UNIVERSITÀ
DEGLI STUDI DI TRIESTE

Master Degree in Functional Genomics
ncRNA Biology Course – 2021/2022

Deregulated Expression of Mammalian lncRNA through Loss of SPT6 Induces R-Loop Formation, Replication Stress, and Cellular Senescence

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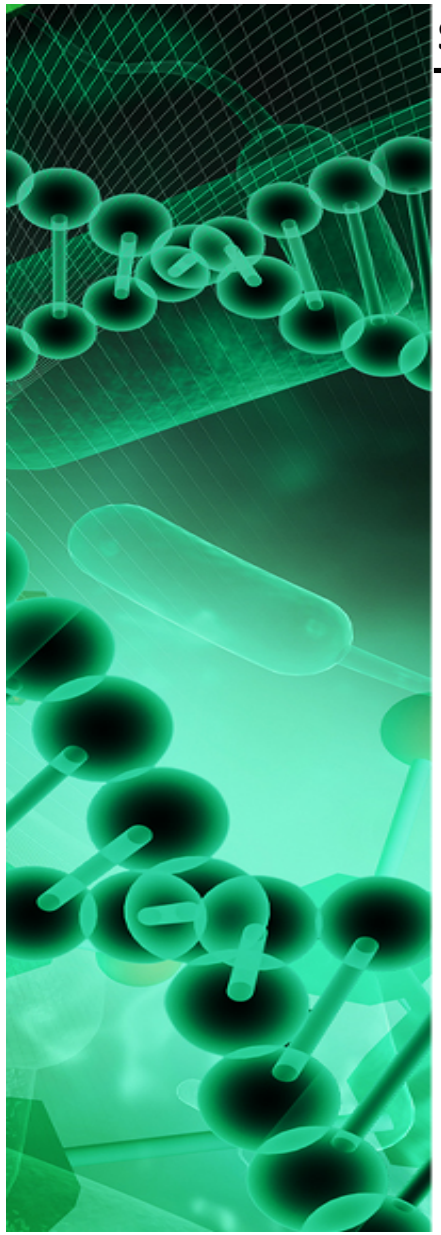


SUMMARY

- **Introduction**
  - lncRNA and differences with mRNA
  - H3K36 and transcription elongation
  - SPT6: features and functions
  - PROMPTs and eRNAs
- **Experimental results**
  - Histone marks distribution analysis
  - How SPT6 affects the H3K36me3 distribution across the genome
  - SPT6 Depletion induces lncRNA Transcription
  - Transcription termination defect: SPT6 role
  - Extended lncRNAs induce R-loop formation and DNA damage
  - Replicative stress and cellular senescence
- **Discussion and Conclusions**

- **> 75% of the human genome is transcribed**
- This widespread transcription events lead to the formation of different types of **long non-coding RNAs (lncRNAs)**
- > 200 nucleotides
- The biogenesis of many lncRNA appears similar to mRNA:
  - mostly transcribed by **RNAPII**
  - capped and mostly polyadenylated
  - splicing
- Compared to mRNA:
  - Less expressed and shorter
  - Less conserved
  - Non significant coding potential

lncRNA can be subdivided on their genomic localization and orientation

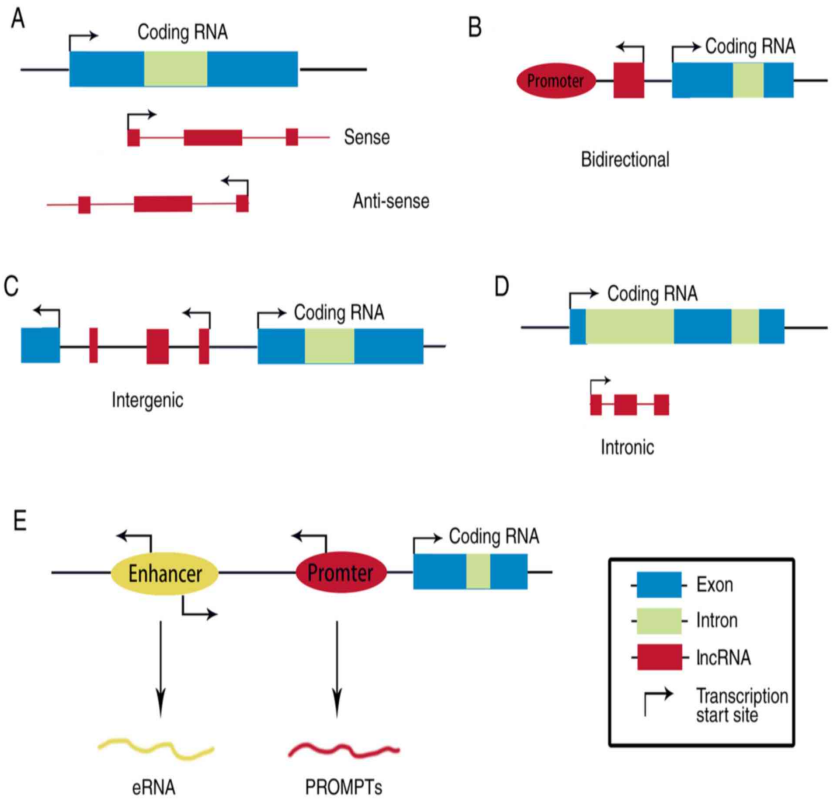
A. Sense/Antisense lncRNAs

B. Bidirectional lncRNAs

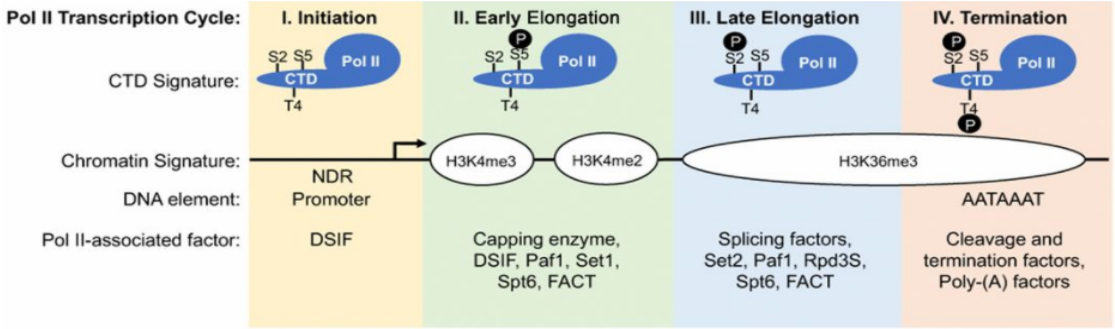
C. Intergenic lncRNAs

D. Intronic lncRNAs

E. PROMPTs and eRNAs



Differences between mRNA and lncRNA transcription



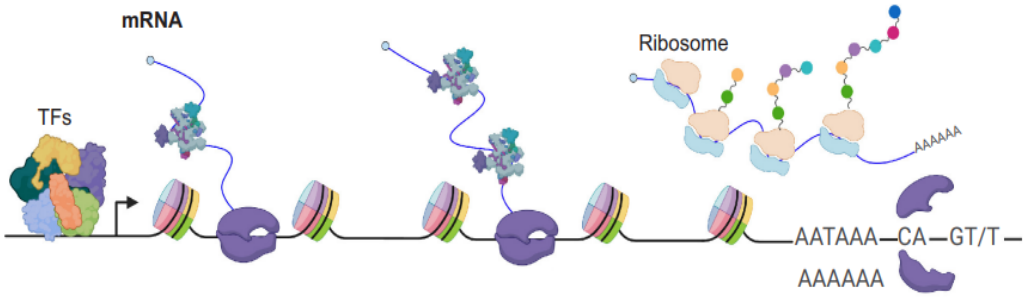
mRNA transcription

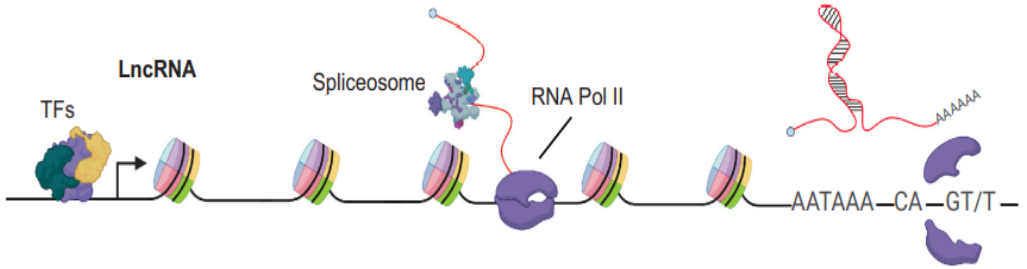
Each transcription phase shows a specific Pol II-CTD phosphorylation

- **S5-P**: early elongation and recruiting of capping enzyme
- **S2-P**: late elongation, related to splicing factor recruitment
- **T4-P**: mRNA transcription termination

lncRNA transcription

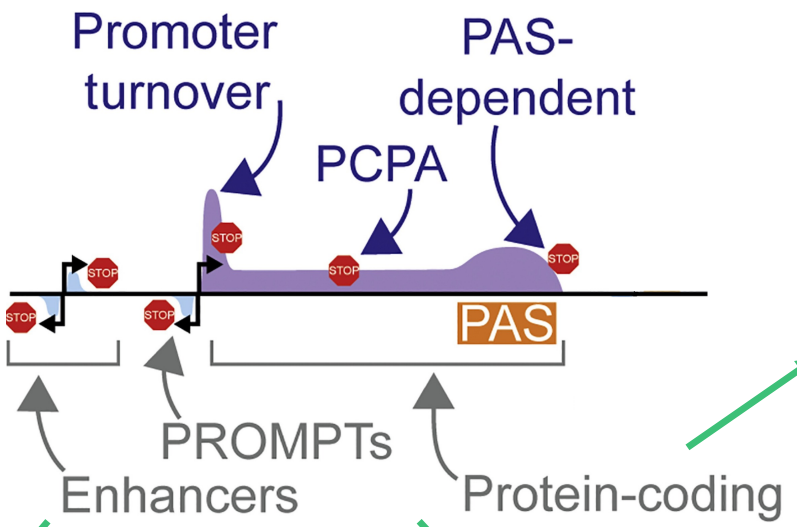
These TUs are characterized by reduced Pol II-CTD phosphorylation: **this leads to a less efficient processing of the transcript and less stability**





INTRODUCTION

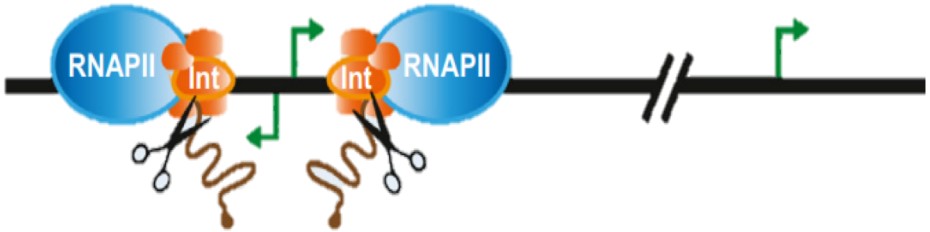
Differences between mRNA and lncRNA transcription

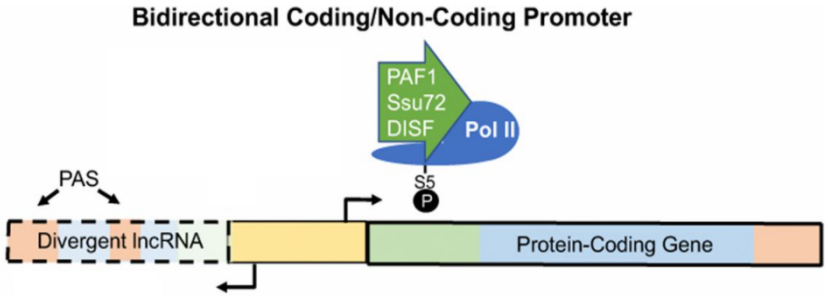


- PAS-CPA termination
- Stable and polyadenylated transcript

- Cleavage by the integrator complex
- Non-polyadenylated transcript

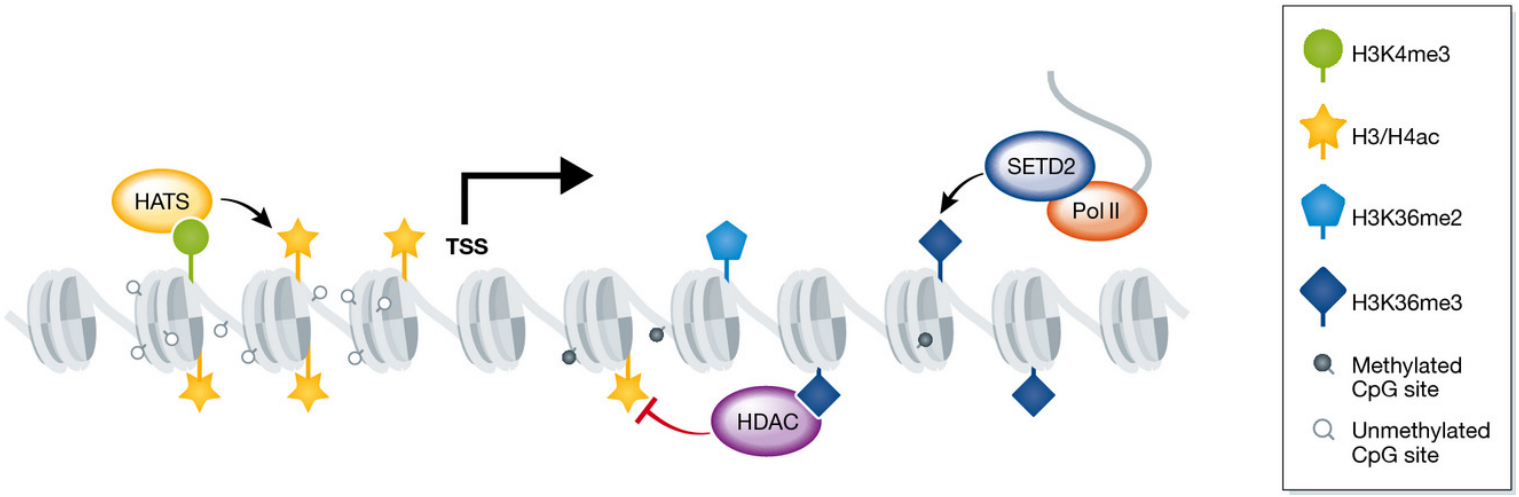
- Cryptic PAS and premature termination
- Polyadenylated transcript





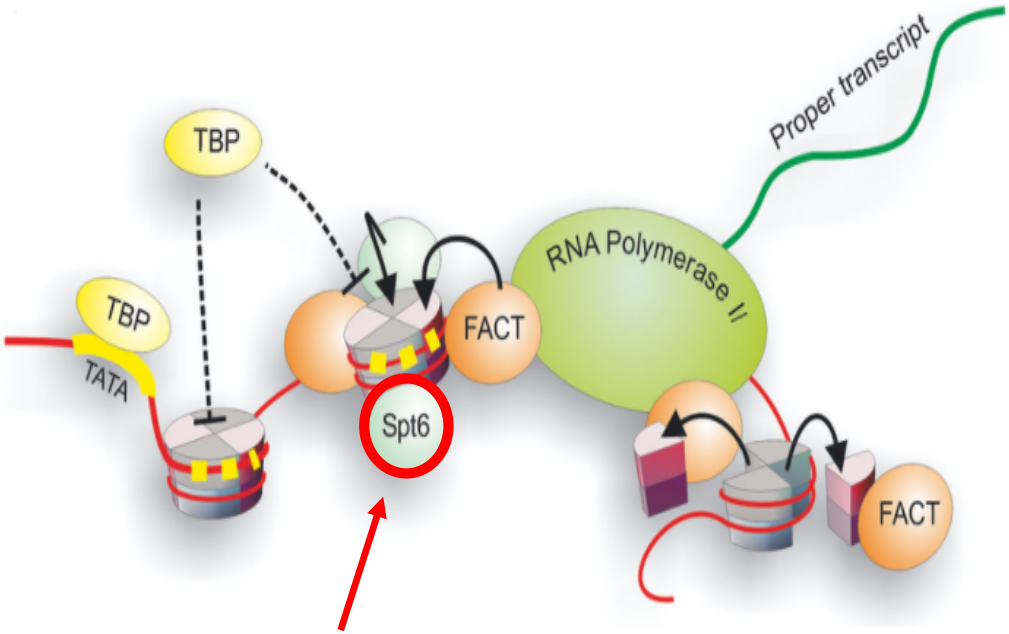
H3K36me3 is important to the mRNA elongation process

- Histone H3K36 methylation is a hallmark of active transcription
- SETD2, the methyltransferase for H3K36me3, is recruited through the Ser-2 phosphorylated C-terminal domain (CTD) of RNA polymerase II (RNAPII) during gene transcription elongation
- H3K36me3 recruits HDACs to sites of active transcription, which deacetylates histones within the gene body
- This histone mark acts to prevent aberrant transcriptional initiation from cryptic gene promoters



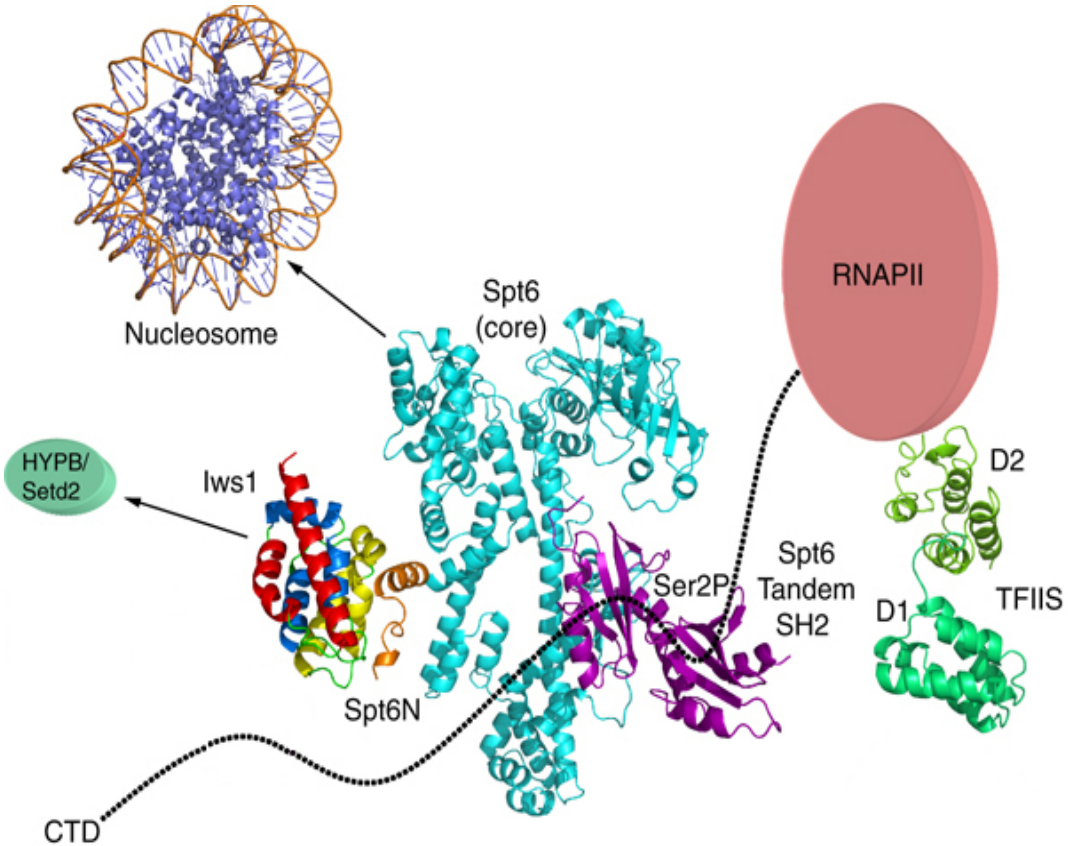
SPT6

- SPT6 is an evolutionary conserved, positive regulator of transcription by RNAPII
- SPT6 is a general transcription elongation factor
- It can interact directly with histones (especially H3) and possesses nucleosome assembly activity (histone chaperon)
- SPT6 is also involved in DNA damage repair process and in the regulation of mRNA turn-over



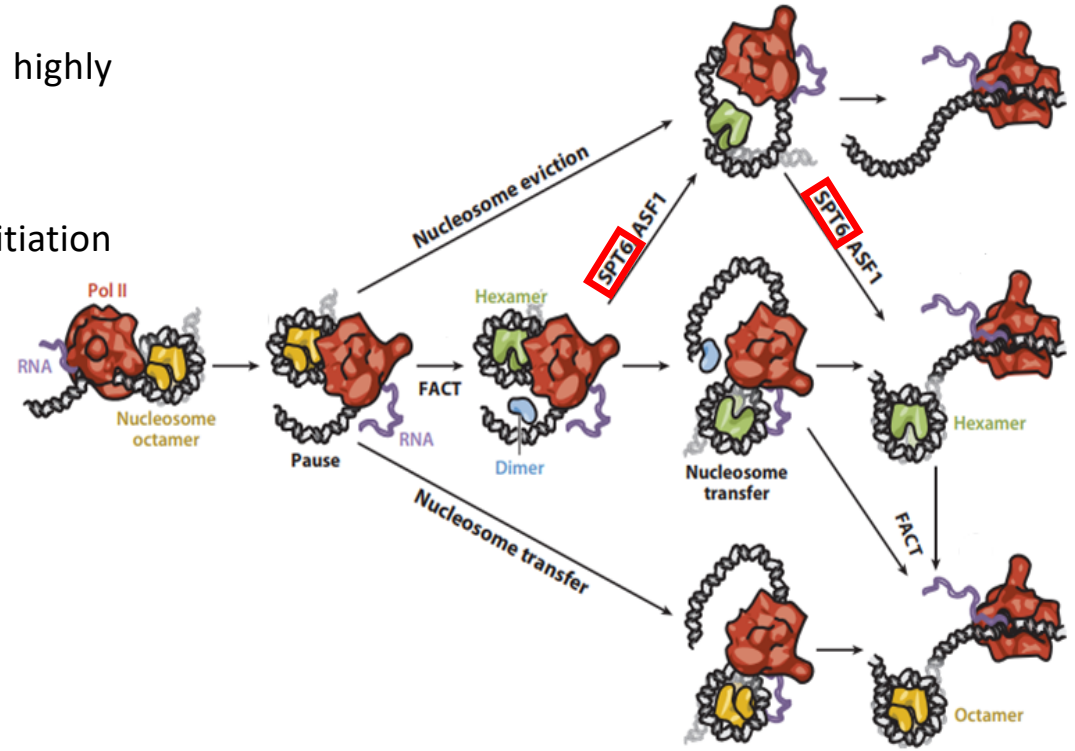
SPT6 interact with Pol II

- SPT6 plays essential roles in the control of transcription and its role is likely to be genome-wide
- It is required both for escape of RNAPII from promoter pausing and subsequent elongation
- The C-terminal domain SH2 of SPT6 mediates the interaction between SPT6 and RNAPII



SPT6: its role during the elongation process

- During transcription elongation, SPT6 is required for the <u>maintenance of the chromatin structure</u>
- Its recruitment seems to occur predominantly on highly transcribed genes
- SPT6 is also able to regulate transcription initiation through its histone chaperoning activities

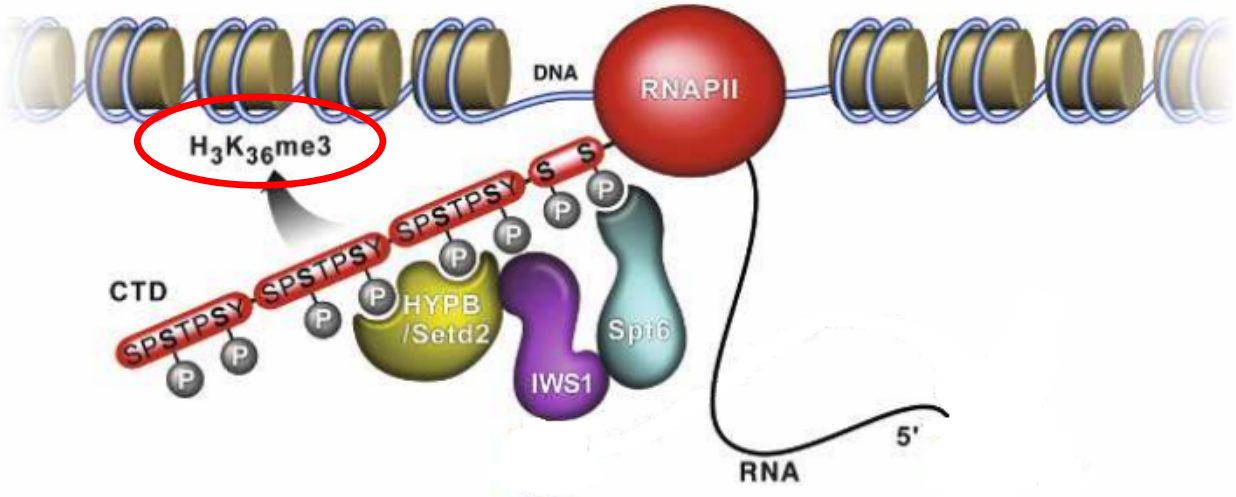


SPT6 and H3K36me3

- SPT6 has the ability to influence the chromatin environment across transcribed genes by affecting histone post-translational modifications

- SPT6 activity has been linked to methylation of lysine 36 of histone H3 (H3K36me)

Can SPT6 and H3K36me3 play a role in lncRNA transcription?

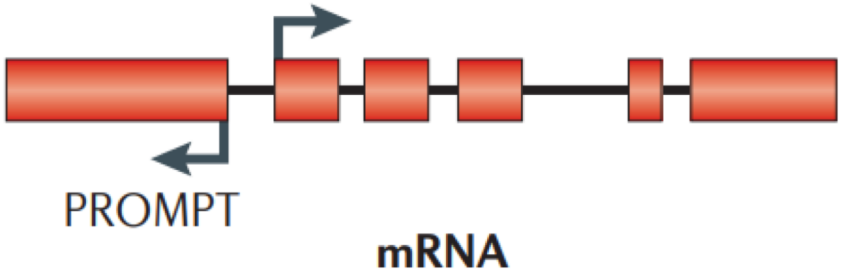


OUTSTANDING QUESTIONS

- Since both transcript classes are transcribed by Pol II, it is unclear how lncRNAs are selected for a different gene expression outcome to protein-coding genes → **What defines these different Transcription Units (TUs)?**
- Considering that SPT6 is a transcriptional elongation factor associated to Pol II → **How its deregulation affect gene expression?**
- Considering that these are short-lived and chromatin restricted RNA molecules, a deregulation of their transcription might compromise the cell functions → **Is important to restrict lncRNA expression?**

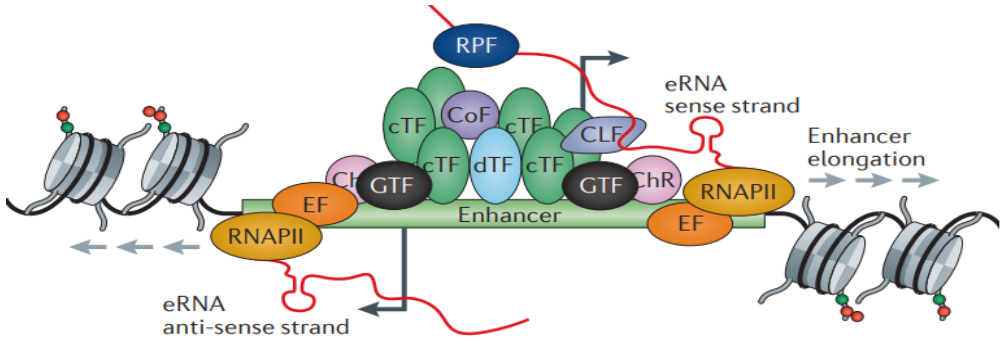
• PROMPTs - Promoter upstream transcripts

- PROMPTs are transcribed on the antisense orientation of bidirectional promoters
- PROMPTs are about 200–600 nt, with 5’-cap structures and 3’-adenosine tails
- They are prematurely terminated by cryptic PAS and eventually by the integrator complex
- PROMPTs levels could be altered under stress conditions and their accumulation may affect the transcription factors binding to promoters
- These transcript may have a functional role in the regulation of the associated protein-coding gene, and their alteration could be linked to cellular stress and tumorigenesis



• eRNA – Enhancer RNA

- eRNAs are usually less than 2000 nt in length and bidirectionally transcribed from enhancers by Pol II and they lack 3’ poly-A tail
- The Integrator complex is involved in the 3’-end cleavage of eRNA primary transcripts
- eRNA can affect the chromatin environment by promoting chromatin accessibility, stimulating the HATs activity of CBP or enhancing the binding of TFs
- They can also stabilize chromatin looping contacts between enhancer and promoters by recruitment of cohesin and can affect the transcription machinery already poised at promoters
- They exhibit tissue and lineage specificity, and serve as markers of cell fate and function



PROMPTs and eRNA are transcribed from loci which are crucial for the gene expression network, so an alteration of their cellular levels may have an important impact on the regulation of their target genes

An accumulation of these chromatin restricted RNAs may compromise the accessibility of the chromatin structure and its general function

Deregulated Expression of Mammalian lncRNA through Loss of SPT6 Induces R-Loop Formation, Replication Stress, and Cellular Senescence

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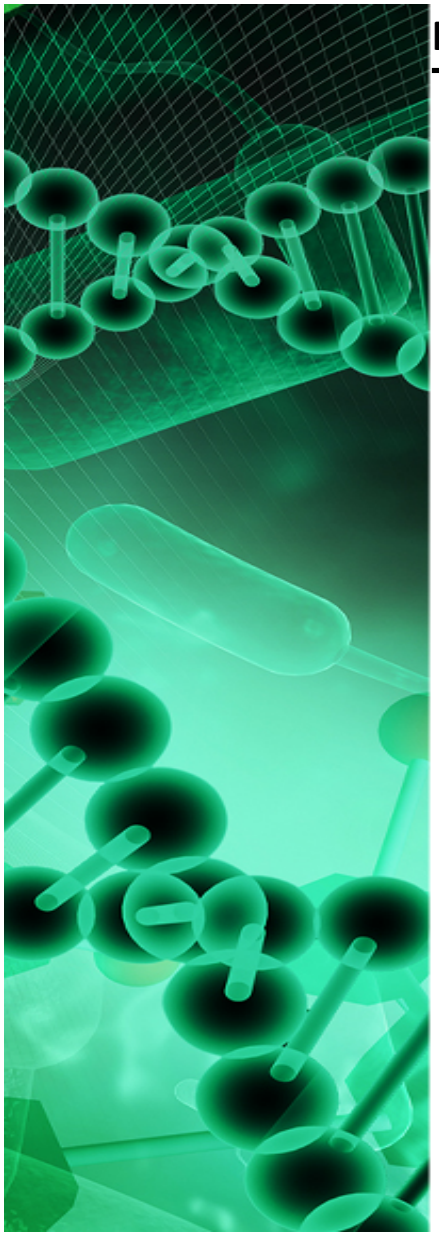
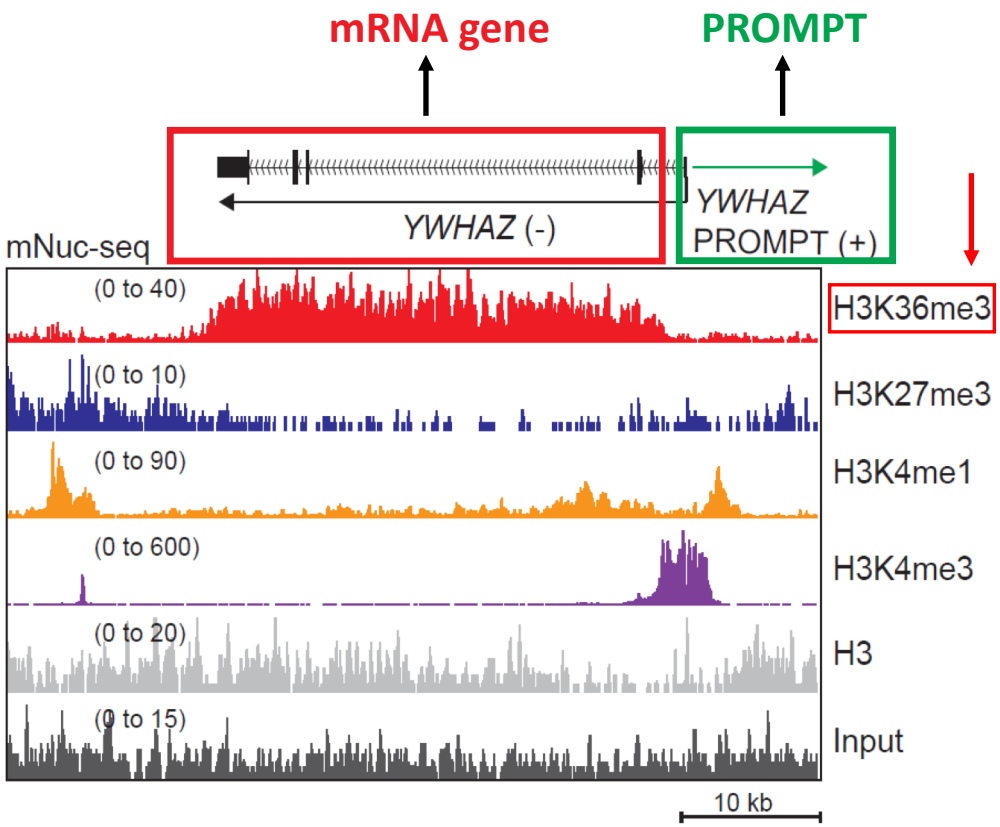


Key points

- **SPT6 promotes the selective distribution of H3K36me3 over protein-coding genes**
- SPT6 loss induce the formation of extended lncRNA transcript
- SPT6 depletion induce R-loop formation and DNA damage
- Deregulated Pol II collides with DNA replisome on lncRNA genes: cellular senescence as a consequence

How H3K36me3 is distributed between protein-coding genes and PROMPTs?



Aim: define the distribution of different histone marks between protein-coding and lncRNA genes produced from bidirectional promoters (PROMPTs, eRNA)

Strategy and methods:

- mNuc-seq: histone marks screening

Results:

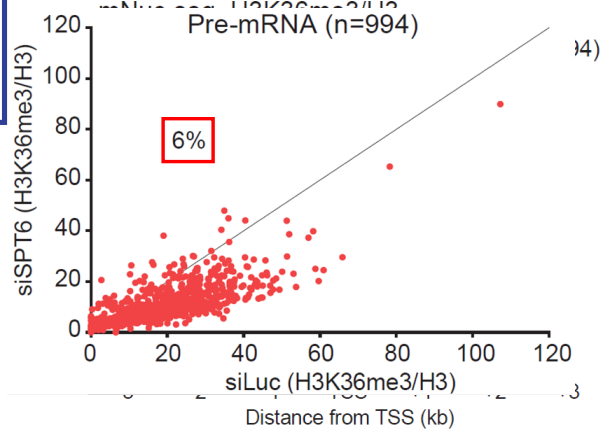
- **H3K36me3:** present over PCG
- **H3K27me3**: anti-correlated to H3K36me3
- **H3K4me1:** enhancer
- **H3K3me3:** promoter regions

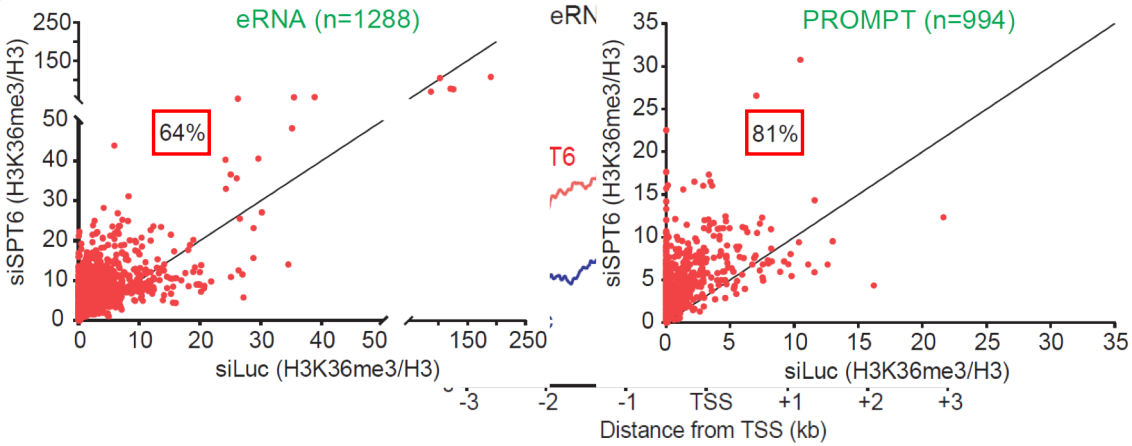
Conclusion: These analysis underline the exclusive presence of **H3K36me3** marks over most protein-coding but not lncRNA TUs

How SPT6 affects the H3K36me3 distribution?

siLuc: untreated HeLa cell

siSPT6: transfected HeLa





Aim: understand SPT6 role in H3K36me3 re-distribution

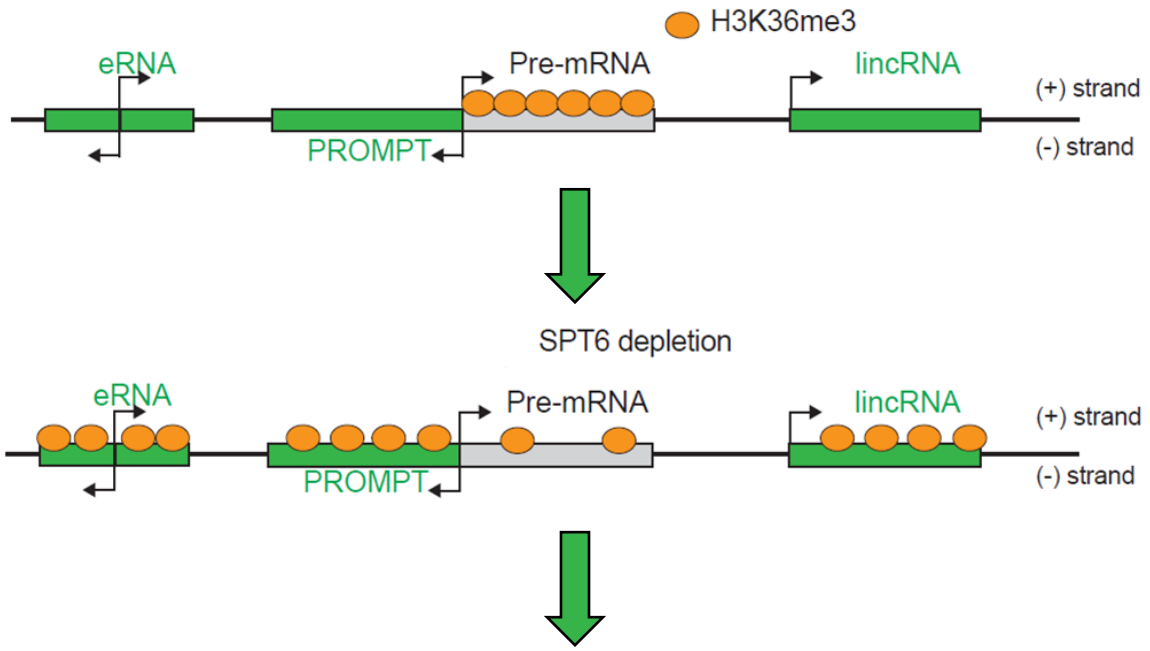
Strategy and methods:

- Cellular model: HeLa cells
- siRNA: SPT6 silencing in order to compare the distribution of the histone mark with or without SPT6

Results: after SPT6 depletion, a variation in H3K36me3 distribution is shown

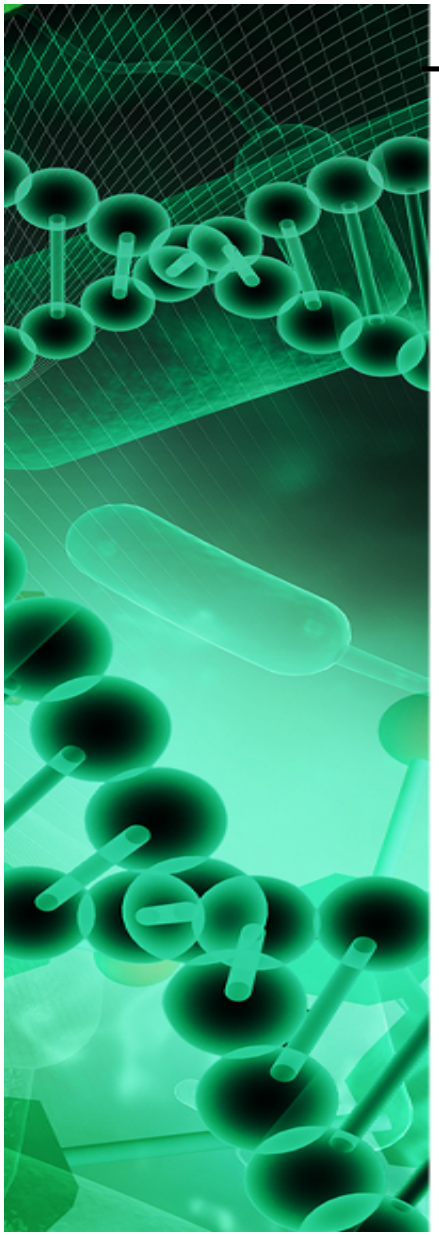
Conclusion: SPT6 depletion induces the loss of H3K36me3 on protein-coding genes associated with divergent TUs, but its increase on PROMPTs and eRNAs

TAKE HOME MESSAGE



SPT6 plays a critical role in defining the specificity of H3K36me3 between pre-mRNA and lncRNA genes

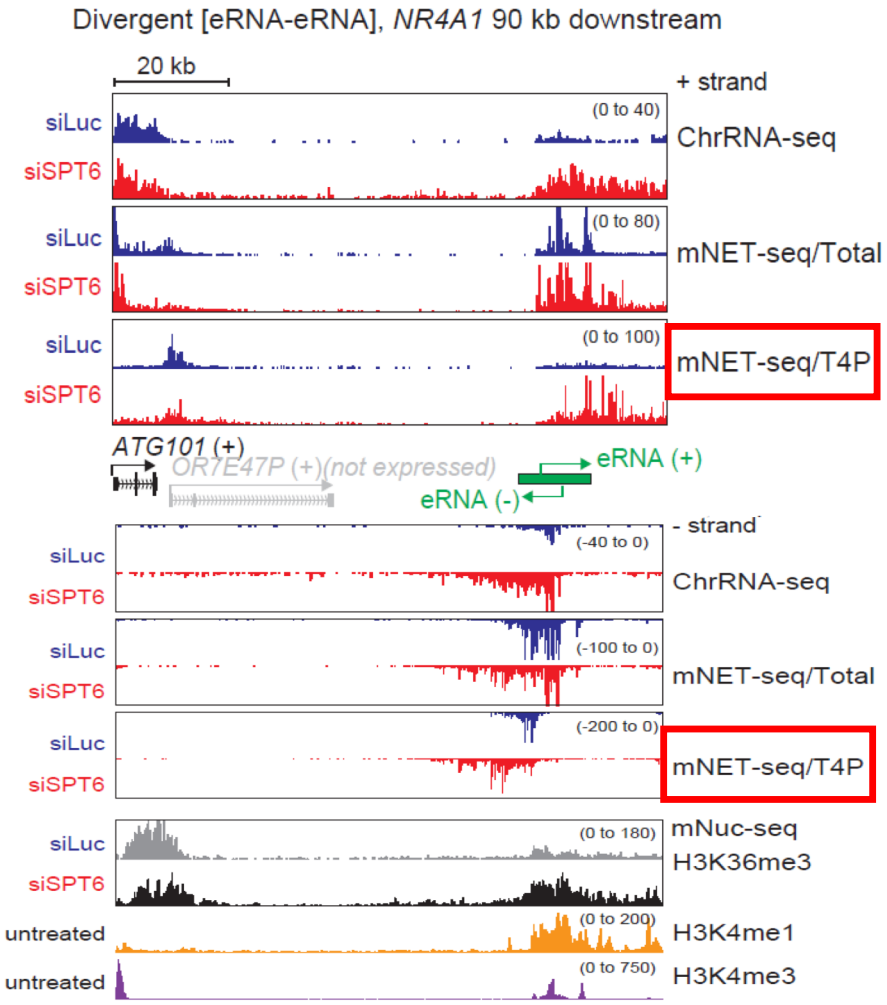
These results suggest that SPT6 depletion oppositely affects protein-coding and lncRNA genes, and that this protein might play a role in defining Pol II TUs across the human genome. This may imply that Pol II can distinguish coding or noncoding TUs by their histone methylation status.



Key points

- **SPT6 loss induce the formation of extended lncRNA transcript**

Does SPT6 depletion affect PROMPTs/eRNA transcription?



Aim: evaluate the rate of lncRNAs transcription in SPT6-depleted cells, across divergent TUs (PROMPTs-pre-mRNA and eRNAs)

Strategy and methods:

- mNET-seq/T4P: total and phopsho-Thr4 (T4P) antibody to establish the transcription termination profile

Results: mNET-seq signals is present over the entire PROMPT/eRNA region

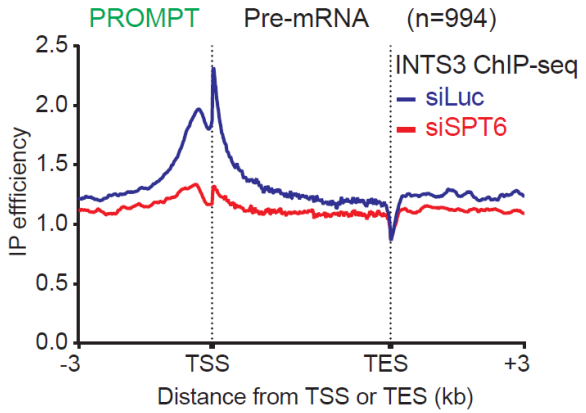
Conclusion: the absence of SPT6 induces a transcription termination defect and enhances the lncRNA expression

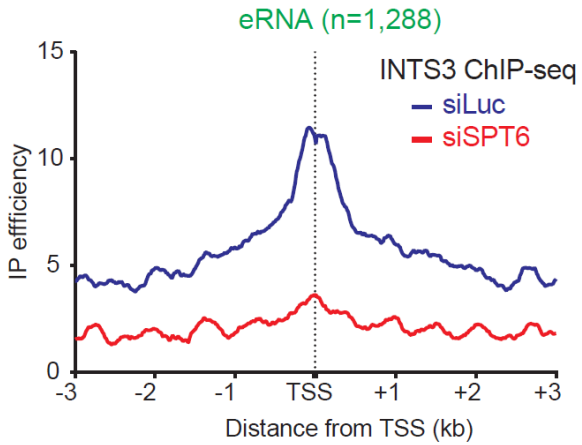
- ✓ **lncRNA (PROMPTs, eRNAs): extended transcription**
- ✓ **SPT6 favors expression of productive pre-mRNA over non-productive lncRNA**

Which is the SPT6 function in lncRNA transcription termination?

siLuc: untreated HeLa cell

siSPT6: transfected HeLa





Aim: describe the putative role of SPT6 in the recruitment of the Integrator complex, in order to allow the lncRNA transcription termination (eRNA, PROMPTs)

Strategy and methods:

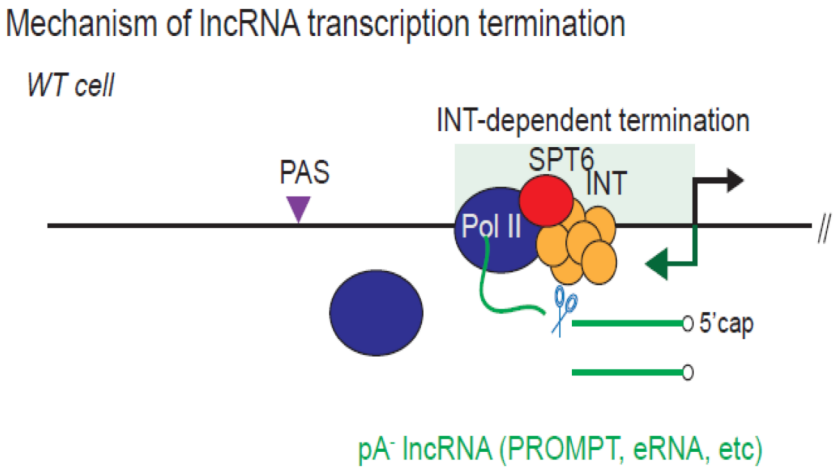
- ChIP-Seq: analysis with specific antibody against a component of the Integrator complex, INTS3

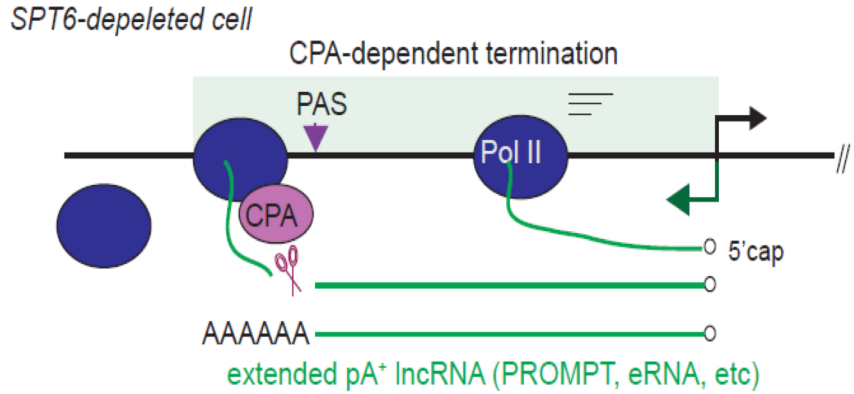
Result: INT3 ChIP-seq peaks are decreased following SPT6 depletion in both PROMPTs and eRNA regions

Conclusion: SPT6 plays an important role in the Integrator complex recruitment at the TSS of the lncRNA TUs and its depletion induces extended lncRNA

TAKE HOME MESSAGE

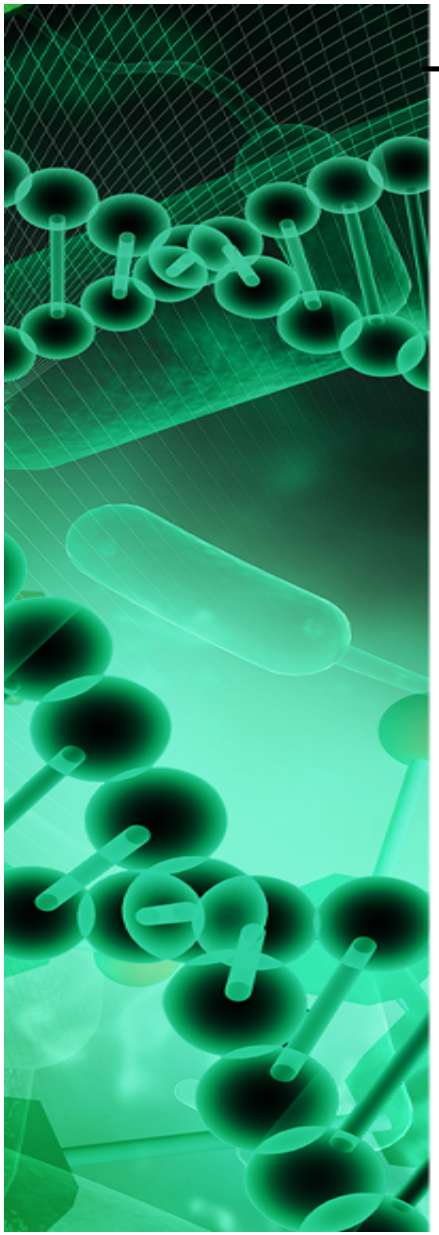
✓ **SPT6 loss leads to formation of extended lncRNAs**





✓ **In wild-type cells, SPT6 facilitates recruitment of The Integrator complex to promote cleavage of lncRNA near their TSS. This generates short pA– lncRNA**

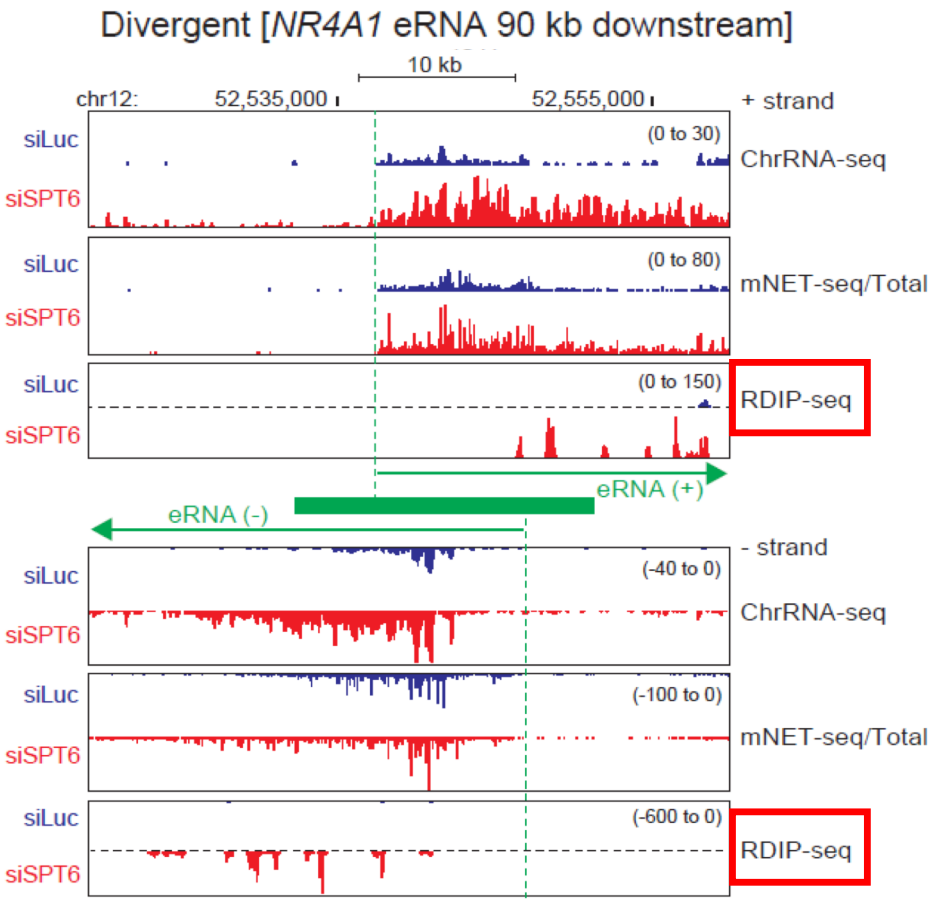
✓ **In SPT6-depleted cells, Pol II fails to recruit the Integrator complex, resulting in extended transcripts. This will result in the formation of polyadenylated transcripts coupled with downstream termination**



Key points

- **SPT6 depletion induce R-loop formation and DNA damage**

Which are the consequences of the termination defect?



Aim: define the correlation between the aberrant transcription induced by SPT6 depletion and R-loop formation, comparing protein-coding and lncRNA genes (PROMPTs, eRNAs)

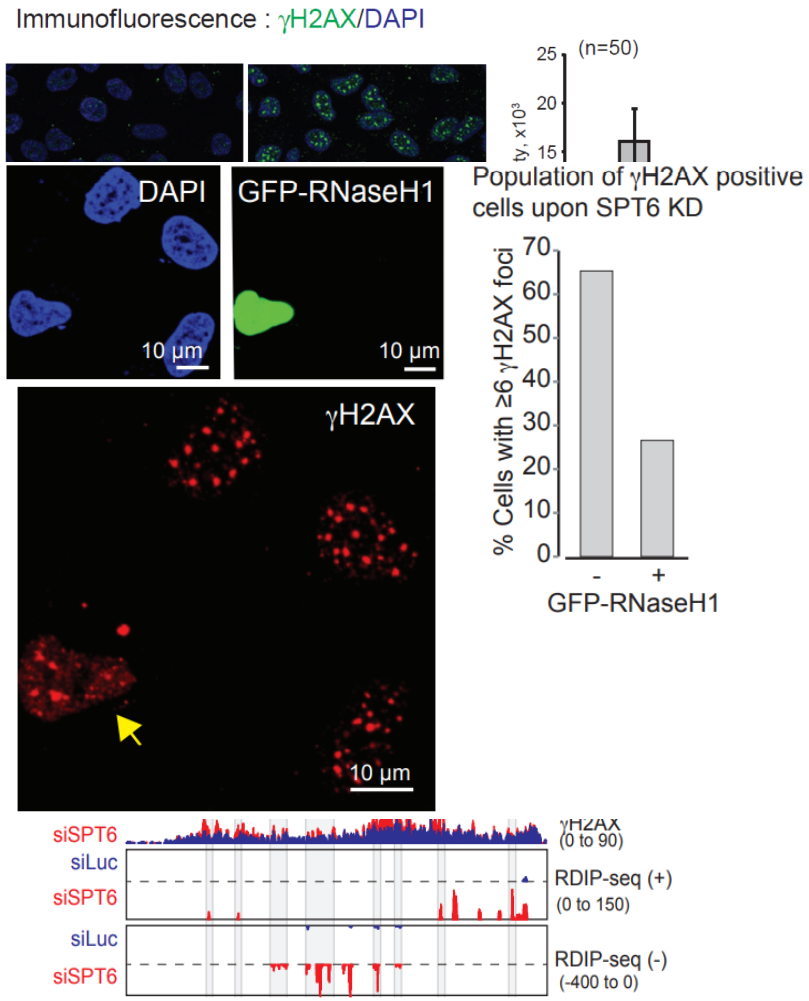
Strategy and methods:

- RDIP-seq: genome-wide analyses of DNA:RNA hybrid (R-loops) on HeLa cells, with or without SPT6

Result: PROMPTs/eRNA show increased RDIP-seq signals over their TUs

Conclusion: SPT6 depletion induces R-loop formation, which occurs selectively on PROMPTs and eRNA regions, but not on protein coding genes

Which are the consequences of the termination defect?



Aim: identify the putative role of SPT6 in genome maintenance

Strategy and methods:

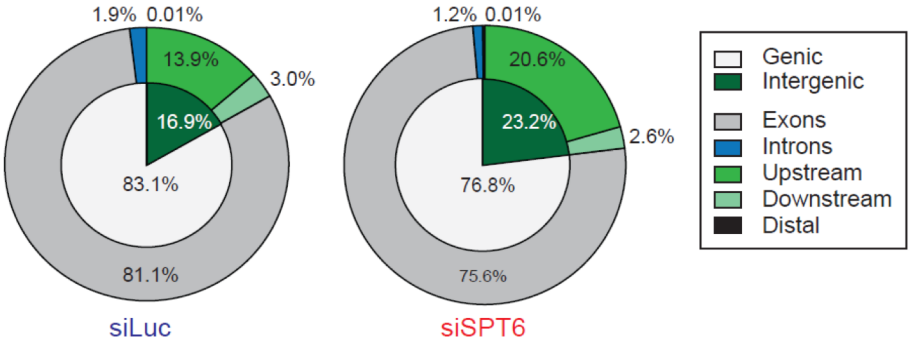
- Immunofluorescence analysis: γ-H2A.X foci increase in SPT6-depleted cells
- ChIP-seq: distribution of γ-H2A.X on chromatin
- Comparison between Chip-seq and RDIP signals in both control and STP6-depleted cells
- RNaseH1 overexpression: confirm that DNA damage is related to R-loop formation

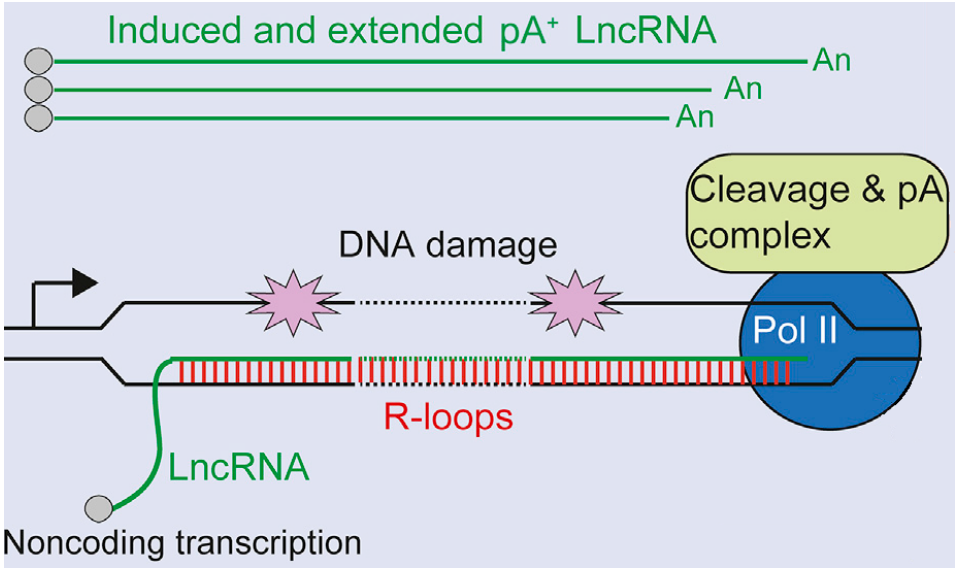
Result: 6-fold increase of γ-H2A.X foci and their distribution overlaps with RDIP-seq signals

Conclusion: we can assess that SPT6 prevents R-loop formation and consequent DNA damage

TAKE HOME MESSAGE

✓ **SPT6 loss leads to formation of extended lncRNAs that are prone to R-loop formation and consequent DNA damage**

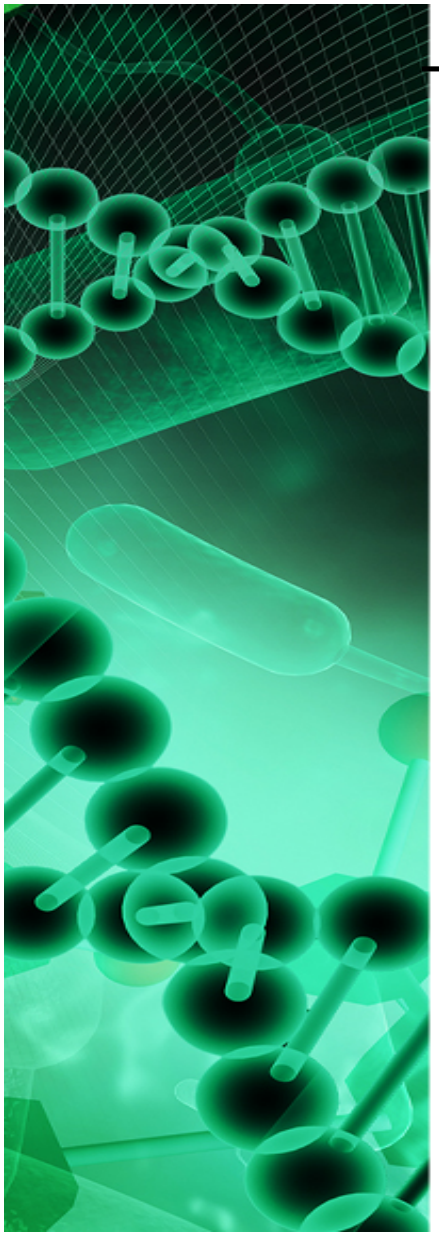




✓ **lncRNAs induction by SPT6 depletion induces R-Loop formation and accumulation**

✓ **The induction of R-loops genome-wide following SPT6 depletion occurs selectively on PROMPT and eRNA regions, but not on protein-coding genes**

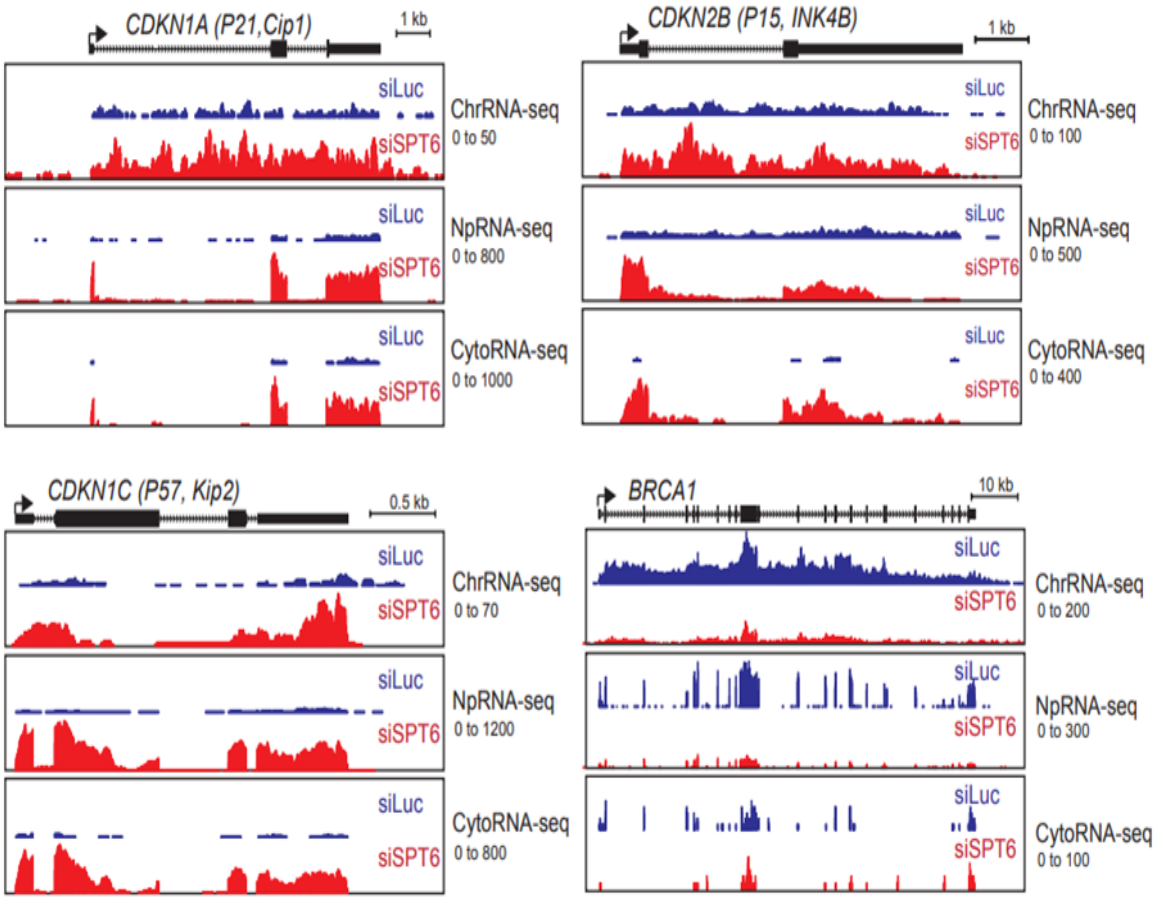
✓ **R-loop induce DNA damage**



Key points

- **Deregulated Pol II collides with DNA replisome on lncRNA genes: cellular senescence as a consequence**

Does DNA damage affect CDK-inhibitors expression pattern?



Aim: understand if the deregulated lncRNA expression leads to a cell-cycle defect

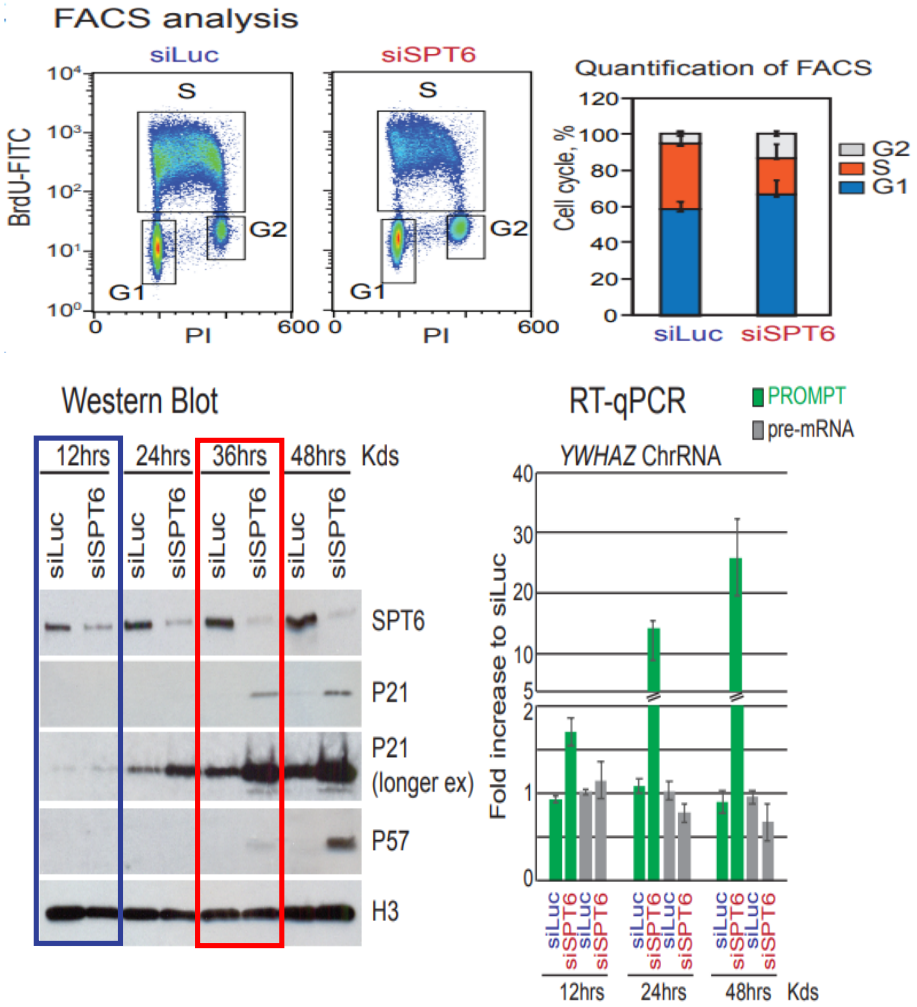
Strategy and methods:

- DEseq2: differential expression analysis of cell-cycle regulators and DNA-damage response genes

Result: 2.722 mRNAs were significantly upregulated based on DESeq2 analysis, in particular cell-cycle inhibitors

Conclusion: SPT6 loss induce enhanced expression of cell-cycle inhibitors and cell-cycle arrest

Is the lncRNAs deregulation that leads to the cell-cycle arrest?



Aim: confirm that the lncRNA aberrant expression is an upstream event to the cell-cycle arrest

Strategy and methods:

- FACS: evaluate the cellular DNA content
- Time-course transfection experiment to verify the relationship between CDK-inhibitors expression and lncRNA transcription

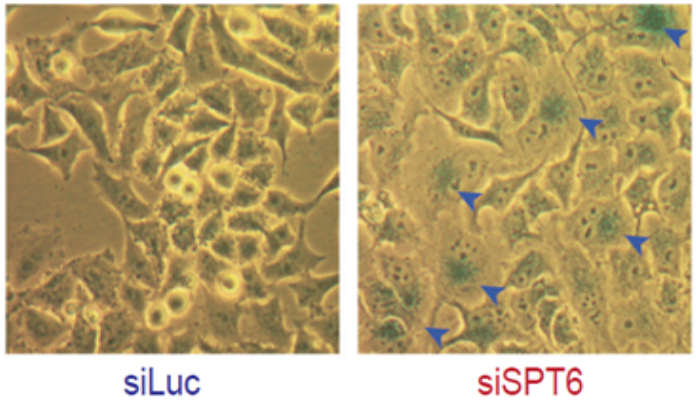
Result:

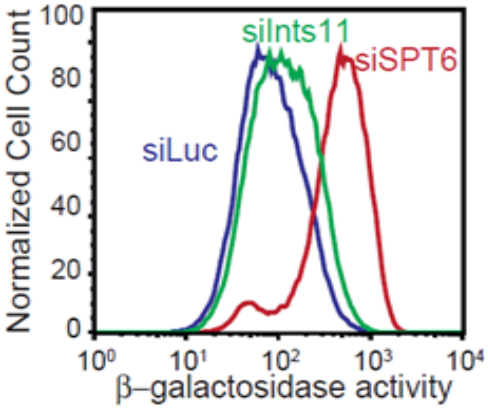
- indicates that SPT6-depleted cells display G1-S and G2-M transition defects, which is consistent with a cell-cycle defect.
- PROMPTs expression occurs by 12h, while p21 and p57 show a slower kinetics response (after 36h)

Conclusion: lncRNA deregulated expression following SPT6 depletion induce the overexpression of CDK-inhibitors

The deregulation of lncRNA expression leads to senescence?

β-galactosidase staining assay (Cellular senescence)





Aim: evaluate the cellular senescence phenotype of STP6-depleted cells

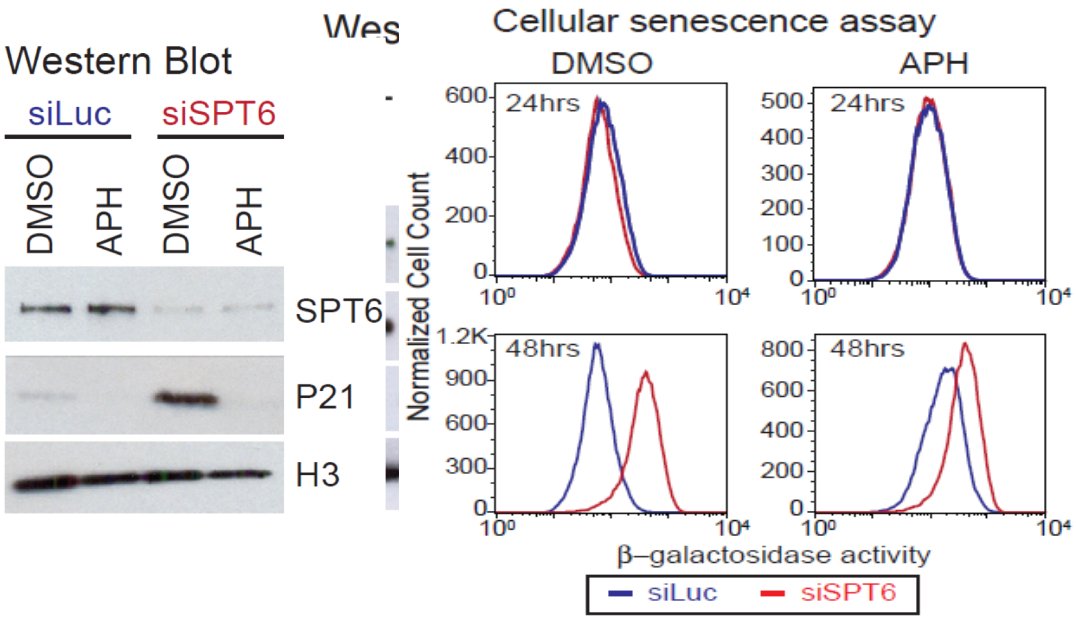
Strategy and methods:

- β-gal activity assay: blue arrows indicate β-gal positive cells

Result: SPT6-depleted cells are detectible by microscopy after β-gal staining and confirmed by cell-sorting

Conclusion: SPT6 depletion leads to cellular senescence phenotype

R-loop or replicative stress: which one leads to the cell-cycle arrest?



Aim: determine the main cause of cellular senescence

Strategy and methods:

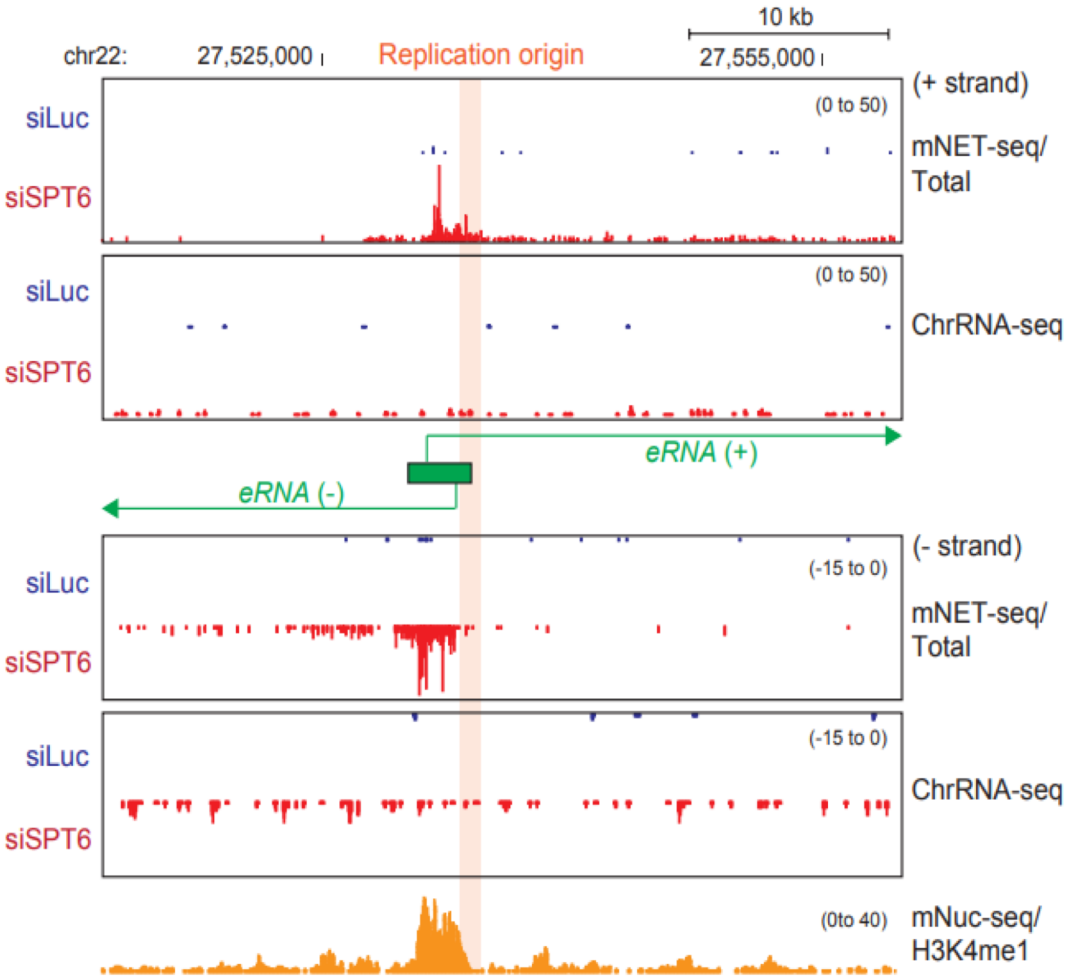
- RNaseH1 overexpression: evaluate if R-loops induce senescence
- APH treatment: inhibition of DNA pol

Result:

- P21 levels are not suppressed by V5-RNase H1 overexpression
- P21 is strongly inhibit after APH treatment
- In addition, β-gal activity is decreased after APH treatment in siSPT6-cells

Conclusion: SPT6 depletion induces cellular senescence in a replication-dependent manner

The extended lncRNA transcription causes a conflict between transcription and replication?



Aim: understand if the replication stress is related to the transcription-DNA replisome collision at intergenic replication origin

Strategy and methods:

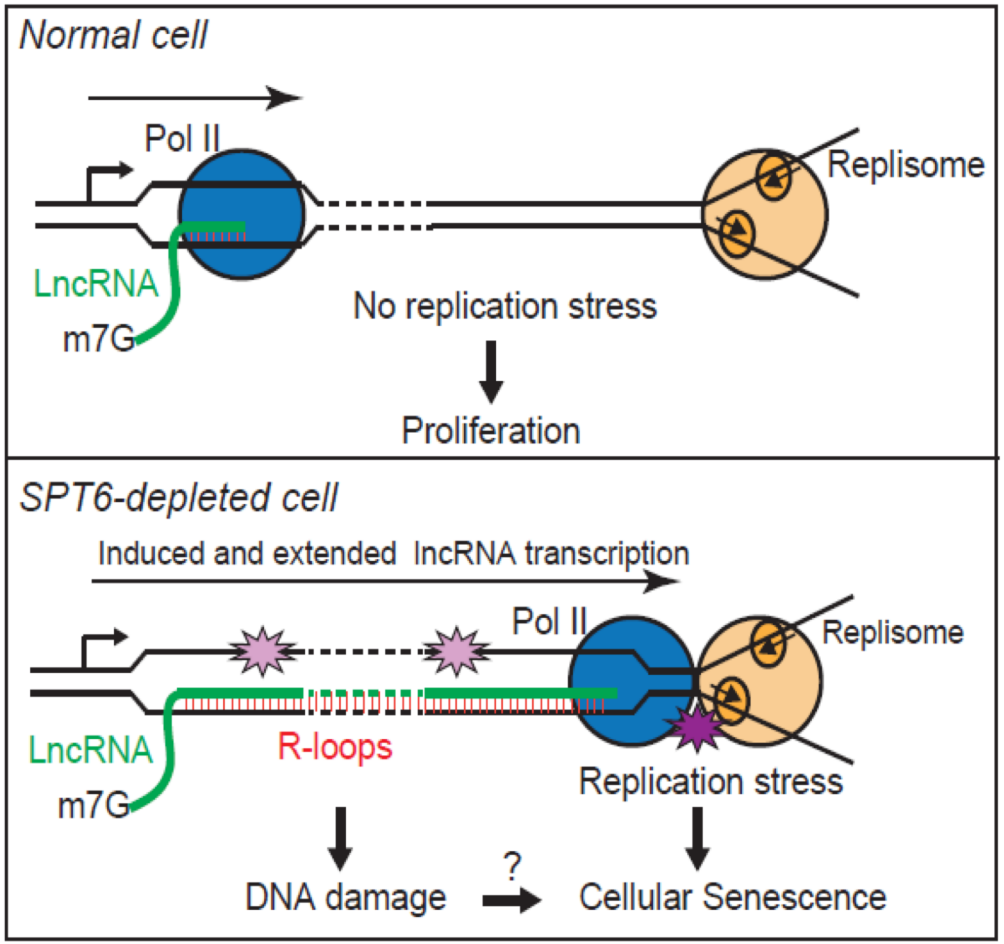
- mNET-Seq: analysis of the elongating Pol II over DNA replication origins in intergenic regions
- The location of intergenic replication origins were compared to the location of PROMPTs and eRNAs annotated in the PrESSTo database

Result: at least half of these origins overlap with extended lncRNA induced by depletion of SPT6

Conclusion: these results connect intergenic transcription-replication conflict with DNA stress and cellular senescence

TAKE HOME MESSAGE

SPT6 loss leads to replication-transcription collision, replicative stress and cellular senescence



- ✓ Intergenic DNA replication origin regions overlap with lncRNA extended transcription
- ✓ These results connect intergenic transcription-replication conflict with DNA stress and cellular senescence
- ✓ Deregulated lncRNA transcription after SPT6 depletion leads to cellular senescence

DISCUSSION AND CONCLUSIONS

- ✓ The expression of many lncRNAs is restricted, implying that their accumulation may be deleterious to the cell

- ✓ In SPT6-depleted cells all major classes of lncRNA considered, show enhanced nascent transcription levels and generate extended transcripts

- ✓ This deregulated transcription events lead to R-loop formation, transcription-replication collision and cellular senescence

- ✓ **SPT6 may acts to prevent such collision and so maintains genome stability**