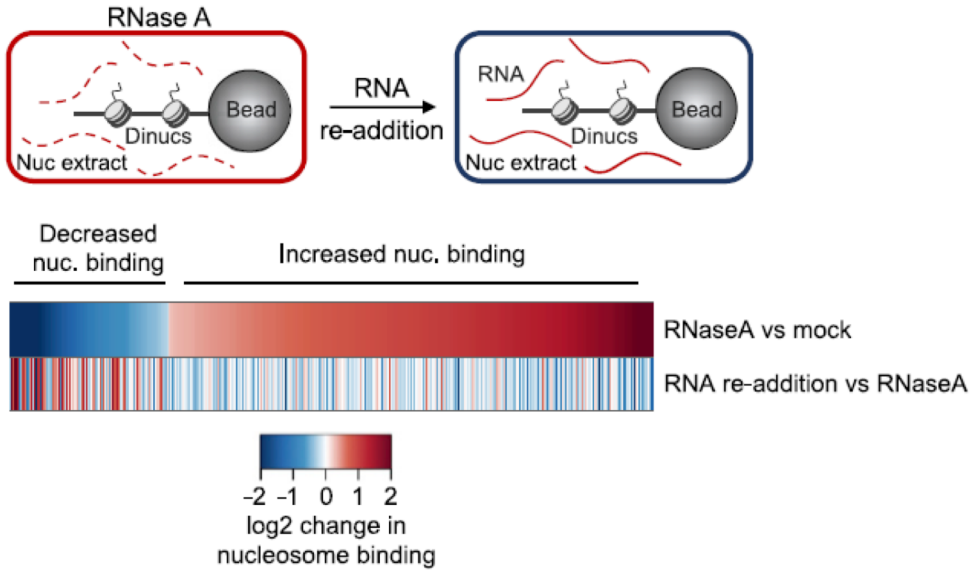


Immunoblots for representative proteins in nucleosome pull-downs (dinucleosomes, or mononucleosomes assembled with 187 or 147 bp DNA) from mock-treated (-) or RNaseA-treated (+) nuclear extracts.

Does the re-addition of RNA reverse the changes?

Nucleosomes purification from nuclear extracts after adding tRNA and RNaseA inhibitor

- PRC2 promiscuously binds complex RNAs
- tRNA can be used to model the competition between RNA and nucleosomes for PRC2 binding



General reversion of the changes in nucleosome binding caused by RNA degradation

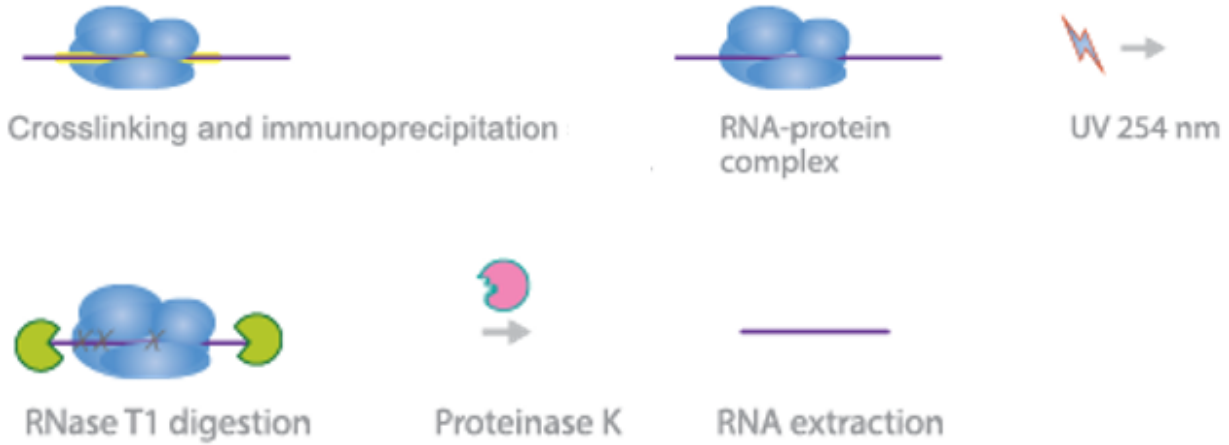
RNA antagonizes the interaction of this set of regulatory proteins with chromatin

Proteins exhibiting either significantly increased or decreased interaction with nucleosomes after RNaseA degradation. Change in nucleosome interaction after tRNA addition is shown below and is anti-correlated

Do regulatory proteins tested directly interact with RNA?

Cross-linking and immunoprecipitation (CLIP) for 8 representative factors

RNA inhibits the interaction of PRC2 with chromatin because it directly competes with nucleosomes for PRC2 binding

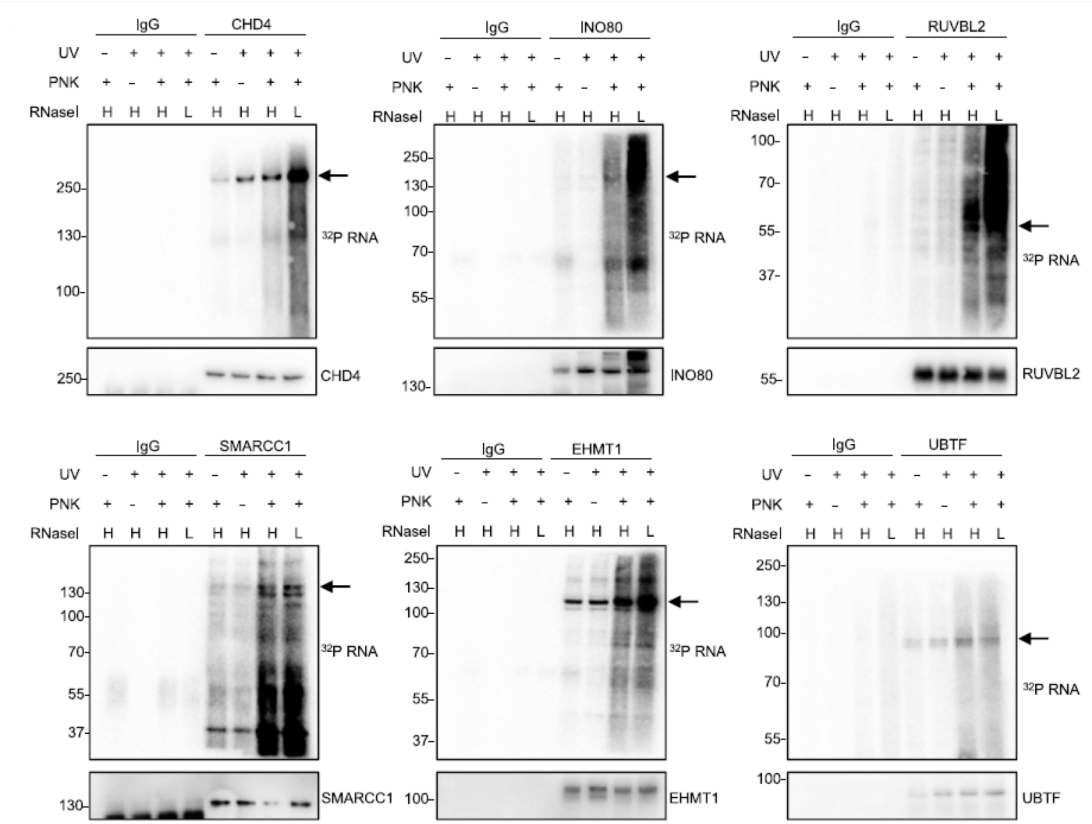


Do regulatory proteins tested directly interact with RNA?

Cross-linking and immunoprecipitation (CLIP) for 8 representative factors

- Detection of RNP of the expected molecular weight
- Smear of trimmed RNA extending above, stronger in +UV and +PNK (polynucleotide kinase) conditions
- The smear diminished as the RNase I concentration was increased

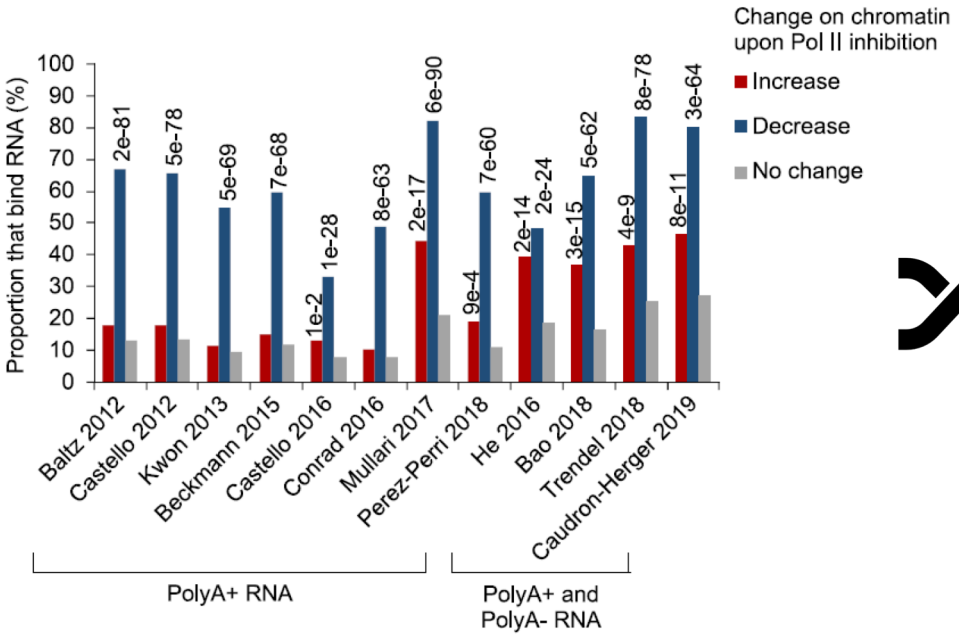
Direct RNA binding for 6 of these 8 proteins



SDS-PAGE for RNPs enriched by CLIP for CHD4, INO80, RUVBL2, SMARCC1, EHMT1, UBTF, and non-specific immunoglobulin G (IgG) controls in ESCs. Autoradiograms of crosslinked ^{32}P-labeled RNA are shown at the top and the corresponding immunoblots below. CLIP was performed with and without UV crosslinking and polynucleotide kinase (PNK) and with high (H; 40 U/mL) or low (L; 4 U/mL) concentrations of RNase I. The arrows indicate the molecular weight of the protein of interest.

To explore this on a more global scale:

we compared the sets of proteins that were depleted or recruited to chromatin upon RNA Pol II inhibition with proteins identified to bind RNA in 12 previous screens:



8 of which identified proteins bound to mature polyadenylated RNA

4 of which could also identify proteins bound to nascent RNA or other non-polyadenylated transcripts.

We found that the set of proteins depleted from chromatin was significantly enriched for RBPs identified by all of the studies

focusing on the set of proteins recruited to chromatin upon RNA Pol II inhibition:

- significantly enriched for RBPs identified by all four studies that could measure non-polyadenylated RNA binding, including a study that specifically identified proteins bound to nascent RNA.

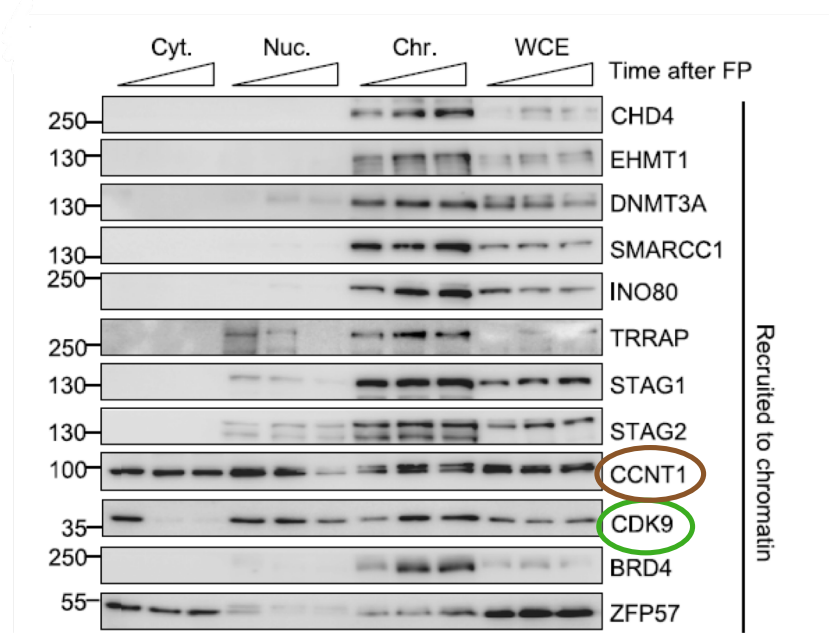
- The set of proteins recruited to chromatin upon RNA Pol II inhibition was also enriched for RBPs

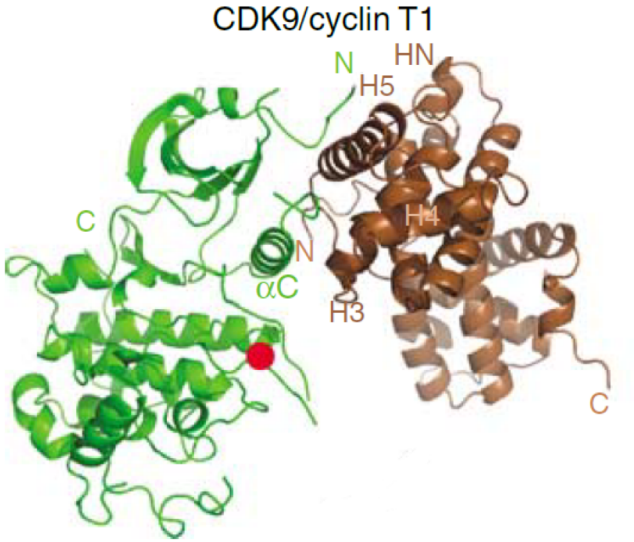
Conclusions:

these results suggest that the inhibitory effect of RNA on the interaction of these proteins with chromatin is due to the interaction of these factors with RNA.

P-TEFb is a Cyc-CDK complex

CDK9 and CycT1 were among the proteins exhibiting the greatest increases in chromatin association upon RNA Pol II inhibition and RNaseA treatment





- Cyclin-dependent kinase 9/cyclin T (CDK9/CycT)
- CDK9 activation requires binding of a T family cyclin (CycT1, CycT2a or CycT2b) or CycK

P-TEFb directly interacts with nascent pre-mRNA in cells

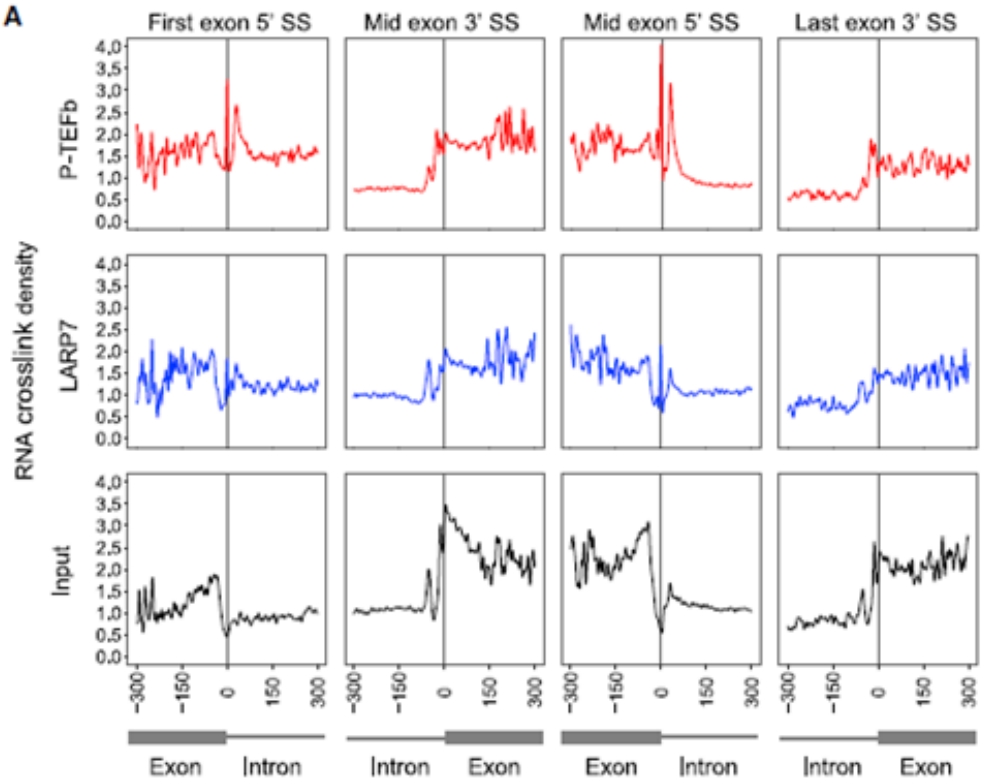
Sequencing of P-TEFb RNA crosslink sites → reveal strong enrichment for 7SK RNA which is able to sequester P-TEFb.

the majority of P-TEFb crosslinks mapped to protein-coding genes with enrichment around 5’ splice sites (5’SS) that was not observed in the background RNA crosslinking from input control samples.

Test and Results: Defining a CLIP experiment for CDK9, there isn’t the co-precipitation of 7SK RNP component LARP7 and crosslinking around 50SS was not observed in iCLIP experiments for LARP7.

This demonstrate that:

- P-TEFb was not binding to pre-mRNA as part of the 7SK RNP.
- the crosslinking detected around 50SS does not reflect the coprecipitation of spliceosome components.

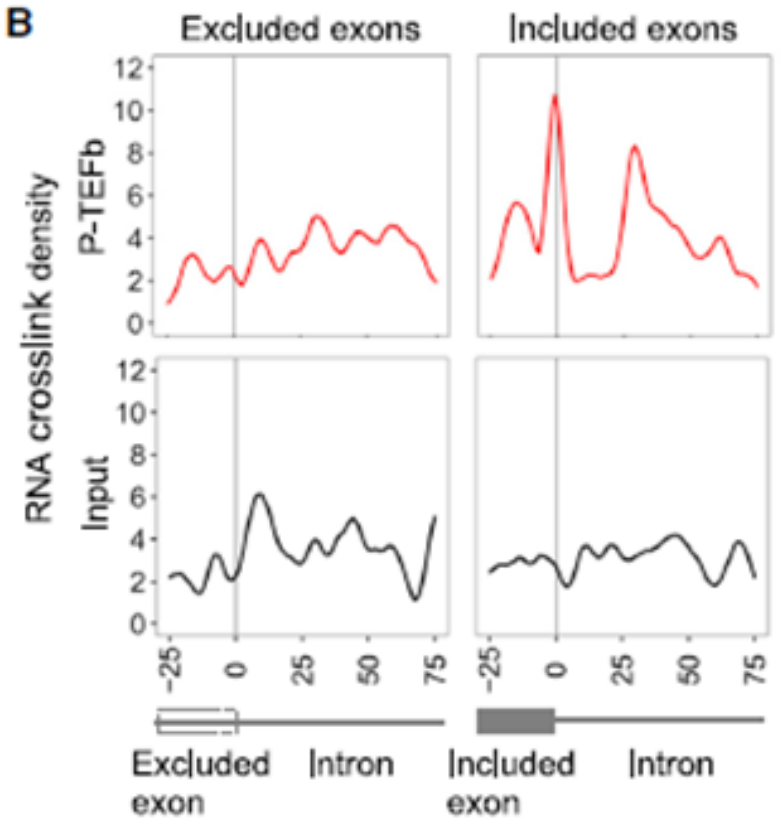


P-TEFB BINDING TO RNA MAY BE DEPENDENT ON SPLICING.

Test
they compared P-TEFb crosslinking at 50SS at exons included in the mature transcript versus exons that were excluded

Results:

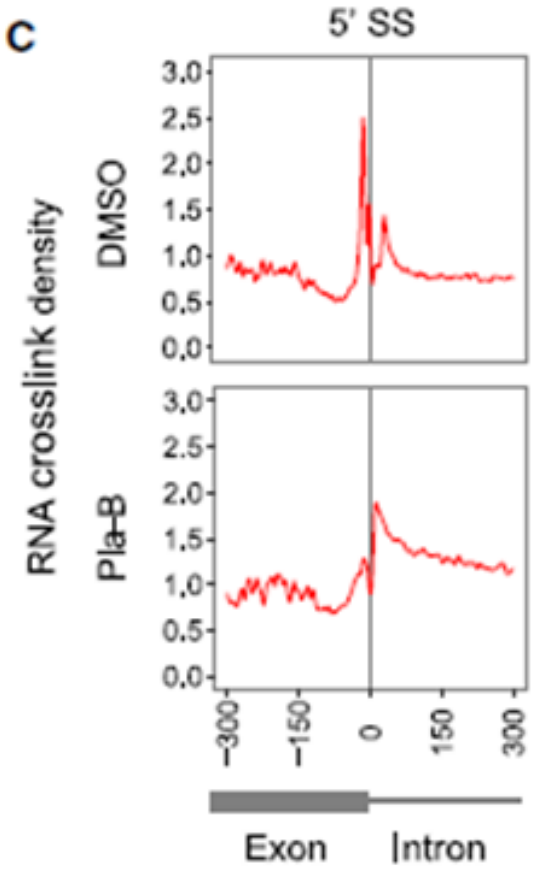
- P-TEFb RNA crosslinking was only apparent at included exons, consistent with this crosslinking being dependent on splicing.
- P-TEFb exhibited reduced crosslinking to RNA transcribed from single-exon compared to multi-exon genes.



- Can P-TEFb directly binds nascent pre-mRNA around 5'SS ?
- Is it dependent on splicing ?

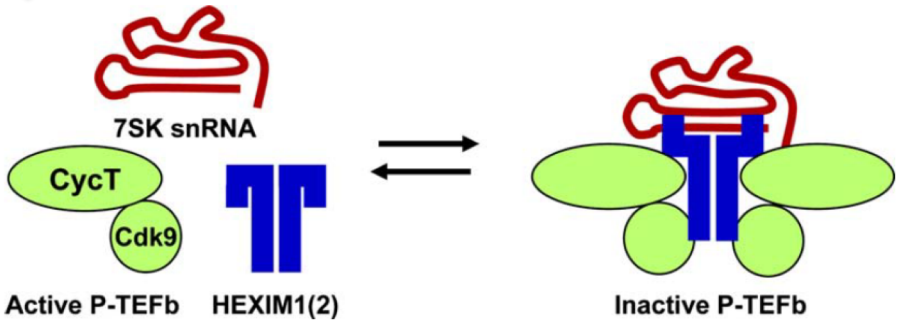
Test:
Repeat CDK9 iCLIP after treatment of cells with the SF3b inhibitor pladienolide B (pla-B, 1 mM for 6 h)

Results:
the specific pattern of P-TEFb crosslinking around 50SS was not observed after treatment with plaB



❑ **Conclusion:** P-TEFb directly binds nascent pre-mRNA around 50'SS and that this is dependent on splicing.

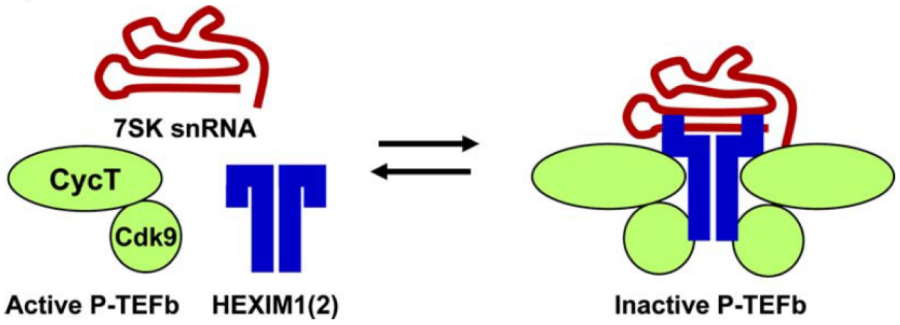
P-TEFb is held in a poised state by the 7SK RNP



Does 7SK antagonize the interaction of P-TEFb with nascent RNA?

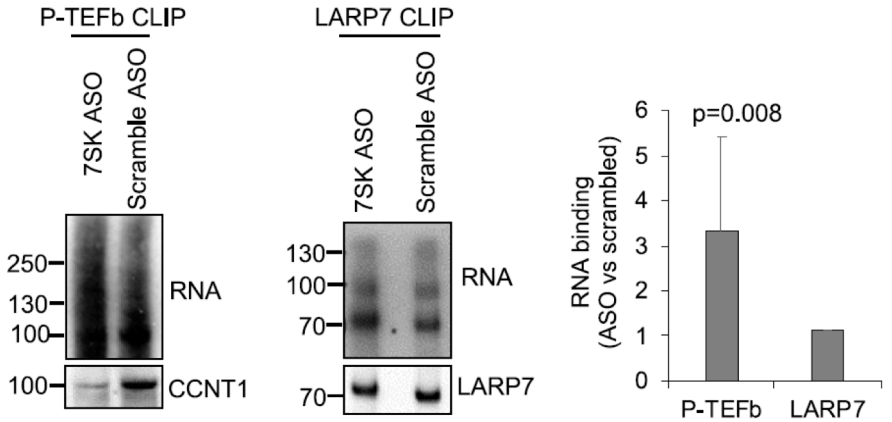
CLIP for P-TEFb in ESCs transfected with ASO specific for 7SK RNA or scrambled ASO

P-TEFb is held in a poised state by the 7SK RNP



- Hypothesis that P-TEFb interacted with nascent RNA in its free, non-7SK associated form
- The depletion of 7SK RNA should increase P-TEFb binding to nascent RNA

7SK knockdown increased P-TEFb RNA binding

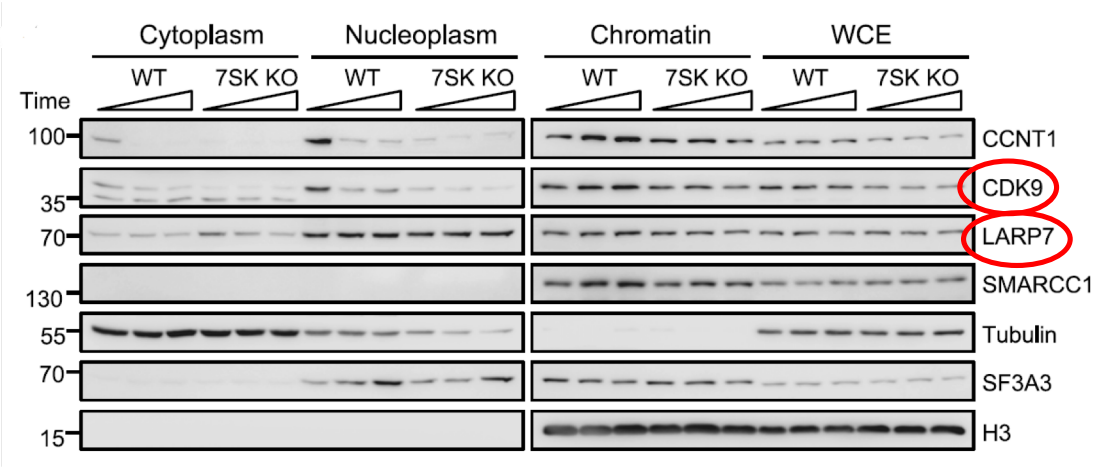


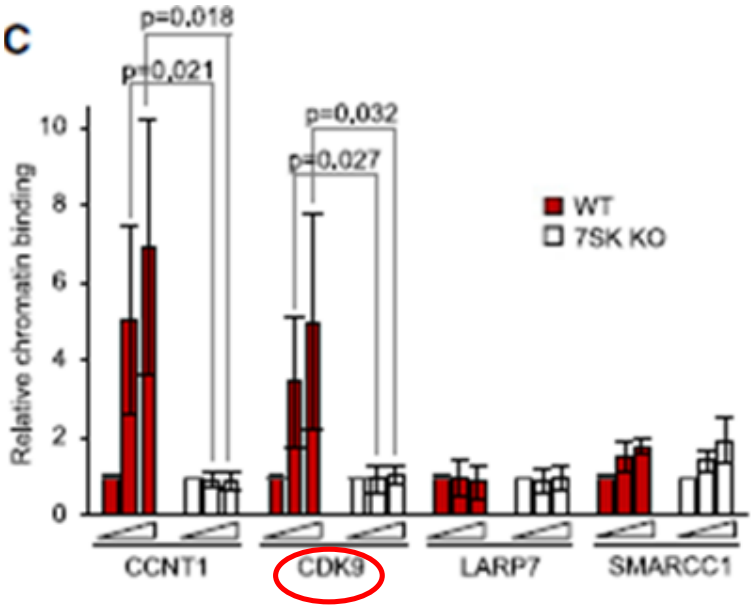
SDS-PAGE for RNPs enriched by CLIP for P-TEFb and LARP7 in ESCs transfected with scrambled antisense oligonucleotide (ASO) or ASO specific for 7SK RNA. Autoradiograms of crosslinked RNA are shown at the top and immunoblots below. Right: quantification of the change in RNA crosslinking (7SK ASO versus scrambled ASO) relative to protein

P-TEFb binds nascent RNA in its free form and this is countered by its interaction with 7SK

Does 7SK regulate the association of P-TEFb with chromatin?

RNA Pol II inhibition with triptolide in wild-type cells and 7SK knockout cells





RNA pol II inhibition increased the association of P-TEFb with the chromatin fraction in WT cells but not in 7SK KO cells

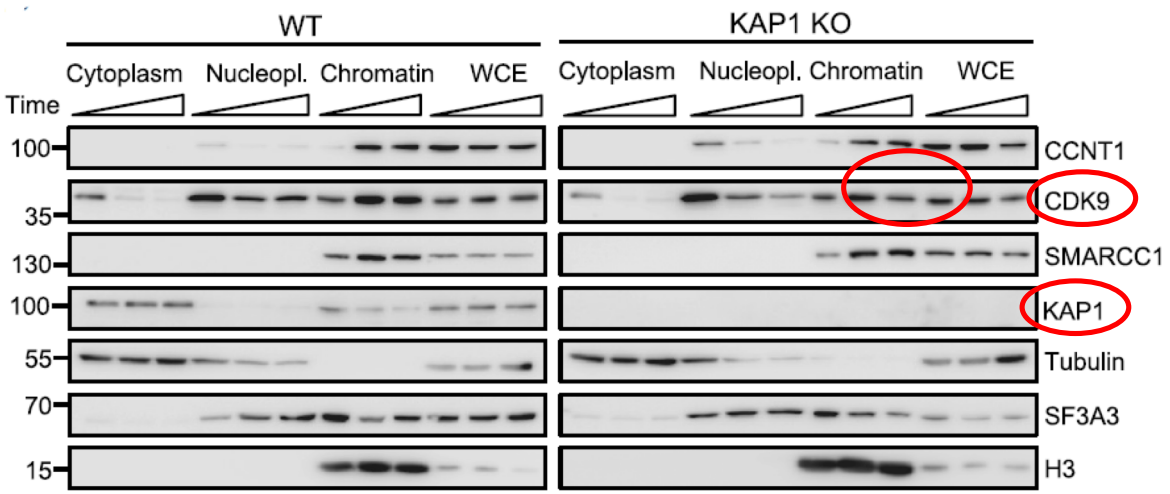
7SK is required for the recruitment of P-TEFb to chromatin upon RNA Pol II inhibition

- Immunoblots for CyclinT1 (CCNT1), CDK9, LARP7, and control proteins in cytoplasmic, nucleoplasmic, and chromatin fractions and WCE from WT and 7SK KO HAP1 cells after incubation with triptolide for 0, 3, and 6 h.
- Quantification of immunoblots shown

Does the transfer of P-TEFb to chromatin upon RNA Pol II inhibition depend on KAP1?

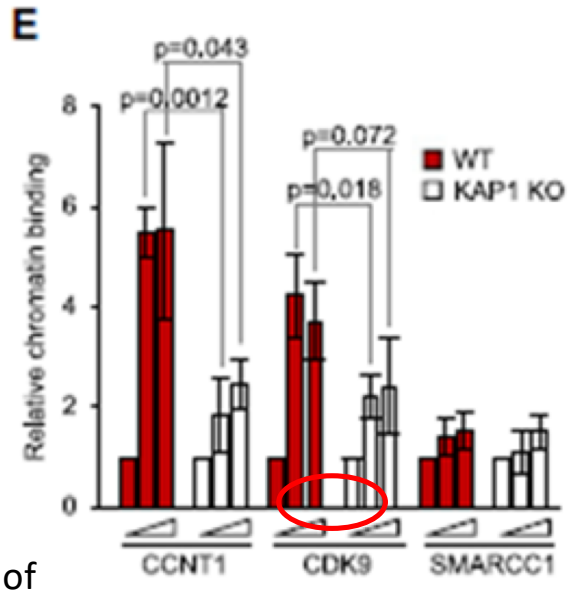
Triptolide treatment on P-TEFb chromatin association in WT and KAP1 KO cells

It's known that 7SK RNP associates with chromatin at active genes through interaction with KAP1



❑ **Results:**

in the absence of KAP1, the extent of P-TEFb recruitment to chromatin was approximately halved



❑ **Conclusion:** the association of 7SK with genes facilitates the recruitment of P-TEFb to chromatin upon RNA Pol II inhibition.

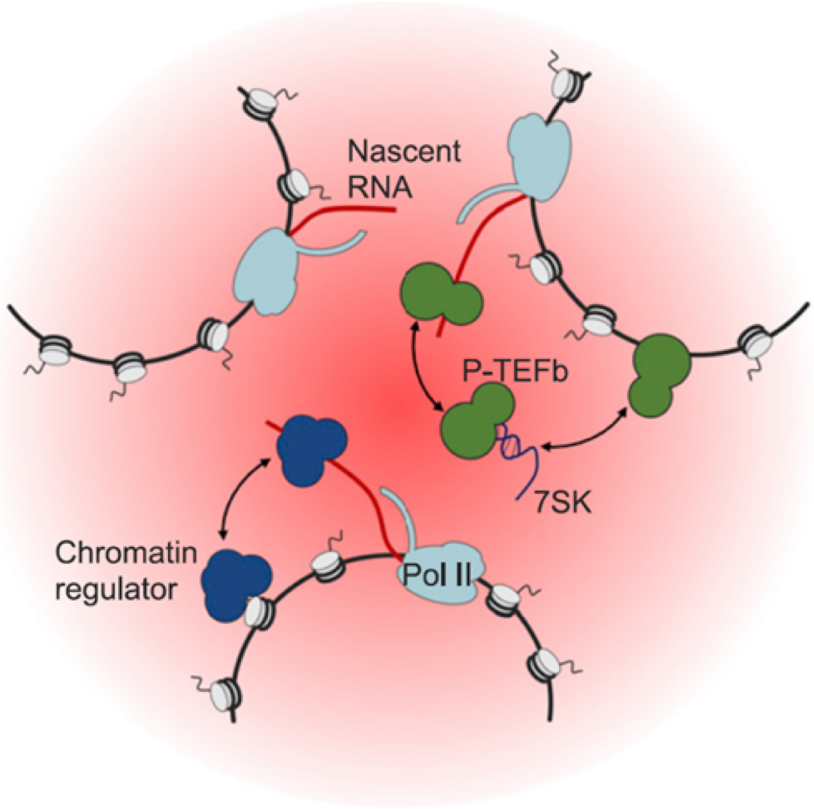
BOTH 7SK AND KAP1 ARE REQUIRED FOR P-TEFB RECRUITMENT ON CHROMATIN: **DOES 7SK INTERACT WITH P-TEFB ON CHROMATIN?**

❑ **Test and results**:
Thanks CyclinT1 immunoprecipitation from the chromatin fraction revealed a reduction in P-TEFb interaction with 7SK RNA and LARP7 after RNA Pol II inhibition

Conclusion:

- ❑ 7SK is necessary for the transfer of P-TEFb to chromatin upon transcriptional inhibition but does not itself constitute the chromatin-associated P-TEFb pool in transcriptionally inactive cells.

- ❑ These data *support a model:* nascent RNA binds to a set of transcriptional and chromatin regulators and inhibits their association with chromatin, which, in the case of P-TEFb, is regulated by the 7SK RNP.



1. INTRODUCTION

2. PLEIOTROPIC EFFECT OF NASCENT RNA ON CHROMATIN REGULATORS

3. PRC2: nucleosome and chromatin

4. P-TEFb and nascent RNA

5. P-TEFb and chromatin

6. CONCLUSION

Summary:

The **antagonistic effect of RNA on the interaction of PRC2** with chromatin has been demonstrated by experiments using RNA Pol II inhibition, RNA degradation, and nucleosome-RNA competition assays

By adapting these methods we have revealed **that the antagonistic effect of nascent RNA on PRC2 function is an example of a broader role for RNA in inhibiting the interaction of transcriptional and chromatin regulator proteins with chromatin.**

The set of proteins identified to be antagonized by RNA is enriched for functions in ESC pluripotency function together in common pathways— for example, PRC2 with NuRD, suggesting that **nascent RNA regulates the interaction of factors with chromatin in a coordinated manner.**

To conclude:

this work demonstrates that:

- ✓ nascent RNA regulates the interaction of a set of chromatin and transcriptional regulatory factors with chromatin and primarily acts to antagonize their interaction with chromatin.
- ✓ These results are consistent with models in which nascent RNA provides direct feedback from gene transcription to chromatin state (Skalska et al., 2017) and provides evidence of a close interplay between RNA and chromatin in gene regulation.

Further studies will be required to:

- ascertain the importance of nascent RNA binding for P-TEFb function.
- Interaction of P-TEFb with nascent pre-mRNA could increase the size of the free PTEFb pool or could specifically direct its activities to particular locations or substrates.
- the importance of RNA binding activity for the function of the proteins in the cell.

Thanks for the attention