Functional transcription promoters at DNA double-strand breaks mediate RNA-driven phase separation of damage-response factors

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COMPARTIMENTALIZATION OF THE CELL

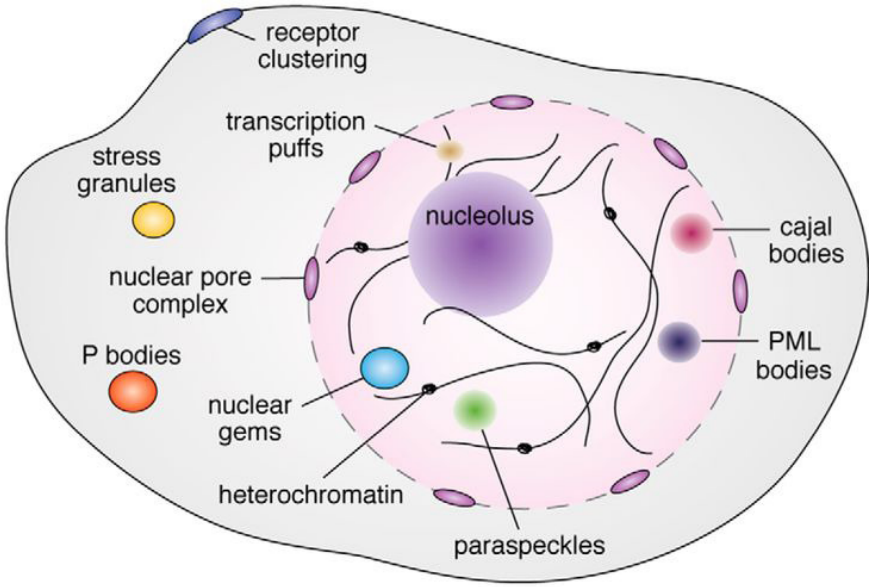
Organelles, defined by a phospholipid membrane:

- Mitochondria
- Lysosome
- Golgi apparatus
- ER
- Nucleus

VS

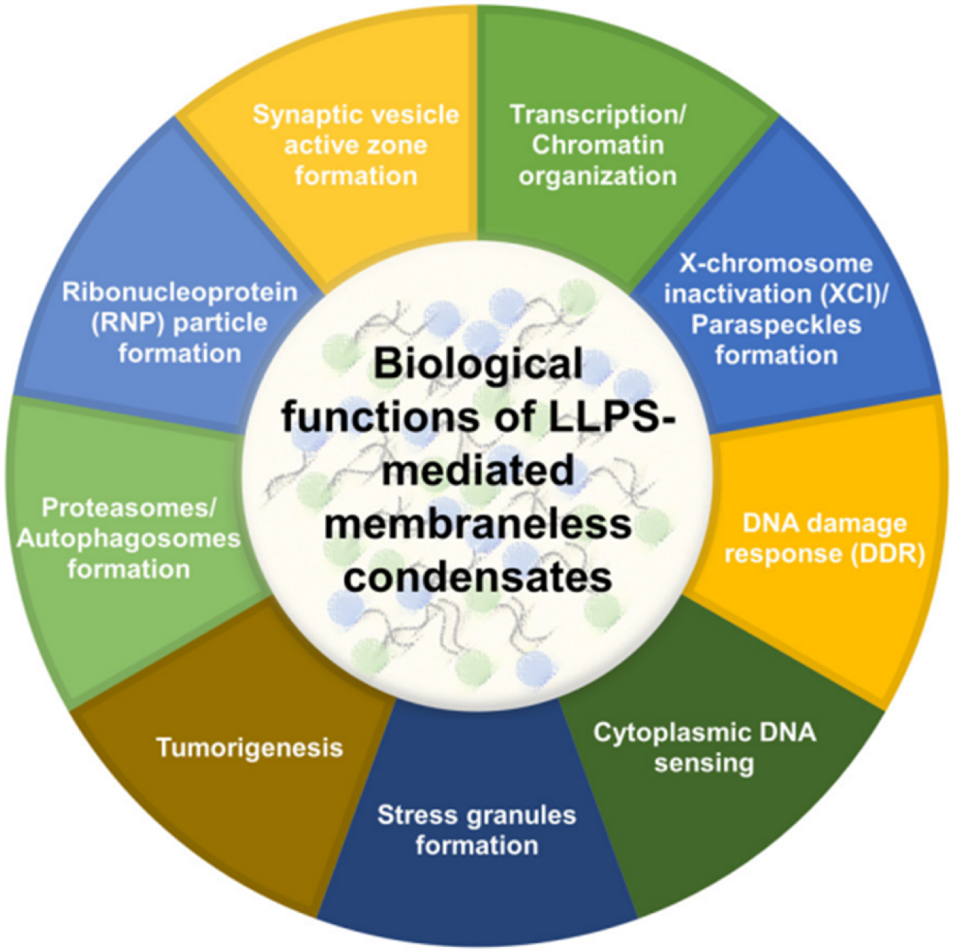
Membrane-less organelles, formed through liquid-liquid phase separation (LLPS):

- DNA damage foci
- Transcription puffs
- XCI compartment
- Cajal body
- Nucleoli
- paraspeckles



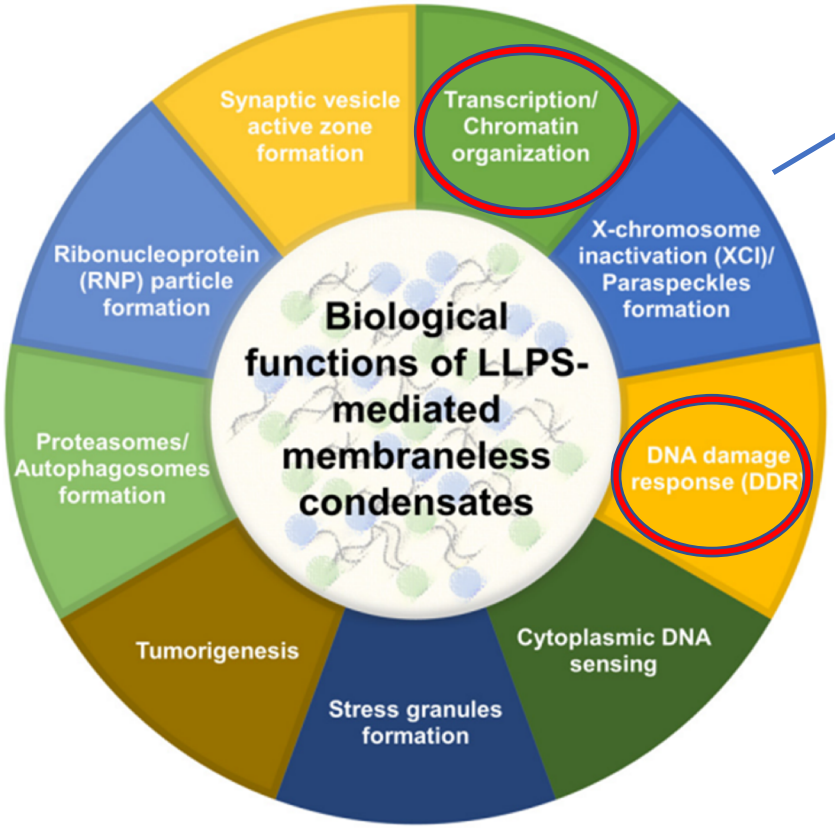
The molecular language of membraneless organelles Published, Papers in Press, July 25, 2018, DOI 10.1074/jbc.TM118.001192
Edward Gomes and X James Shorter1 From the Department of Biochemistry and Biophysics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104

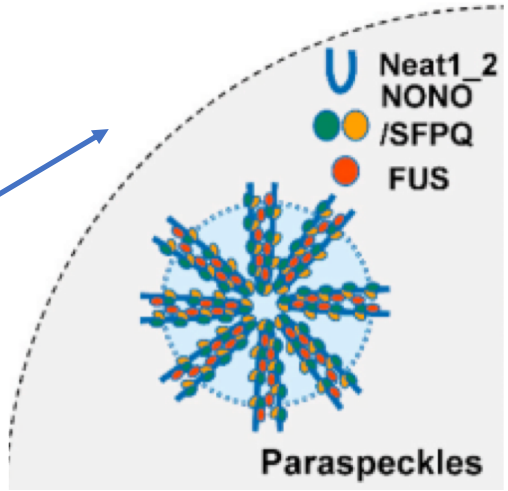
WHAT ARE THE MAIN CHARACTERISTICS OF MEMBRANELESS ORGANELLES ?



- Generated through Liquid-liquid phase separation
- Represent a well defined space inside the cell
- Enriched for specific proteins and/or RNAs
- Defined function

EXAMPLE OF LLPS CONDENSATE AND THEIR FUNCTION



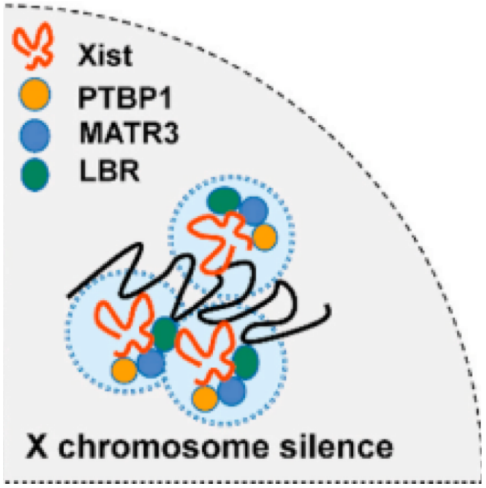


Control gene expression through nuclear retention of RNA

Possible role in regulation of:

- Differentitation
- Viral infection
- Stress response

Deregulation of Neat1 and/or FUS lead to Frontotemporal dementia and ALS.



Induce **heterochromatinization** of the X chromosome and thus its silencing.

BIOCHEMISTRY OF LLPS

A Homogenous solution **spontaneously demixes** into two liquid phases:

-**Dense phase**, enriched for specific molecules
-**Diluite phase**, depleted for specific molecules

Generation of a **boundary** between the two phases

-generation of **functional compartment**
-that allows **diffusion** of selective molecules

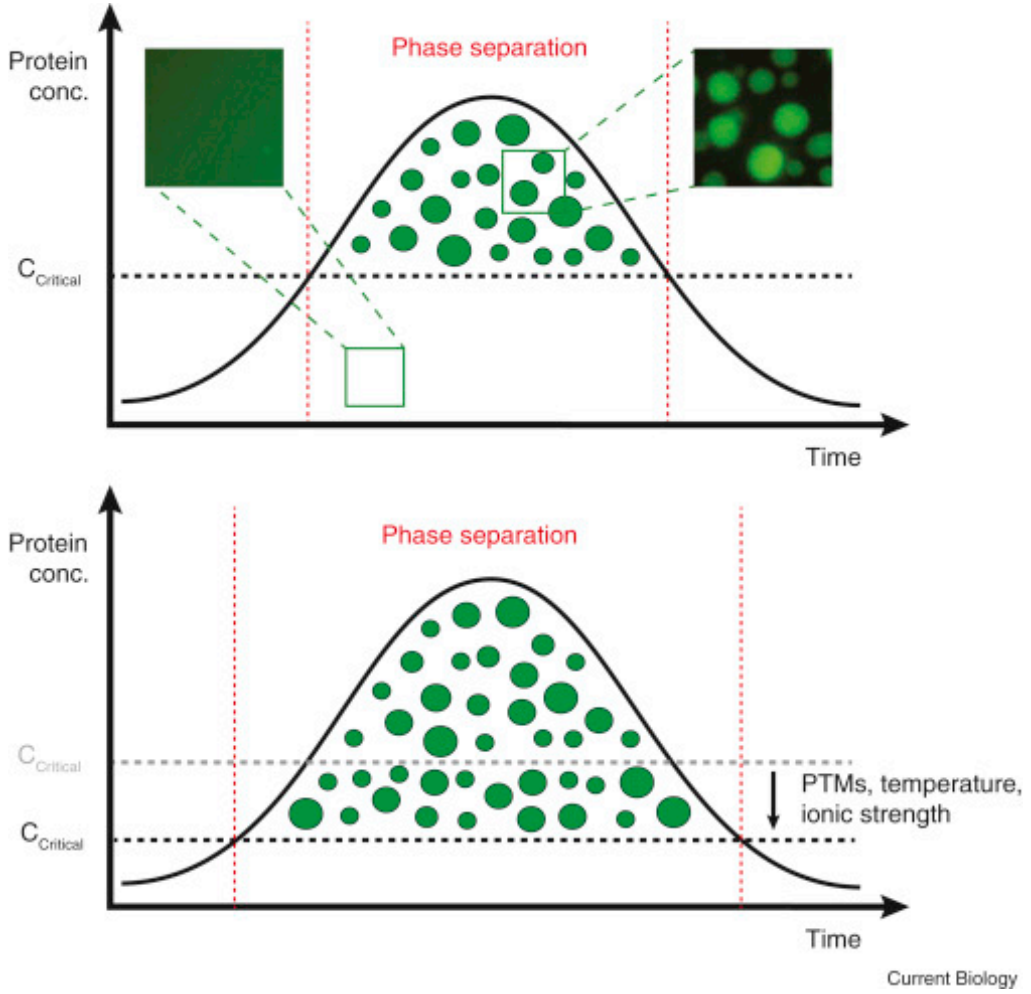
Phase separation is **dependent** on:

-Protein's concentration (Critical Concentration)
-Valency
-Solubility

Can also be **affected** by:

-Post Translational Modifications (**PTMs**)
-**Temperature**
-**Ionic strength**

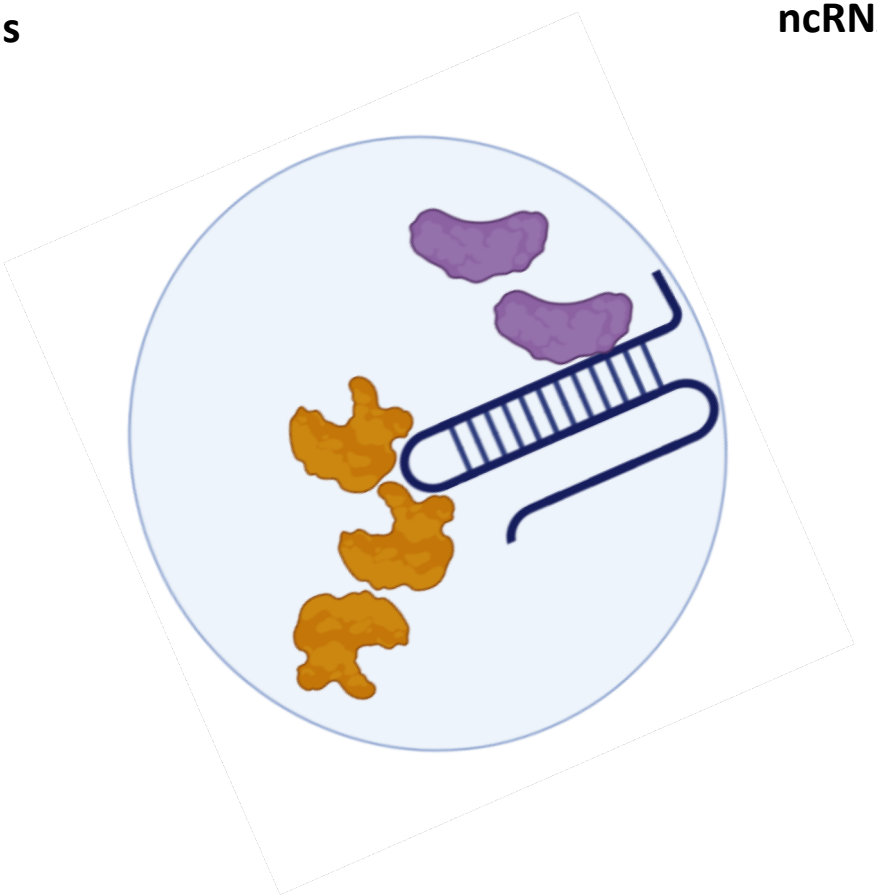
-> Able to lowering the Critical concentration threshold



LIQUID-LIQUID PHASE SEPARATION COMPONENTS

Proteins

ncRNAs



LIQUID-LIQUID PHASE SEPARATION COMPONENTS: *PROTEINS*

Condensate in the cell enriched for specific proteins

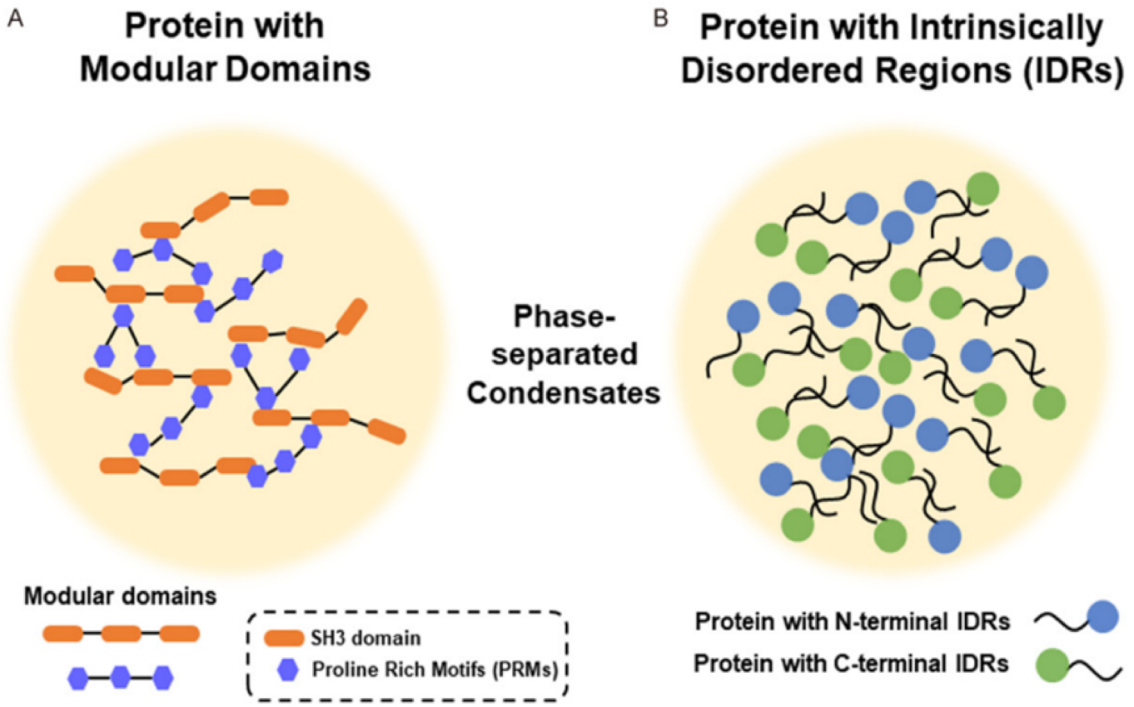
PROTEINS:

Have either:

A. **Modular domains** (SH3) between proline rich motifs (**PRMs**)
B. **I**ntrinsically disordered regions **(IDRs)** such as **Prion like domain (PrLDs)** or an arginine/glycine rich sequnce (**RGG/RG).**

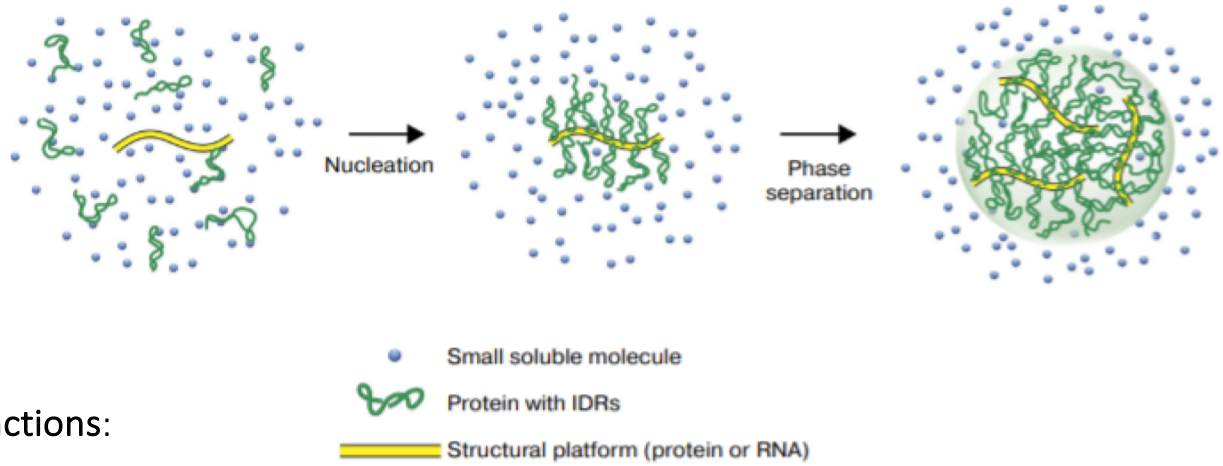
- **These domains make multiple weak electrostatic interaction between different proteins or with ncRNAs**

- **Post-trancriprional modifications (PTMs)** can modulate these interactions by affecting the proteins charges



LIQUID-LIQUID PHASE SEPARATION COMPONENTS: *ncRNAs*

Condensate in the cell enriched for specific ncRNAs



LONG NON CODING RNA:

lncRNAs based on :

- Abundance
- Sequence
- Length
- Secondary structure

Can have different Functions:

1. **Molecular scaffold** to bind **RNA binding proteins (**RBPs), forming phase separation droplets
2. Seed to **recruit** specific RBPs that can recruit additional protein to form LLPS and control different function of LLPS
3. Tune the physical features of phase separated condensate including size, shape, viscosity, surface tension and molecular composition
4. **Buffer** RBPs in nucleus keeping them soluble

PHASE SEPARATION IN TRANSCRIPTION: *ENHANCE ACTIVATION*

Recent reports showed the involvement of LLPS during transcription thanks to different components:

Super-enhancers (SEs)

Enhancer **cluster** that can **bind** to high-density **proteins** and **RNAs**

Enhancer RNAs (**eRNAs**)

Heterogeneous class
Have a role in phase separation

Transcription Factors (TFs)

bind to **enhancers** and **promoters**

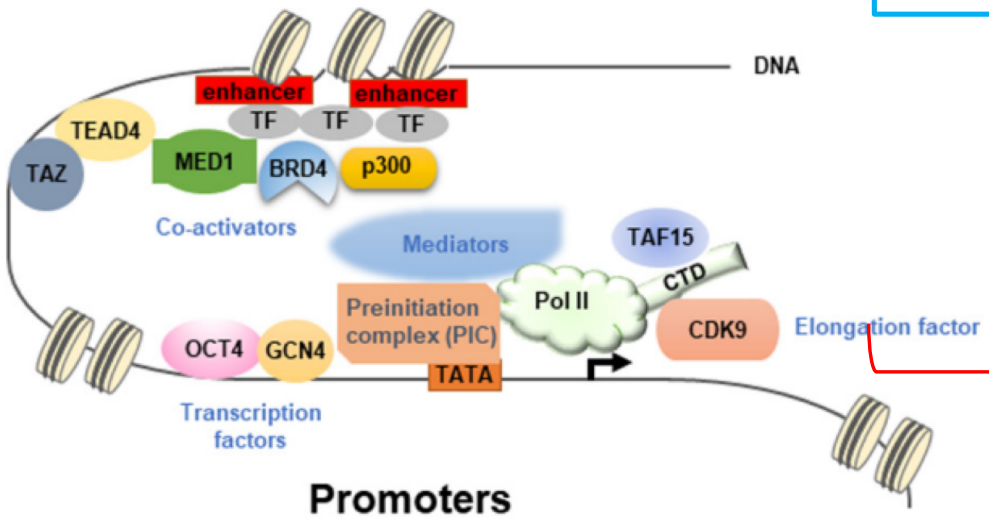
Co-Activators

Capable to **bind** to **TFs**

IDRs can form transcriptional condensate via LLPS
Ex**: BRD4** and **MED1**

Mediator complex

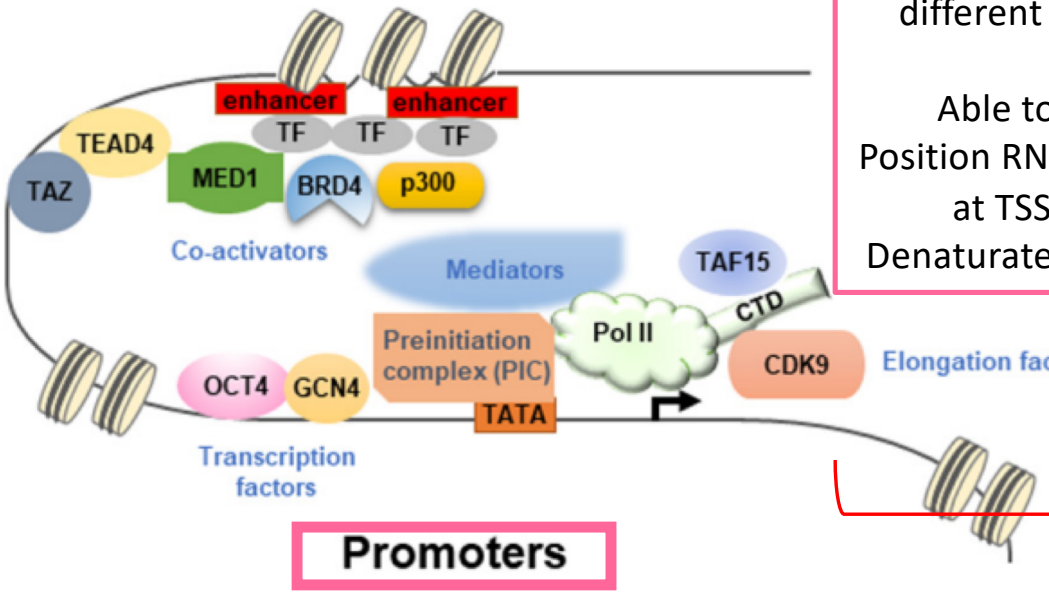
Multiprotein complex function as co-activator binding TF;CoActivators;RNApolII and Pre Initation complex (PIC)



Formation of phase separated droplets from SEs

PHASE SEPARATION IN TRANSCRIPTION: *PROMOTERS*

Recent reports showed the involvement of LLPS during transcription thanks to different components:



Mediator complex

TFs

Complex of different TFs

Able to:
Position RNApolII at TSS
Denaturate DNA

Pre Initiation Complex (PIC)

RNApolII

Carbossi Terminal Domain (CTD) -> IDR

- Able to interact with low complexity domains of **TFs** promoting llps
- Phosporylation levels mediate different condensate during different phases of transcription

FET proteins
TAF15-EWS-FUS

PrLDs

Interact with **RNApolII-CTD** favoring llps enhancing transcription

Formation of phase separated droplets from promoters

TAZ-MEDIATED GENE EXPRESSION TROUGH LLPS

HIPPO pathway ‘OFF’

↓

TAZ activation

↓

Phase separated nuclear condensate with:

-TEAD4

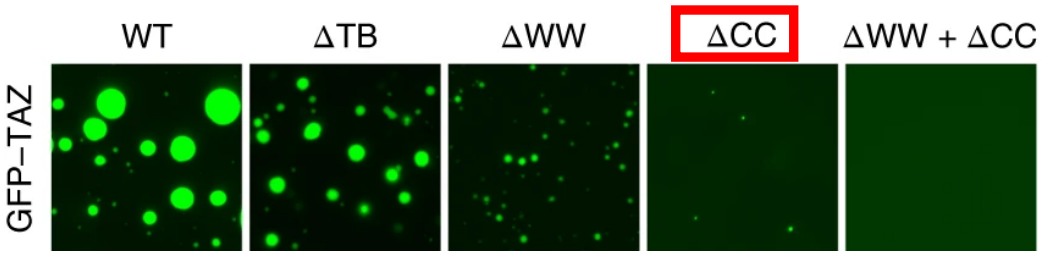
-BRD4

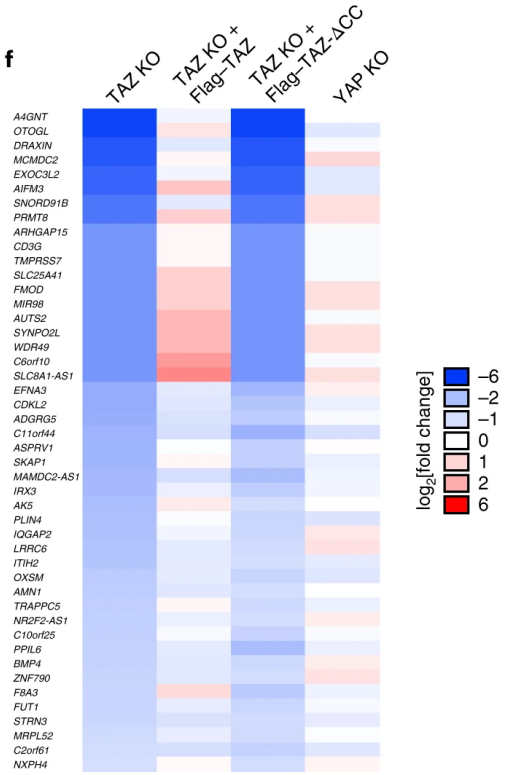
-MED1

-CDK9

↓

ACTIVATION OF TAZ REGULATED GENES





HIPPO pathway ‘ON’

↓

TAZ phosporylation via LATS

↓

Change in TAZ **coiled-coil (CC) domain**

⊣

TAZ activation

⊣

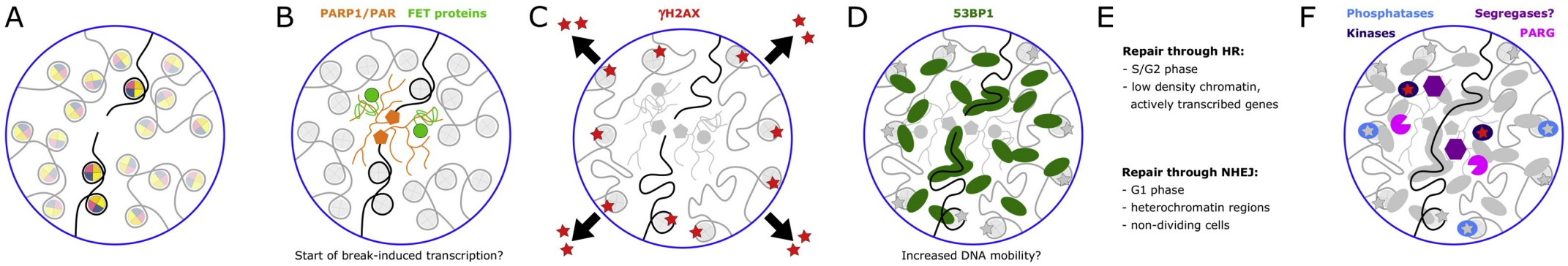
phase-phase separation

⊣

ACTIVATION OF TAZ REGULATED GENES

OVERVIEW ON LLPS IN DNA DAMAGE FOCI FORMATION

- Most severe lesion that can occurs at DNA is the double strand break (**DSB**)
- **Repair** of DSB starts with a **signaling cascade** and the consecutive **recruitment** of **repair factors** to the damage site
- **Formation of repair focus**: accumulation of markers and repair proteins
- **Phase separation** allows control of **early response** to DNA damage



PHASE SEPARATION IS PRESENT AT MULTIPLE LEVELS IN DDR

A

Double strand DNA break (DSB)

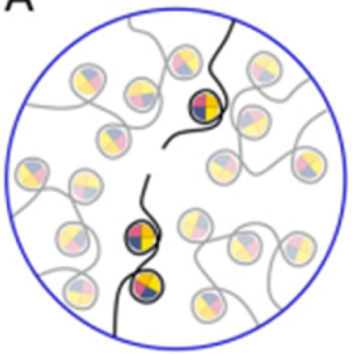
B

PolyADPribosepolymerase1 PARP1

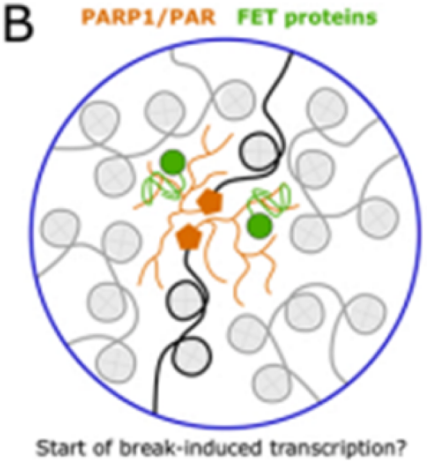
B

FET proteins recruitment and **dilncRNAs** trancription

A



B



Early responder at DNA damage

Produce long and branched PolyADP-Ribose chains (**PAR**) that:

a. Act as signal for DNA repair proteins

b. Lead to **phase separation** around the damage site thanks to negative charges interacting with proteins IDRs

Resulting in control of which proteins get recruited to the DSB condensate

Recruited thanks to PrLDs

Functions in transcription, binding to RNApolII-CTD recruited independently

Resulting in amplification and stabilization of phase separation; and in RNApolII **transcription** of **dilncRNAs**

PHASE SEPARATION IS PRESENT AT MULTIPLE LEVELS IN DDR

C

ATM phosporilate H2AX

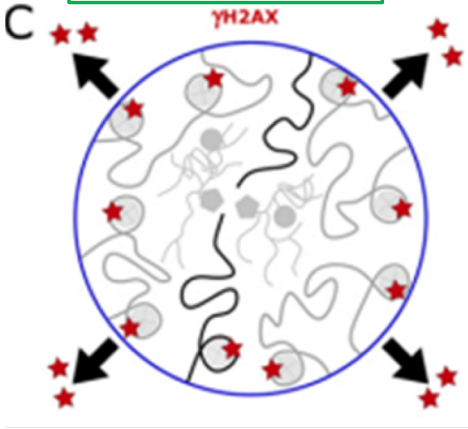
D

Spread of γH2AX

D

MDC1 recruits **53BP1**

C

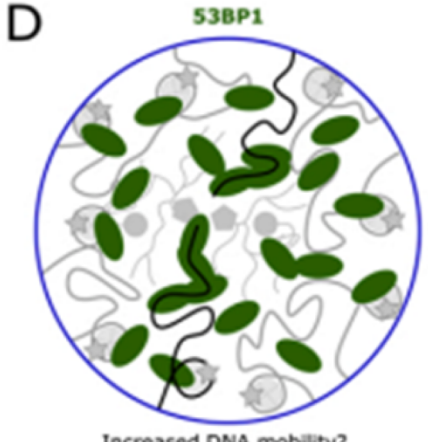


promotes chromatin decondensation

Modification of phase separated compartment around the break site allowing more proteins in

Resulting in recruitment of **MDC1**

D



Tudor domain

- Bind dimethylated histone H4K20, more accessible thanks to the decondensation of the chromatin
- interact with **dilncRNAs**

BRCT domains

- important for its phase separating capabilities and foci formation

Resulting in nucleation and growth of **53BP1 foci** dependent on **dilncRNAs**

PHASE SEPARATION IS PRESENT AT MULTIPLE LEVELS IN DDR

E

DSB repair

F

DNA repair foci dissolution

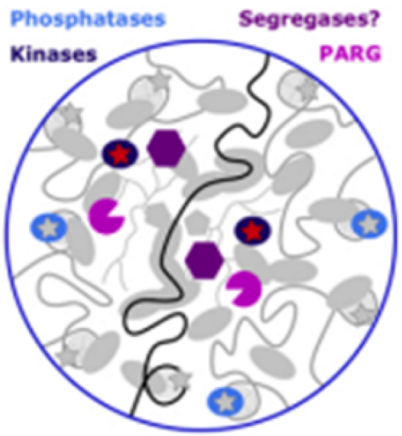
An important take-away message

E

Repair through HR:
- S/G2 phase
- low density chromatin, actively transcribed genes

Repair through NHEJ:
- G1 phase
- heterochromatin regions
- non-dividing cells

F



PAR glycohydrolase (PARG) break down PAR chains

PARP1 **autoPARylation** prevent excessive growth of foci

ATM can phosphorylate:
- **FUS -> less prone to aggregation**
- **RNApolII-CTD ->** regulating RNApolII aggregation and dilncRNAs transcription
- **HP1 ->** chromatin condensation

Phosphatase 2A and **WIP1** act on γH2AX resolving the focus

RNApolII with its CTD is able to interact and promote phase separation by itself.

Moreover the dilncRNAs transcribed by the RNApolII are fundamental for the formation and stability of DDR foci.

indipendent studies showed that the absence of dilncRNAs lead to foci dissipation

ARTICLES
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nature cell biology

Functional transcription promoters at DNA double-strand breaks mediate RNA-driven phase separation of damage-response factors

Fabio Pessina[1], Fabio Giavazzi[2], Yandong Yin[3], Ubaldo Gioia[1], Valerio Vitelli[1], Alessandro Galbiati[1], Sara Barozzi[1], Massimiliano Garre[1], Amanda Oldani[1], Andrew Flaus[4], Roberto Cerbino[2], Dario Parazzoli[1], Eli Rothenberg[3] and Fabrizio d'Adda di Fagagna[1,5*]

Damage-induced long non-coding RNAs (dilncRNA) synthesized at DNA double-strand breaks (DSBs) by RNA polymerase II are necessary for DNA-damage-response (DDR) focus formation. We demonstrate that induction of DSBs results in the assembly of functional promoters that include a complete RNA polymerase II preinitiation complex, MED1 and CDK9. Absence or inactivation of these factors causes a reduction in DDR foci both in vivo and in an in vitro system that reconstitutes DDR events on nucleosomes. We also show that dilncRNAs drive molecular crowding of DDR proteins, such as 53BP1, into foci that exhibit liquid–liquid phase-separation condensate properties. We propose that the assembly of DSB-induced transcriptional promoters drives RNA synthesis, which stimulates phase separation of DDR factors in the shape of foci.

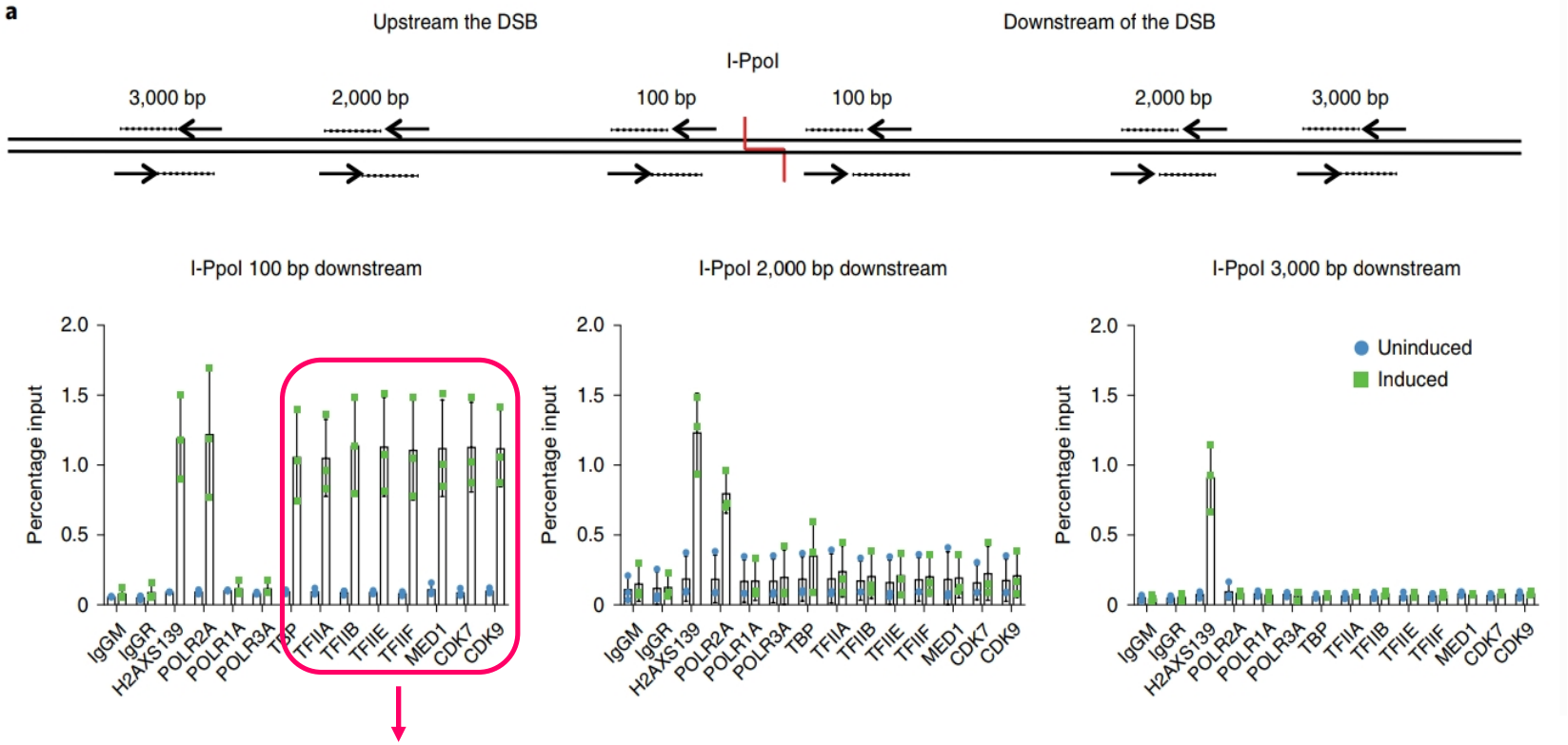
NATURE CELL BIOLOGY | VOL 21 | OCTOBER 2019 | 1286–1299 | www.nature.com/naturecellbiology

Can PIC complex and associated dilncRNA synthesis at DSB have a role in DNA damage response? Is LLPS of DDR factors such as 53BP1 involved?

IS PMC COMPLEX RECRUITED TO DSBS TOGETHER WITH POLR2A?

PMC: Preinitiation Complex (PIC) + MED1 + CDK9

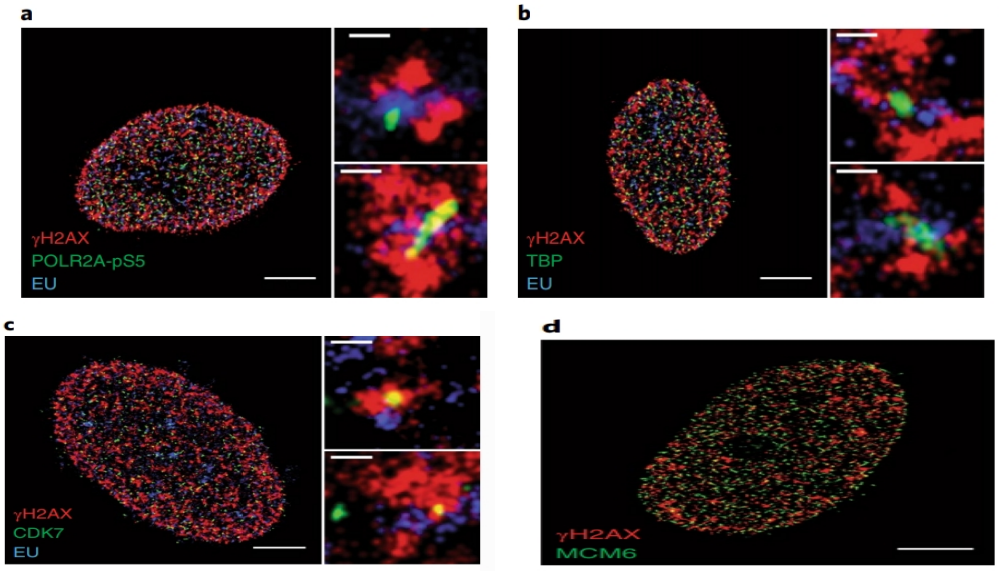
HeLa cells whit a specific endogenous locus cleaved by I-PpoI endonuclease to induce DSB → ChIP analyses at different distances to the cut

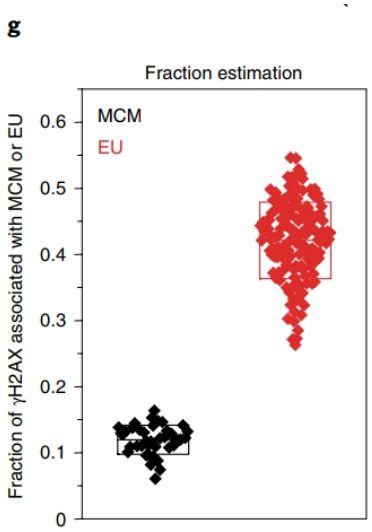


PMC components and RNAPII are strongly associated only in proximity of the DSB (100bp) → **Active transcription at DSB?**

THERE IS ACTIVE TRANSCRIPTION AT DSB?

U2OS cells, treated with neocarcinostatin (NCS) to induce DSB → labelled with Ethynil uridine (EU) to detect nascent transcripts → Super resolution imaging analysis





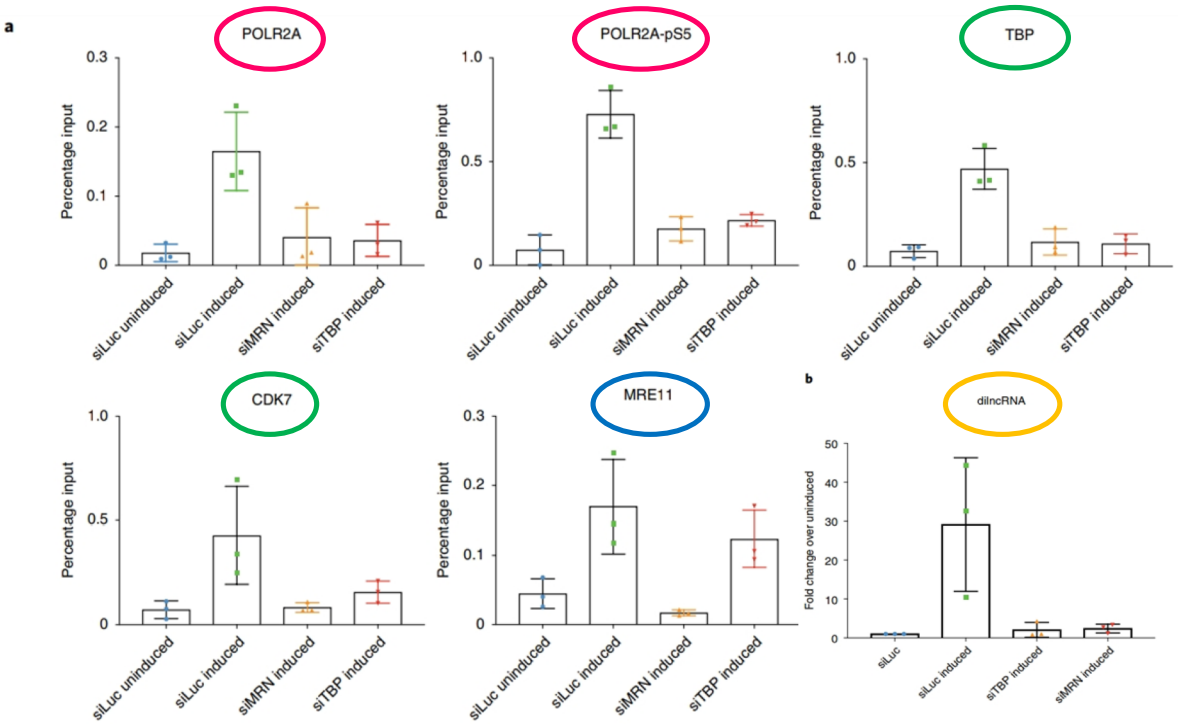
γH2AX, PIC component TBP or CDK7, POL2A-p5S colocalize with EU staining → RNA transcription after DSB occur at DSB

EU - γH2AX colocalization levels increase in NCS-treated cells proportionally with increased γH2AX signal.

PMC components, RNAPOLII, assemble at DSB and coexist with local RNA synthesis

How is RNAPII recruited ad DSB site?

HeLa cells with locus cleaved by I-PoI endonuclease → RNA interference with injection of siMRN (MRN complex) and siTBP (PIC complex)



siTBP and siMRN inhibited the accumulation of RNAPII

Similar to their assembly at promoters:

- CDK7 recruitment depends on TBP and MRN
- TBP recruitment depends on MRN

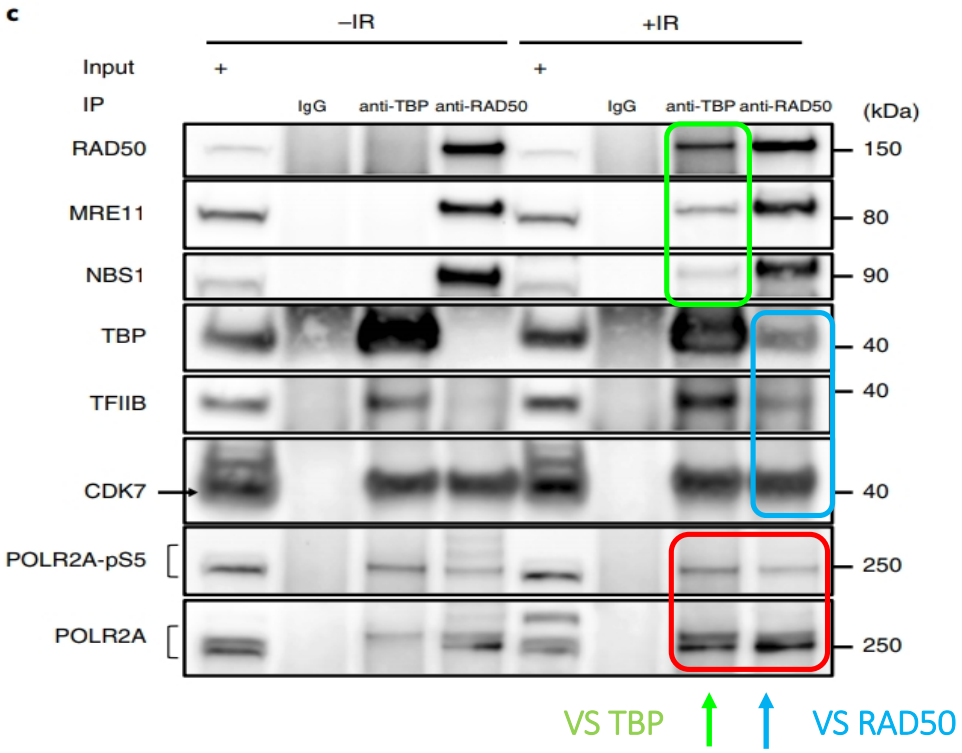
MRN recruitment does NOT depends on TBP

siTBP and siMRN inhibited dilncRNA synthesis

MRN and PIC are important for recruitment and stabilitazion of RNAPII and also impact each other

How MRN and PIC complexes act to localize RNAPII at DSBs?

HeLa cells before and after IR exposition, that induce DSB → CoIP with antibodies anti-RAD50 (MRN complex) or anti-TBP (PIC complex)



Anti-TBP pulled-down:
- MRN component tested
- POLR2A

Anti-RAD50 pulled-down:
- PIC component tested
- POLR2A

MRN acts as a tethering factor at DSBs for PIC, with which it forms a complex. PIC has a crucial role in dilncRNA synthesis.

Does PIC complex impact on DDR signalling?

U2OS cell exposed to IR to induce DSB are treated with:

RNA interference VS PIC components:

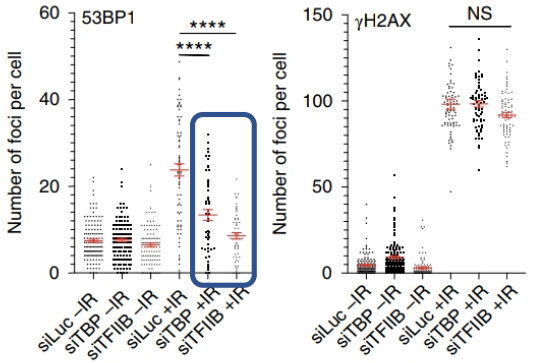
- siTBP
- siTFIIB

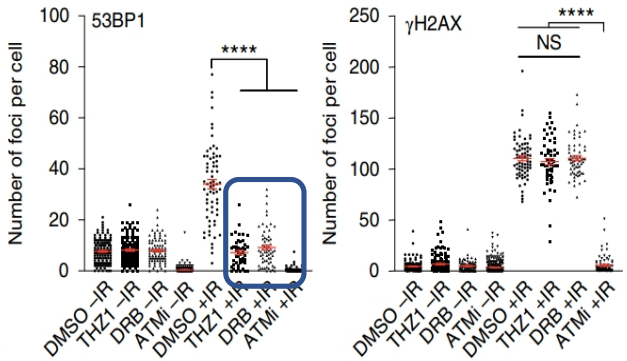
small molecules:

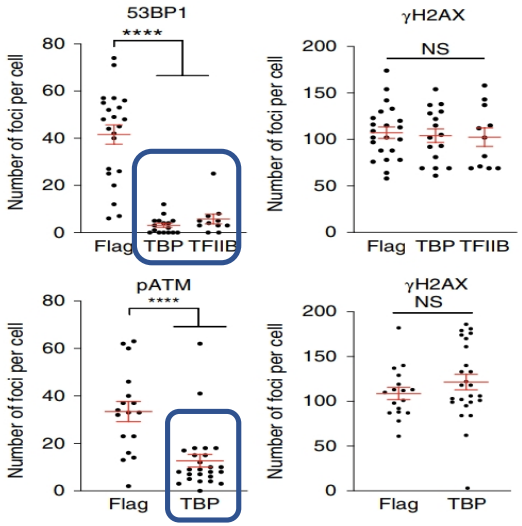
- THZ1 (CDK7 inhibitor)
- DRB (CDK7 – CDK9 inhibitor)
- ATMi

inhibitory antibodies:

- Anti-TBP
- Anti-TFIIB







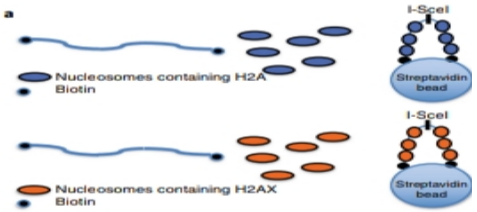
PIC inactivation reduce DDR signalling, despite γH2AX foci are unaffected in number and endogenous levels of DDR protein are unalterated

PIC impact on DDR signalling thanks to his function in RNA transcription?

Does RNA transcription impact on DDR signalling?

EX 1. RNA transcription active induction impact on DDR signalling?

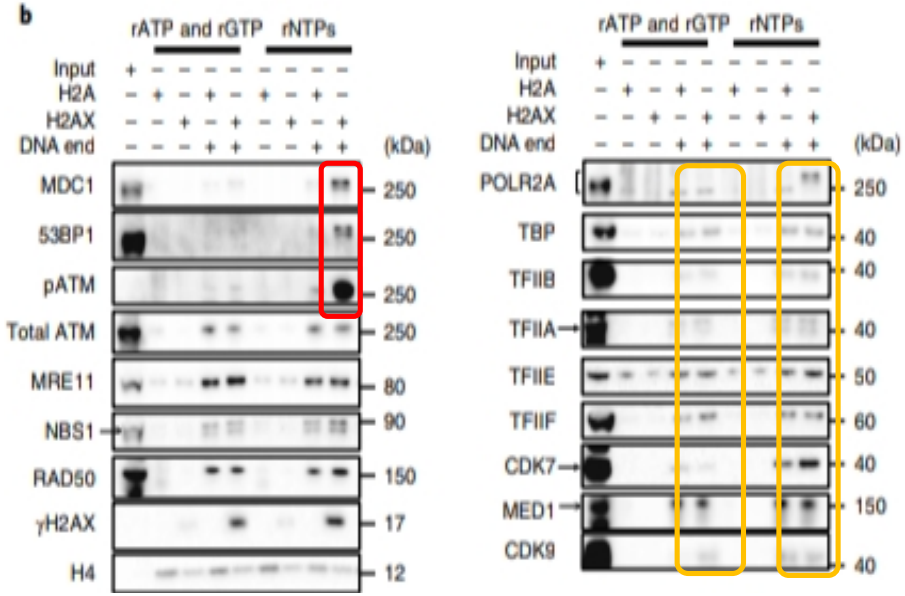
IN VITRO ASSAY:



→ Incubated with HeLa nuclear extract
In NO TRANSCRIPTION condition:

- With rGTP and rATP for energy
- No other rNTP

→ Supply the complete rNTP pool,
ALLOW TRANSCRIPTION



When the complete rNTP is supply is observed a H2AX – dependent accumulation of DDR FACTORS

PIC and RNAPII recruitment at nucleosome is independent of RNA synthesis

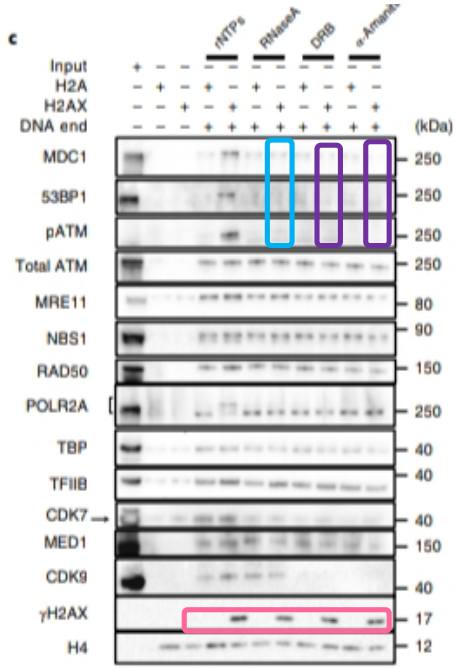
RNA transcription is necessary for secondary DDR factors recruitment

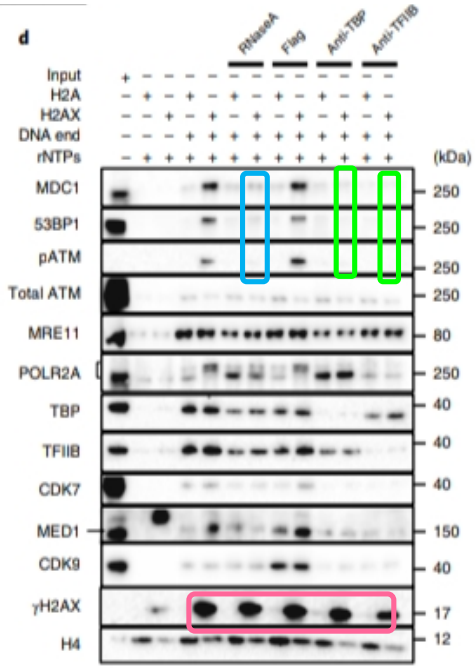
Does RNA transcription impact on DDR signalling?

EX 2. RNA transcription inhibition impact on DDR signalling?

Same in vitro assay but at the end treated with:

- RNaseA (degrade RNA)
- DRB or α-amanitin (prevent RNA synthesis)
- Antibody VS TBP or TFIIB (prevent recruitment of downstream PMC components)



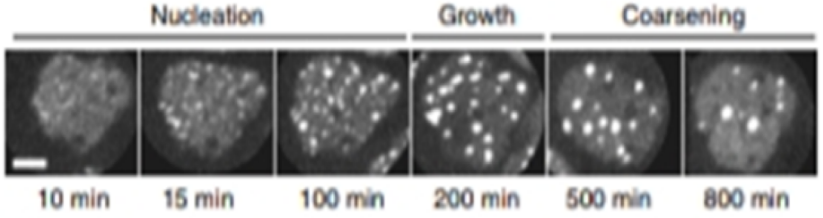


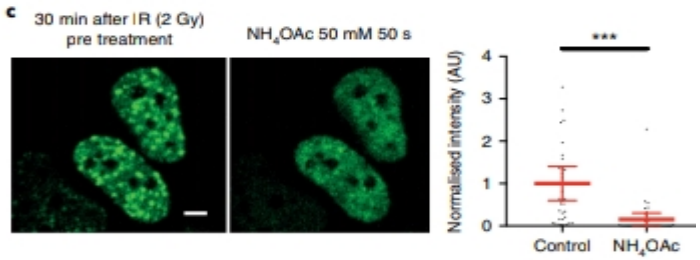
RNA inhibition reduce pATM, 53BP1 and MDC1 (DDR secondary factors) recruitment, despite unchanged γH2AX levels

RNA synthesis, supported by PIC components, is essential for H2AX-dependent recruitment of DDR factors.

Do DDR factor 53BP1 foci show LLPS characteristics?

foci formation analysis with time-laps live cell microscopy reveal LLPS foci formation phases

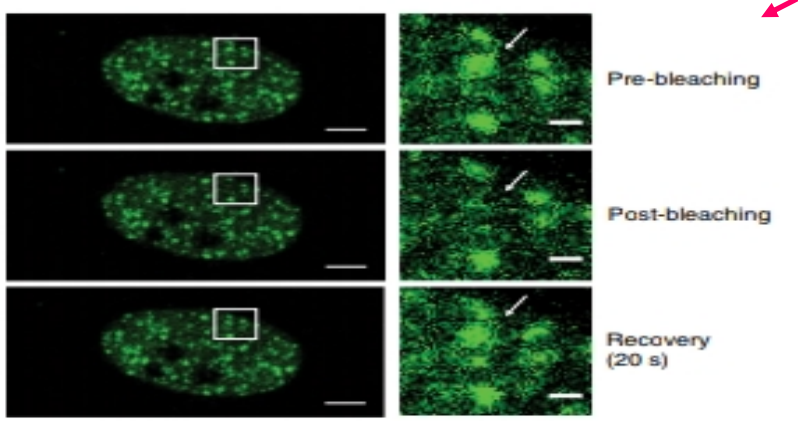




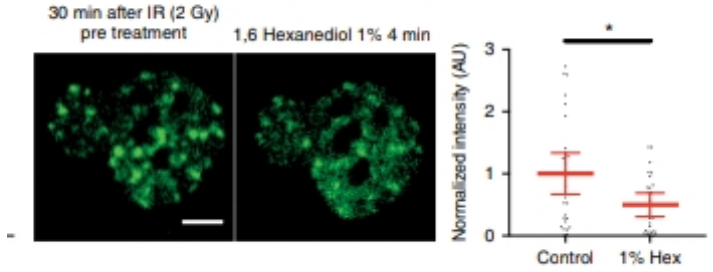
U2OS cells:

- stably expressing near-endogenous levels of 53BP1-GFP
- IR irradiated to induce DSB

Foci condensate properties analysis are confirmedwith Fluorescent Recovery After Photobleaching (FRAP)



53BP1 foci dissolve after treatment with ammonium acetate and 1,6 – hexanediol

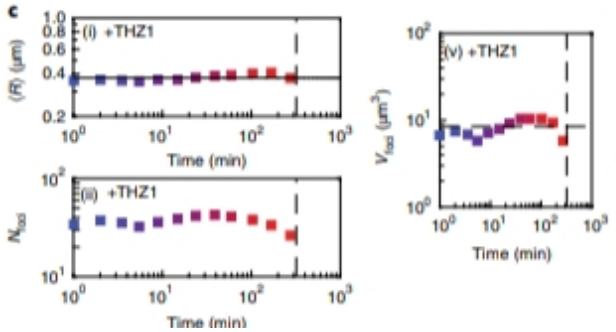


53BP1 foci are LLPS compartments

Can dilncRNA transcription impact on LLPS of 53BP1?

1. U2OS cells:
   - stably expressing near-endogenous levels of 53BP1-GFP
   - IR irradiated to induce DSB

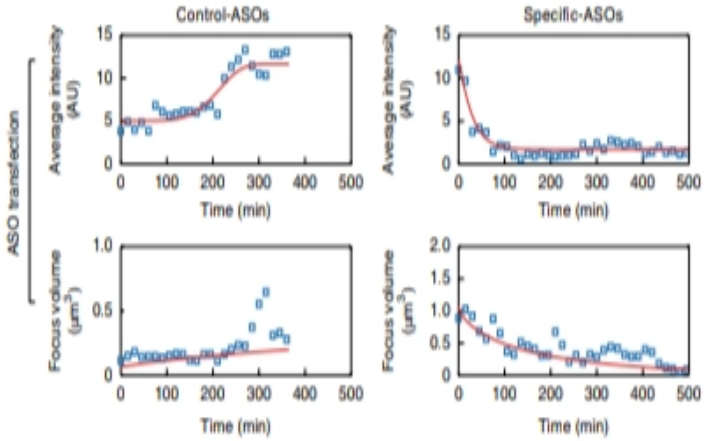
→ Treated with THZ1 (CDK7 inhibitor)
STOP TRANSCRIPTION



foci maturation arrested during nucleation phase

2. NIH 2/4 cell, in which is induced DSB

→ Transfected with specific oligonucleotide VS dilncRNA
or control oligonucleotide



Specific-ASO cause the 53BP1 focus to disappear; << average intensity during a time scale that is 10X faster than the size reduction, typical of liquid/viscous objects

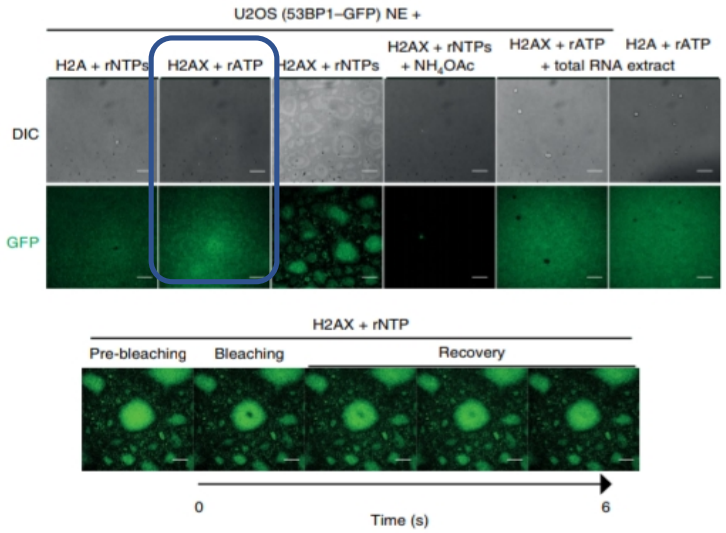
53BP1 foci are LLPS compartment dependent on dilncRNA through time

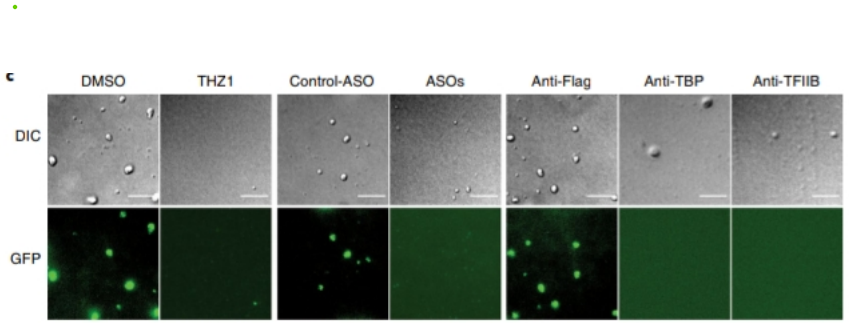
Can dilncRNA transcription promote LLPS formation?

in vitro assay incubated with U2OS 53BP-GFP nuclear extract, free of rNTPs

↓

Supply the complete rNTP pool, ALLOW TRANSCRIPTION





Only the combination of H2AX-containing nucleosome array + rNTP:

- enable the formation of 53BP1-GFP-containing condensates, that show recovery after photobleaching

→ Treatment with

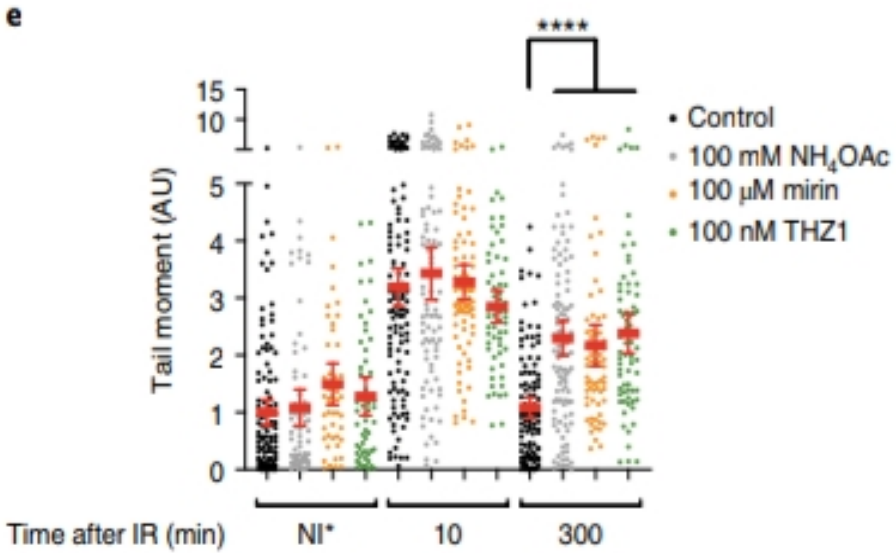
- transcriptional inhibitors
  - THZ1
  - Anti-TBP
  - Anti-TFIIB

  prevent the formation of 53BP1-GFP droplets and disrupted the droplets once they are already formed
- ASO vs dilncRNA

dilRNA and PIC component have a role in promoting 53BP1 LLPS events

Can phase separation and de novo transcription impact on DBS repair?

HeLa cells, irradiated IR to induce DSB → Treated with → Ammonium acetate (Impact on LLPS) / THZ1 (Impact on transcription) → Neutral comet assay



Treatment with THZ1 (VS transcription) and ammonium acetate (VS phase separation) result in increase comet tail moment, indicative of impaired DNA repair

PIC components and LLPS positively contribute to DSB repair

CONCLUSIONS