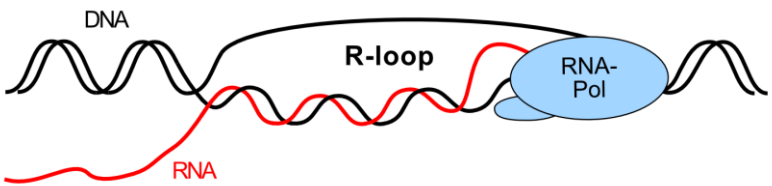
Live-cell imaging of R-loops –

Towards an imaging-based R-loop screening platform

Robert M. Martin

Drug Discovery Platform - Sérgio Almeida Lab
GIMM
November 5, 2024 - 1st TRIAD Meeting

Background



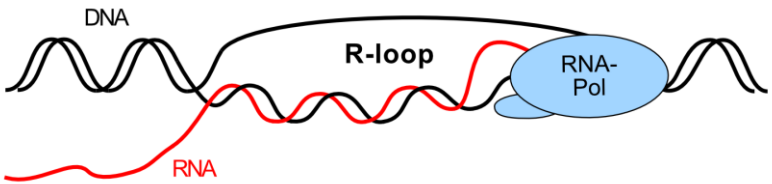
- R-loops are three stranded nucleic acid structures
- DNA/RNA hybrid & a displaced ssDNA
- Form mostly co-transcriptional - thread back model

- R-loops are dynamic structures with important roles in regulating gene expression & pose a threat to genome stability if not kept in check

- Specific enzymes regulate R-loops by degrading the RNA moiety (RNaseH1/2) or unwinding the hybrid (helicases such as DEAD/H-box)

- **R-loop** detection by **imaging** (IF or live cells) **was** basically **unavailable** due to artefacts produced by the S9.6 antibody
  - R-loop levels in single cells or nuclear substructures such as telomeres were inaccessible

Background



- R-loops are three stranded nucleic acid structures
- DNA/RNA hybrid & a displaced ssDNA
- Form mostly co-transcriptional - thread back model

R-loops have implications in **human disease**

ARTICLE

Received 13 Jun 2014 | Accepted 10 Sep 2014 | Published 21 Oct 2014 DOI: 10.1038/ncomms6220 OPEN

RNaseH1 regulates TERRA-telomeric DNA hybrids and telomere maintenance in ALT tumour cells

Rajika Arora[1], Yongwoo Lee[1], Harry Wischnewski[1], Catherine M. Brun[1], Tobias Schwarz[1] & Claus M. Azzalin[1]

Trends in Cancer

Review

R Loops and Their Composite Cancer Connections

James P. Wells,[1,3] Justin White,[1,2,3] and Peter C. Stirling[1,2,*]

CellPress REVIEWS

> Cell. 2024 Jun 20;187(13):3390-3408.e19. doi: 10.1016/j.cell.2024.04.025. Epub 2024 May 15.

ARID1A suppresses R-loop–mediated STING–type I interferon pathway activation of anti–tumor immunity

Matthew B Maxwell [1], Marianne S Hom-Tedla [2], Jawoon Yi [3], Shitian Li [1], Samuel A Rivera [1], Jingting Yu [4], Mannix J Burns [3], Helen M McRae [3], Braden T Stevenson [3], Katherine E Coakley [2], Josephine Ho [3], Kameneff Bojorquez Gastelum [3], Joshua C Bell [3], Alexander C Jones [3], Ramez N Eskander [5], Emily C Dykhuizen [6], Gerald S Shadel [7], Susan M Kaech [8], Diana C Hargreaves [9]

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Review

Out of Balance: R-loops in Human Disease

Matthias Groh, Natalia Gromak*

Sir William Dunn School of Pathology, University of Oxford, Oxford, United Kingdom

MOLECULAR & CELLULAR ONCOLOGY 2021, VOL. 8, NO. 1, e1848233 (3 pages) https://doi.org/10.1080/23723556.2020.1848233 Taylor & Francis

AUTHOR'S VIEWS OPEN ACCESS Check for updates

Inducing DNA damage through R-loops to kill cancer cells

Fred C. Lam [a,b,c,d], Yi Wen Kong [b,c,d], and Michael B. Yaffe [b,c,d,e]

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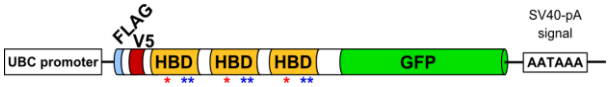
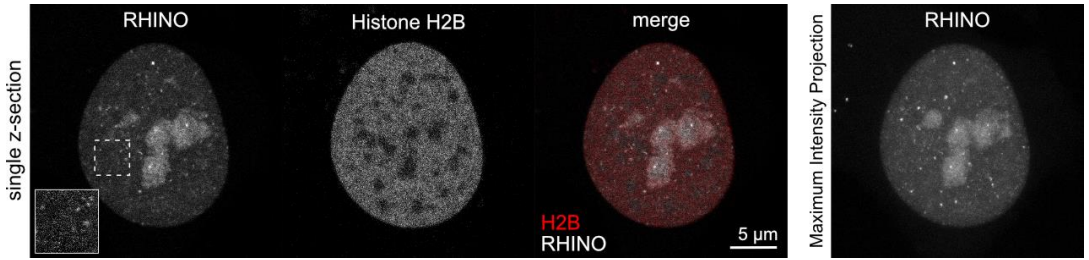
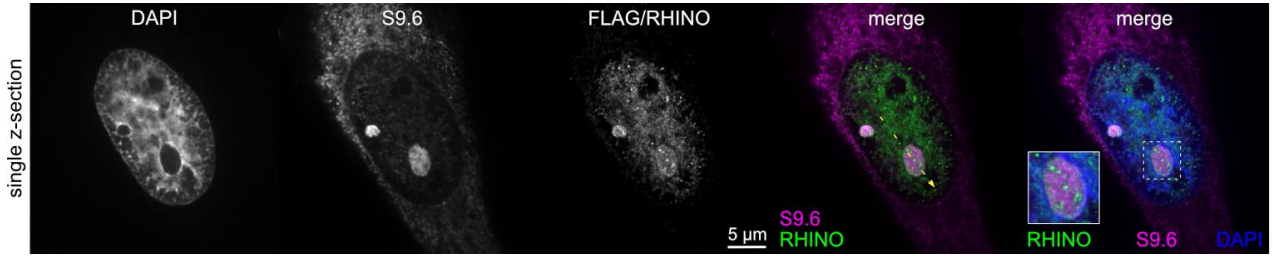
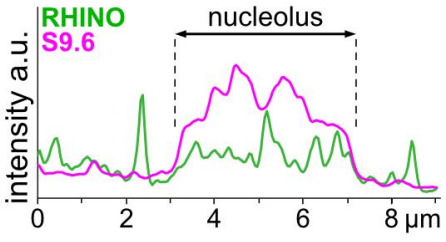
RHINO - A novel tool to detect R-loops by live cell imaging

- synthetic tool: RNA Hybrid ImagiNg tOol (RHINO)
- triple tandem of a DNA/RNA hybrid binding domain
- fused to a bright fluorophore









- RHINO is exclusively in cell nucleus, displays numerous foci (live & fixed)
- some nucleolar accumulation with additional bright foci
- little to no colocalization with S9.6 staining

What are the foci marked by the out tool? Which structures or sequences are labeled?

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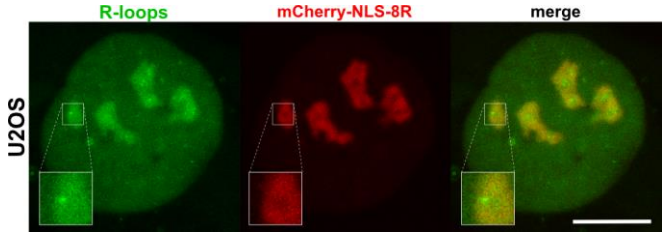
Labeling of nucleolar R-loops

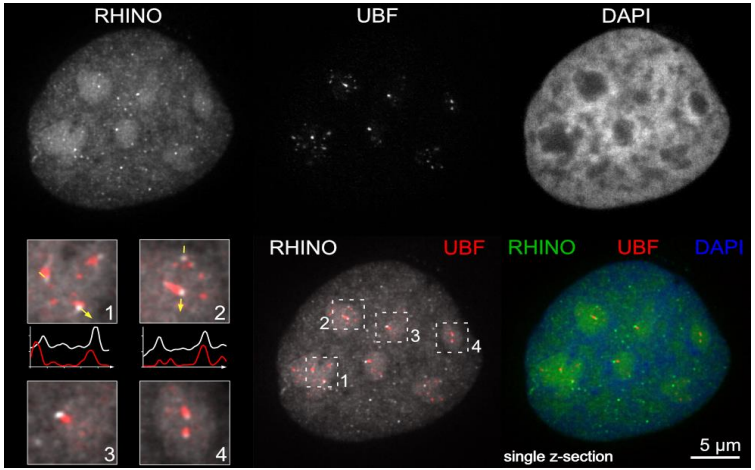


Genes Dev. 2010 Jul 15; 24(14): 1546–1558.
doi: 10.1101/gad.573310
PMCID: PMC2904944
PMID: 20634320

Loss of Topoisomerase I leads to R-loop-mediated transcriptional blocks during ribosomal RNA synthesis

Aziz El Hage,[1] Sarah L. French,[2] Ann L. Beyer,[2] and David Tollervey[1,3]

VT-iSIM



UBF is a ribosomal gene transcription factor for RNAPI

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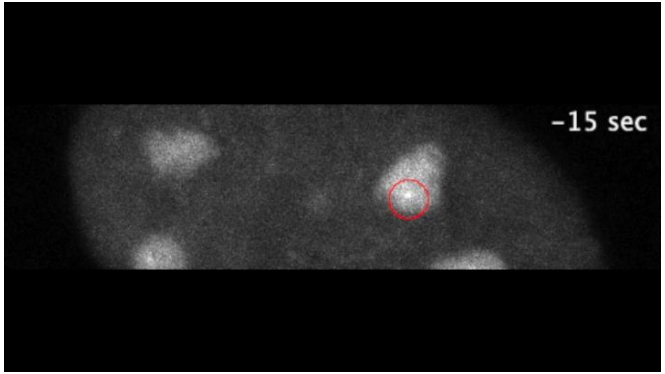
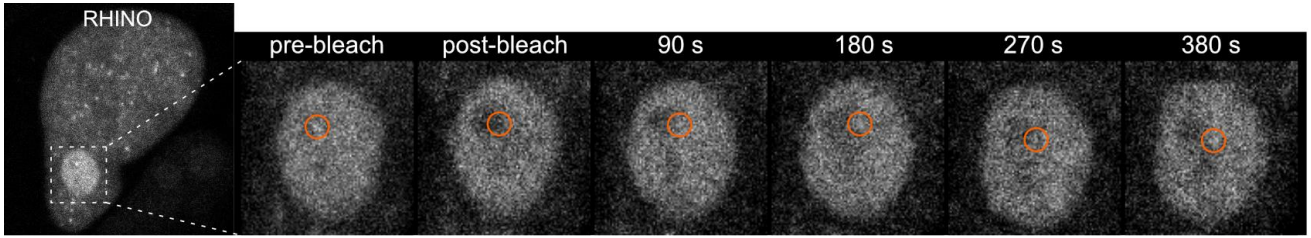
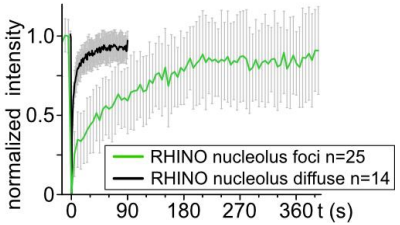
Labeling of nucleolar R-loops







- foci of labeled R-loops present in center region of nucleoli
- nucleolar R-loops overlap with ribosomal gene transcription factor UBF (RNA Pol I)
- nucleolar R-loop foci FRAP recovery to 80% in 135 sec; elongation phase of RNAPI around 182 sec

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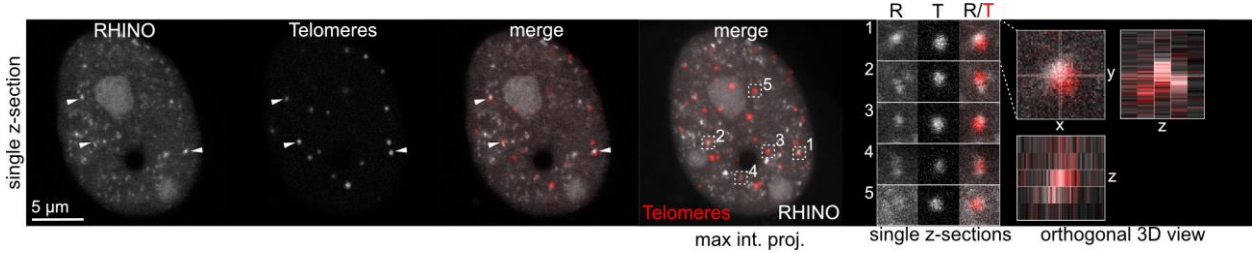
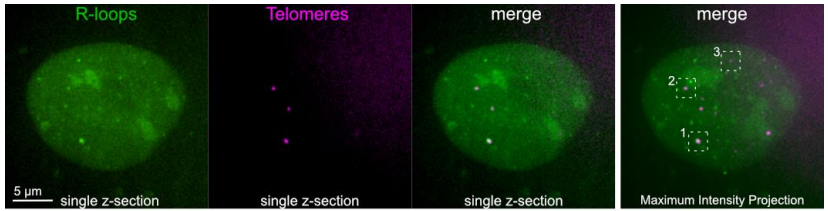
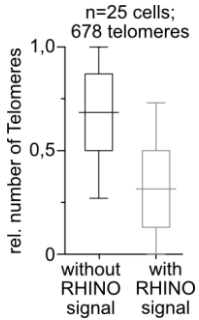
Labeling of telomeric-R-loops







> Nat Commun. 2014 Oct 21;5:5220. doi: 10.1038/ncomms6220.

RNaseH1 regulates TERRA–telomeric DNA hybrids and telomere maintenance in ALT tumour cells

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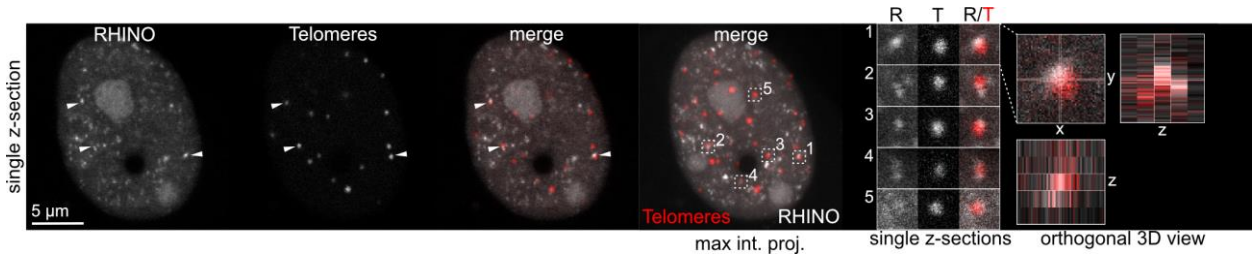

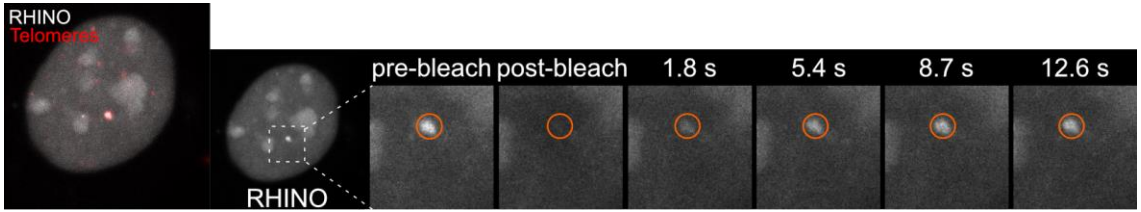

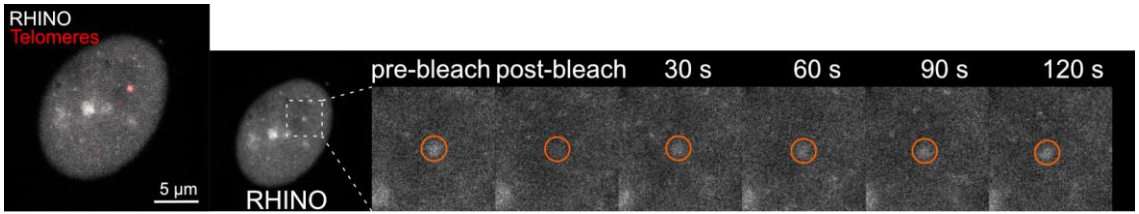

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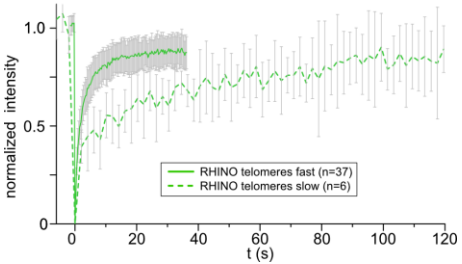

- RHINO hybrid sensor labels telomeric R-loops, would allow a quantification
- dynamics of R-loops differ at telomeres (2 fractions) and between different genomic loci

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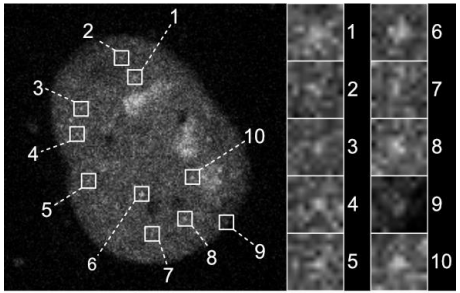
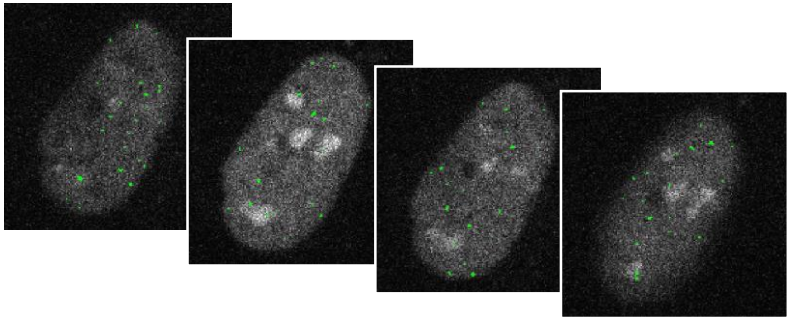
Quantification of R-loop levels in live cells



single confocal z-section

Imaging of cells with similar fluorescence intensity level in 3D → ICY Bioimage analysis → 3D Spot Detector plugin →



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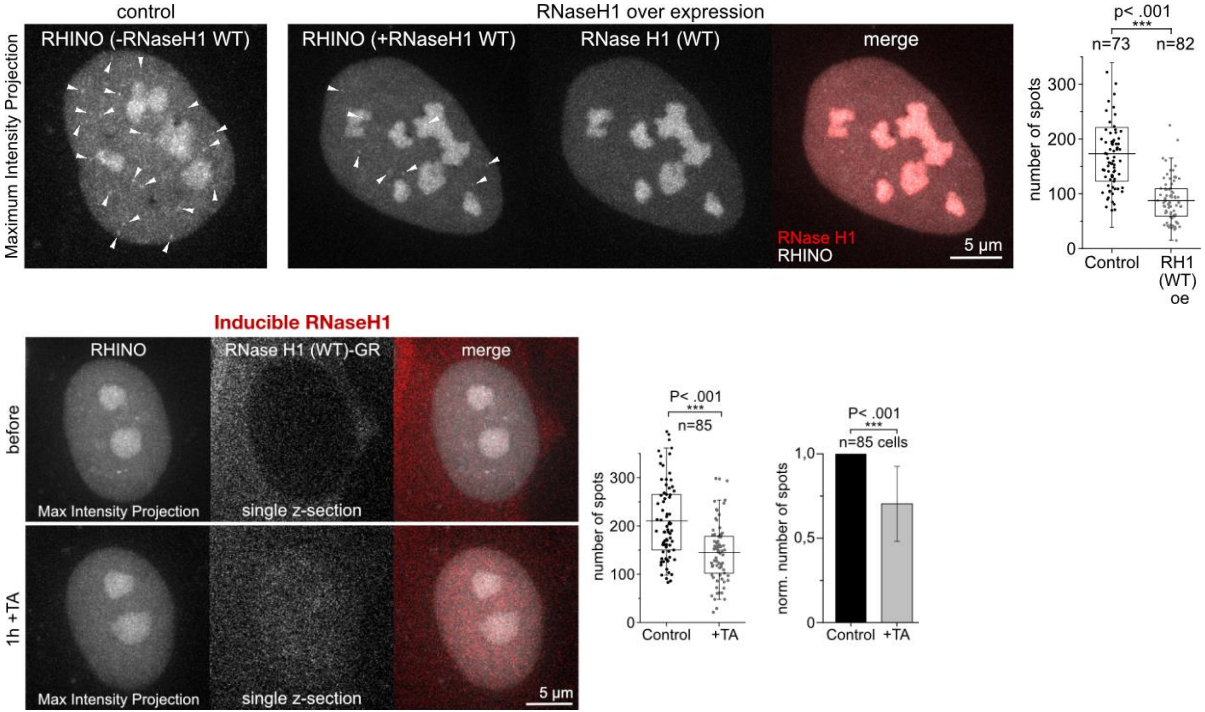

- Number of RHINO foci (RNA/DNA hybrid labeling) decrease upon RNaseH1 overexpression / induction
- Tool to quantify the effect of gene k.o. / overexpression and compounds interfering with R-loops

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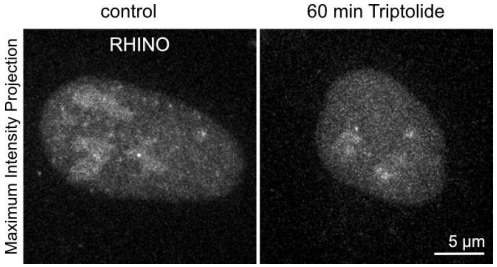
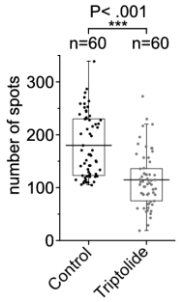
R-loop levels decrease upon transcription inhibition





- reduced number of R-loop signals
- loss of nucleolar foci
- nucleolar disintegration

Triptolide inhibits
RNAP I + II initiation.

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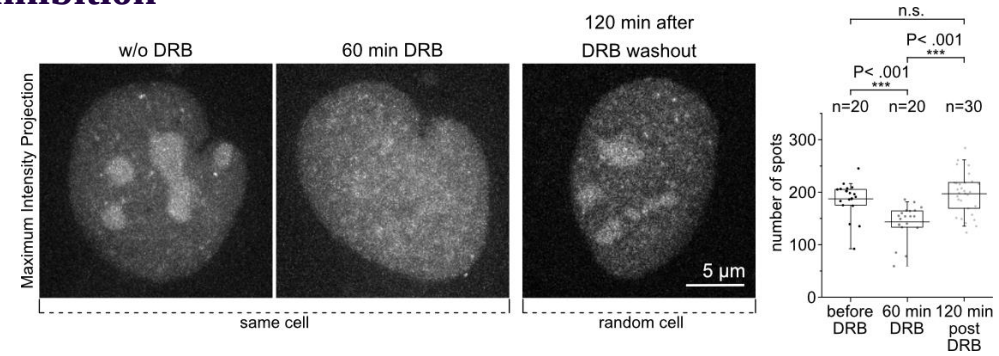
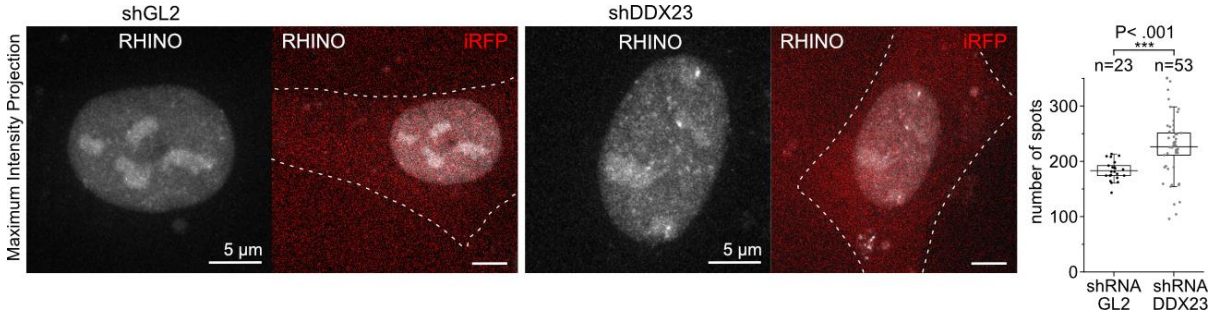
R-loop levels decrease upon transcription inhibition



DRB inhibits (reversibly)
RNAP II elongation & rRNA processing.



- Quantification of R-loop levels upon manipulation of R-loop formation or removal
- RHINO can be used to search for molecules altering R-loop levels

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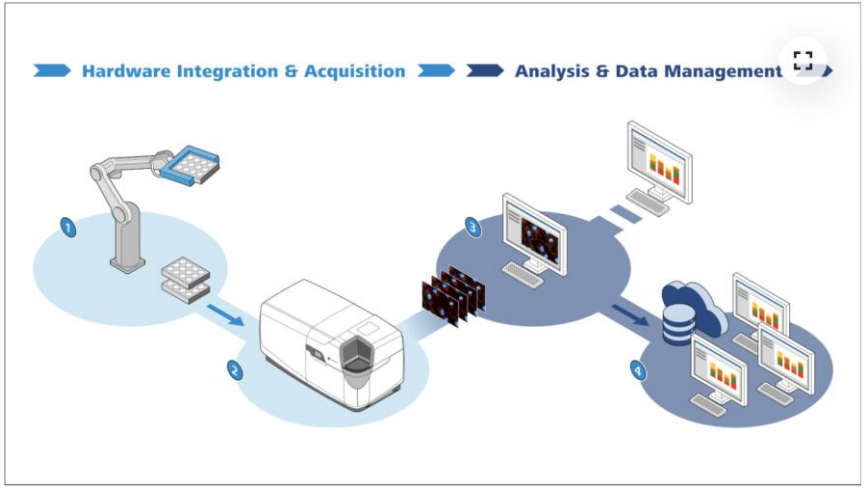
A RHINO based imaging platform to screen for drugs affecting R-loop levels

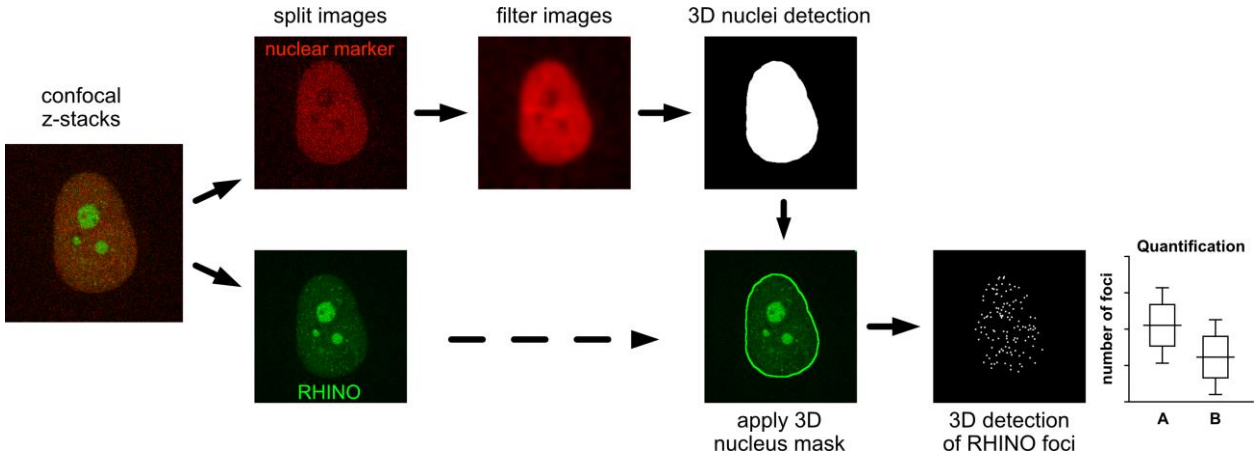
- Establish a high content imaging approach using RHINO (live cell R-loop quantification)
- Zeiss Cell Discoverer 7 with incubation, robotic arm and liquid handling system for 96-well plates
- Automated imaging with high resolution (AiryScan); data storage & handling hub and imaging analysis pipeline



- Screening of drug libraries to identify candidates, which increase or suppress R-loop levels, induce unscheduled R-loop accumulation / cytoplasmic R-loops

A RHINO based imaging platform to screen for drugs affecting R-loop levels

- Several RHINO cell lines were established: **hTert-RPE** (immortalized, normal); **HeLa** (cancer), **U2OS** (Alt-cancer)
- Additional cell lines planned: pairs of cell lines from normal / cancerous tissue (breast, skin, lung)
- Cell lines allow inducible (Dox) co-expression of RHINO + marker for cell nucleus
- Allows streamlined automated imaging analysis pipeline to detect nuclear + extra-nuclear R-loops and DNA/RNA hybrids



- Allows streamlined automated imaging analysis pipeline to detect nuclear + extra-nuclear R-loops and DNA/RNA hybrids

Conclusions

- RHINO tool was developed to label DNA/RNA hybrids in live cells,

  in structures known to form R-loops (nucleoli, telomeres, actively transcribed genes)
- RHINO allows live cell measurements of DNA/RNA hybrids/R-loop dynamics
- Quantification of R-loops in different cell types, upon drug treatments and upon gene k.o. + over expression
- RHINO is now utilized for an imaging-based drug screening platform to find candidates of potent R-loop modulators

Perspectives

- Screening for Tel-R-loop modulators (with Azzalin lab)
- use the RHINO screening platform for genetic screens to identify novel R-loop modulators / interactors (CRISPR libraries; siRNA libraries)
- Open the RHINO screening platform for external commercial library screens

Summary / Conclusions

- RHINO labels DNA/RNA hybrids in live cells,

  in structures known to form R-loops (nucleoli, telomeres, actively transcribed genes)
- RHINO allows live cell measurements of DNA/RNA hybrids/R-loop dynamics
- Quantification of R-loops in different cell types, upon drug treatments and upon gene k.o. + over expression

Future perspectives

- study R-loop dynamics in live cells (transcription of coding genes, Tel-R-loops, upon stimuli, development)
- establish RHINO based R-loop labeling as diagnostic tool in drug/candidate gene screens aiming at manipulating R-loops

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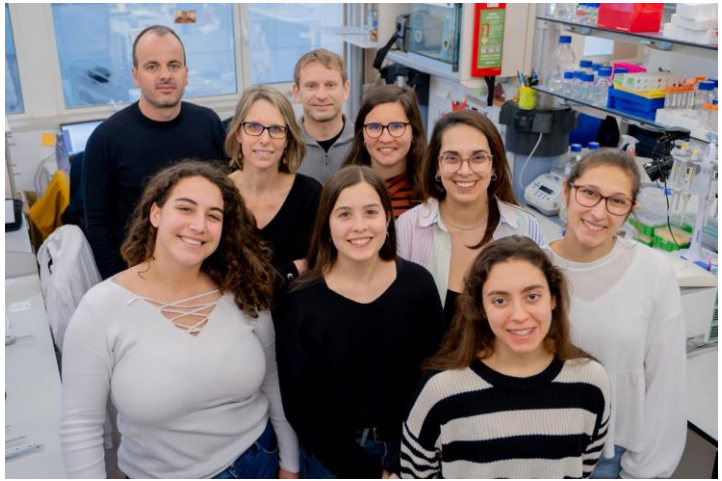

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José Rino and all facility members

GIMM Technology Transfer Platform

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