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Dipartimento di
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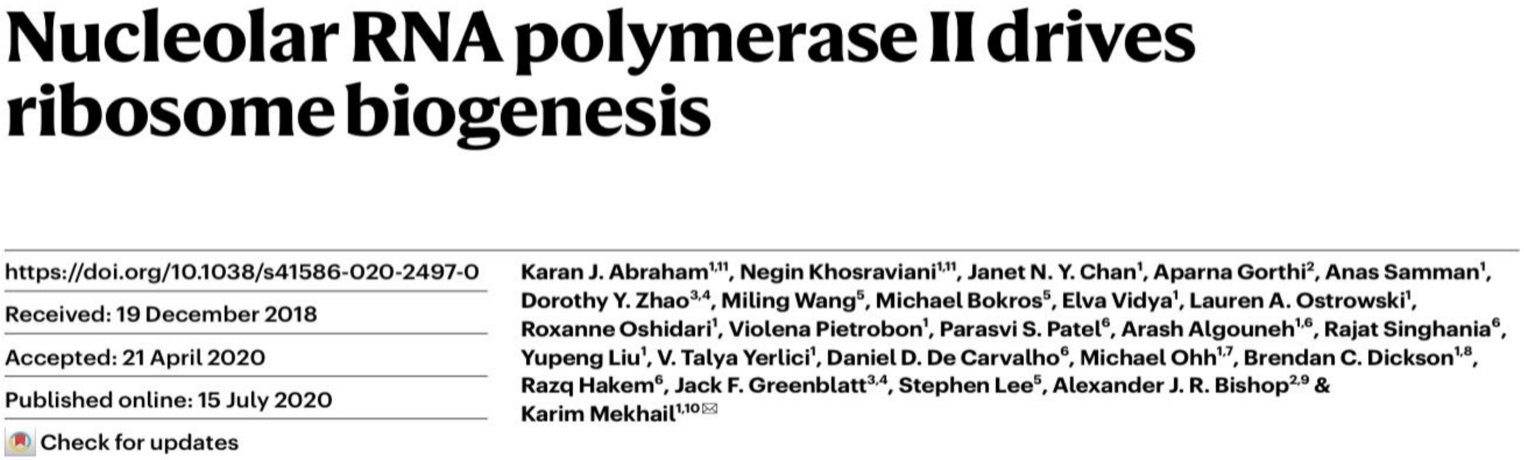
Nucleolar RNA polymerase II drives ribosome biogenesis

Biz Giulia, Rinaldin Mirko



Nucleolar RNA polymerase II drives ribosome biogenesis

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Karan J. Abraham[1,11], Negin Khosraviani[1,11], Janet N. Y. Chan[1], Aparna Gorthi[2], Anas Samman[1], Dorothy Y. Zhao[3,4], Miling Wang[5], Michael Bokros[5], Elva Vidya[1], Lauren A. Ostrowski[1], Roxanne Oshidari[1], Violena Pietrobon[1], Parasvi S. Patel[6], Arash Algouneh[1,6], Rajat Singhania[6], Yupeng Liu[1], V. Talya Yerlici[1], Daniel D. De Carvalho[6], Michael Ohh[1,7], Brendan C. Dickson[1,8], Razq Hakem[6], Jack F. Greenblatt[3,4], Stephen Lee[5], Alexander J. R. Bishop[2,9] &
Karim Mekhail[1,10] ✉

Outline

Introduction → Experimental results → Discussion → Conclusion

- Nucleolar localisation of Pol II;
- Pol II operates directly at the level of IGS by generating asyncRNA;
- Pol II forms an antisense R-loop that restricts the synthesis of Pol-I-dependent syncRNAs;
- SyncRNA accumulation drives nucleolar disorganisation;
- Senatassin supports the R-loop shield;

Ribosome biogenesis: the nucleolus

Main components of the **nucleolus**:

- **Fibrillary center (FC)**: involved in the transcription of rRNA genes;
- **Dense fibrillar component (DFC)**: contains the rRNA processing machinery;
- **Granular component (GC):** Accumulation site of ribonucleoprotein particles involved in the ribosomal assembly process.

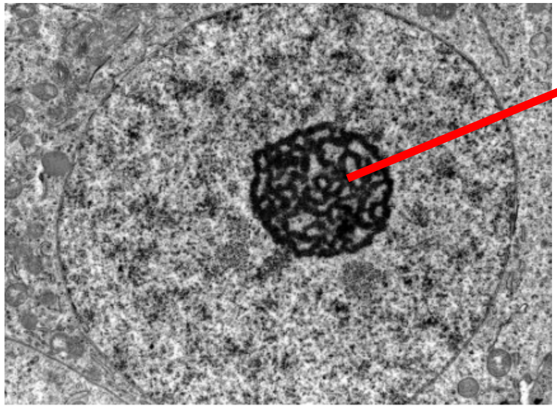
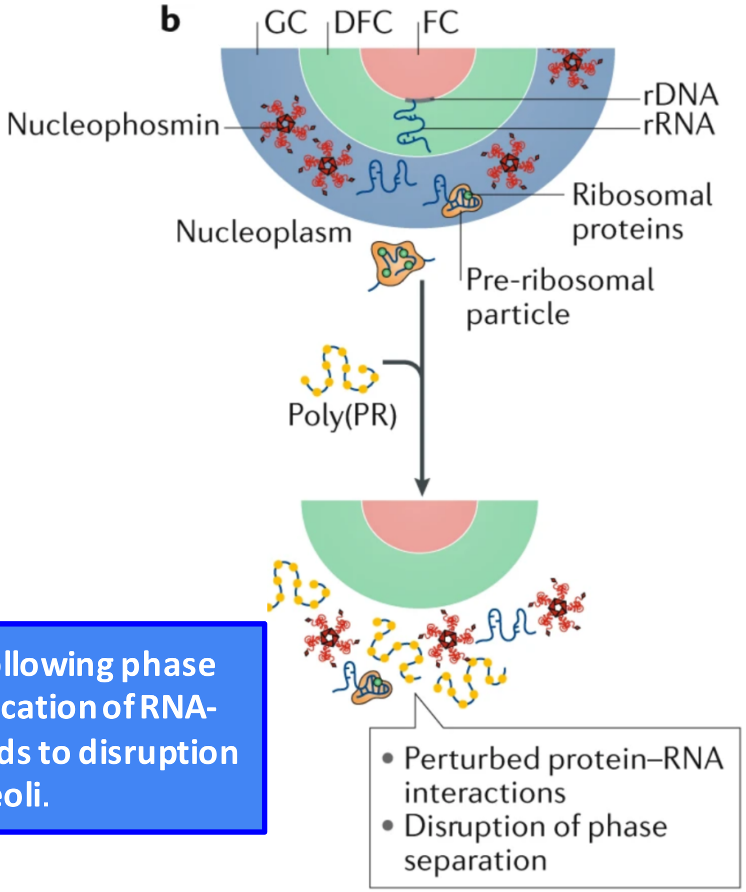


Fig.1 Histological representation of the nucleolus

The nucleolus arises following phase separation: the modification of RNA-protein interactions leads to disruption of the nucleoli.



Ribosome biogenesis: major characters

1. rDNA and ribosomal subunit

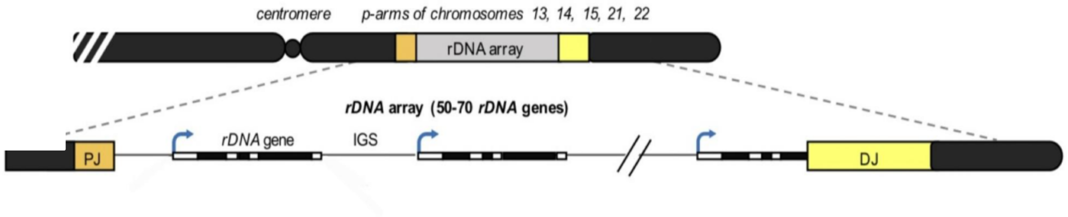


Fig.3 Schematic representation of the rDNA array on the p-arms of five acrocentric chromosomes.

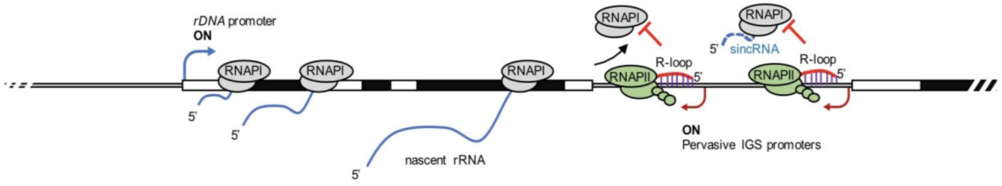


Fig.4 Localization of R-loops in IGS of rDNA array

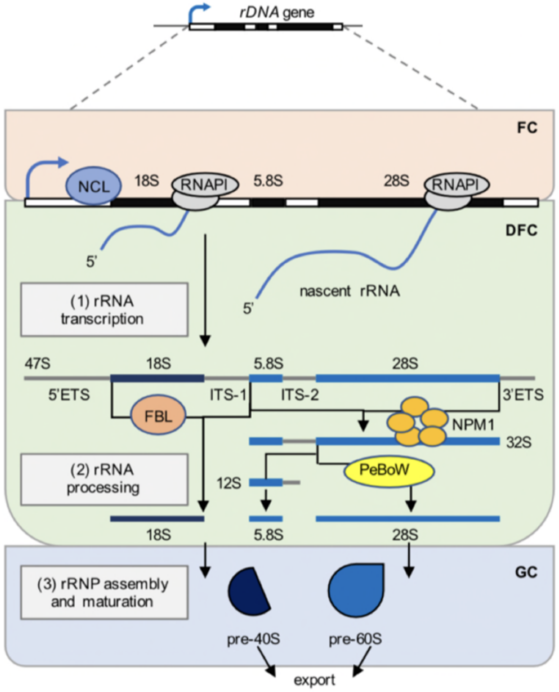


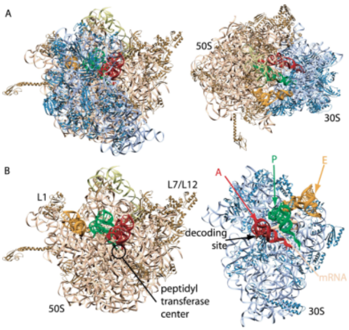
Fig.5 Pre-rRNA maturation in human cells. Simplified processing pathway of the 40S and 60S pre-rRNA in human cells.

Ribosome biogenesis: major characters

2. Polymerase

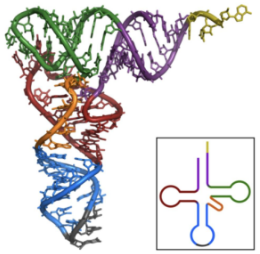
Polymerase I

rRNA



Polymerase III

tRNA



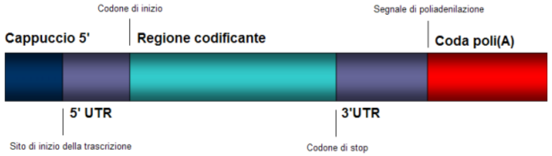
rRNA

5S

Polymerase II

snRNA

mRNA



Regulatory function?

Non-protein coding genes

Protein coding genes

Ribosome biogenesis: major characters

3. RNAPII-dependent small nucleolar RNAs (snoRNAs).

After transcription, nascent rRNA molecules (called pre-rRNAs) undergo a series of processing steps to generate the mature rRNA molecule. These include methylations(C/D box) and pseudouridylations(H/ACA), driven by snoRNAs.

Each snoRNA associates with at least four basic proteins in an RNA/protein complex called a small nucleolar ribonucleoprotein particle (snoRNP).

⇩

The proteins associated with each RNA depend on the type of snoRNA molecule and catalyze a certain modification.

⇩

The snoRNA molecule contains an antisense element that is complementary to the sequence surrounding the nucleotide to be modified in the pre-RNA molecule.

⇩

Once the snoRNP has bound to the target site, associated proteins catalyze the chemical modification of the target base.

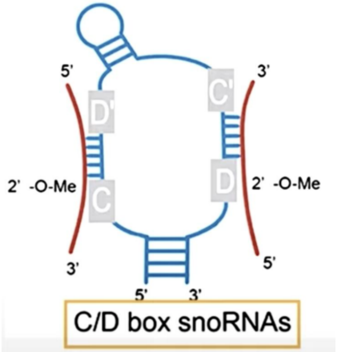


Fig.6 Schematic representation of the C/D snoRNAs

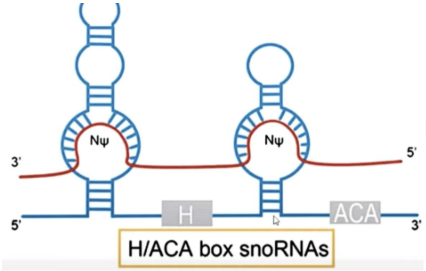


Fig.7 Schematic representation of the H/ACA snoRNAs

Ribosome biogenesis: major characters

4. Ribosomal production and processing factors

Nucleolin (NCL)

Nucleolin is one of the most abundant nucleolar proteins.

Nucleolin has several function:

- It induces chromatin decondensation (binding to histone H1);
- It is involved in the process of transcriptional elongation and it can act as a transcriptional coactivator
- Nucleolin could act as a carrier for ribosomal proteins from the cytoplasm to the nucleolus and as an adaptor for specific binding of ribosomal proteins to rRNA

Nucleophosmin (NPM)

The nucleophosmin are a family of nuclear chaperones → ensure the correct assembly of nucleosomes and the correct formation of higher-order chromatin structures. NPM1 lead the accumulation of the late processing machinery of the large ribosomal subunits in the nucleolus.

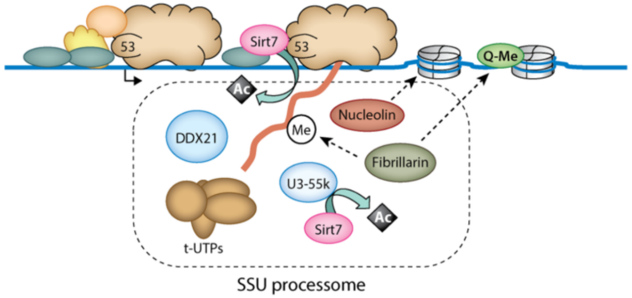
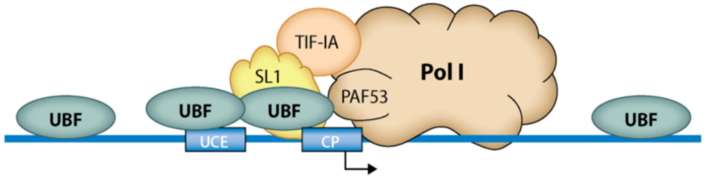


Fig.8 The SSU processome contains several associated factors with dual functions in Pol I transcription and early rRNA maturation steps.

Upstream binding factor (UBF)

UBF molecules are involved in both accessible transcription and have an architectural role → They bind to the entire ribosomal gene and generate a loose ribosomal chromatin structure



- Fig.9 Factors mediating Pol I initiation and coordination of pre-rRNA synthesis with processing.

Ribosome biogenesis: assembly pathway

1. Synthesis of pre-ribosomal RNA (pre-RNA) and **r** proteins.
2. Modification of the bases of the pre-rRNA.
3. Pre-rRNA folding.
4. Assembly of pre-rRNA with **r** proteins.
5. Exonucleolytic processing of pre-rRNA to remove external and internal transcribed spacer rRNAs (5′-ETS, ITS1, ITS2, 3′-ETS).

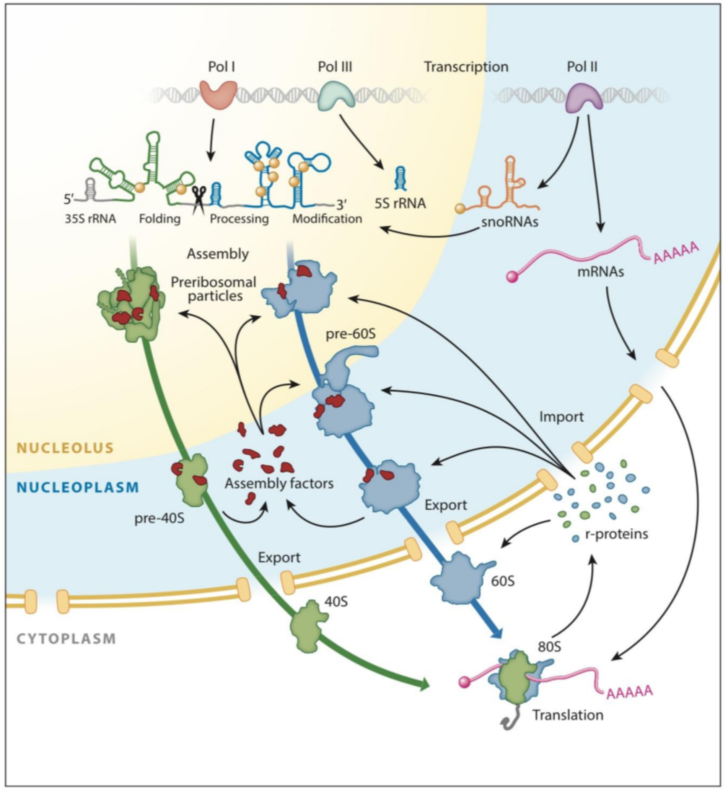


Fig.10 Overview of the eukaryotic ribosome assembly pathway.

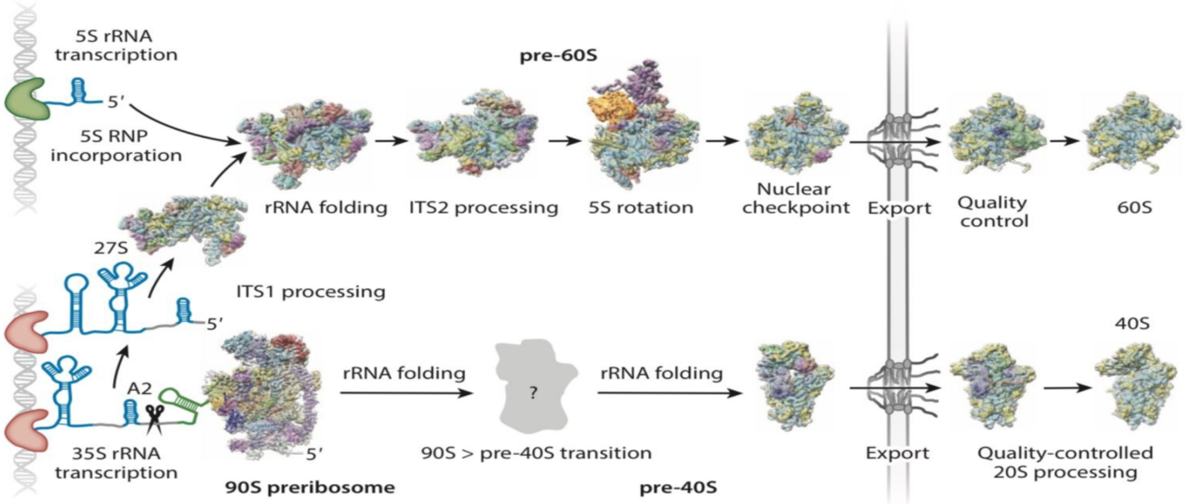


Fig.11 Structural snapshots of the eukaryotic ribosome assembly pathway.

Ribosome biogenesis: the regulation

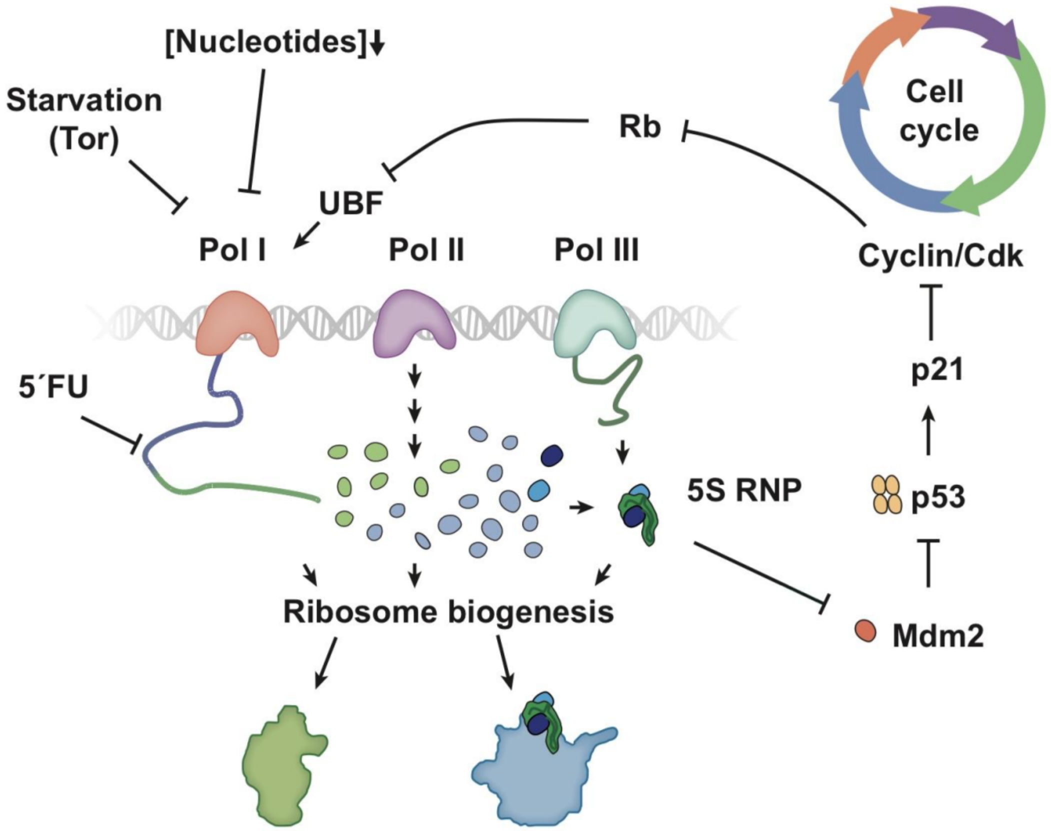


Fig.12 Ribosome biogenesis depends on the activity of all three RNA polymerases.

- CDK1
- When is downregulate and when is upregulate?
- And in what phases?

Experimental observation

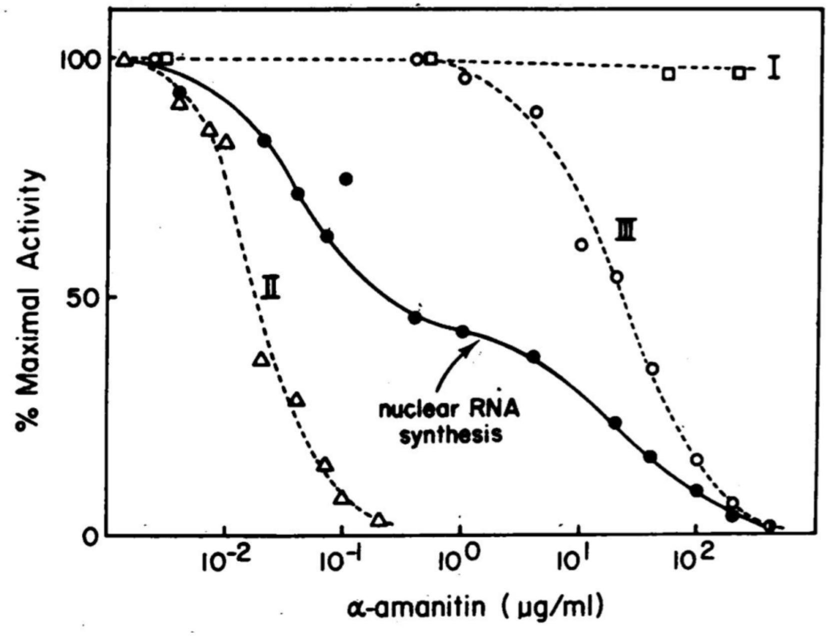


Fig.13 Effect of α-amanitin concentration on purified RNA polymerases and on endogenous RNA polymerase activity in isolated nuclei.
(Oliver H. Lowry, May 22 ,1974)

RNAPII inhibition by the fungal venom α-amanitin destroys nucleoli, although RNAPI activity is not affected by the drug.

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Proteins are manufactured by ribosomes—macromolecular complexes of protein and RNA molecules that are assembled within major nuclear compartments called nucleoli[1,2]. Existing models suggest that RNA polymerases I and III (Pol I and Pol III) are the only enzymes that directly mediate the expression of the ribosomal RNA (rRNA) components of ribosomes. Here we show, however, that RNA polymerase II (Pol II) inside human nucleoli operates near genes encoding rRNAs to drive their expression. Pol II, assisted by the neurodegeneration-associated enzyme senataxin, generates a shield comprising triplex nucleic acid structures known as R-loops at intergenic spacers flanking nucleolar rRNA genes. The shield prevents Pol I from producing sense intergenic noncoding RNAs (sincRNAs) that can disrupt nucleolar organization and rRNA expression. These disruptive sincRNAs can be unleashed by Pol II inhibition, senataxin loss, Ewing sarcoma or locus-associated R-loop repression through an experimental system involving the proteins RNaseH1, eGFP and dCas9 (which we refer to as 'red laser'). We reveal a nucleolar Pol-II-dependent mechanism that drives ribosome biogenesis, identify disease-associated disruption of nucleoli by noncoding RNAs, and establish locus-targeted R-loop modulation. Our findings revise theories of labour division between the major RNA polymerases, and identify nucleolar Pol II as a major factor in protein synthesis and nuclear organization, with potential implications for health and disease.

QUESTION 1 : Is the nucleolar disruption, established with α-amanitin, due to an actual presence of Pol II at the level of nucleoli?

Experimental approach

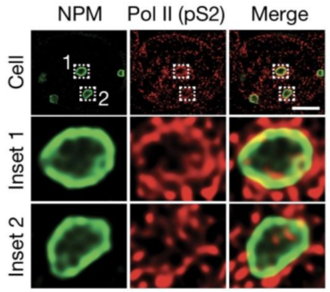
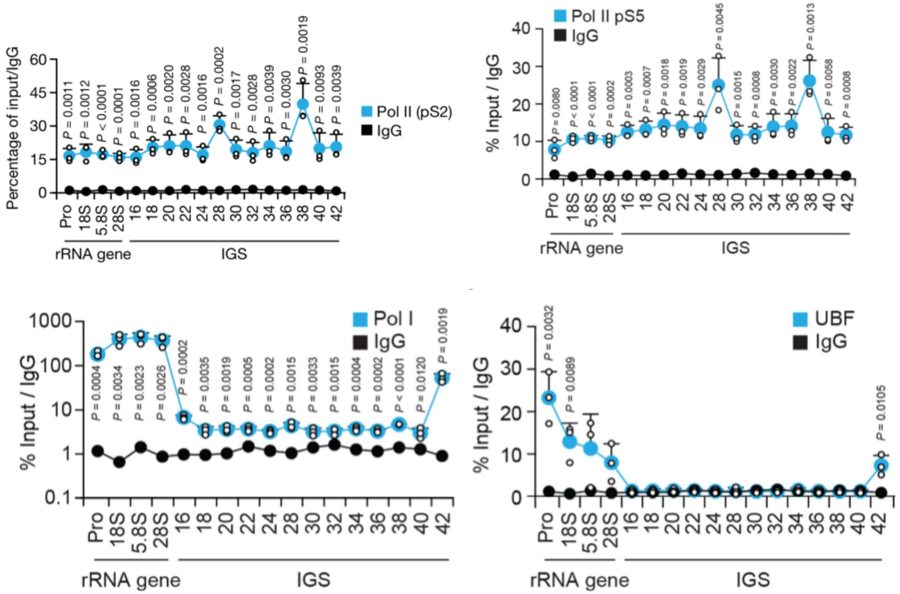


Fig.14 Immunofluorescence coupled with super-resolution microscopy to detect NPM and Pol II (pS2).

It was observed that within the nucleoli, delineated by nucleophosmin (NPM), there are foci corresponding to active Pol II (pS2)



Observations following chromatin immunoprecipitation (ChIP):

- Pol II (pS2) and Pol II (pS5) are enriched throughout the rDNA, with the highest levels at IGS28 and IGS38.
- Pol I and its initiation factor (UPF) are mainly located in rRNA genes, although low levels of Pol I exist in all IGS.
- Pol II was overrepresented compared with Pol I only within IGSs.

Results: These data suggest that rDNA loci are cohabited by Pol I and Pol II.

Fig.15. Pol I and Pol II localize to rDNA IGSs.

QUESTION 2: Is Pol II activity directly involved in rRNA expression?

Is Pol II activity directly involved in rRNA expression?

Methodological approach: RNA Pulse-Chase assay

Fig.16 Cell-population-based RNA pulse-chase assay used to assess pre-rRNA synthesis and processing.
EU→Ethylene uridine

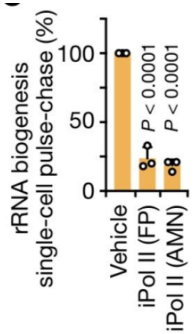


Fig.17 Effect of a 3-hour Pol II inhibition (iPol II) using FP and AMN

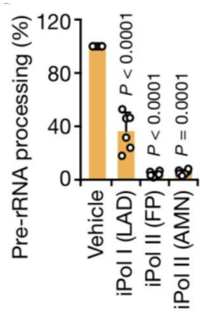


Fig.18 RNA pulse-chase assays were used to assess pre-rRNA processing following a 3-hour inhibition of Pol I or Pol II

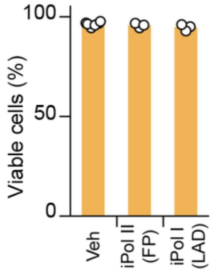


Fig.19 Trypan blue exclusion assay confirms that the 3-hour Pol II inhibition (iPol) do not compromise cell viability.

Results:
Pol II could directly support nucleolar rRNA expression.

<u>Inhibition of Pol II</u> with α-amanitin or flavopiridol <u>perturbed global ribosomal biogenesis</u>

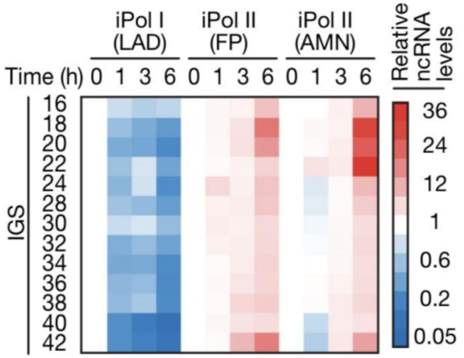
Pol II inhibition almost completely abolished pre-rRNA processing → <u>Direct function of Pol II through its enrichment in rDNA</u>

Cell viability and overall protein levels remained unchanged after Pol II inhibition → <u>no indirect effects</u>

QUESTION 3: How is Pol II involved in the regulation of rRNA expression?

How is Pol II involved in the regulation of rRNA expression?

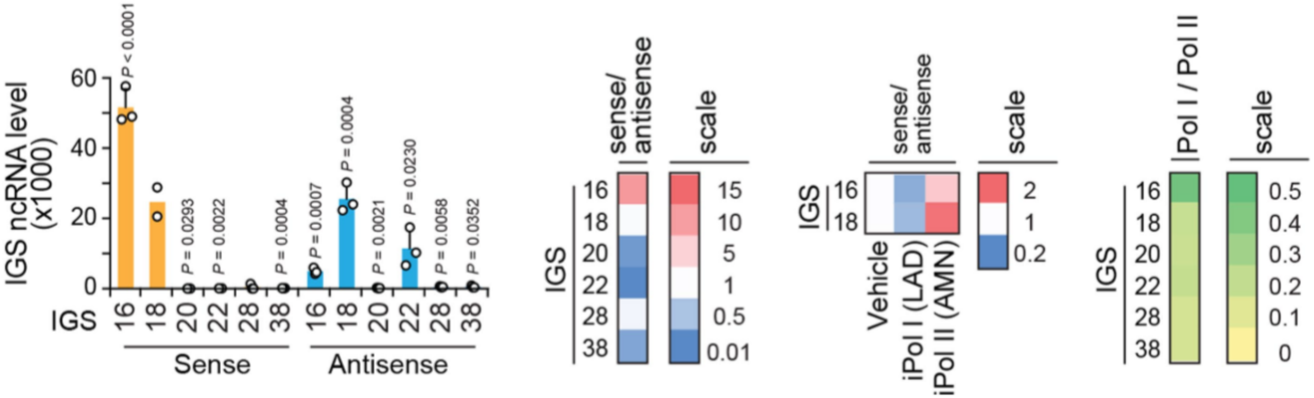
Fig.20 RT-qPCR analysis shows that Pol I promotes ncRNA synthesis on IGS while Pol II represses them



1. Inhibition of Pol I → decrease in abundance of IGS non-coding RNA (ncRNA)

2. Inhibition of Pol II → IGS ncRNAs were markedly induced and transcribed de novo

Fig.21 Pol I inhibition reduces the sense(sinc)/antisense(asinc) ratio, while Pol II inhibition increases it.



Analysis of IGS-specific transcripts identified intergenic sense ncRNAs (sincRNA →Pol I) and intergenic antisense ncRNAs (asincRNA →Pol II).
The syncRNA/asincRNA ratio parallels the enrichment of Pol I/Pol II in IGSs.

Results: Pol II operates directly through IGS, where it generates asyncRNA and limits spurious synthesis of syncRNAs by Pol I.

QUESTION 4: What is the molecular mechanism by which Pol II constitutively represses Pol I-dependent syncRNAs?

How does Pol II molecularly repress Pol I-dependent syncRNAs?

Observation: Nucleoli naturally have an enrichment of R-loops → Hypothesis: Basal R-loop levels in IGSs may modulate Pol I-Pol II cross-talk.

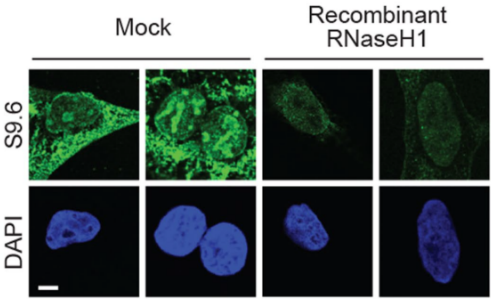


Fig.22 In vitro treatment with recombinant RNase H1 with S9.6 (an antibody against DNA–RNA hybrids).

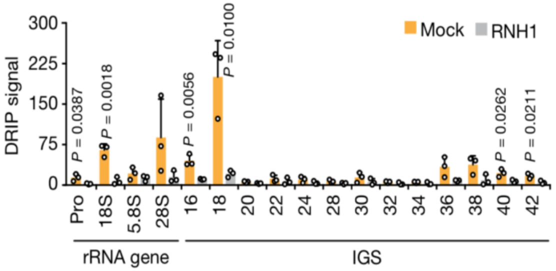


Fig.23 DRIP analysis shows RNase H1-sensitive R-loop peaks at rDNA.

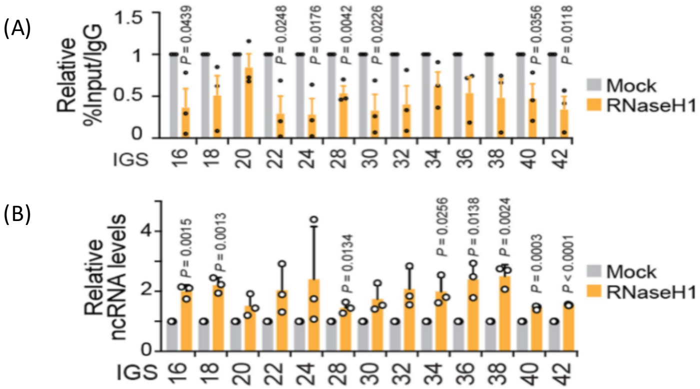


Fig.24 RNase H1 overexpression partly lowers R-loop levels (A) and increases ncRNA levels at the IGS (B).

DNA-RNA hybrid immunofluorescence (DRIF) revealed nucleolar R-loops partially repressed:

- by inhibition of Pol II
- by the repressor of recombinant RNase H1 (Fig.14)

DNA-RNA hybrid immunoprecipitation (DRIP) revealed R-loop signal peaks at the junctions between rRNA genes and IGSs

Repression of R-loop by overexpression of RNase H1 increased syncRNA expression at most of the IGS sites analyzed.

Results: R-loops are important molecular mediators of syncRNA repression by Pol II.

How does Pol II molecularly repress Pol I-dependent syncRNAs?

RNase H1 is often not enriched at the loci studied, so the phenotypic changes observed could also be due to R-loop repression elsewhere → **The RED–LasRR system**

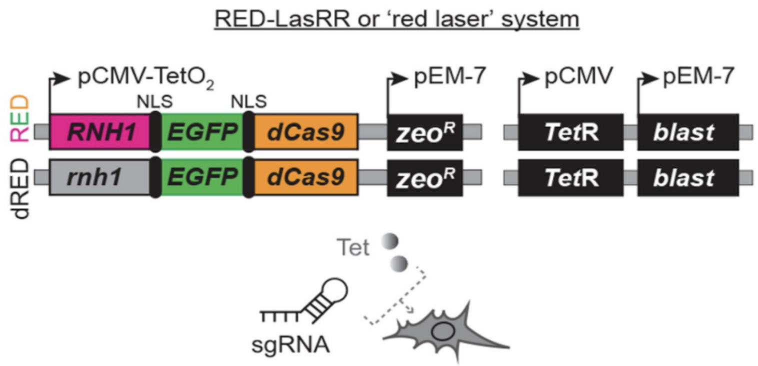


Fig.25 Design details for the RED/dRED–LaSRR systems created to achieve inducible locus-associated R-loop repression.

Full-length human RNase H1 was fused with eGFP and deactivated Cas9 from Streptococcus pyogenes.

- **rnh1**→RNase H1 with point mutation (non-functional protein)→dRED
- **dCas9** →protein lacking endonuclease activity, but still able to bind to its guide RNA (sgRNA) and target DNA strand;
- **zeoR** →The zeocin resistance gene was used for stable cell line generation;
- **tetR** → tetracycline repressor;
- **blast** →the blasticidin-resistance gene for selection of the tetracycline repressor (TetR);

Tet-On system → tetracycline induces transcription of the gene of interest

1. The immediate promoter of cytomegalovirus (CMV) early genes promotes transcription of tetR (tetracycline repressor).
2. In the presence of tetracycline tetR binds the tetO2 operator together with the minimal promoter (CMV) promoting transcription of downstream genes

How does Pol II molecularly repress Pol I-dependent syncRNAs?

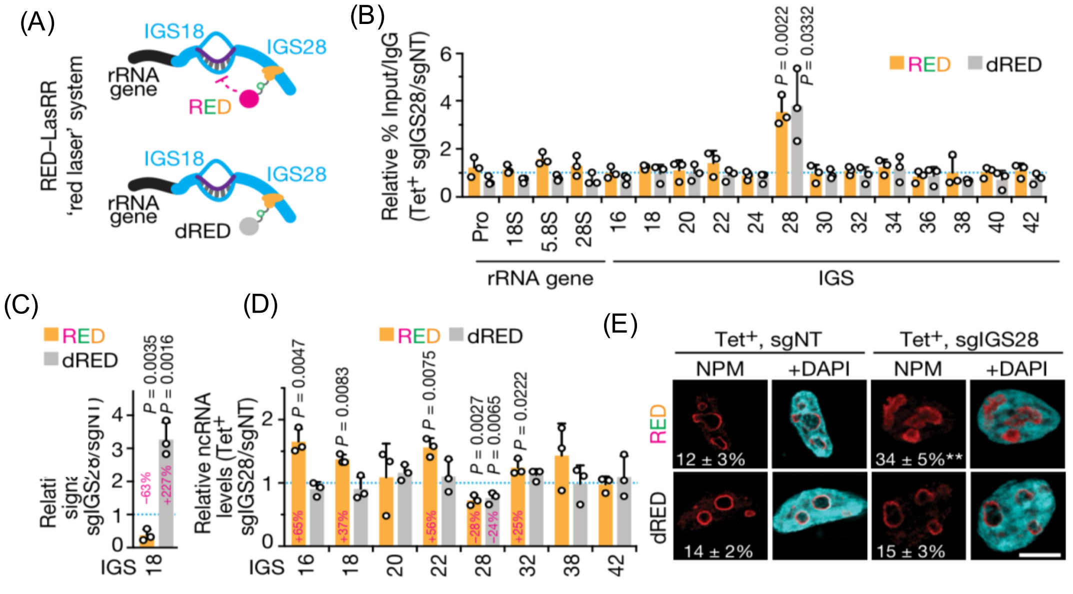


Fig.26 Repression of an IGS R-loop shield disrupts nucleoli.

Preliminary observation: Within the IGS, constitutive chromatin looping juxtaposes IGS27/28 sites with IGS16/18 sites (A).

Hypothesis: could a pool of three sgRNAs (short guide RNA for IGS28) targeted to IGS28 enrich RED at IGS28 and suppress the strong R-loop spikes at IGS16/18?

Results: Using RED or dRED together with sgIGS28 respectively decreased or increased R-loop levels at IGS18 (C).
RED sgIGS28 induced ncRNA levels (D) and disrupted NPM localization (E).

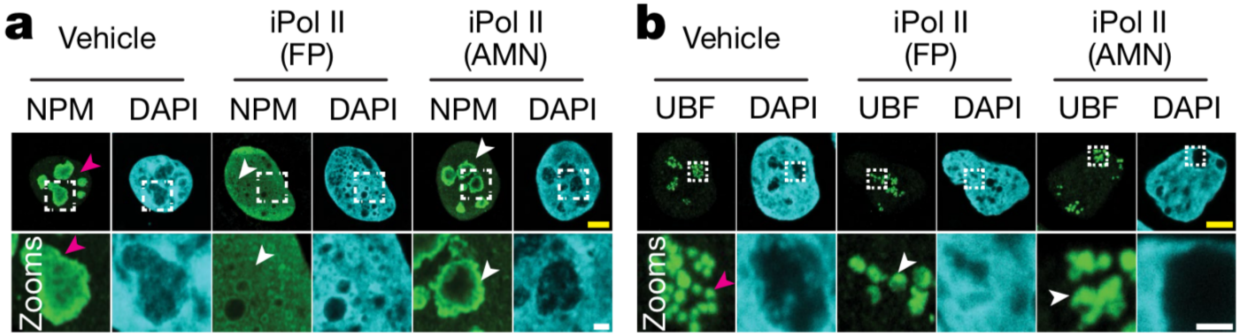
Results:

- An R-loop antisense shield (produced by Pol II) limits the synthesis of Pol-I-dependent syncRNAs
- Small increases in syncRNA levels can significantly alter the structure of nucleoli.

QUESTION 5: What are the consequences of inhibition by polymerase II and how is it reflected in sincRNA levels?

Experimental approach: Pol II inhibition

Fig.27 Effects of a 3-hour Pol II inhibition on NPM (a) and UBF (b) localization, as shown by immunofluorescence microscopy.



Pol II inhibition abrogated the phase separation of NPM, which was quickly reorganized into ruffled bodies before undergoing complete mixing with the nucleoplasm

Pol II inhibition resulted in the relocation of UBF to the nucleolar periphery, where UBF formed large spheres, rings or crescent-shaped bodies

Results:
Pol II inhibition partially and strongly disrupts the organisation of rRNA synthesis and processing → in the nucleolar space aberrant liquid-to-solid phase transitions occur.

Experimental approach: formation of amyloid bodies by heat shock

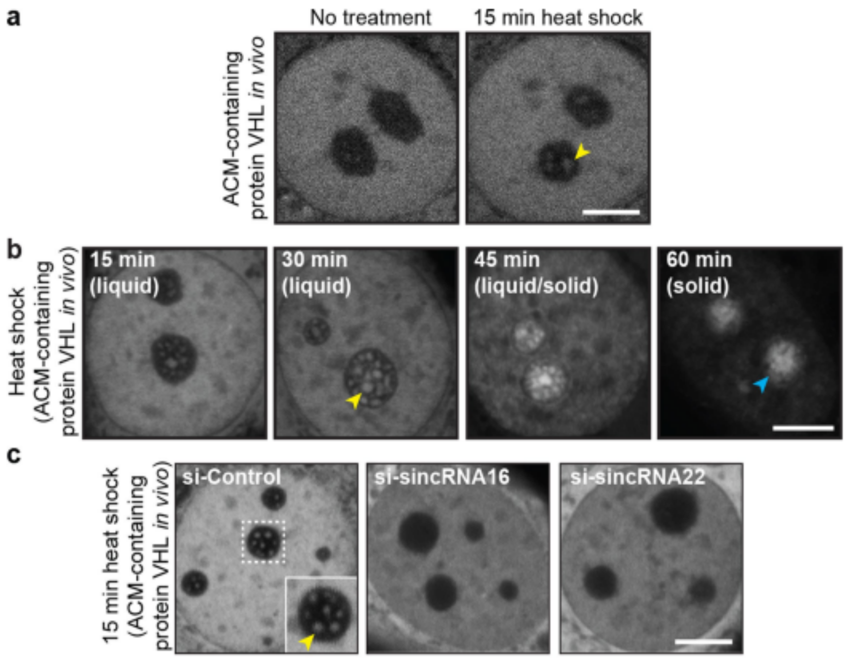


Fig.28 Heat shock limits a sincRNAs and triggers sincRNA-dependent nucleolar phase transitions

Results:

- Environmental stress represses asyncRNA levels and promotes syncRNA-dependent nucleolar remodelling.
- SyncRNAs induce liquid droplets in vitro and promote liquid droplets and subsequent amyloid-like solid bodies in vivo.

Experimental approach: restoring nucleolar organisation after sincRNA inhibition

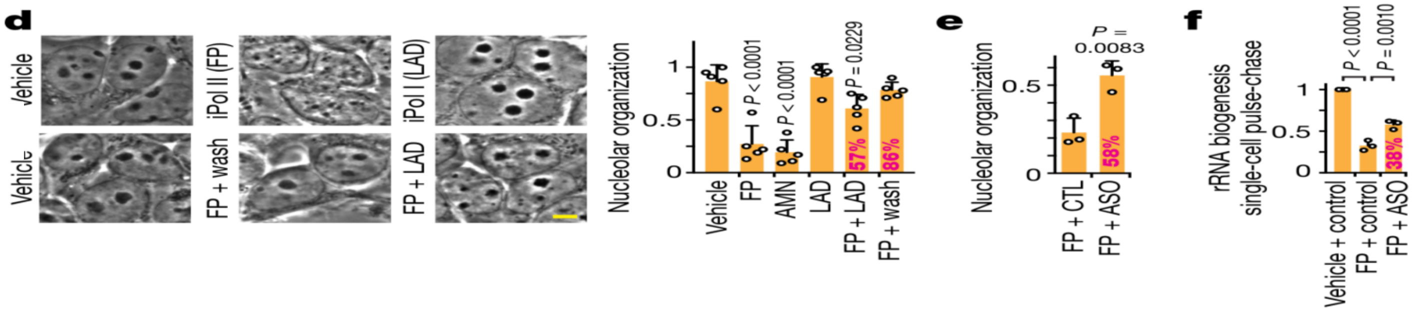


Fig.29 Pol II represses sincRNAs to maintain nucleolar structure and function.

Nucleolar organisation was restored after:

- Wash-off of the Pol II inhibitor.
- Co-inhibition of Pol I.
- Direct repression of syncRNA levels with antisense oligonucleotides (ASO)

Results:
The accumulation of syncRNA drives nucleolar disorganisation

QUESTION 6: Are there molecular factor that can modulate Pol II and thus influence nucleolar disorganisation?

Experimental approach: knock-out of SETX

- Senataxin(SEXT) supports the R-loop shield and is linked to neurodegeneration and modulation of transcription, including Pol II loading and R-loop repression20-22.
- SETX is enriched in all human IGS, especially in IGS28, and has a nucleolar localization.

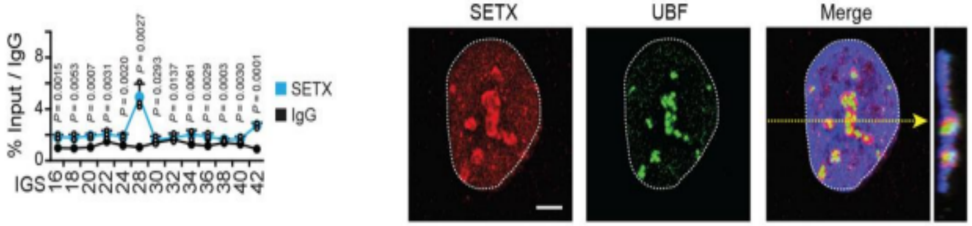


Fig. 30 ChIP showing SETX enrichment at the IGS. SETX has a nucleolar/nucleoplasmic localization

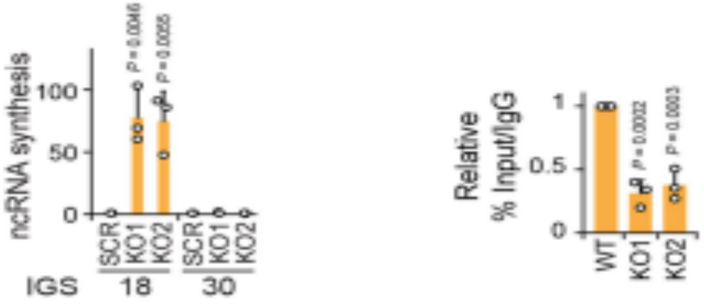
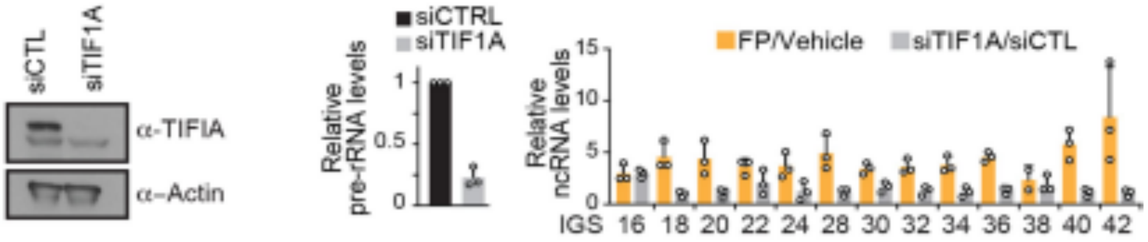


Fig.31 SETX KO induces IGS ncRNA synthesis and decreases Pol I enrichment at the rRNA gene (5′-ETS region)

Results:
Sequential ChIP revealed that SETX was preferentially co-enriched with Pol II over Pol I at IGS28.
Knockout of SETX reduced intergenic enrichment of Pol II and its R-loops.
This change was accompanied by increased intergenic enrichment of Pol I, elevated syncRNA synthesis, and decreased localization of Pol I to rRNA genes.

Experimental approach: knock-down of TIF1A



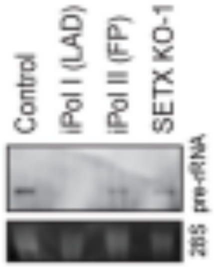


Fig.32 siRNA-mediated knockdown of TIF1A lowers Pol-I-dependent pre-rRNA levels but fails to induce IGS ncRNAs.
Different experiment but are shown here for better visual comparison

Fig.33 Northern blotting reveals that Pol II or SETX disruption does not induce rRNA gene read-through transcripts.

Results:
The R-loops of IGS act more as a shield that prevents Pol I recruitment, rather than a barrier that limits read-through transcription.
Data indicate that SETX is co-enriched with the Pol II IGS and supports it in repressing a subset of Pol-I-dependent syncRNAs that can disrupt nucleolar organization and function. SETX could achieve this effect by promoting efficient loading and release of Pol II into an intergenic promoter of IGS28.

Discussion

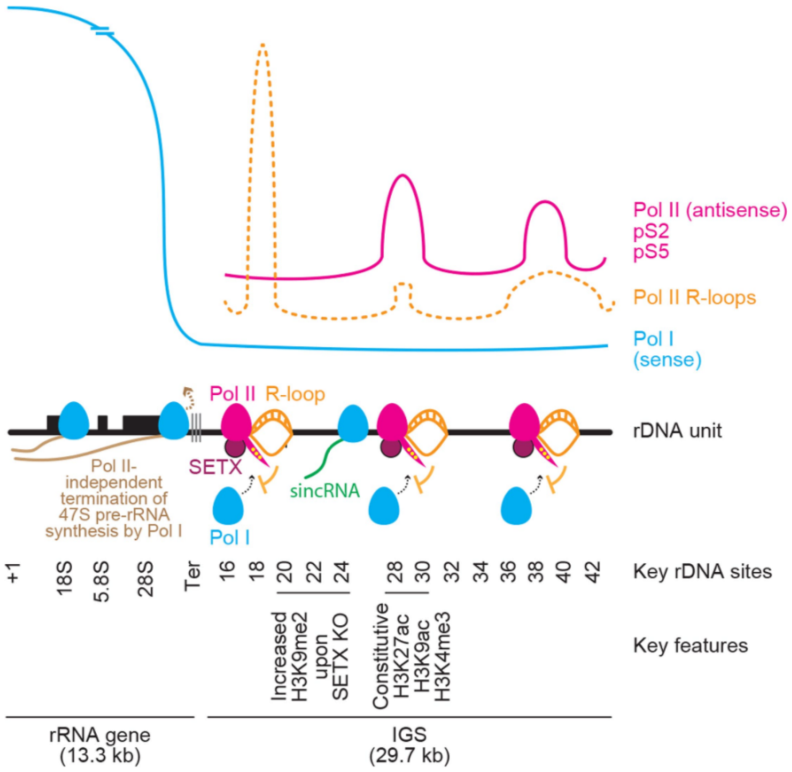


Fig.34 Detailed model illustrating how nucleolar Pol-II-dependent R-loops shield the IGS from sincRNA synthesis by Pol I.

Together these results demonstrate that **Pol II constitutively represses several Pol-I-dependent syncRNAs to prevent unscheduled nucleolar phase transitions** and to maintain endogenous nucleolar condensates, which are essential for rRNA biogenesis.

The data indicate that **SETX is co-enriched with Pol II IGS and supports it in repressing a subset of Pol-I-dependent syncRNAs** that can disrupt nucleolar organisation and function. SETX could achieve this effect by promoting efficient loading and release of Pol II in an intergenic promoter of IGS28.

Conclusion

- Nucleolar organization, which is intimately linked to cell growth and viability, can be an aid in the diagnosis and treatment of some tumors.
- Nucleolar disruption following Pol II dysregulation is similar to constitutive disorganization of nucleoli in human Ewing sarcoma tumors.
- EWS cells showed increased levels of ncRNA and R-loops in all IGS.
- The natural increase in syncRNA levels may explain the aberrant nucleolar morphologies commonly observed in cancer.
- The increase in R-loops in this context may reflect the selection of cells that have compensated for the increased syncRNA levels.

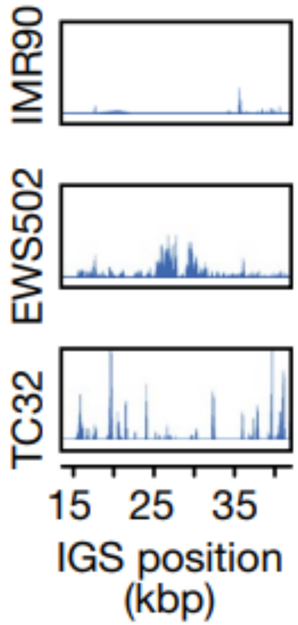


Fig.35 RNA-seq data indicate increased ncRNA levels at the IGSs of EWS502 and TC32 cells, as compared with IMR90 control cells

Thank you for your attention!

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