

**Damage-induced lncRNAs  
control the DNA damage  
response through interaction  
with DDRNAs at individual  
double-strand breaks**

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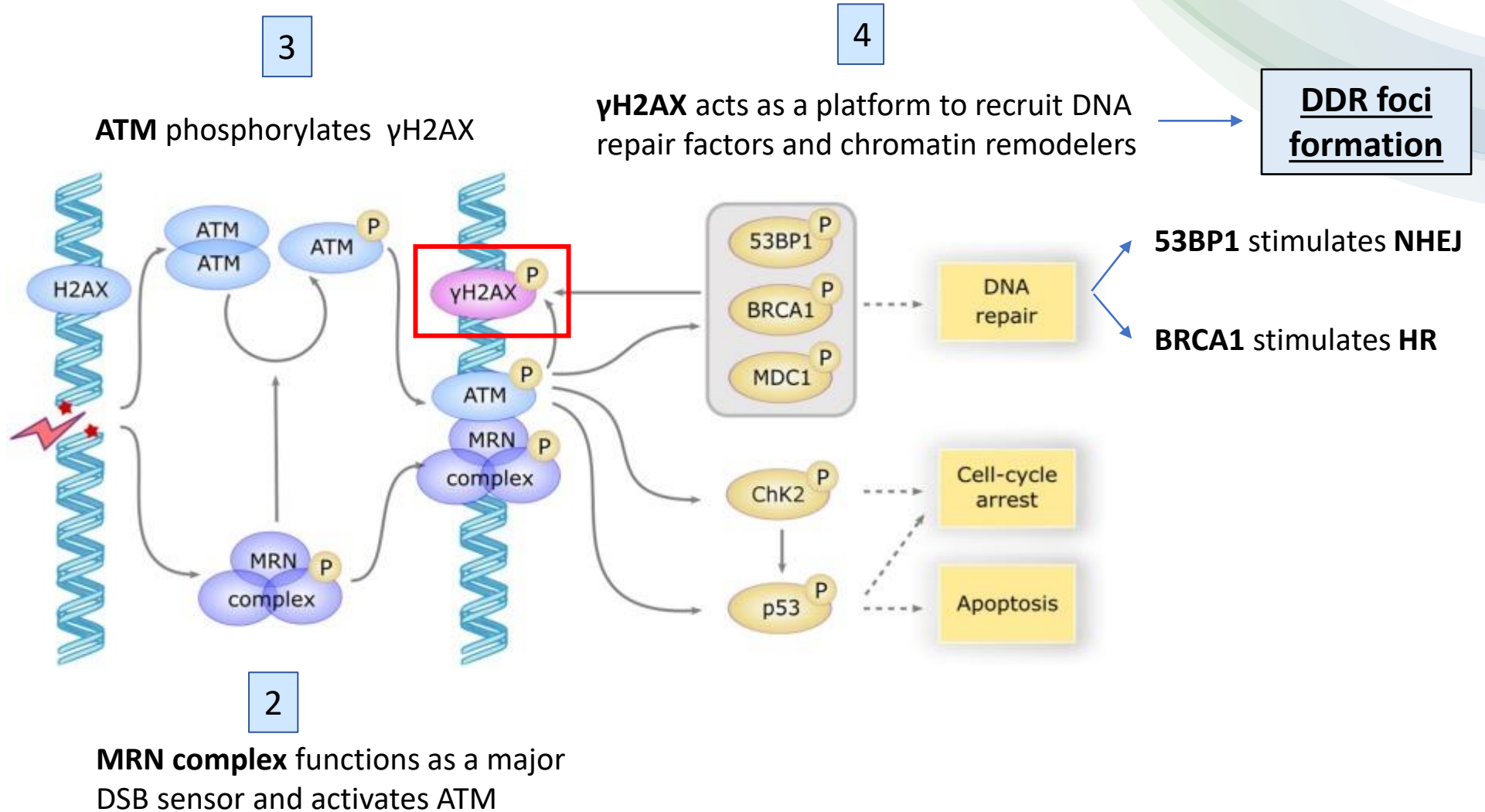
# INTRODUCTION

- DNA damage response (DDR) and DDR-foci formation.
- sncRNAs play a role in DSB response in:
  - *Arabidopsis thaliana*
  - Mammals
  - *Drosophila melanogaster*
- MRN complex and RNAPOLII association regulates the transcription initiation of diIncRNAs in mammals.
- Role of DICER;

# DNA-Damage response (DDR)

1  
Exogenous and endogenous factors induce:

- SSBs
- DSBs

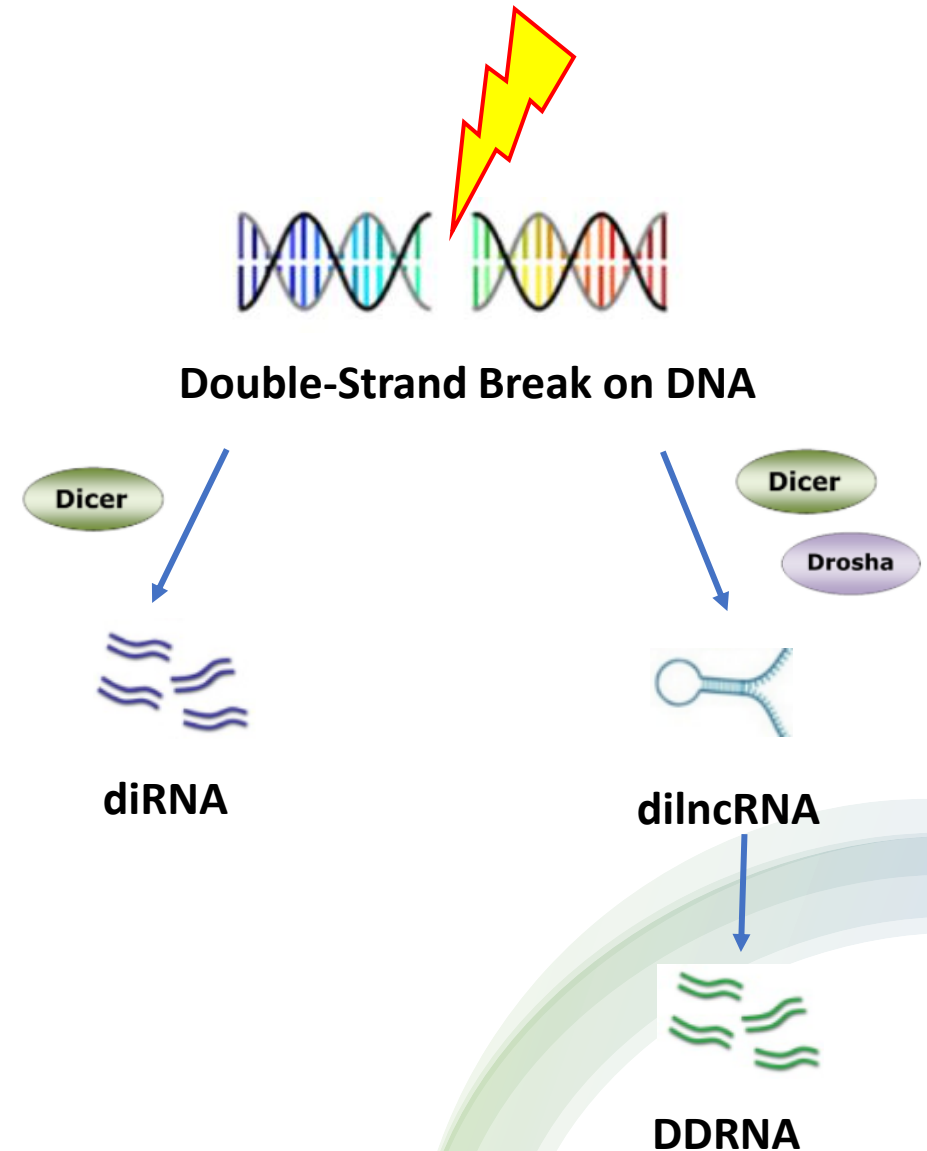


# Biogenesis of Small Noncoding RNA in Response to DSBs

Direct link between DNA damage and small noncoding RNA (sncRNA) production.

These sncRNAs produced in response to DSBs are termed:

- **Double-strand break-induced RNAs (diRNAs)**  
DSB site induces diRNA production
- **DNA Damage Response Small RNAs (DDRNs)**  
the sensing of DNA damage through interaction between MRN complex and RNAPII  
↓  
induce the production of a primary RNA transcript, called **damage-induced long noncoding RNAs (dilncRNA)**  
↓  
dilncRNA are processed by Dicer and Drosha into DDRNA that have a role in DDR

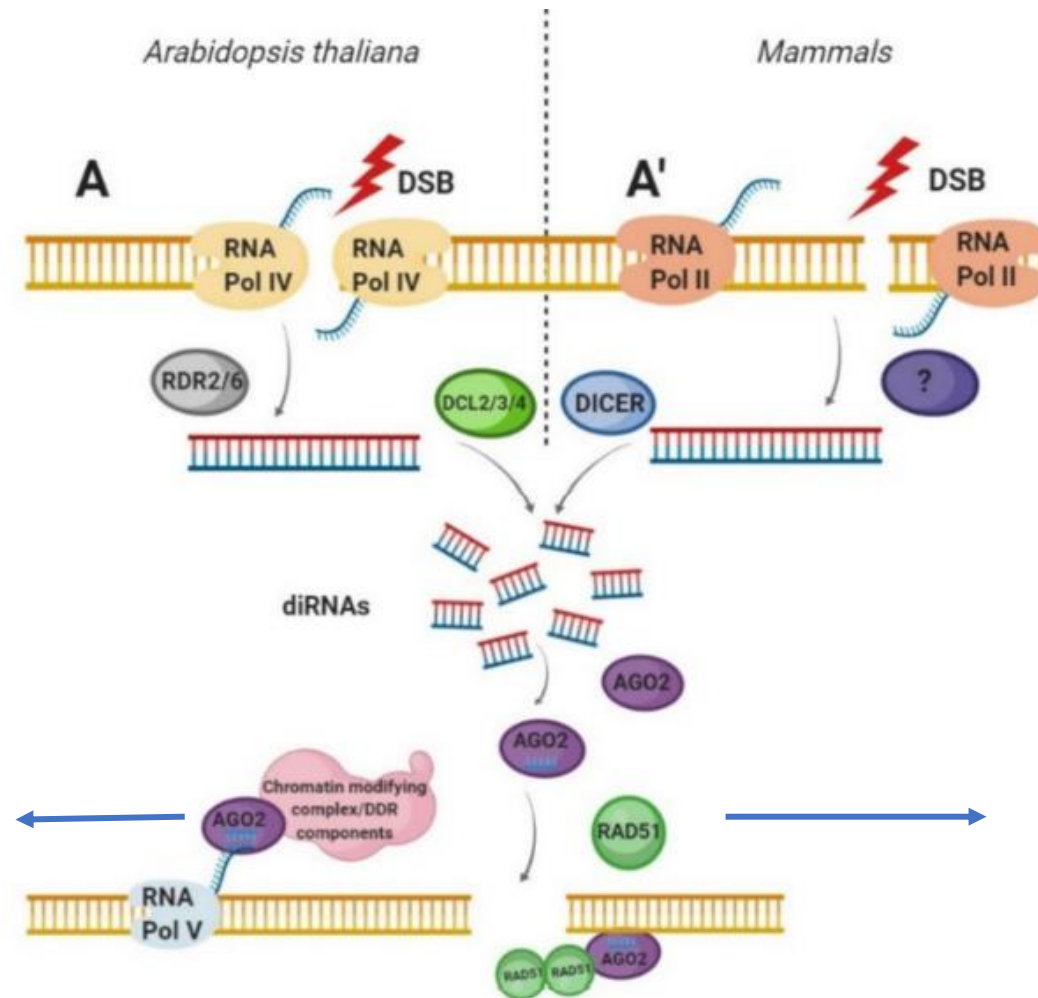


# *Arabidopsis thaliana* vs Mammals

## diRNA BIOGENESIS

### *Arabidopsis thaliana*

- 1) Transcription: **RNA Pol IV**
- 2) dsRNA synthesis: **RDR2/6**
- 3) diRNA production:  
**DCL2/3/4**
- 4) **AGO2—diRNA complexes**



### Mammals

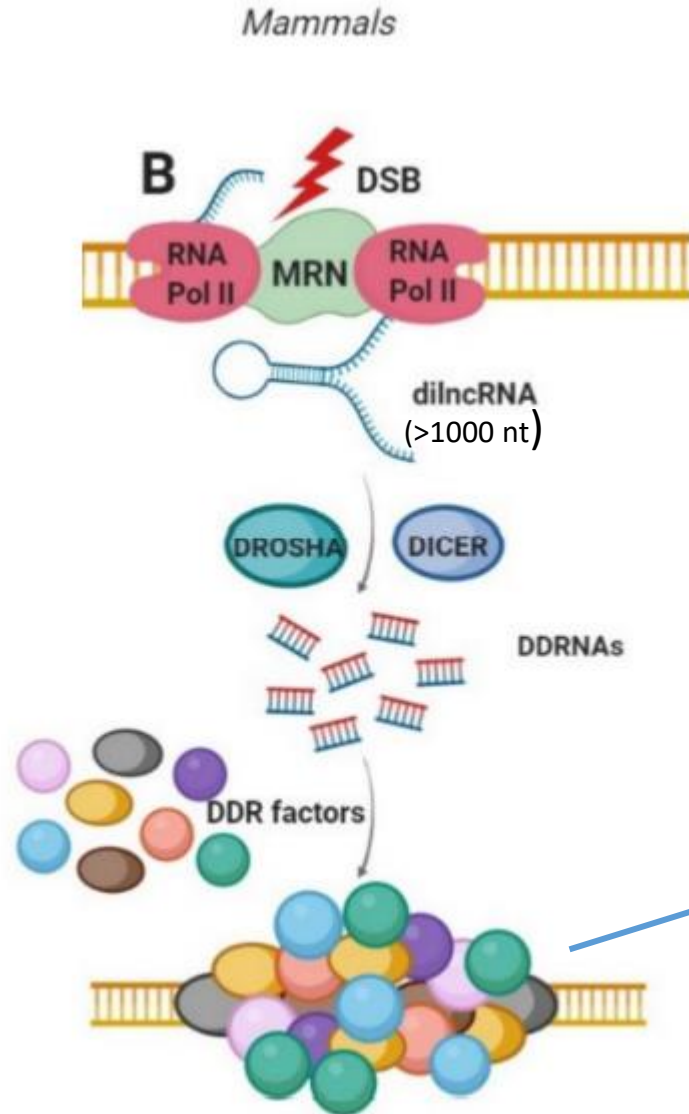
- 1) Transcription: **RNA Pol II**
- 2) dsRNA synthesis: **???**
- 3) diRNAs production: **Dicer**
- 4) **AGO2—diRNA complexes**

DDR foci formation

diRNA are required for RAD51 foci formation at DNA damaged sites in a homology-dependent manner



# DDRNAS BIOGENESIS IN MAMMALS



- 1) Sensor of DSB: **MRN complex**
- 2) **diIncRNA** synthesized by RNA Pol II
- 3) Generation of **DDRNs** by diIncRNAs cleaving mediated by **Dicer** and **Drosha**

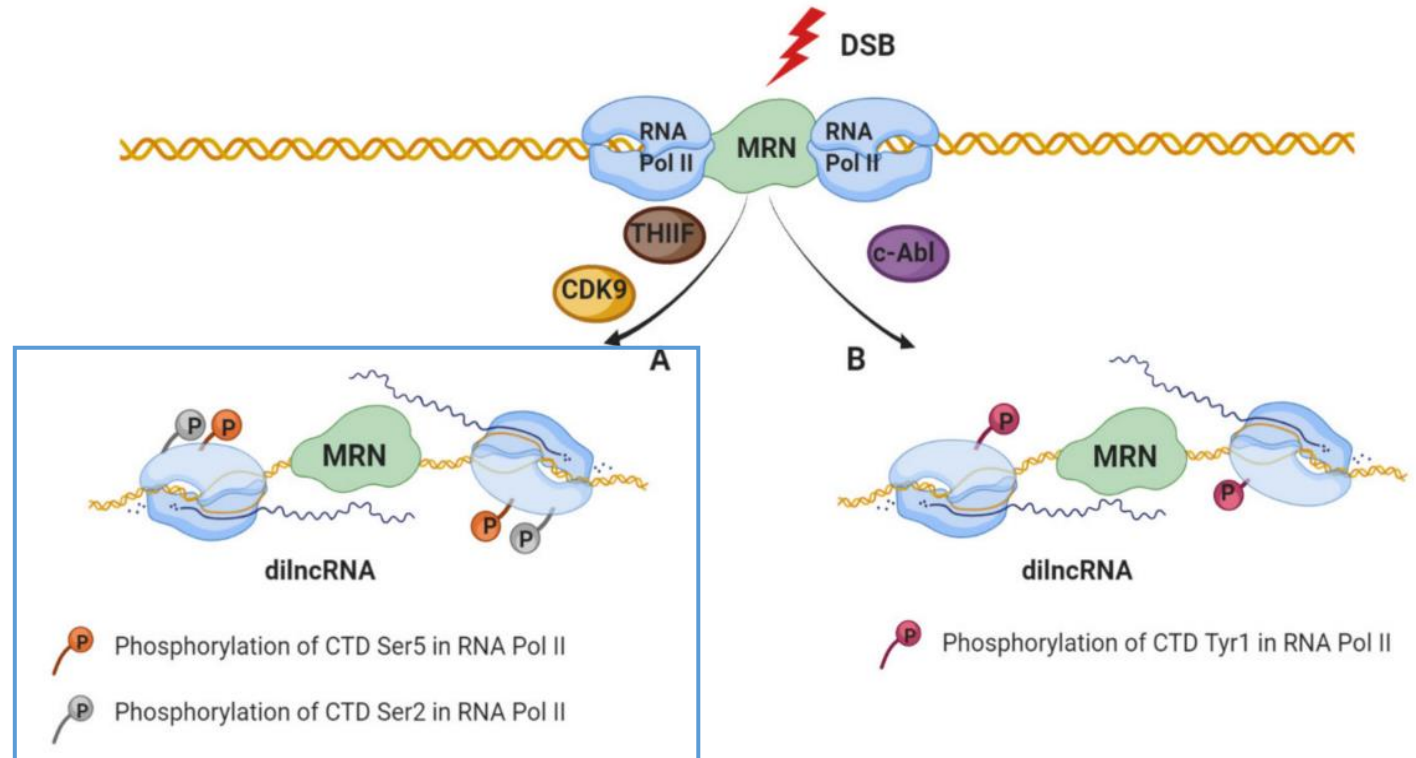
diIncRNAs interact with DDRNs and guide them to the damaged DNA, resulting in the DDR foci formation

# MRN complex and RNAPOLII association regulates the transcription initiation of diIncRNA

The transcription initiation of diIncRNA at the broken DNA is regulated by the MRE11-RAD50-NBS1 (MRN) complex which directly binds to the RNA Pol II

Two models of diIncRNAs production:

- A. Transcription factor IIH (TFIIH) and Cyclin-dependent kinase 9 (CDK9)**
- B. C-Abl**



# Role of DICER

## HYPOTESIS: Biogenesis of Dicer-dependent and Dicer-independent diRNAs

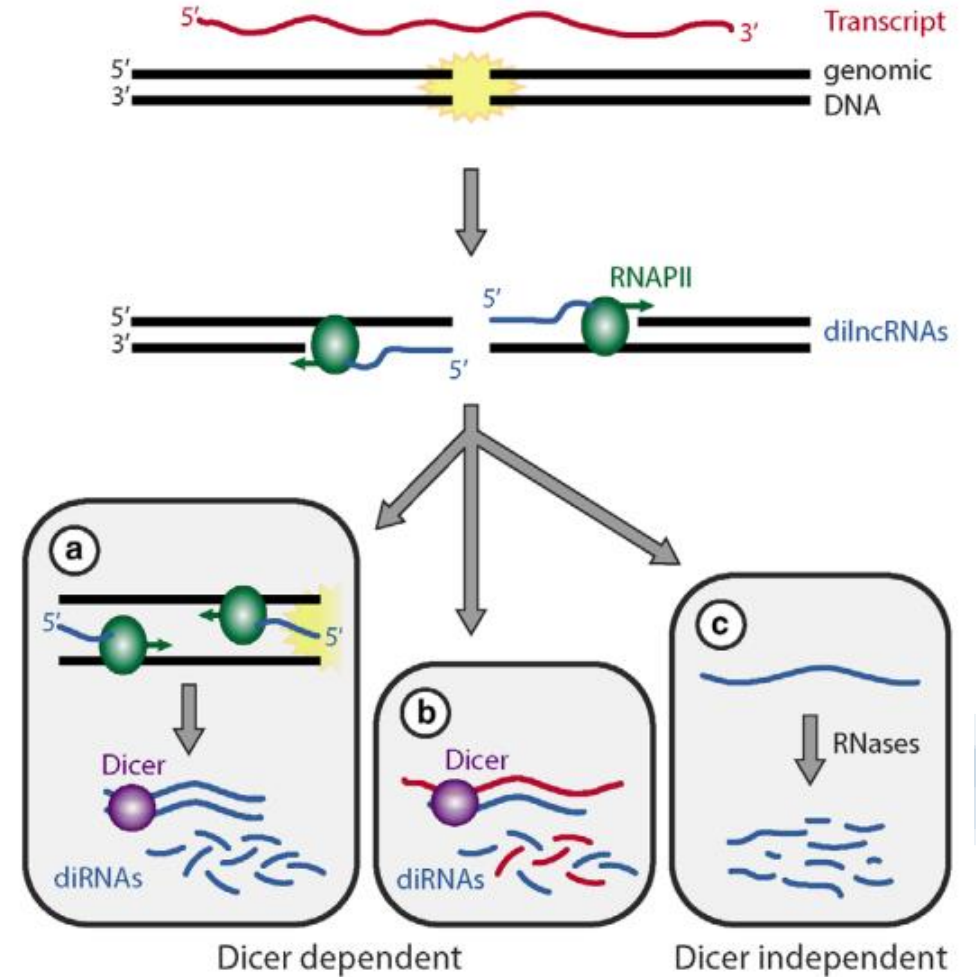
Dicer-Independent

Dicer-dependent

a) **Bidirectional transcription** by RNAPII → generation diIncRNA which formed dsRNA → dsRNA is cleaved by Dicer into short diRNAs.

b) diIncRNAs base-pair with a **pre-existing transcript**

c) **RNases** acting on ssRNA can cleave diIncRNAs producing a heterogeneous population of diRNAs.





# What happens in *Drosophila melanogaster* ?

From DSBs there are the formation of 21 nt **sncRNAs** named **endo-siRNAs**, *via* the activity of the **Dicer-2 protein**

The perturbation of siRNA biogenesis does not effect DNA repair efficiency.

*In Drosophila, are sncRNAs as important for DDR as they are in other species analysed?*

**A connection of endo-siRNAs with DDR is not yet clear in *Drosophila*!**



- 1) HOW ARE diIncRNAs TRANSCRIBED? WHO TRANSCRIBES diIncRNAs?
- 2) HOW ARE DDRNAs PROCESSED?
- 3) WHAT IS THE LOCALIZATION AND ROLE OF DDRNAs IN THE DDR?



ARTICLES

nature  
cell biology

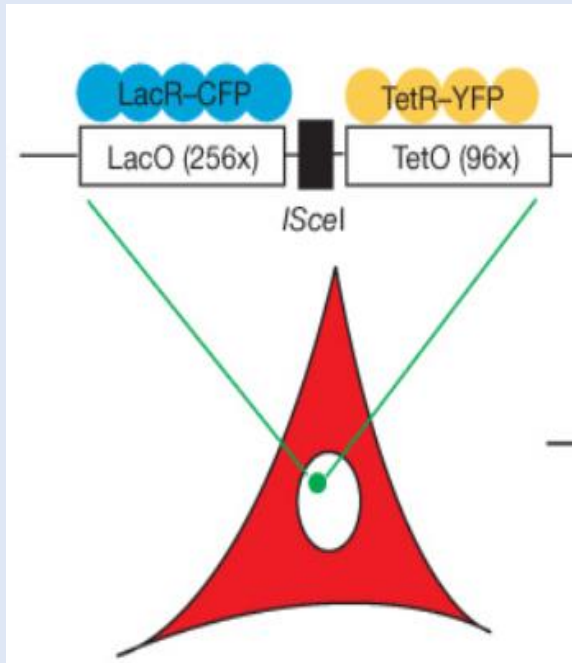
## Damage-induced lncRNAs control the DNA damage response through interaction with DDRNAs at individual double-strand breaks

Flavia Michelini<sup>1</sup>, Sethuramasundaram Pitchiaya<sup>2,5</sup>, Valerio Vitelli<sup>1</sup>, Sheetal Sharma<sup>1</sup>, Ubaldo Gioia<sup>1</sup>, Fabio Pessina<sup>1</sup>, Matteo Cabrini<sup>3</sup>, Yejun Wang<sup>4</sup>, Iliaria Capozzo<sup>3</sup>, Fabio Iannelli<sup>1</sup>, Valentina Matti<sup>1</sup>, Sofia Francia<sup>1,3</sup>, G. V. Shivashankar<sup>1,4</sup>, Nils G. Walter<sup>2</sup> and Fabrizio d'Adda di Fagagna<sup>1,3,6</sup>

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# **1. HOW ARE diIncRNAs TRANSCRIBED?**

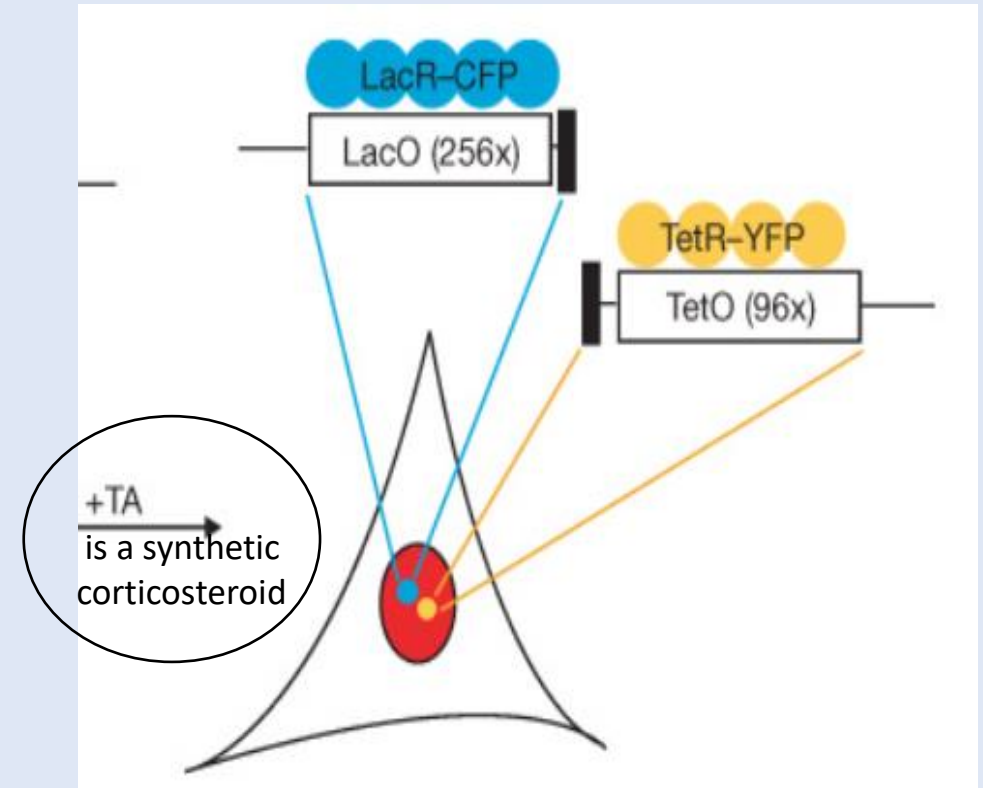
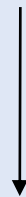
# NIH2/4 - MOUSE CELL LINE CARRYING THE Lac-*I*Scel-Tet construct



The Lac- and Tet-arrays were visualized simultaneously by expression of

- CFP-LacR
- YFP-TetR

To control the induction of a DSB at the Lac-*I*Scel-Tet array

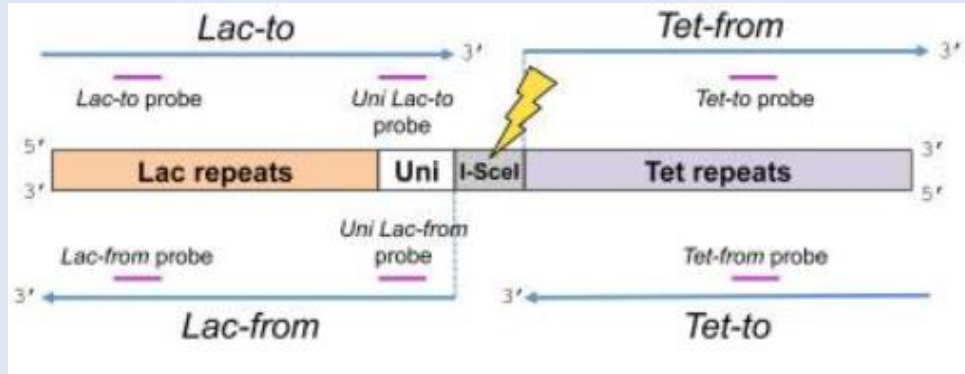


A chimera between:

- *I*Scel restriction endonuclease
- the glucocorticoid receptor (GR) domain that binds the ligand in frame with monomeric RFP

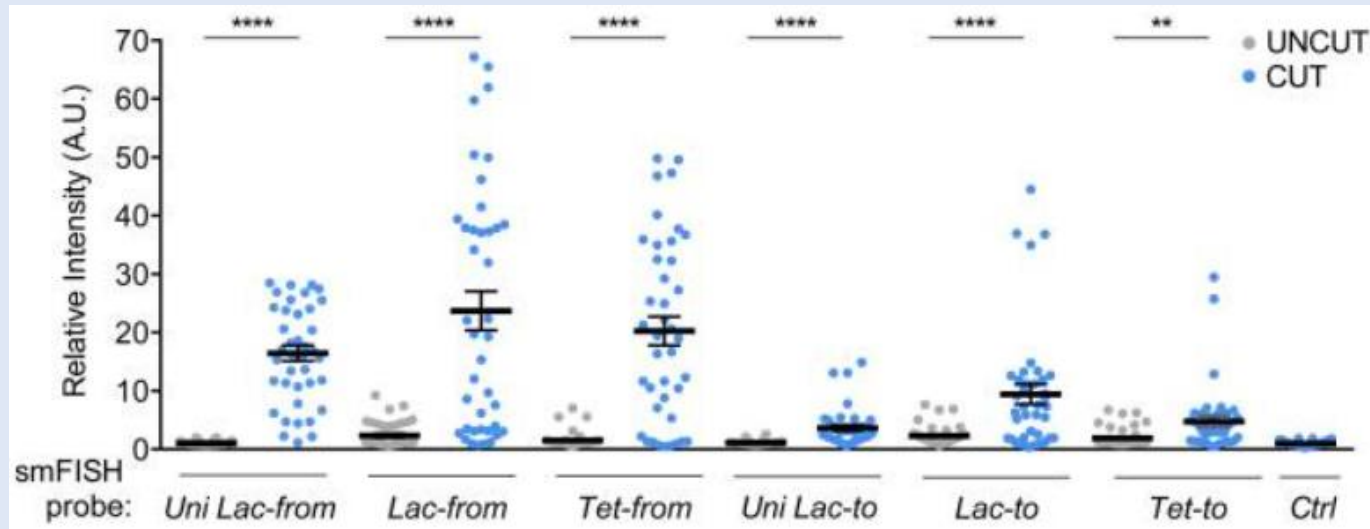
→ ***I*Scel-GR**

# HOW ARE diIncRNAs TRANSCRIBED?



Converging to the DSB (Lac-to and Tet-to)

Diverging from the DSB (Lac-from and Tet-from)

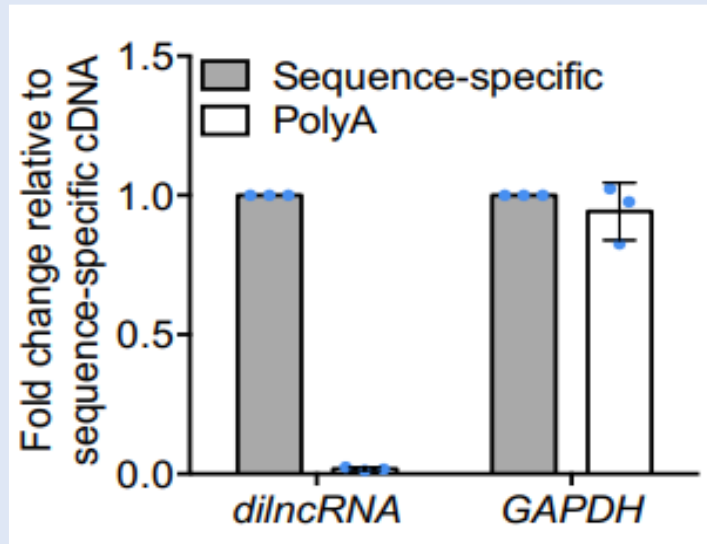


**SmFISH** – Single-molecule fluorescent in situ hybridization: with strand-specific DNA probes revealed a stronger signal at the GFP-LacR.

**CONCLUSION** → THE TRANSCRIPTION IS BIDIRECTIONAL, BUT IT IS MORE DIVERGENT FROM DSB THAN CONVERGENT TO DSB



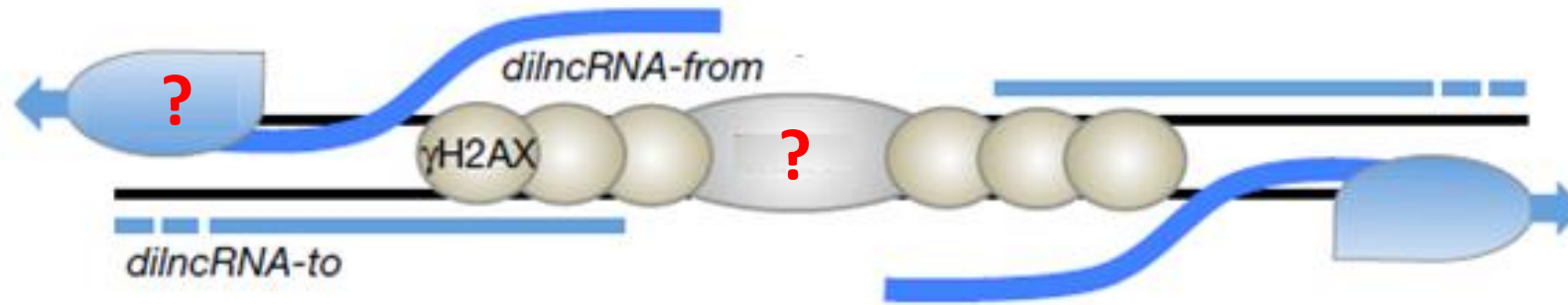
# ABOUT THE POLYADENYLATION?



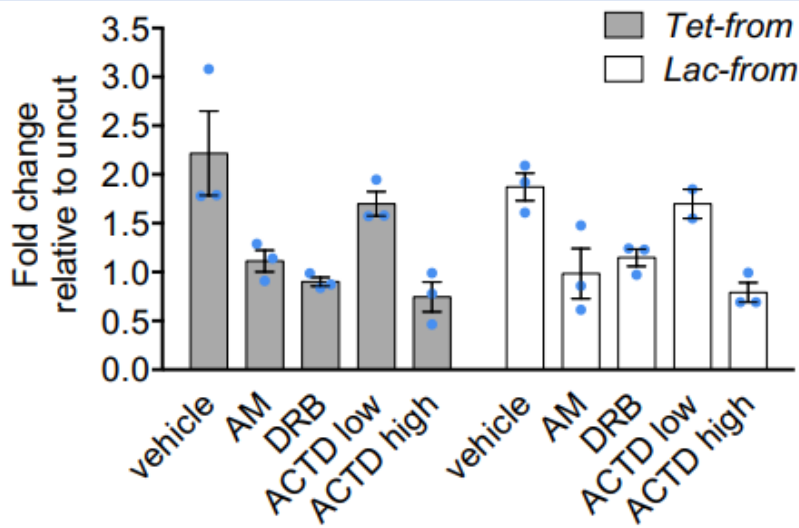
With strand-specific **RT-qPCR** in NIH2/4 using **oligo-dT primers**, we cannot detect *dilncRNAs*, indicating that *dilncRNAs* lack polyadenylation

**CONCLUSION → THE TRANSCRIPTS LACK OF POLYADENYLATION**

# 1. WHO TRANSCRIBES diIncRNAs?



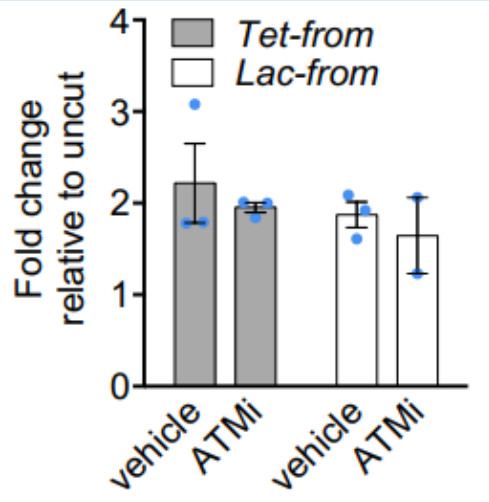
# WHICH RNA POLYMERASE IS INVOLVED?



NIH2/4 treated with:

- **AM** → Specific RNAPII inhibitor
- **DRB** → inhibitor of RNAPII elongation
- **ACTD high dose** → inhibitor of RNAPII
- ACTD low dose → inhibitor of RNAPI

} **RNAPII INHIBITION** →  
**LOWER diIncRNA**  
**INDUCTION**

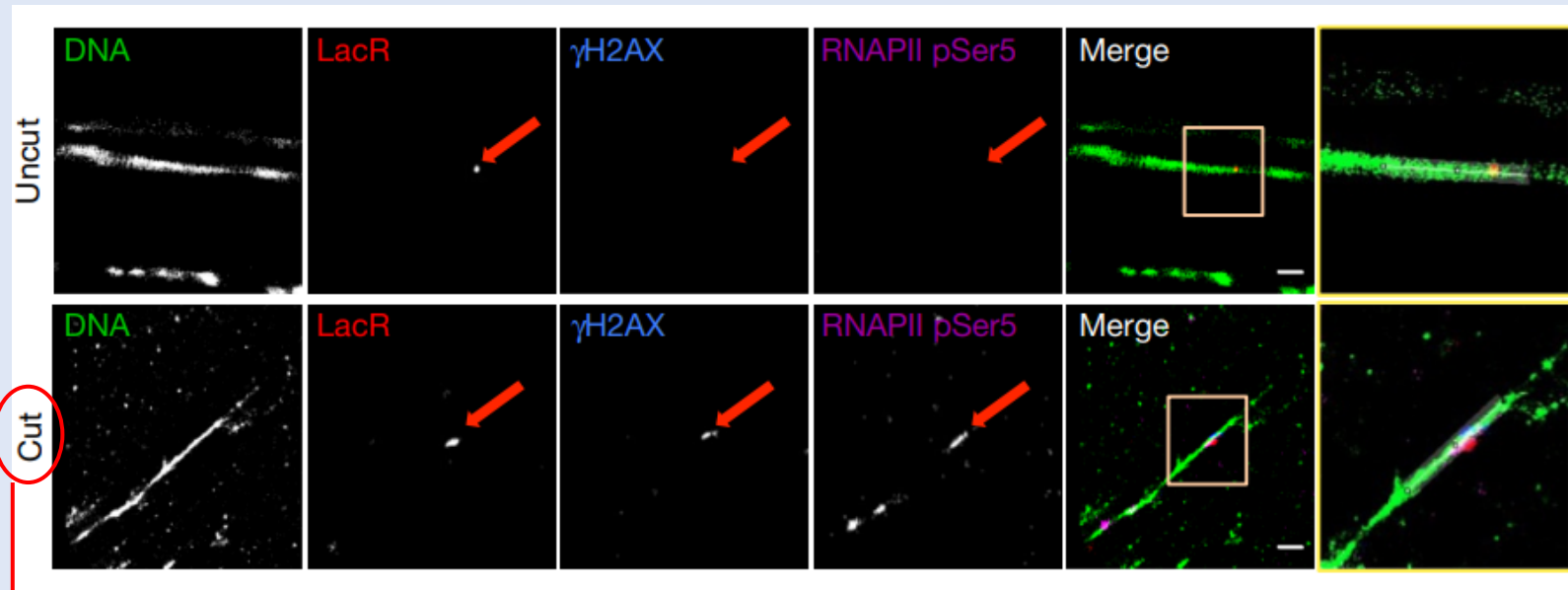


**BY CONTRAST, ATM INHIBITION DID NOT AFFECT diIncRNA GENERATION**

## CONCLUSION

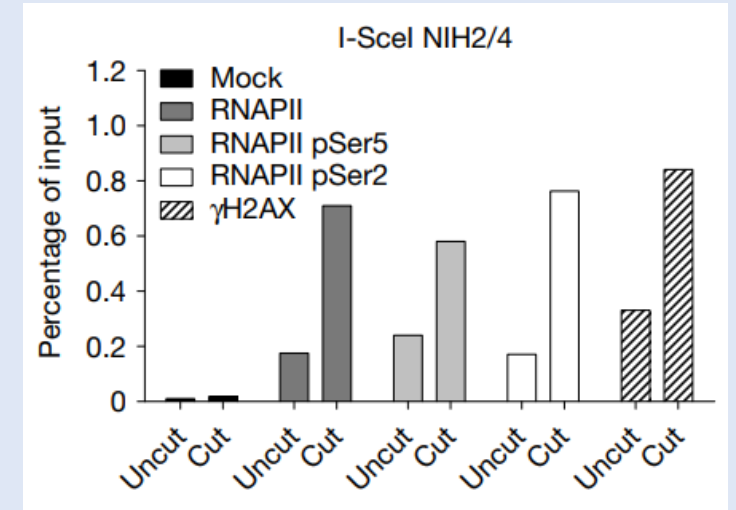
1. **DAMAGE-INDUCED TRANSCRIPTION IS DEPENDENT ON RNAPII**
2. **DAMAGE-INDUCED TRANSCRIPTION IS INDIPENDENT ON DDR FACTORS**

# IS RNAPII RECRUITED AT DSBs?



Detection of  $\gamma$ H2AX and RNAPII pSer5 at the DNA damage locus by imaging chromatin fibers with super-resolution **Binding-Activated Localization Microscopy (BALM)**.

Transfected with Scl1



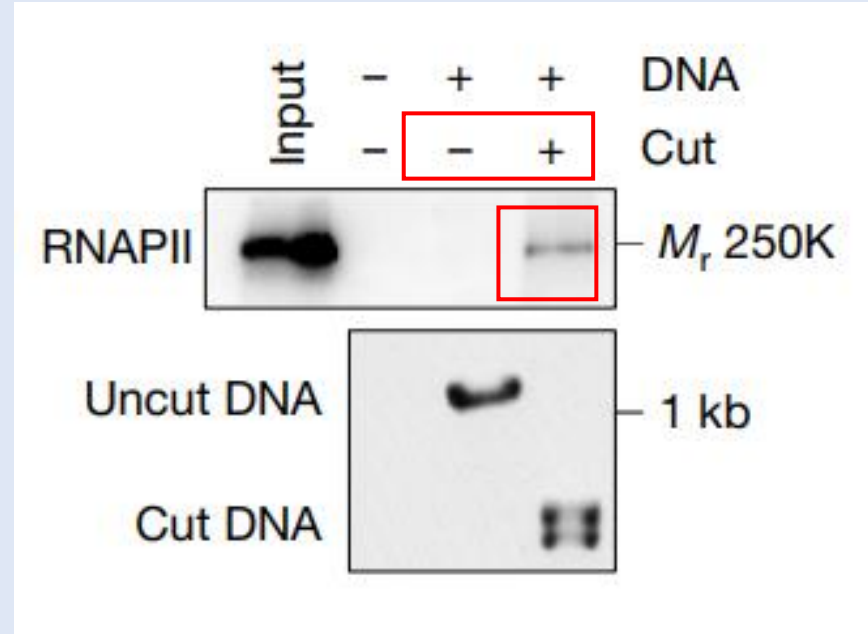
**Chromatin immunoprecipitation (ChIP) analyses in NIH2/4**

**CONCLUSION → RNAPII ACCUMULATES AT DSBs**

# IS RNAPII ABLE TO RECOGNIZE DNA DOUBLE-STRAND ENDS?

Pull-down samples probed for total RNAPII:

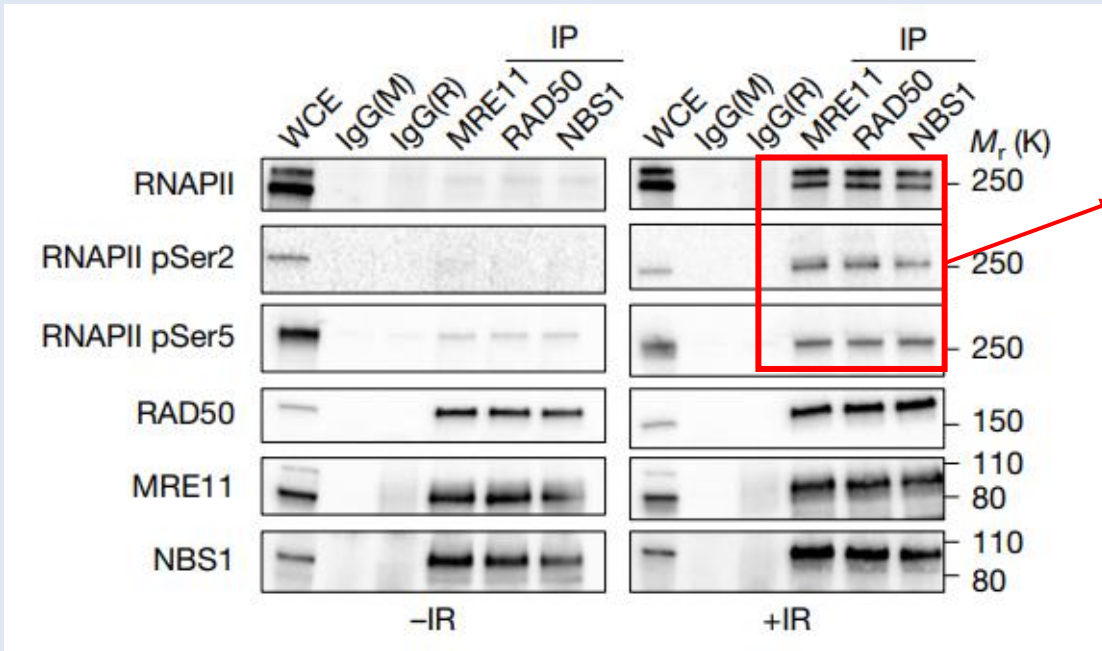
- Uncut DNA → **NO** RNAPII detection
- Cut DNA → RNAPII detection



**CONCLUSION → RNAPII HAS THE AFFINITY FOR DOUBLE-STRAND DNA ENDS**



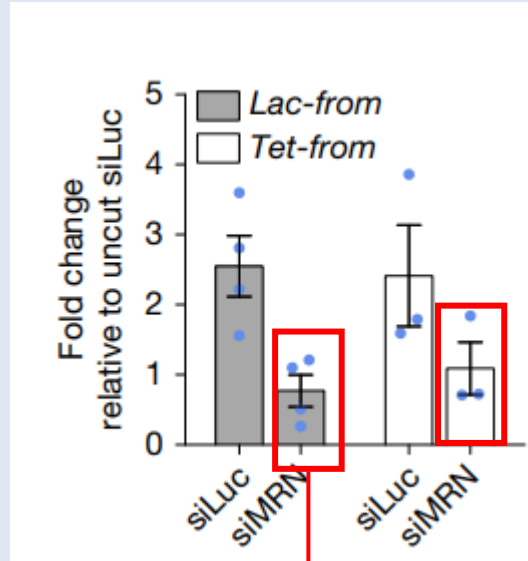
# RNAPII and MRN COMPLEX: DO THEY WORK TOGETHER?



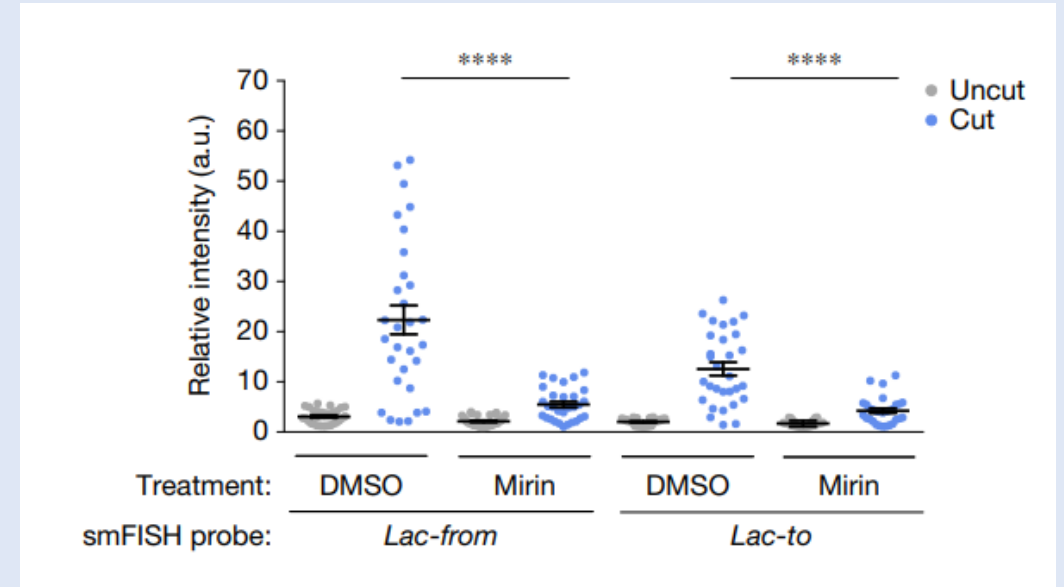
**Co-immunoprecipitation** of RNAPII and its phosphorylated forms with the MRN complex, following IR exposure in **HEK293T** cells.

**CONCLUSION → THERE IS AN INTERACTION BETWEEN RNAPII AND MRN**

# IS MRN COMPLEX INVOLVED IN TRANSCRIPTION?



MRN knockdown in NIH2/4  
→ Reduction of diIncRNA  
induction following I-SceI cutting



smFISH with the MRN inhibitor Mirin  
→ DSB-induced transcription is reduced  
by Mirin.

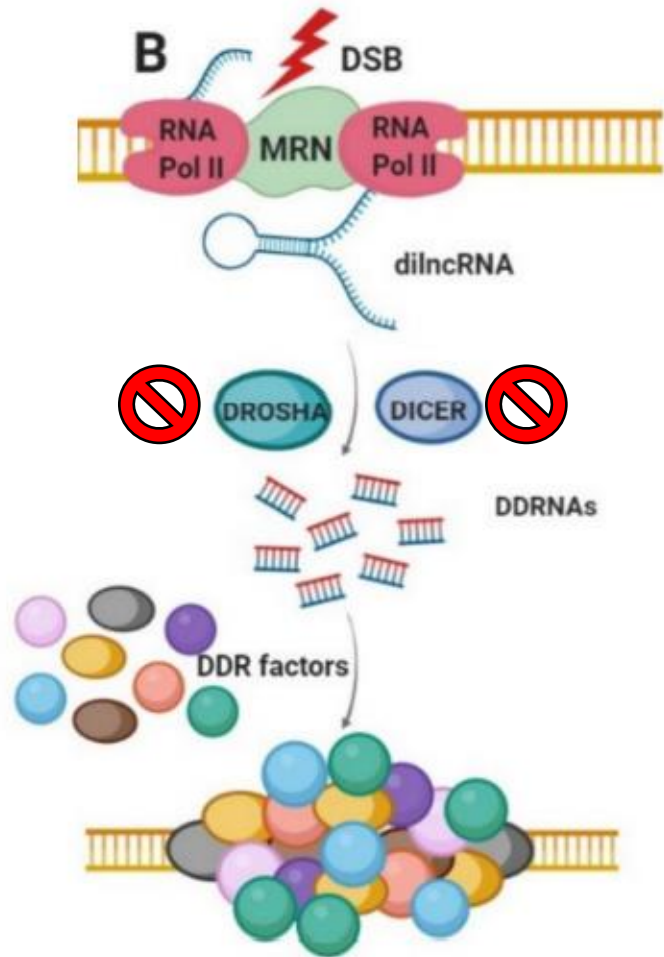
**CONCLUSION → DSB-INDUCED TRANSCRIPTION IS DEPENDENT ON MRN**

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## **2. HOW ARE DDRNAs PROCESSED?**

# diIncRNAs ARE DDRNA PRECURSORS?

Mammals



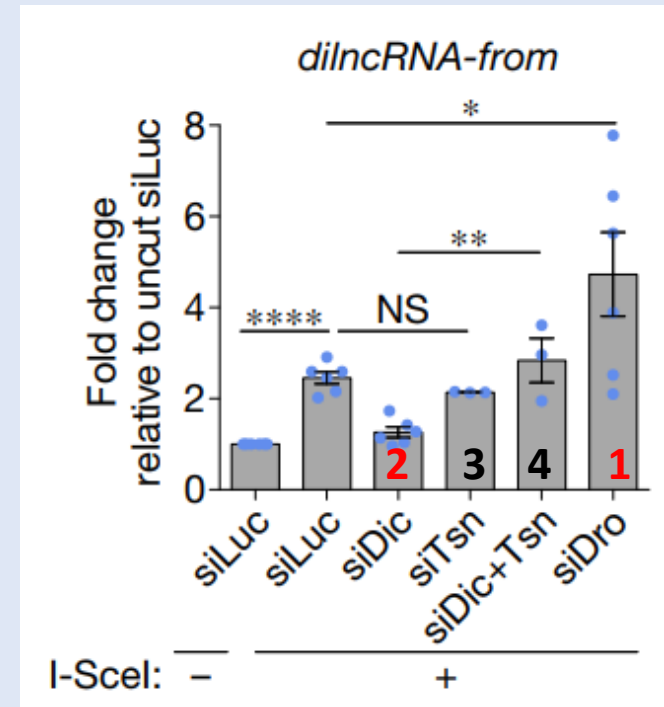
1) Drosha knockdown → accumulation of diIncRNA in cut cells

2) Dicer knockdown → there isn't an accumulation of diIncRNAs in cut cells



3) Translin Knockdown → there isn't an accumulation of diIncRNA in cut cells

4) Translin + Dicer Knockdown → accumulation of diIncRNA



**CONCLUSION → diIncRNA ARE REALLY PROCESSED BY DROSHA AND DICER**

# CAN WE SUPPOSE THAT miRNA AND DDRNA HAVE SIMILAR PROCESSING MECHANISMS?

1) If we knock down Drosha



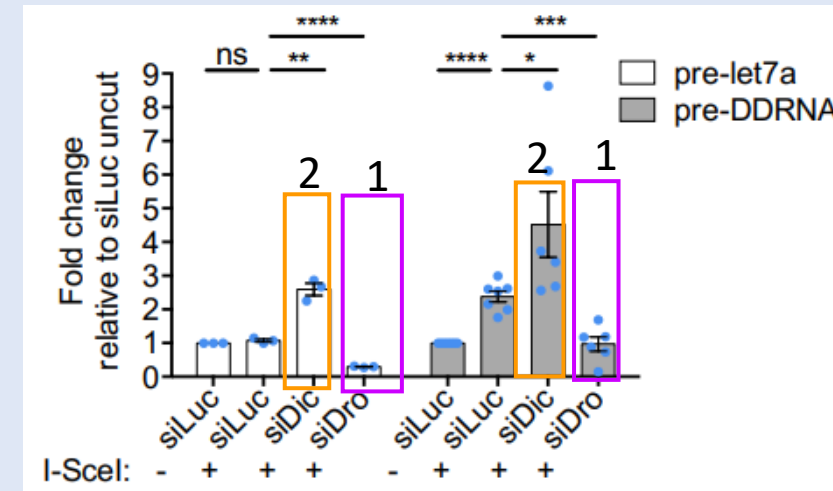
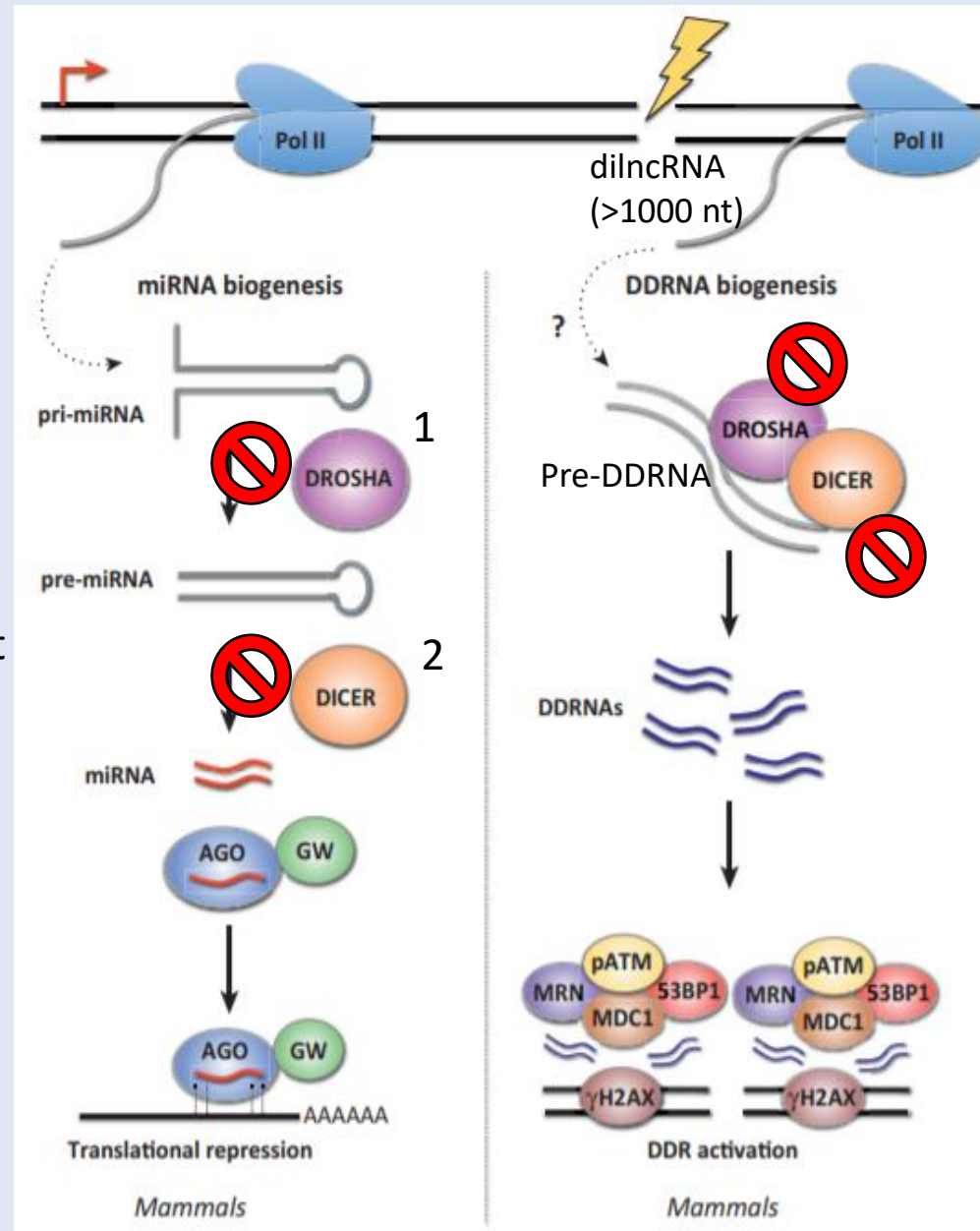
pre-miRNA and pre-DDRNA are **not formed**.

2) If we knock down Dicer



pre-miRNA and pre-DDRNA are not processed

so they accumulate



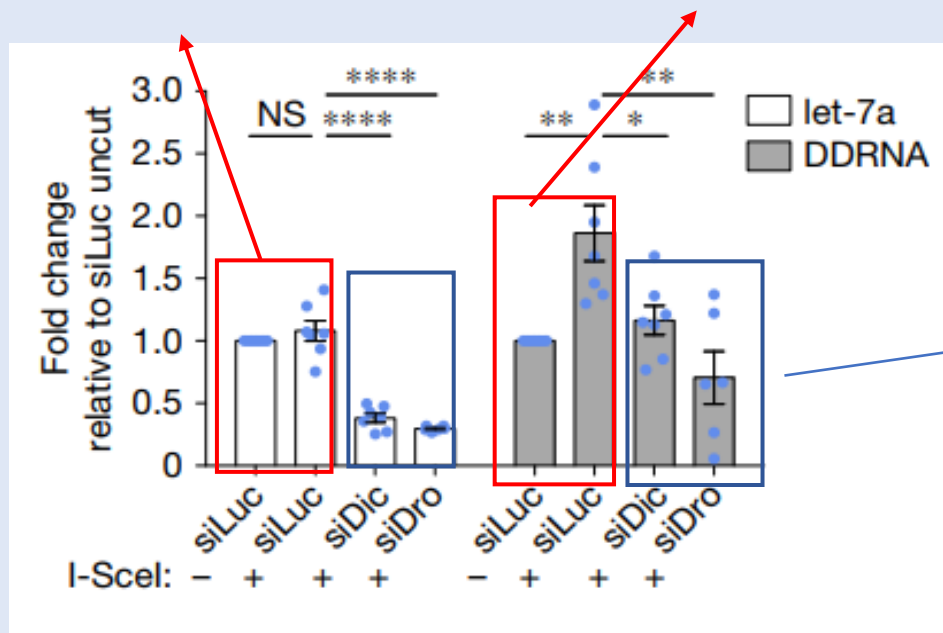
To isolate **pre-DDRNAs** and **pre-miRNA** it has been purified **long fractions RNA** by gel-extraction (40-200nt).



## NOW, WE CAN ANSWER THE LAST QUESTION

1) In cut cells (siLuc/I-SceI+) → miRNA levels doesn't increase respect the control which the cell are uncut

2) In cut cells (siLuc/I-SceI+) → DDRNAs levels increase respect the control which the cells are uncut



Purification of **RNA short fraction 15-40 nt** long containing **mature miRNA (let-7a) and DDRNA** by gel extraction

In cut cells with siDic and siDro there are decreasing of:

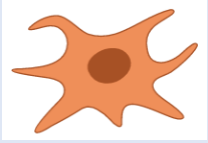
- DDRNA
- miRNA (let-7a)

**CONCLUSION → THIS EVIDENCE MAKES US THINK THAT THE PROCESSING OF DDRNAs AND miRNAs IS SIMILAR**

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### **3. WHAT IS THE LOCALIZATION AND ROLE OF DDRNAs IN THE DDR?**

# DOES DDRNA LOCALIZE AT THE DAMAGE SITE?



NIH2/4 mouse cell line

- transfected with **GFP-LacR**
- carrying an integrated **LacO-I-SceI-TetO** construct

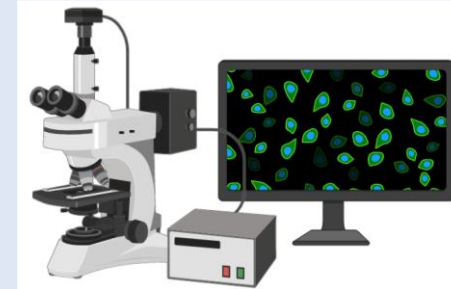


Microinjection of:

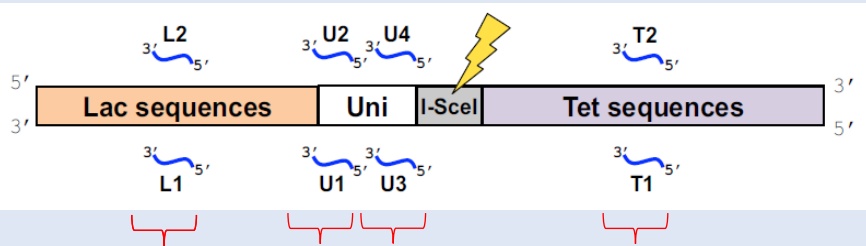
- Pooled double-stranded DDRNAs-Cy5**
- Or **two control miRNAs:**

Together with:

- BSA**
- Or **I-SceI**



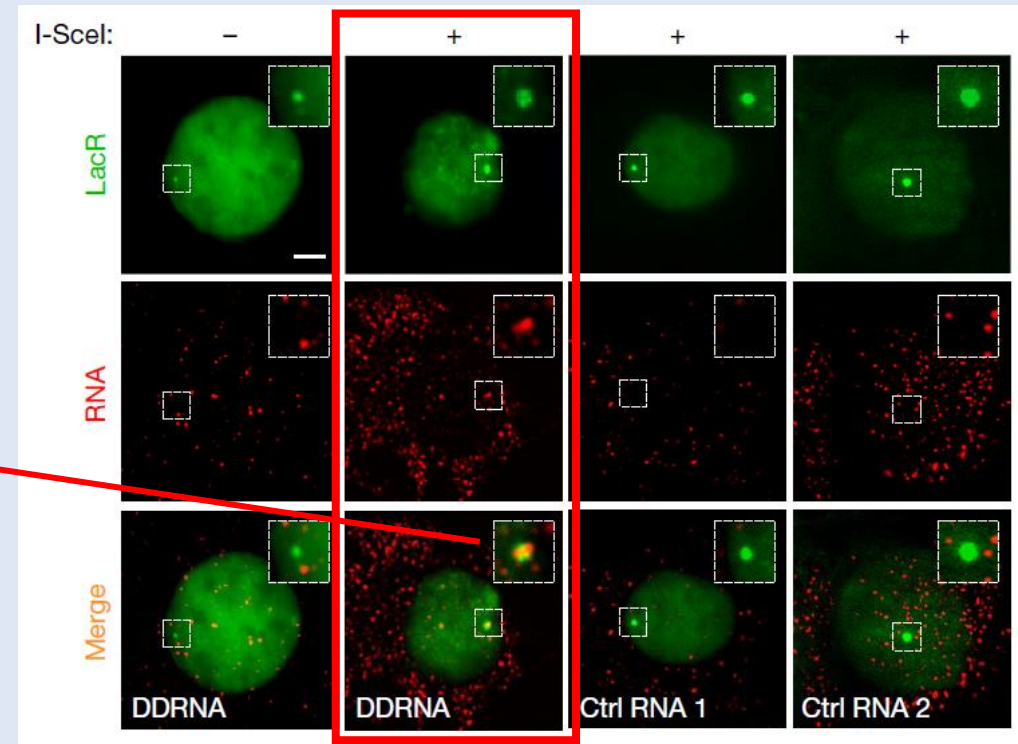
Single-molecule  
fluorescence  
microscopy analysis



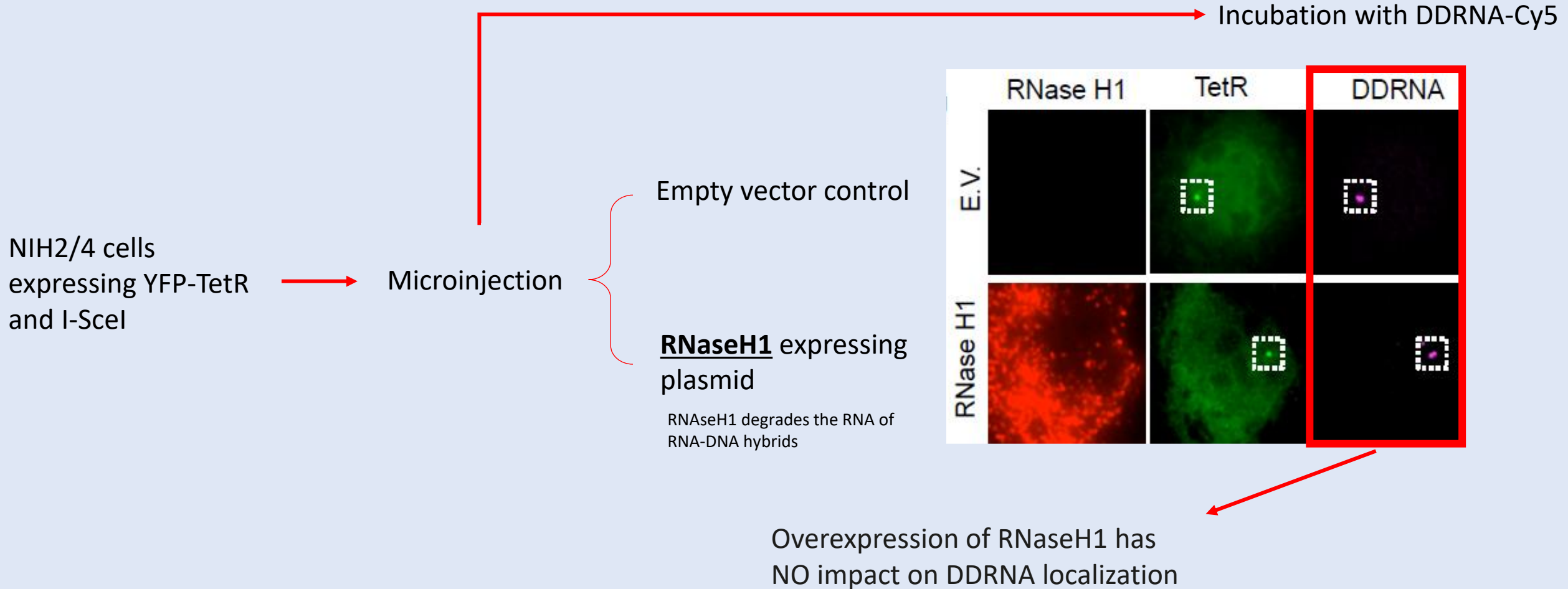
Four 3'-fluorophore-labelled double-stranded DDRNA pairs (DDRNA-Cy5)

**CONCLUSION → DDRNAs ARE LOCALIZING AT THE DAMAGE SITE**

CUT CELLS

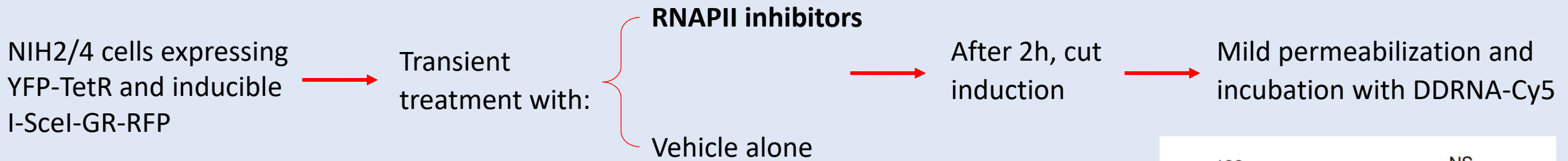


# DOES DDRNA INTERACT WITH DNA?



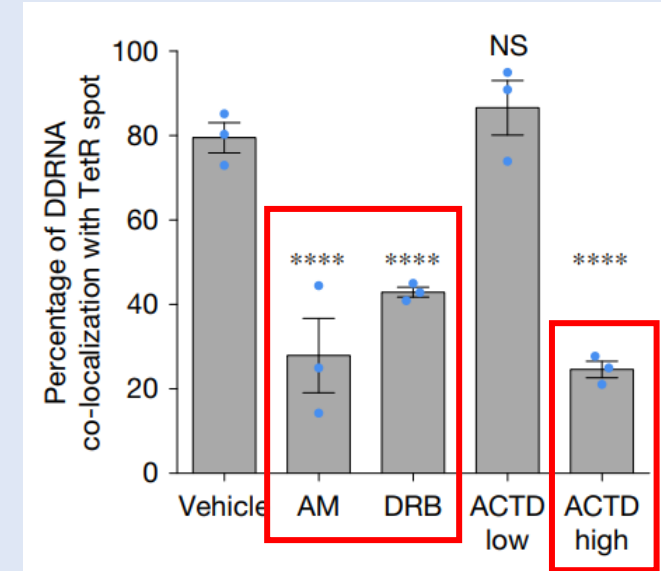
CONCLUSION → DDRNA LOCALIZATION DOES NOT DEPEND ON DDRNA:DNA PAIRING

# DOES DDRNA INTERACT WITH RNA?



**CONCLUSION → DDRNA LOCALIZATION DEPENDS ON RNA:RNA BASE PAIRING**

**WHAT ARE THESE RNAs?**



**RNAPII inhibition reduces DDRNA localization to the damage site (TetR spot)**



# HYPOTHESIS: COULD THIS RNA BE **dilncRNA**?

NIH2/4 cells expressing  
inducible I-SceI-GR

→ Transfection with:

**Biotinylated DDRNAs**  
(*btn-L1:L2*, *btn-U1:U2*)

Biotinylated miRNA  
(*btn-let-7a*)

Negative control

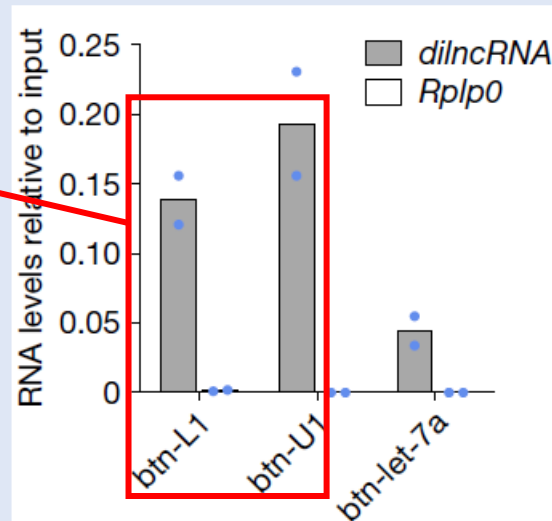
→ RNA pull down assay

↓  
Cell lysate + streptavidin coated Dynabeads

↓  
Isolation of bound RNAs

↓  
RT-qPCR

Enrichment of dilncRNAs bound to  
DDRNAs compared with control RNA



**CONCLUSION → DDRNA localization depends on RNA-RNA base pairing with dilncRNA in a RNAPII-dependent manner.**

# CAN DDRNAs ACTIVATE DDR?

NIH2/4 cells  
expressing YFP-TetR  
and I-SceI

→ **Dicer or Drosha  
knockdown**

→ Cell permeabilization with  
a mild detergent

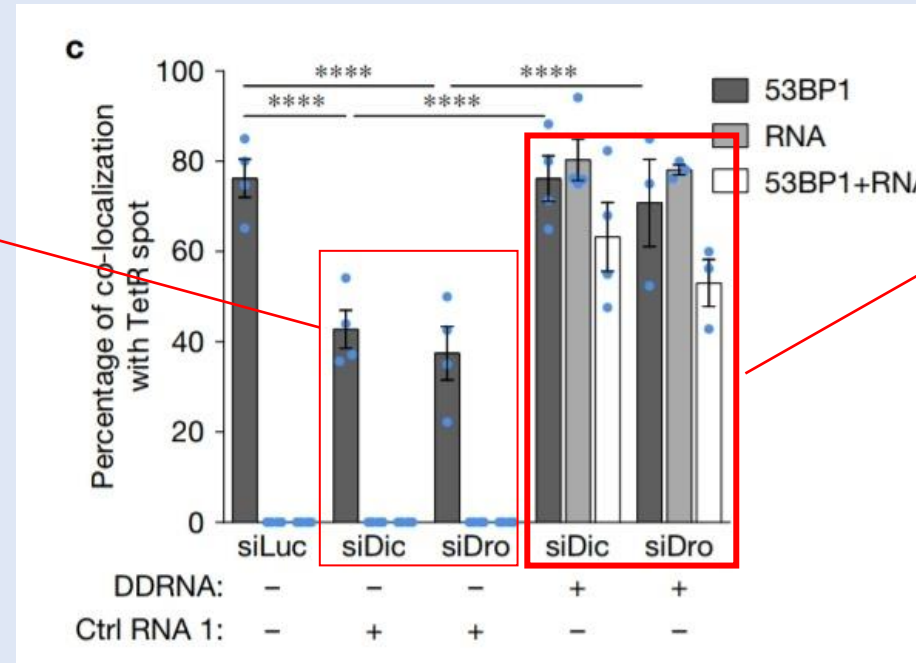
→ Incubation with:

DDRNs-Cy5 (*DDRNA*)

miRNA CXCR4-Cy5 (*Ctrl  
RNA 1*) as a control

→ Cell staining for  
DDR markers:  
**53BP1**

- Dicer or Drosha knockdown
- Incubation with **Ctrl RNA 1**



- Dicer or Drosha knockdown
- Incubation with **DDRNs-Cy5**

53BP1

RNA

**(DDRNA-Cy5)**

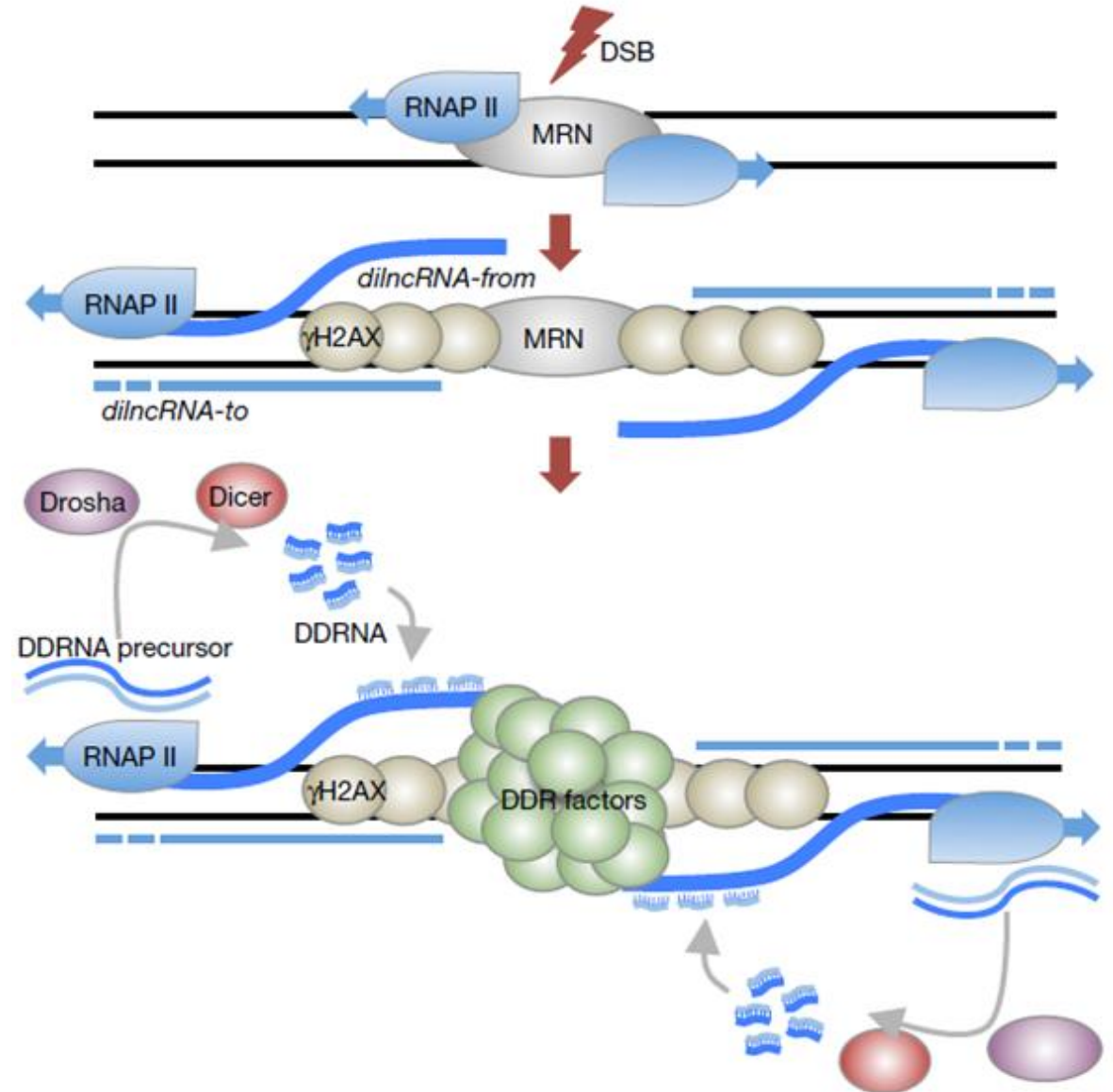
53BP1+RNA

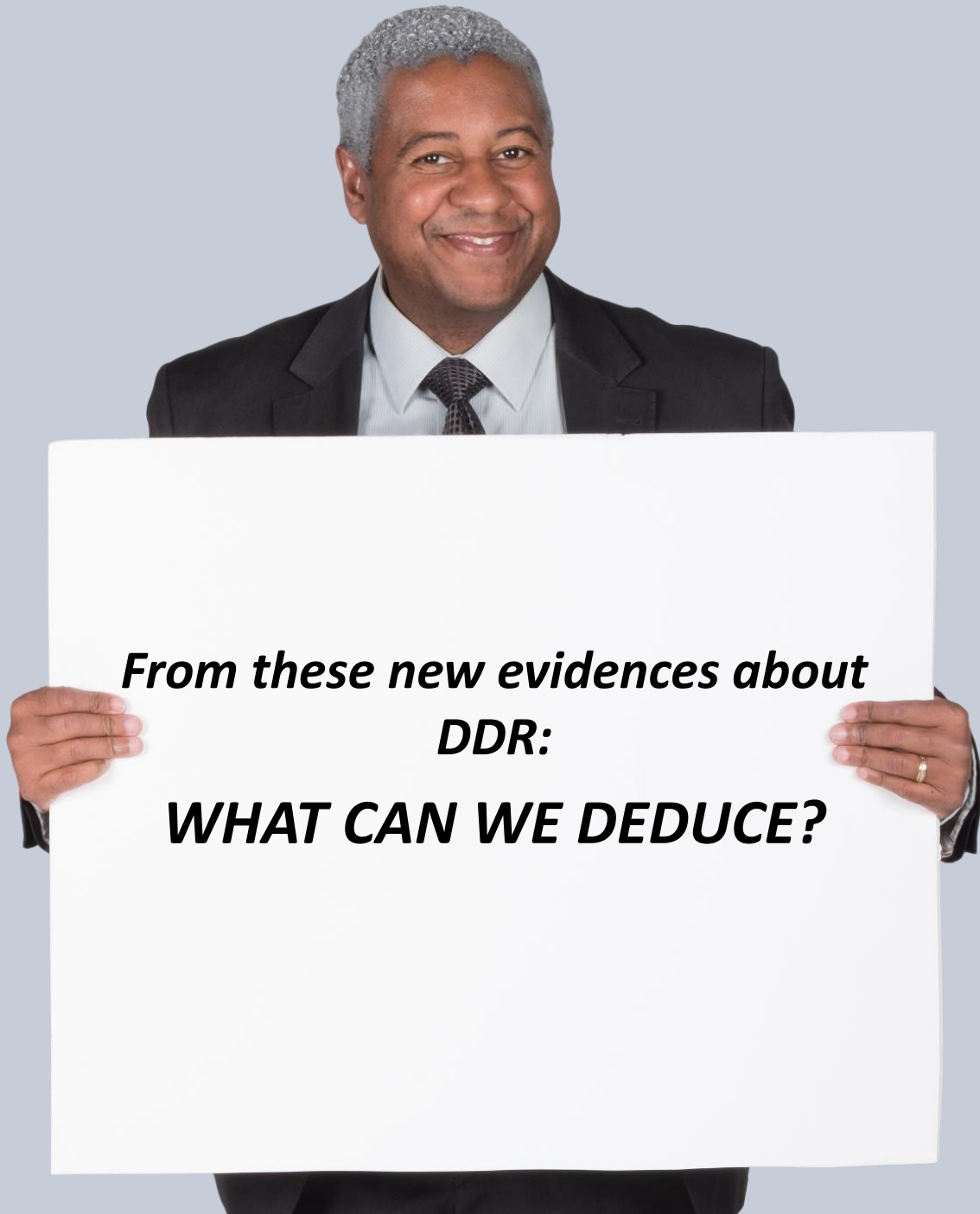
→ Co-localization with TetR

**CONCLUSION → DDRNAs ARE LOCALIZING AT THE DAMAGE SITE TO CONTRIBUTE TO  
DDR FOCI FORMATION**

# CONCLUSIONS

- MRN complex
- RNAPII recruitment
- Bidirectional synthesis:
  - dilncRNA-from (*blue*)
  - dilncRNA-to (*light blue*)
- Processing by DROSHA and DICER → comparable with miRNA processing
- DDRNAs:dilncRNAs interaction





***From these new evidences about  
DDR:***

***WHAT CAN WE DEDUCE?***

## **FUTURE PERSPECTIVES:**

Development of new therapeutic approaches which target the localization of DDRNAs

THANK YOU  
FOR YOUR  
ATTENTION

