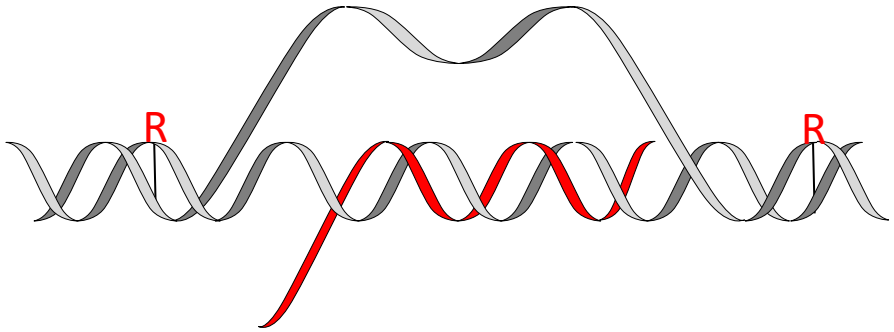
RNA-DNA hybrids

Form, Flexibility and Function



Brian Luke
IMB Mainz
Johannes Gutenberg Universität, Mainz

RNA-DNA hybrids

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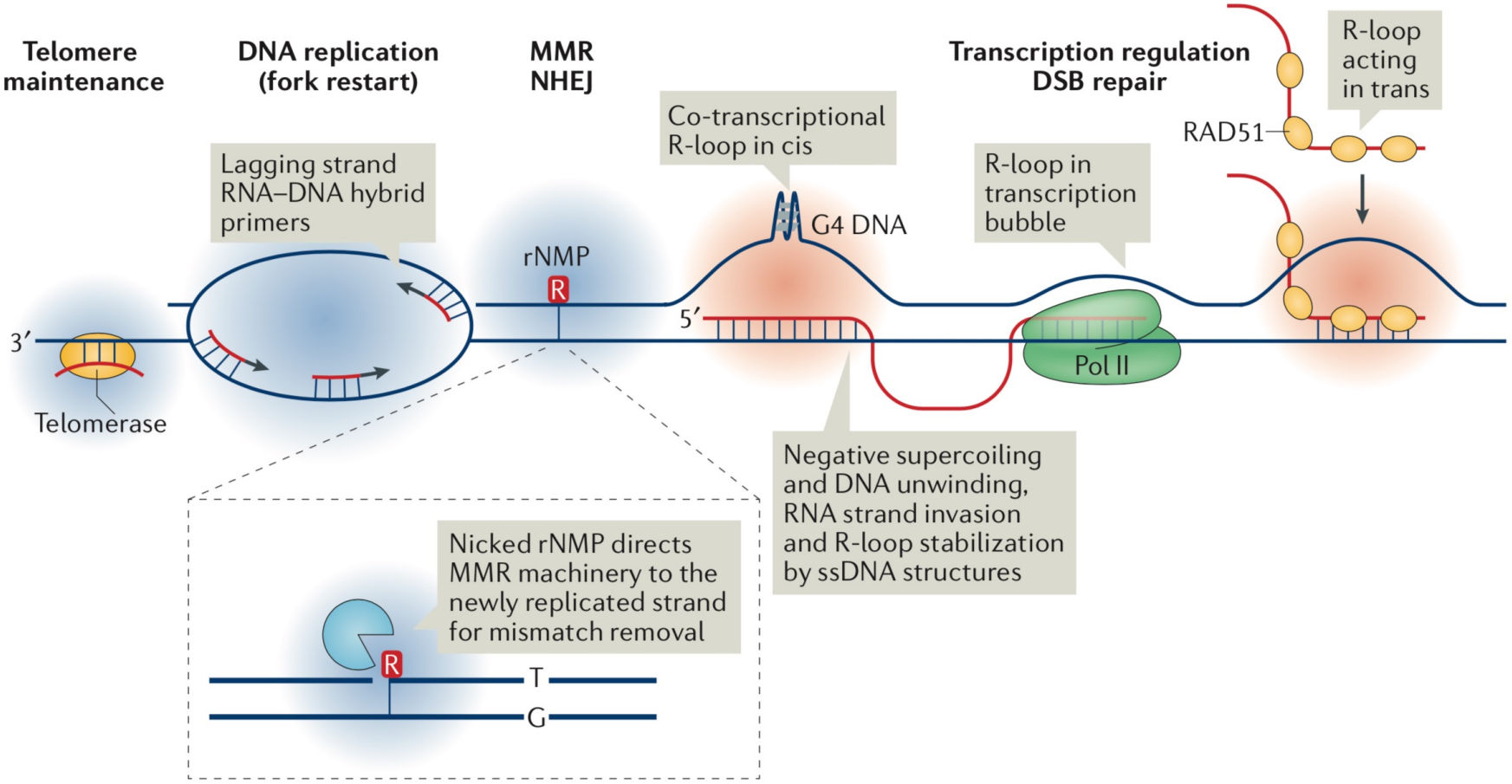
3. R-loops, their formation, detection regulation and distribution
4. Functional R-loops
5. R-loops at telomeres and the timing of RNA-DNA hybrid removal by RNase H
6. R-loops as regulators of DNA methylation

Many types of RNA-DNA hybrids exist on genomic DNA



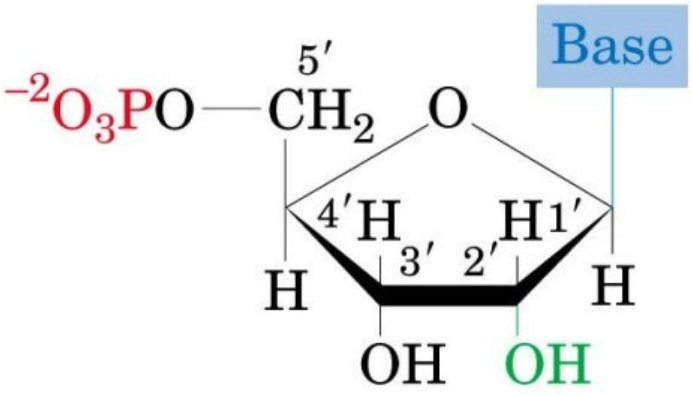
Niehrs and Luke, 2020, *Nat. Rev. Mol. Cell Biol*

-also CRISPR/Cas9 makes RNA-DNA hybrids

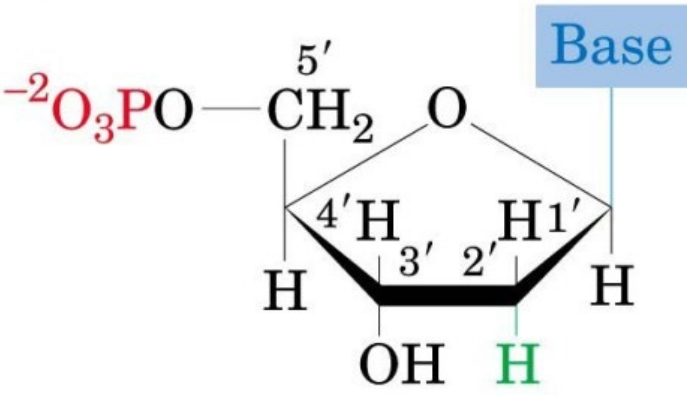
Brian Luke

b.luke@imb-mainz.de

RNA-DNA hybrids – the building blocks of RNA and DNA



Ribonucleotides

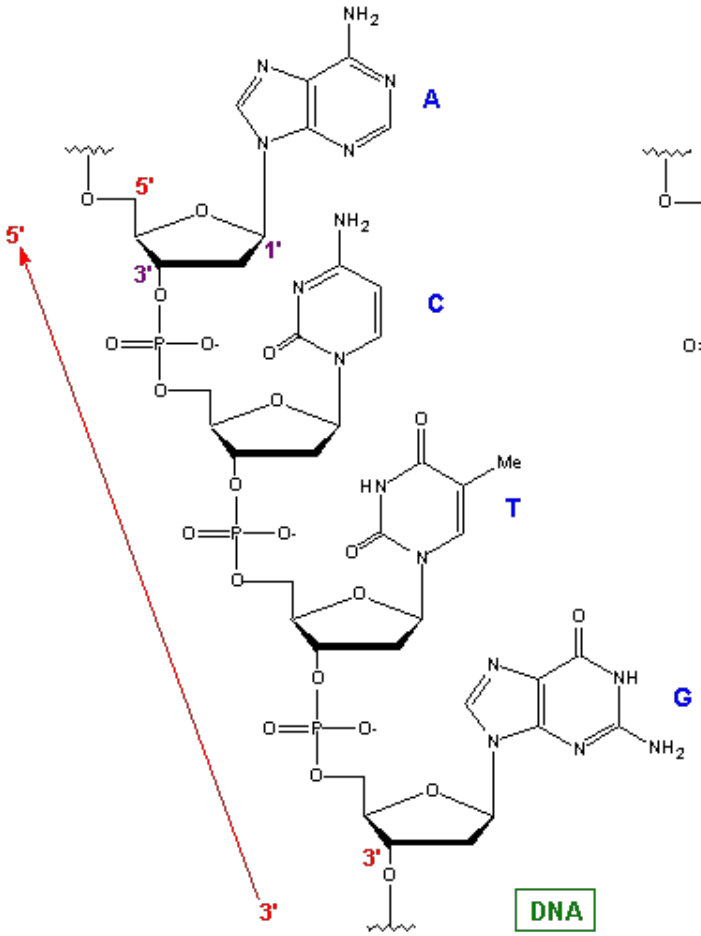


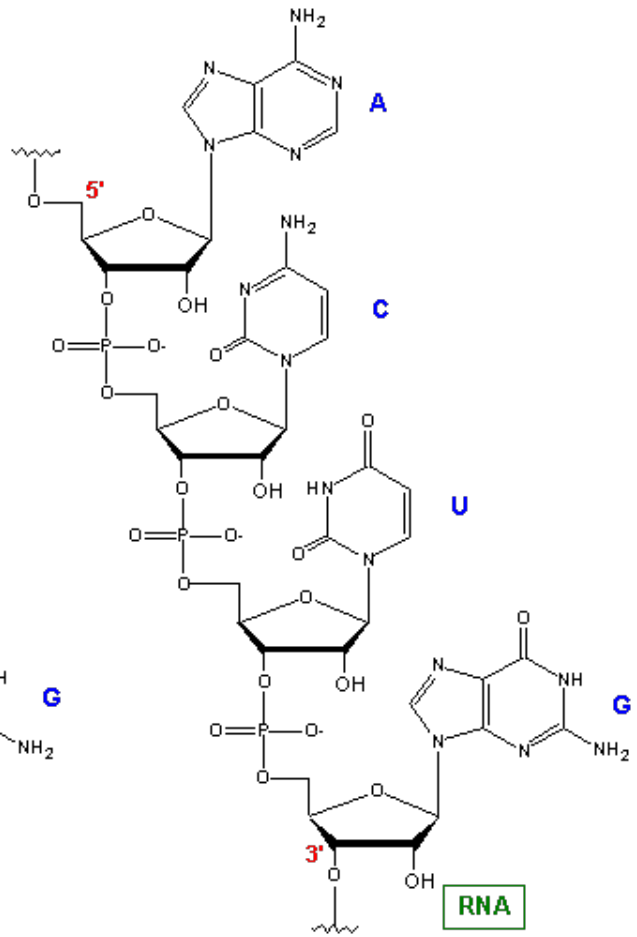
Deoxyribonucleotides

RNA-DNA hybrids – ss polymerized RNA and DNA molecules

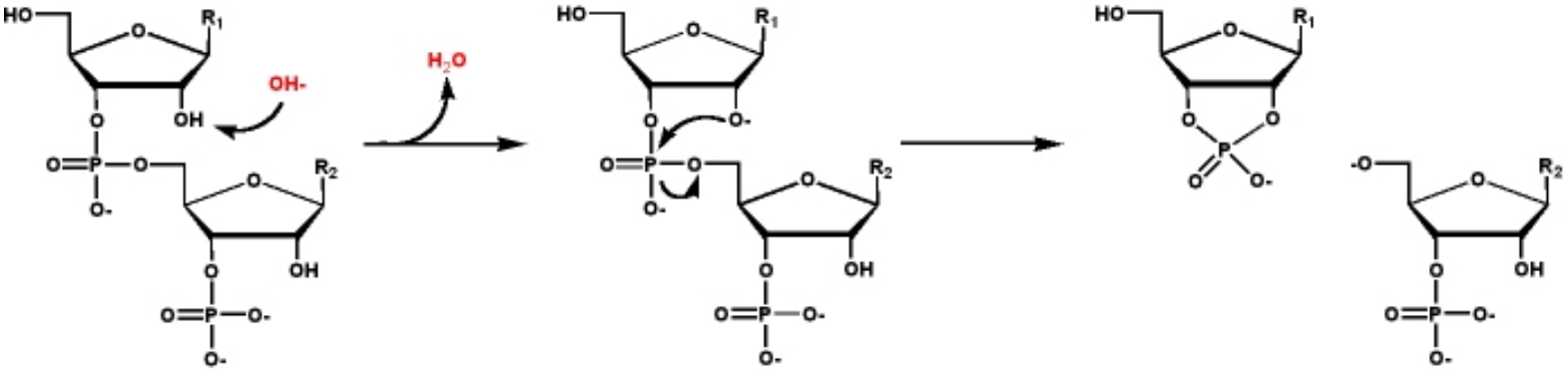
- DNA is relatively stable compared to RNA

-The 2‘OH of ribonucleotides can hydrolyze the sugar backbone by nucleophilic attack on the phosphate bond





The presence of 2‘OH makes RNA susceptible to hydrolysis



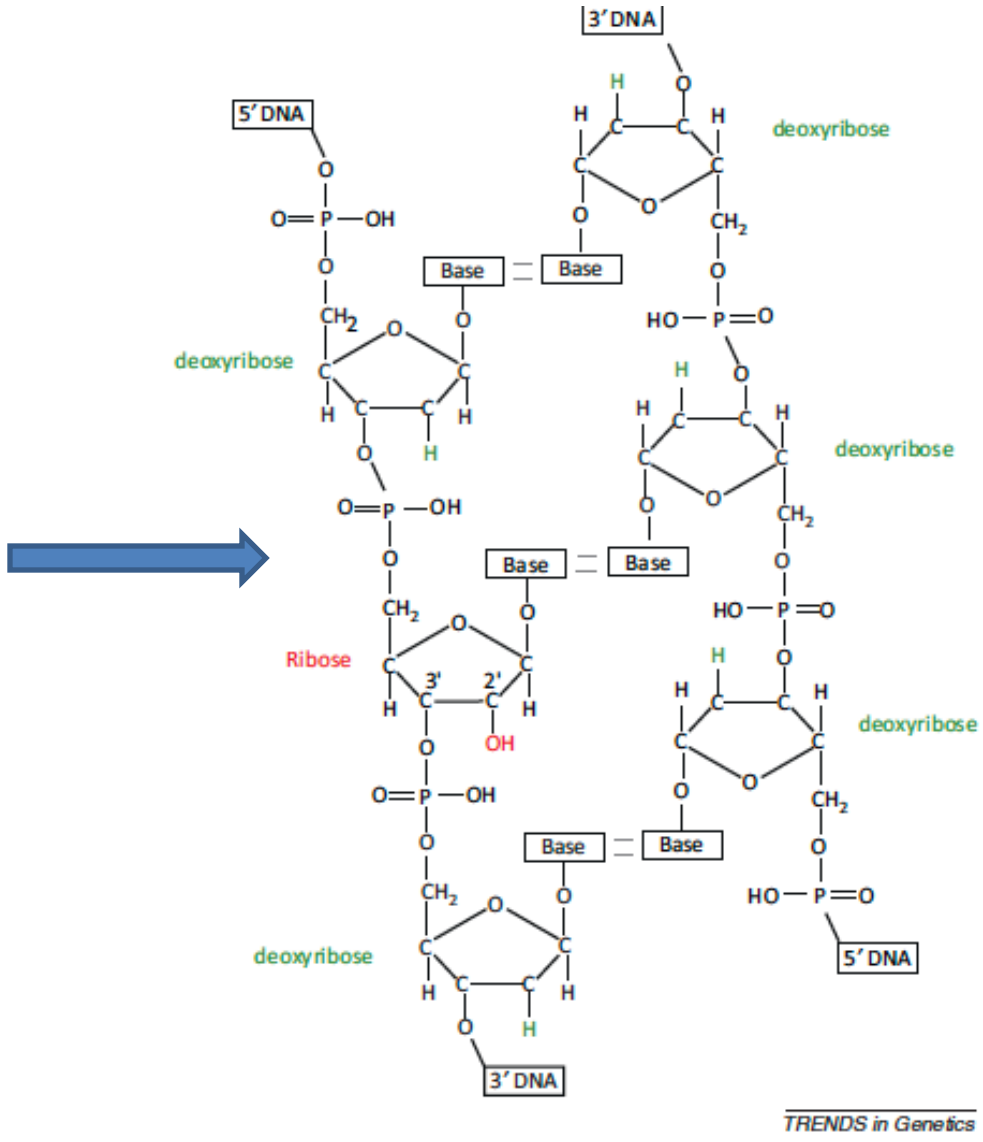
- Hydrolysis of RNA occurs and this is particularly frequent in alkaline conditions (high pH)

RNA-DNA hybrids – ribonucleotides in the context of dsDNA

- Ribonucleotides can be inserted into the DNA backbone and basepair with deoxyribonucleotides

-**this is considered an RNA-DNA hybrid molecule**

-the effect is that the stability of the DNA is now compromised



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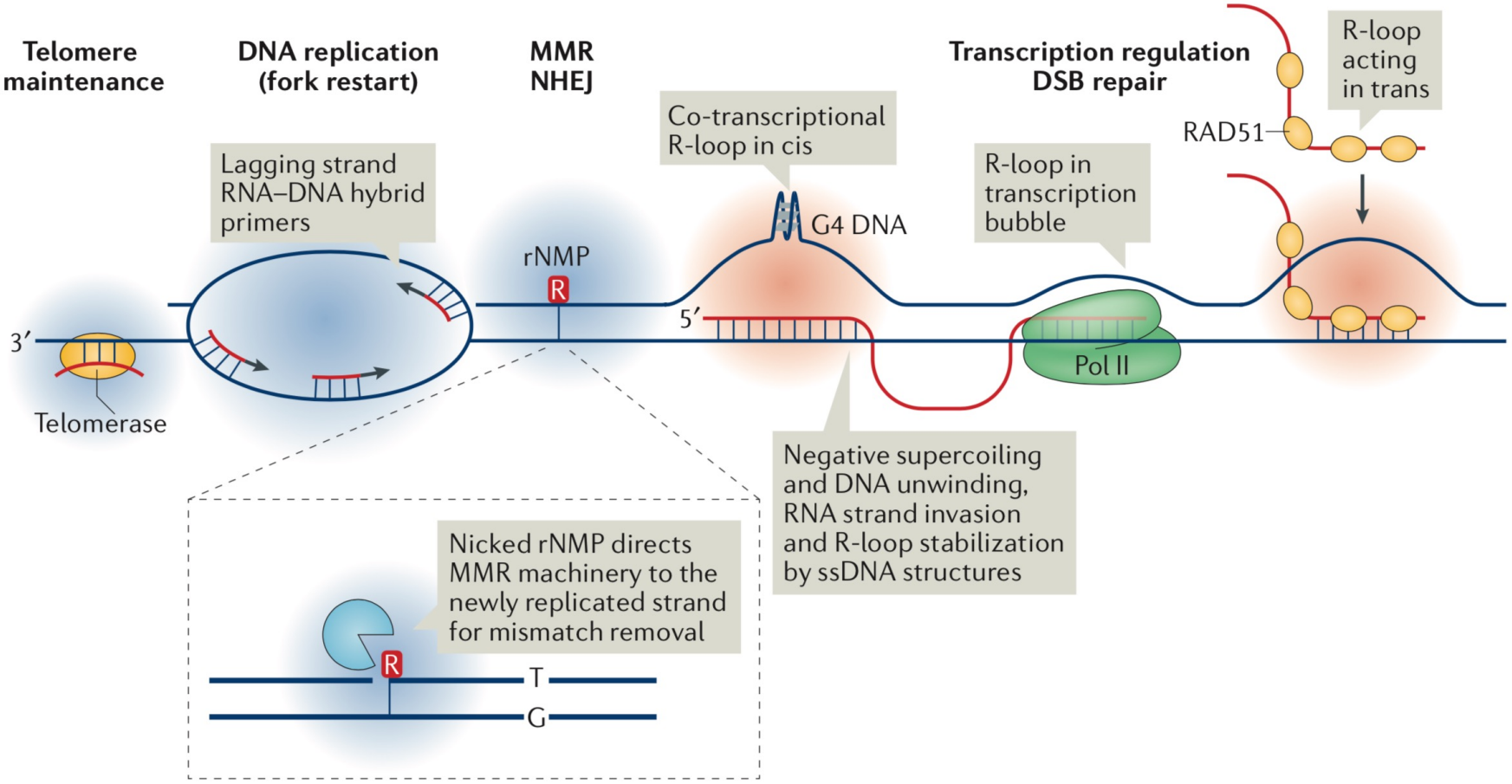

Niehrs and Luke, 2020, *Nat. Rev. Mol. Cell Biol*

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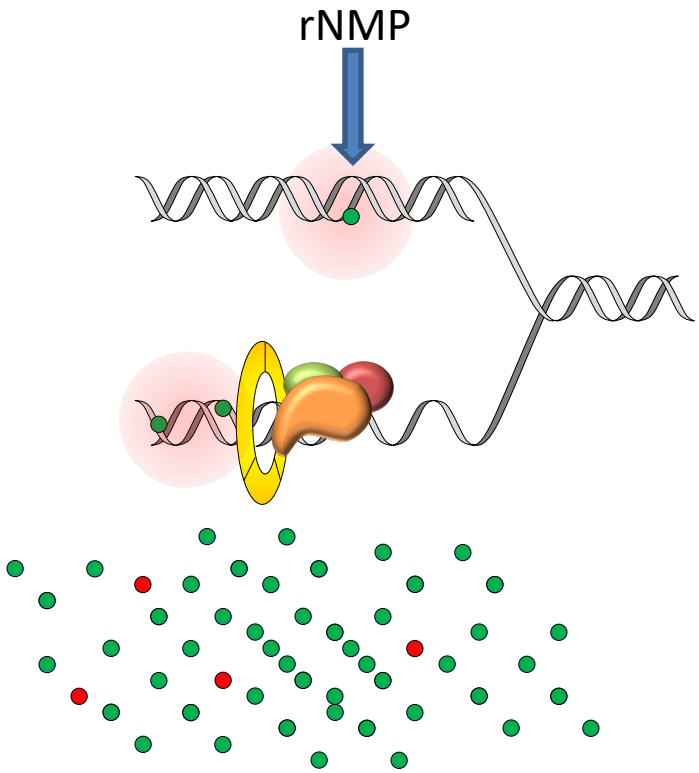
rNTPs are frequently incorporated into DNA by the replicating polymerases

- DNA polymerases accidentaly incorporate rNTPs into DNA which are then called rNMPs (ribonucleoside monophosphate)
- Why does this happen?
- DNA polymerases have a tyrosine steric gate that recognizes the 2‘OH of rNTPs and prevents their entry into the catalytic site
- However the gate is not perfect rNTPs are used
- This is in part due to the high concentration of rNTPs compared to dNTPs



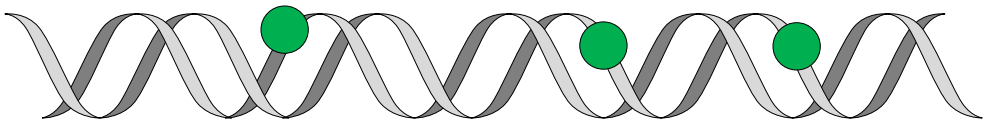
- Depending on the base, rNTPs are between 30 and 200-fold more concentrated that dNTPs in the cell

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

rNTPs incorporation is frequent

- In yeast approximately 10, 000 rNMPs are incorporated into dsDNA per S phase
- this means approximately one rNMP per 6500 bases of DNA
- This makes ribonucleotides the most frequently occurring of all types of DNA damage
- Numbers are similar in human cells



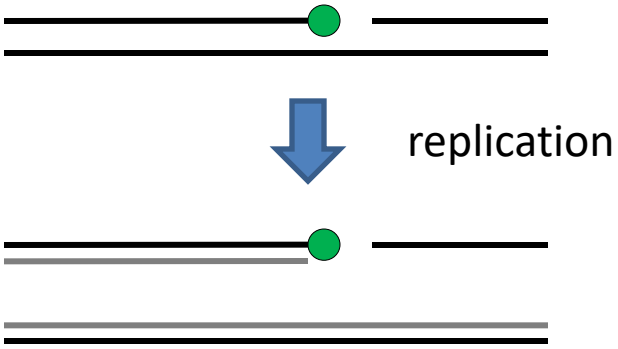
So what is the problem with rNMP insertions?

Brian Luke
b.luke@imb-mainz.de

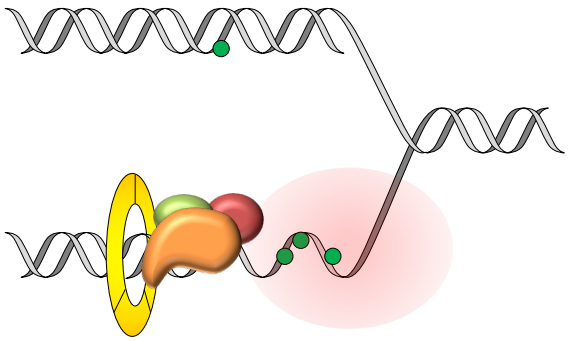
imb Institute of Molecular Biology

rNMPs can lead to genome instability

- rNMPs are inherently prone to hydrolysis which would leave ss nicks in the DNA
- Upon DNA replication ss nicks are converted into ds breaks



- Moreover, the presence of ribonucleotides leads replication stress and polymerase stalling
- Most importantly, rNMPs are acted on by Top1 which can lead to mutagenesis (see later)



Replication stalling/stress

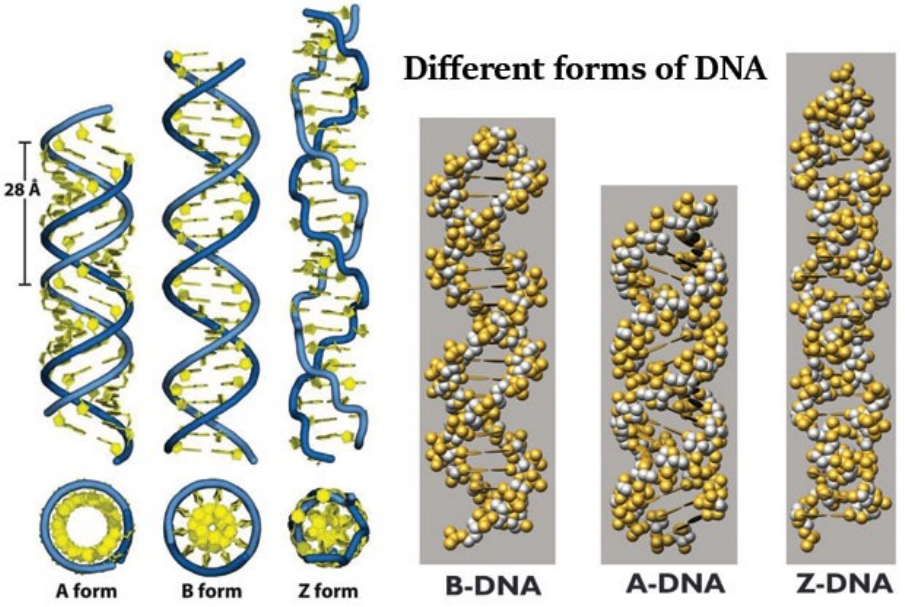
Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

rNMP hybrids may lead to other alterations as well

- RNA-DNA hybrids form A form DNA instead of the usual B-form.

- This likely also prevents nucleosome assembly and may affect local epigenetic marks

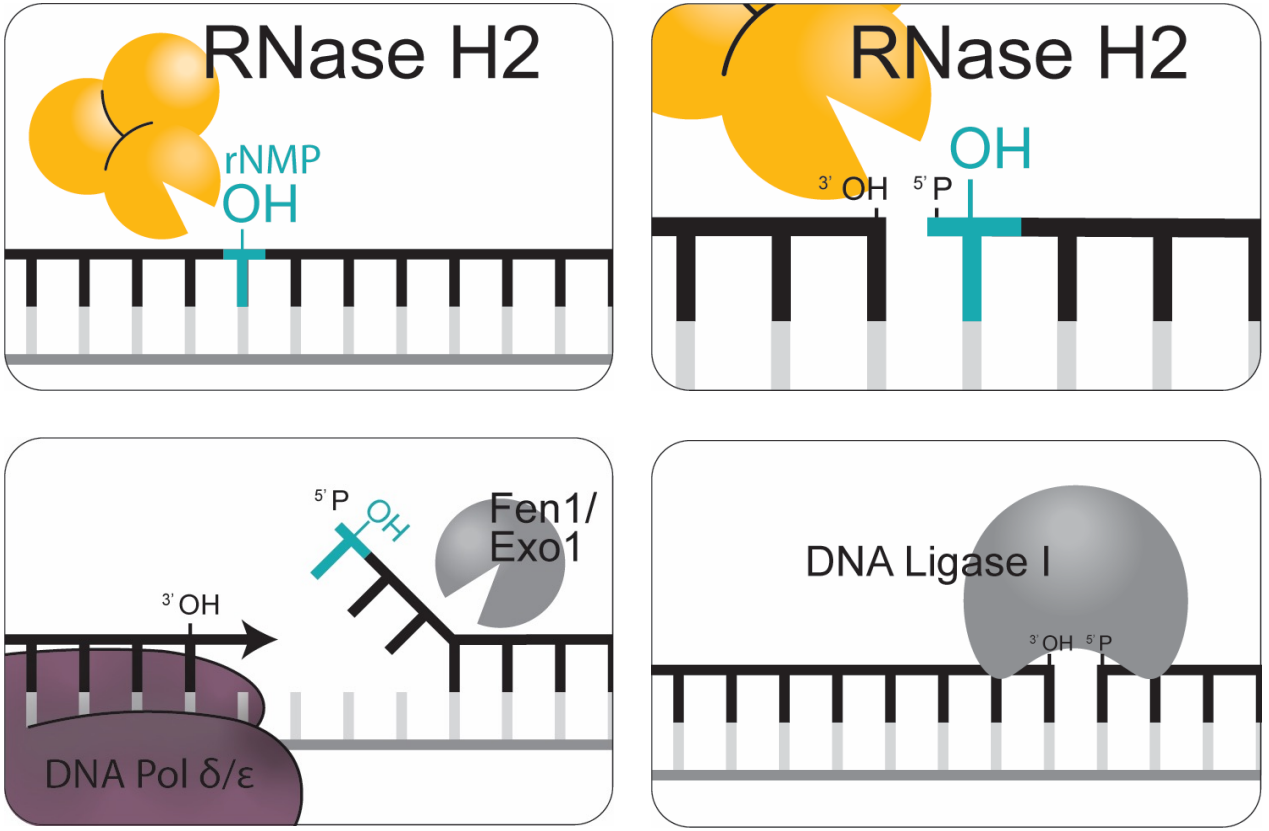


So how do we get rid of rNMPs that have been inserted into the genome?

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

RER: RNase H2-initiated, faithful ribonucleotide excision repair



Brian Luke
b.luke@imb-mainz.de

Kellner and Luke, 2019, EMBO J.

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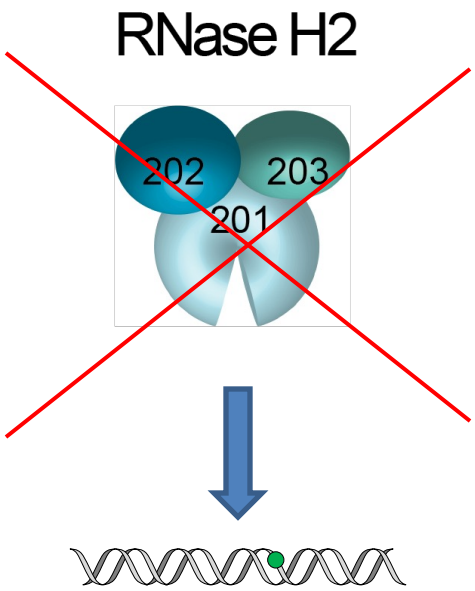
Ribonucleotide excision repair (RER)



Ribonucleotide insertions

-Mutations in RNase H2 result in a neurological syndrome called Aicardi Goutières Syndrome (AGS)

-neurodegeneration with severe ataxia

-auto immune

-high levels of genome instability

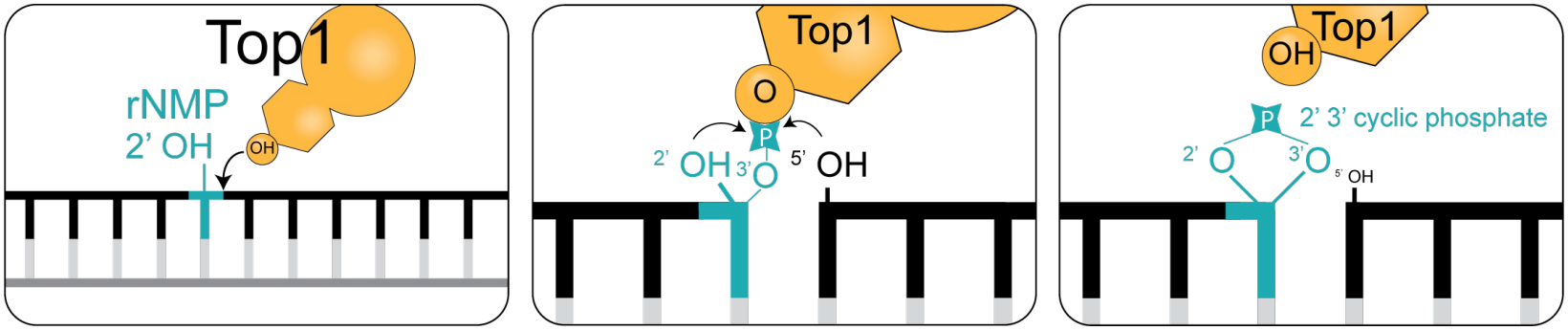
-RNase H2 is also mutated in metastatic castration-resistant prostate cancer and in CLL

There is an RER „back-up“ mechanism that is responsible for a lot of the problems when RNase H2 is missing

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

Topoisomerase 1 (Topo1) as a Ribonucleotide excision repair (RER) backup



Bottom line: RNase H2 repairs rNMPs in an error –free manner

When RNase H2 doesn't work

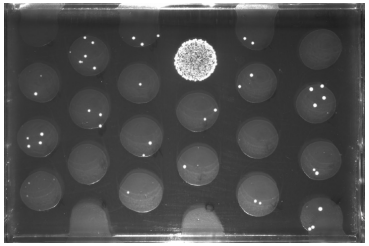
Top1 steps in, but makes mistakes and leads to more mutations

Brian Luke
b.luke@imb-mainz.de

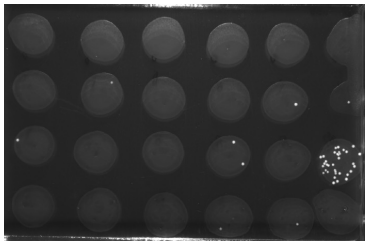
imb Institute of Molecular Biology

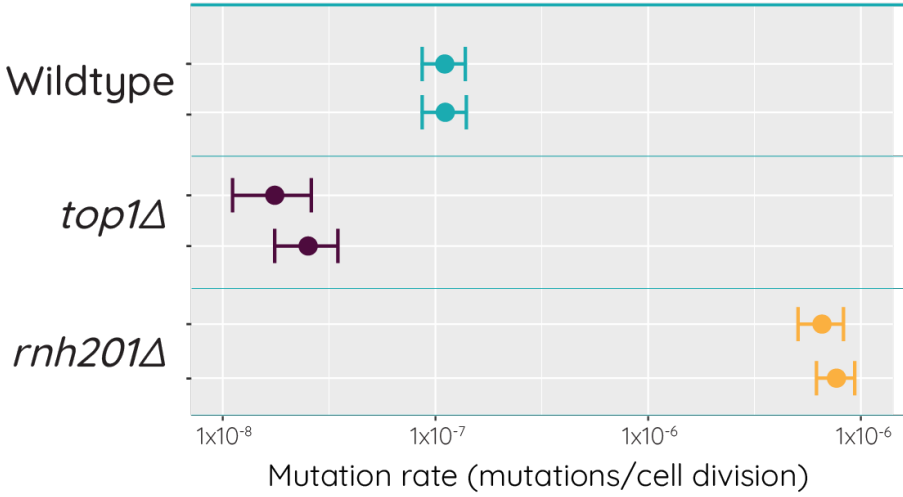
We can measure the mutations that Top1 makes

Wildtype



top1Δ





	Mutation rate*	Confidence interval*
Wildtype	11.1	8.67 - 13.8
	11.1	8.67 - 14.0
top1Δ	1.76	1.11 - 2.61
	2.52	1.75 - 3.48
rnh201Δ	657	507 - 829
	768	619 - 935

Could these Top1 mutations increase with age?

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

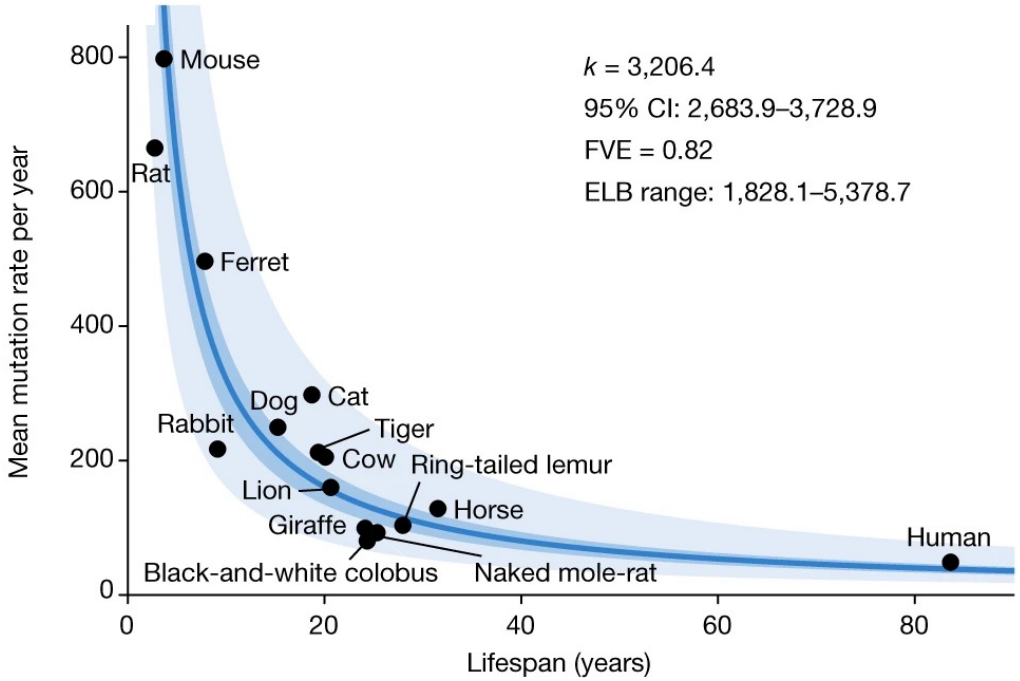
Low mutation rate = Longer lifespan

Article

Somatic mutation rates scale with lifespan across mammals

https://doi.org/10.1038/s41586-022-04618-z
Received: 17 August 2021
Accepted: 7 March 2022
Published online: 13 April 2022
Open access
Check for updates

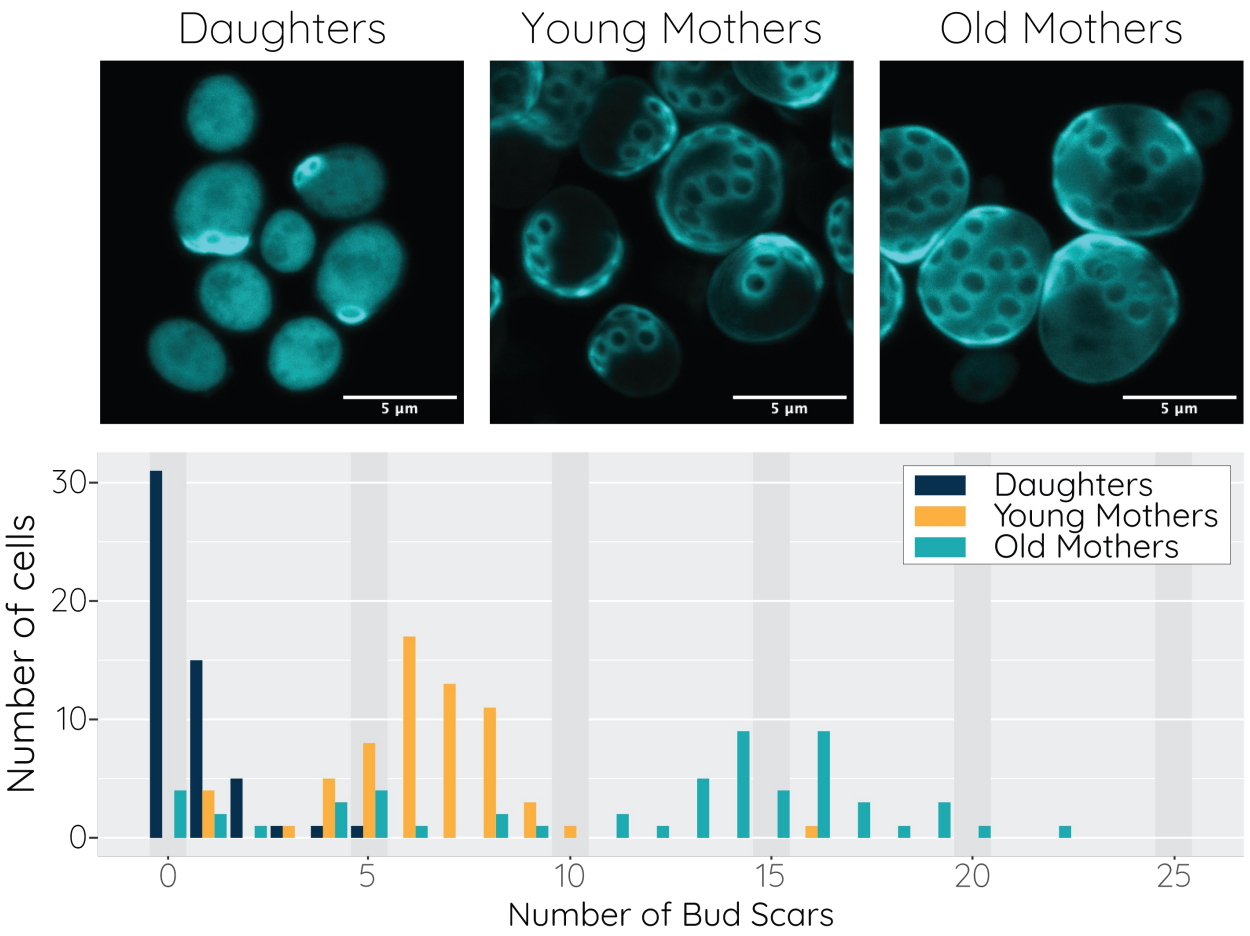
Alex Cagan[1,15], Adrian Baez-Ortega[1,15], Natalia Brzozowska[1], Federico Abascal[1], Tim H. H. Coorens[1], Mathijs A. Sanders[1,2], Andrew R. J. Lawson[1], Luke M. R. Harvey[1], Shriram Bhosle[1], David Jones[1], Raul E. Alcantara[1], Timothy M. Butler[1], Yvette Hooks[1], Kirsty Roberts[1], Elizabeth Anderson[1], Sharna Lunn[1], Edmund Flach[3], Simon Spiro[3], Inez Januszczak[3,4], Ethan Wrigglesworth[3], Hannah Jenkins[3], Tilly Dallas[3], Nic Masters[3], Matthew W. Perkins[5], Robert Deaville[5], Megan Druce[6,7], Ruzhica Bogeska[6,7], Michael D. Milsom[6,7], Björn Neumann[8,9], Frank Gorman[10], Fernando Constantino-Casas[10], Laura Peachey[10,11], Diana Bochynska[10,12], Ewan St. John Smith[13], Moritz Gerstung[14], Peter J. Campbell[1], Elizabeth P. Murchison[10], Michael R. Stratton[1] & Iñigo Martincorena[1]



Vanessa Pires
vanpires@uni-mainz.de

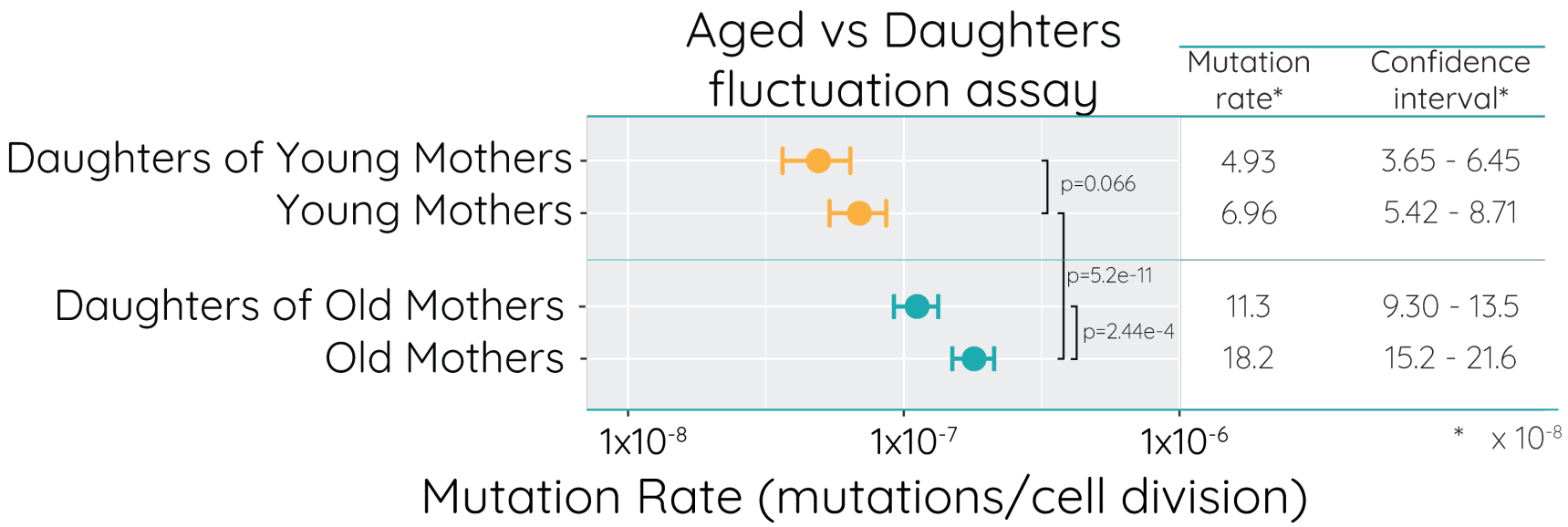
Institute of Molecular Biology

We collected old cells ……..



Brian Luke
b.luke@imb-mainz.de

….and measured Top1 mutations at rNMPs



-Top1 mutations increase with age
-Why…..do rNMPs increase with age, do Top1 levels increase with age?
-do RNase H2 levels decrease……these are ongoing questions that are lab is trying to answer

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

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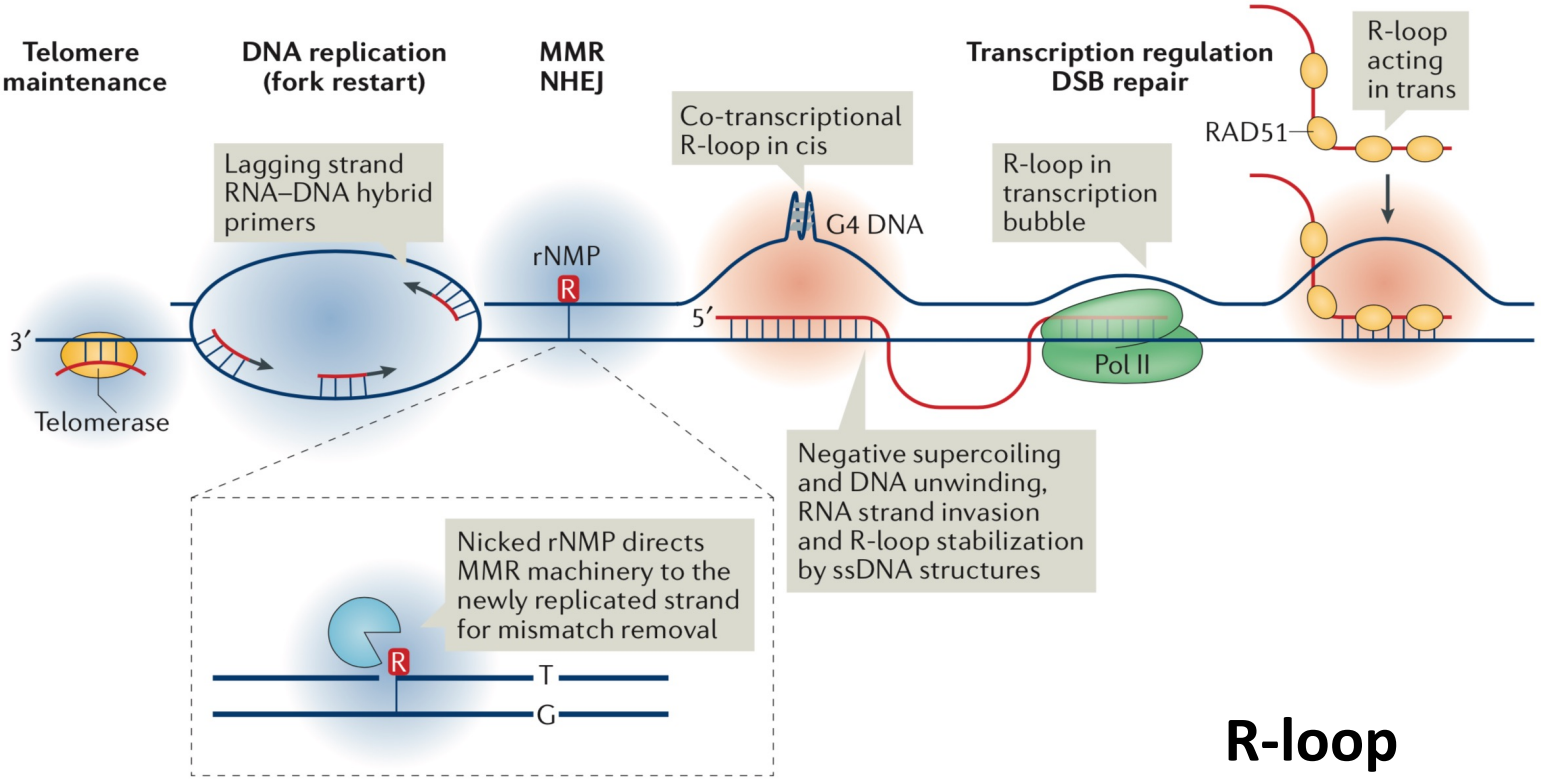
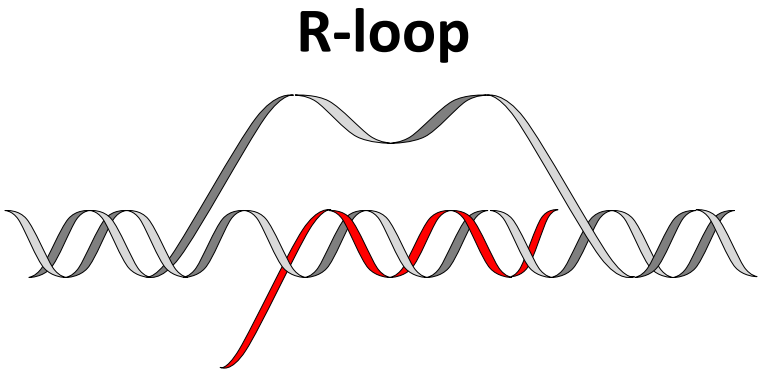


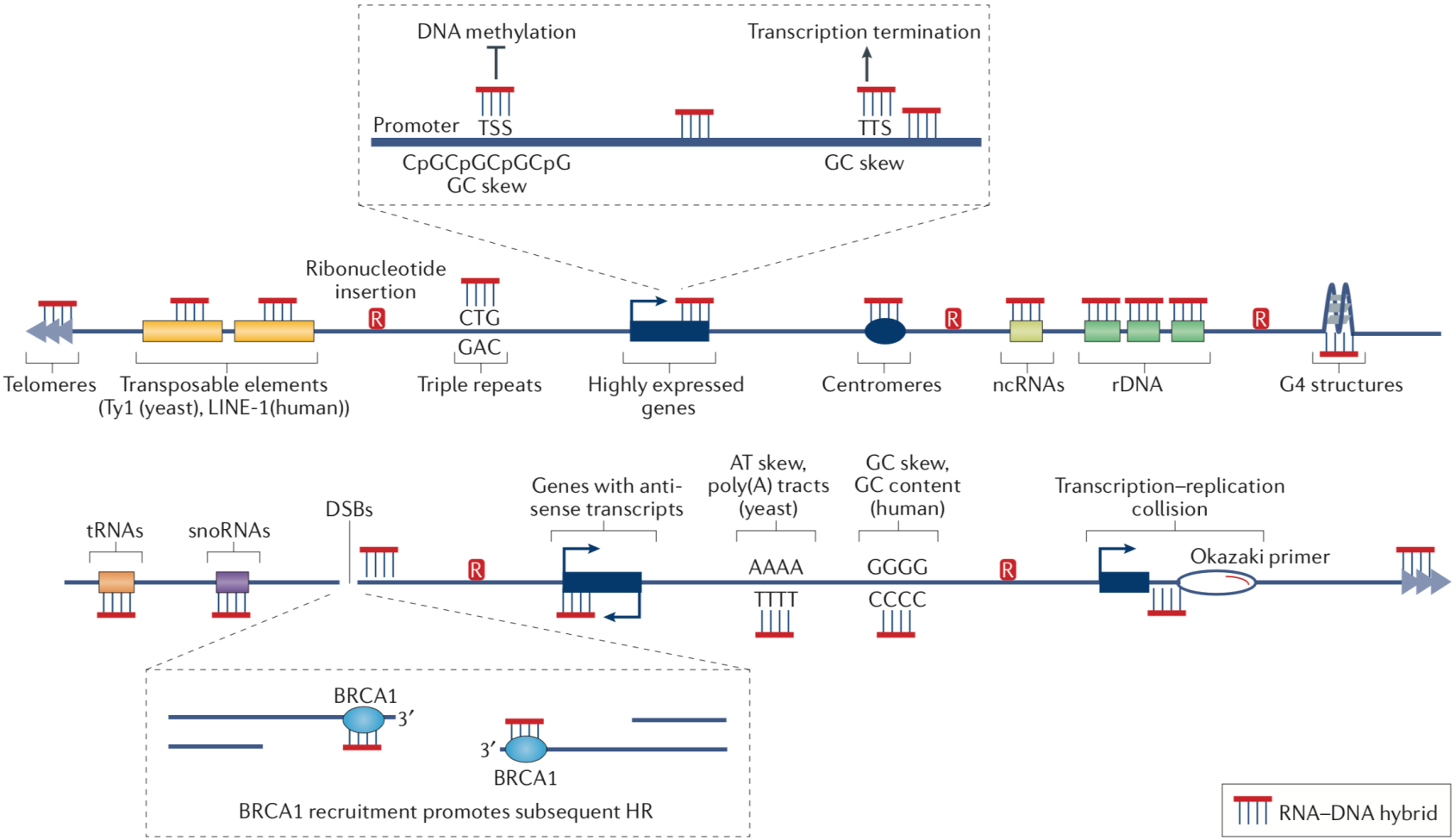
R-Loops refer to RNA that is base-paired to DNA in a Watson-Crick manner resulting in one strand of DNA being displaced.



Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Where do we find R-loops?



Niehrs and Luke, 2020, *Nat. Rev. Mol. Cell Biol*

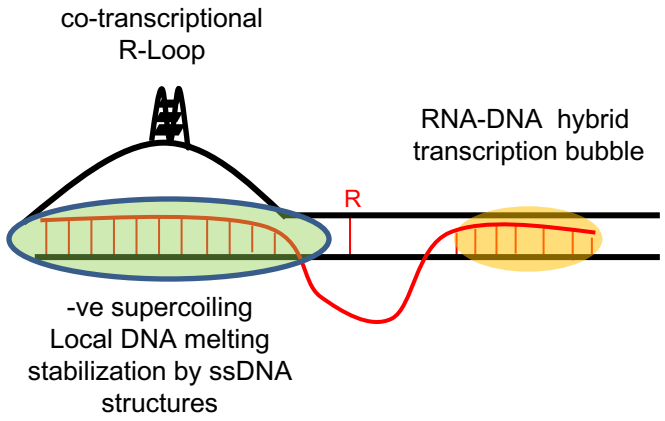
Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

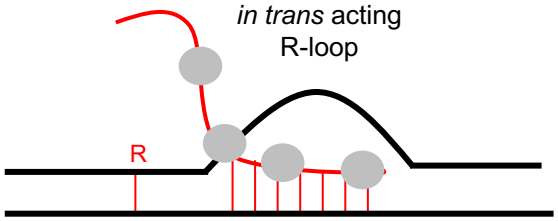
How are R-loops formed?

There are likely many ways that R-loops form and there is no clear consensus on this

1. Prevailing view is that R-loop formation is transcription coupled and that the transcript basepairs with the DNA template behing the transcription machinery.
Here there is **negative supercoiling** and more chance for helix melting



2. That R-loops can form *in trans.* There is evidence that **R-loops can become coated with Rad51 and do strand invasion.** Plasmid expressed RNA can form R-loops on chromosomes at homologous sequences. Therefore R-loops can form *in trans*.....do they?

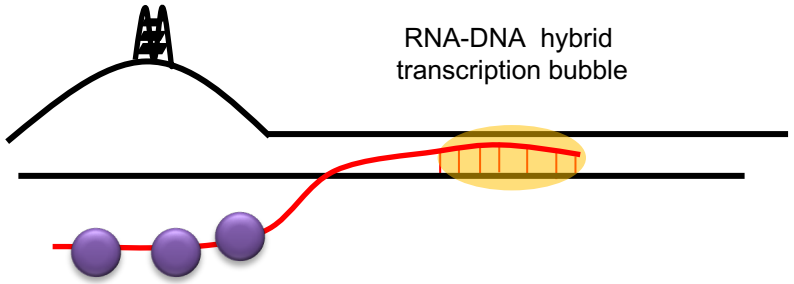


Brian Luke
b.luke@imb-mainz.de

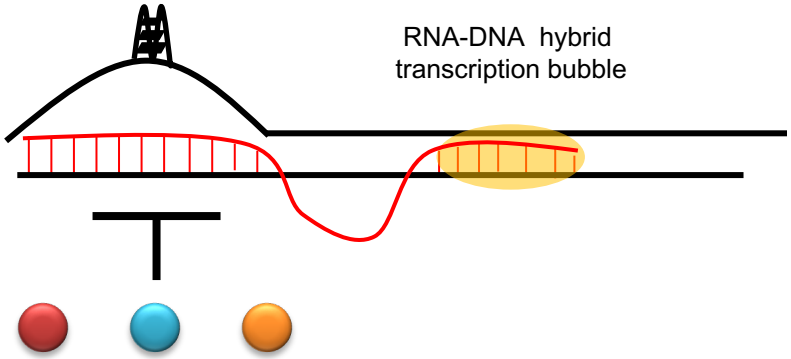
imb Institute of Molecular Biology

How are R-loops regulated?

1. Prevent them from forming



2. Remove them once they have formed



Brian Luke
b.luke@imb-mainz.de

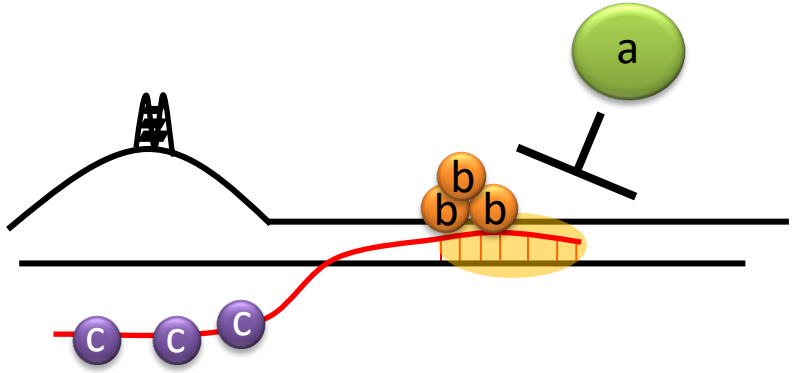
How are R-loops prevented from forming?

Prevent them from forming

a. **Transcriptional repressors** – suppressing transcription especially at repetative regions prevents an RNA and hence an R-loop

b. **Elongation factors** – transcription stalling can also facilitate R-loop formation – therefore progressive elongation is critical

c. **RNA binding** – processing factors that promote RNA maturation, splicing and export prevent the RNA from re-annealing



Brian Luke
b.luke@imb-mainz.de

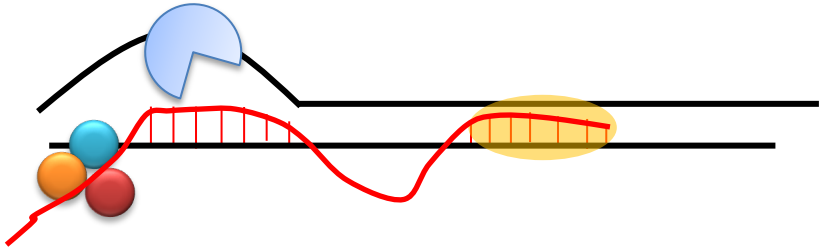
How are R-loops regulated?

Remove them once they have formed

a. **helicases**– some helicases have preference for unwinding RNA-DNA hybrids *in vitro*, and may also do so *in vivo* e.g. **BLM, SETX, AQR, DHX9** and a host of other factors that have been identified as R-loop interactors

a. **Nucleases** – The RNase H enzymes (RNase H1 and RNase H2) are the primary enzymes responsible for R-loop cleavage

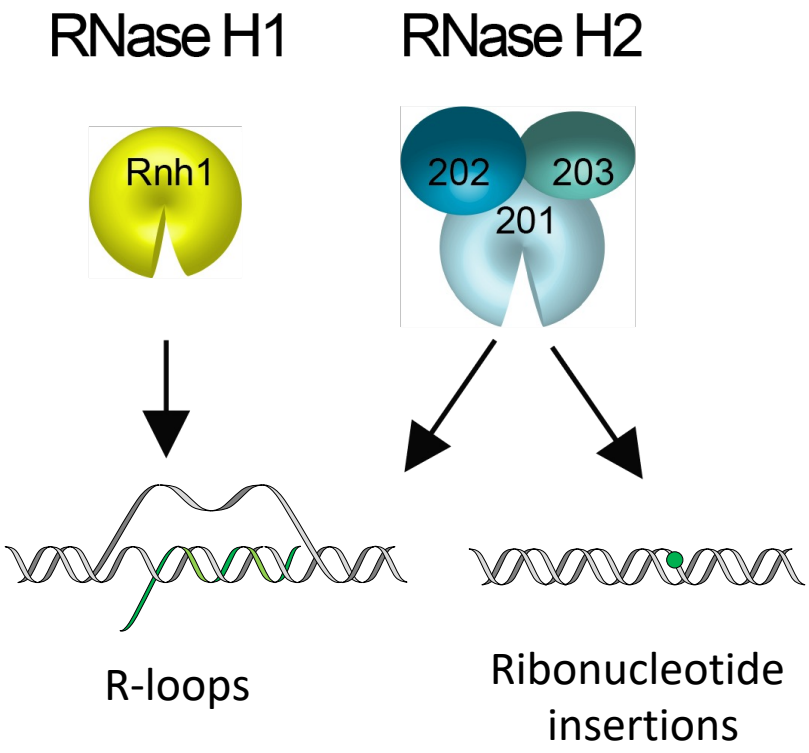
RNase H for R-loop cleavage



Helicases for R-loop unwinding

Brian Luke
b.luke@imb-mainz.de

How are R-loops regulated?



RNases H1 and H2 can hydrolyze the RNA moiety of R-loops

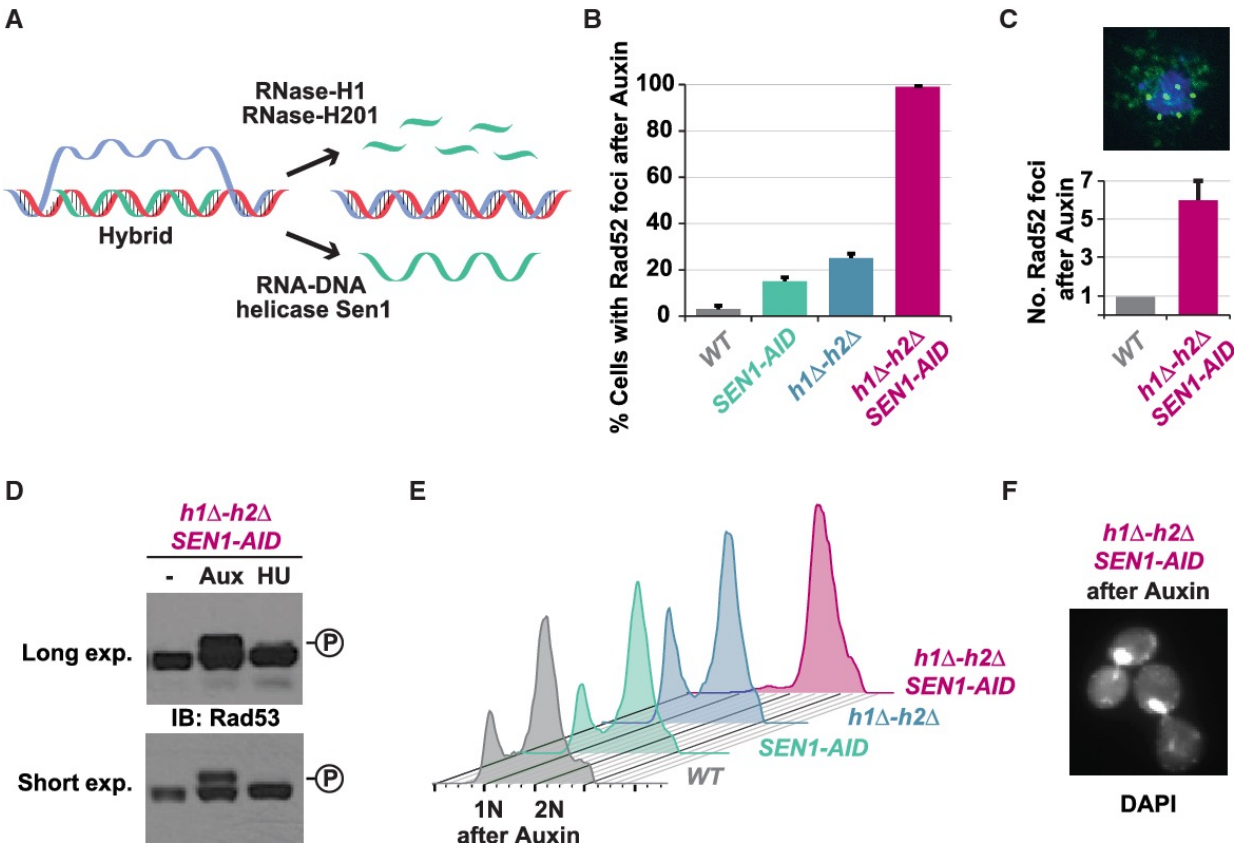
RNase H2 accounts for most of the RNase H activity in the cell

Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Why do R-loops need to be removed?

The stabilisation of R-loops leads to increased DNA damage and genome instability.



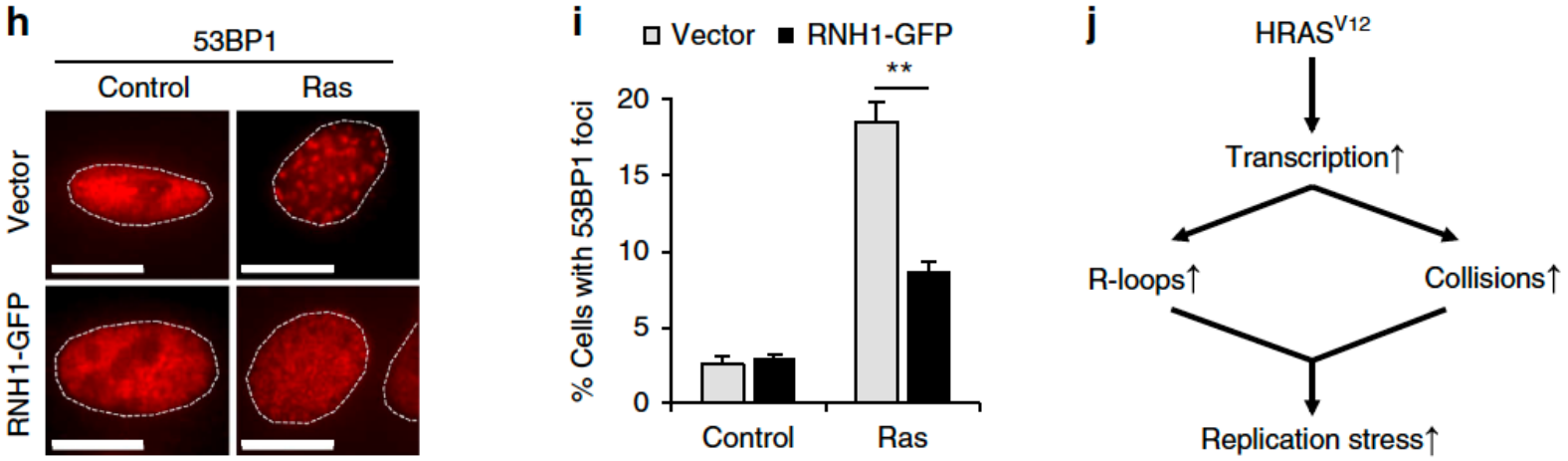
Example of yeast mutants accumulating R-loops
Costantino et al, 2018, Mol Cell

Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Why do R-loops need to be removed?

Kotsantis et al, 2016, Nat Comm

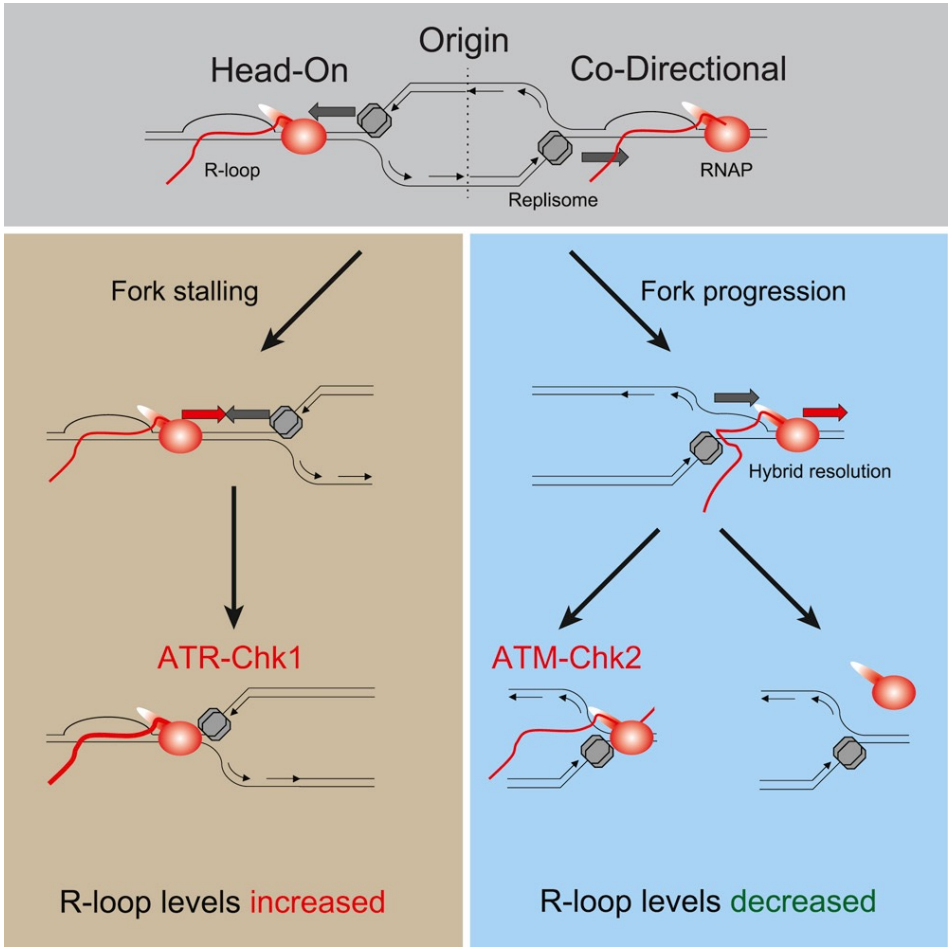


Brian Luke
b.luke@imb-mainz.de

Why do R-loops need to be removed?

R-loops collide with replication

- Causes replication fork stalling and then processing of DNA
- May not be the RNA but rather the transcription machinery itself (i.e. a crash with the polymerase)
- Also the displaced strand is more vulnerable
- In general it is thought that head-on collisions are more detramental than co-directional collisions
- It has also been suggested that the RNA-DNA hybrid *per se* is not the problem but rather the local compaction of the chromatin



Hamperl et al, 2017, Cell

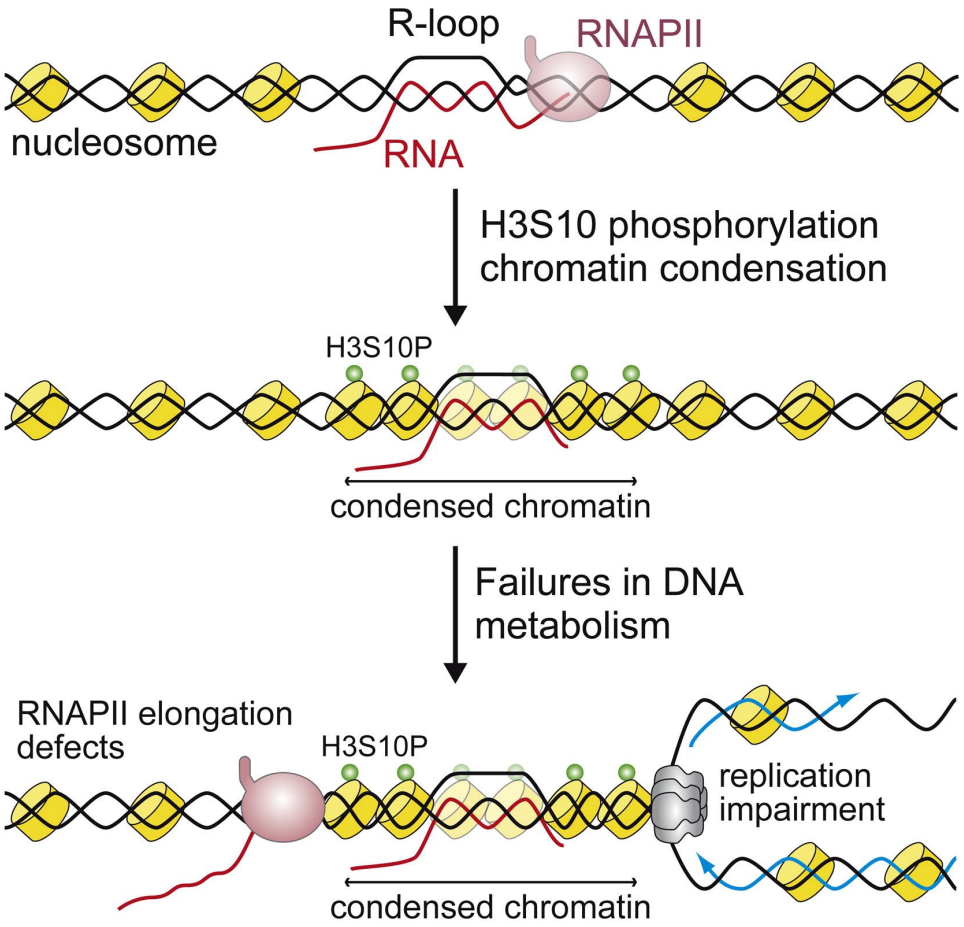
Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Why do R-loops need to be removed?

R-loops collide with replication

- R-loops lead to local increases in H3S10 phosphorylation and hence chromatin compaction
- This prevents proper replication and repair
- Mutants that prevent compaction can rescue the phenotypes despite the fact that the R-loops persist



Castellano-pozo et al, 2013, Mol Cell

Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Why do R-loops need to be removed – disease associated with R-loops

Disease	R-Loop Factor/Locus	Proposed Mechanism	References
Breast/Ovarian	Estrogen	Estrogen-induced R-loops cause DNA damage and genome instability.	Stork et al., 2016
	BRCA1	BRCA1 interacts with SETX and suppresses R-loops and DNA breaks at gene terminators.	Hatchi et al., 2015
		RNAPII pausing contributes to BRCA1-associated R-loop accumulation and breast cancer development.	Zhang et al., 2017
		BRCA1 is sequestered in cells expressing heterochromatin-associated non-coding RNAs, leading to genome instability.	Zhu et al., 2018
	BRCA2	BRCA2 depletion elevates R-loop levels and causes genome instability.	Bhatia et al., 2014
		Aldehydes deplete BRCA2 and cause R-loop-dependent genome instability.	Tan et al., 2017
		BRCA2 depletion causes transcription stress at gene promoters and R-loop-mediated DNA damage.	Shivji et al., 2018
Ewing's sarcoma	EWS-FLI, BRCA1	R-loops cause transcriptional stress, resulting in functional depletion of BRCA1 and subsequent DNA damage.	Gorthi et al., 2018
Myelodysplastic syndromes (MDS)	SRSF2, U2AF1	R-loops induced by splicing factor mutations cause replication stress and impair bone cell function.	Chen et al., 2018
Multiple myeloma and Burkhitt's lymphoma	TRD3-TOP3B	TRD3-TOP3B complex relieves negative supercoiling and reduces R-loop levels at *c-MYC* and *Igh* to suppress chromosomal translocations.	Yang et al., 2014
Alternative lengthening of telomeres (ALT)-dependent cancers	Telomeric repeat-containing RNA (TERRA)	TERRA R-loops are upregulated in cancer cells and promote homologous recombination to preserve telomeres by the ALT pathway.	Arora et al., 2014
Fanconi anemia (FA)	FANCM, FANCD2	FA factor deficiency leads to increased R-loop levels, exacerbating TRCs and causing genome instability.	Schwab et al., 2015; García-Rubio et al., 2015
AOA2	SETX	SETX resolves R-loops in neuronal cells; R-loops are elevated in neural progenitor cells from AOA2 patients with SETX mutations.	Becherel et al., 2015

Etc.....

Crossley et al, 2019, Mol Cell

Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

How are R-loops detected?

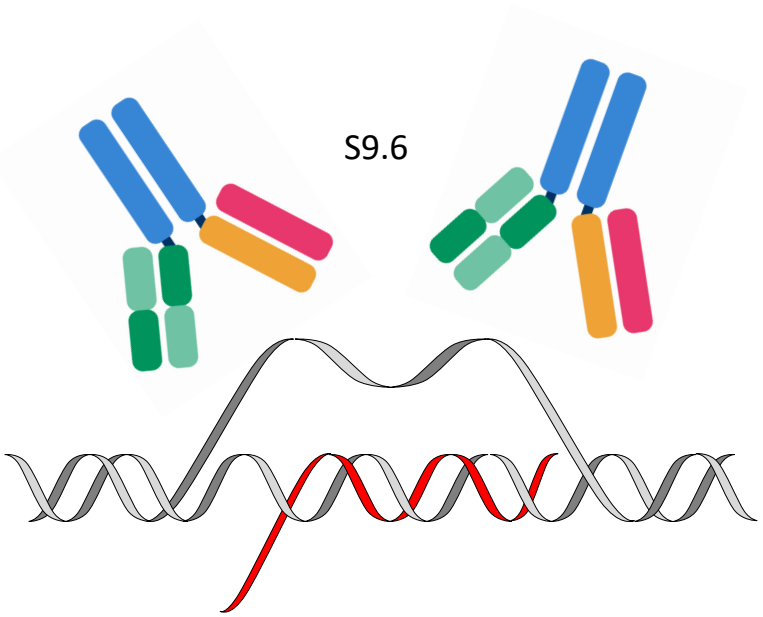
All R-loop detection to date is based on two different methods...

1. The S9.6 monoclonal antibody.

-This antibody was developed to increase the hybridization of RNA to DNA for microarray experiments, was later determined to bind to RNA-DNA hybrid in a structure specific and sequence independent manner

-epitope is a hybrid of at least 6 base-pairs

-easy to handle
-specificity issues, the antibody recognizes dsRNA and other structured RNAs....many controls required



Used for DRIP (DNA-RNA IP), to pull down the hybrid and analyse by qPCR or NGS

-can be used for IF as well as for Southwestern dot blotting

Brian Luke
b.luke@imb-mainz.de

How are R-loops detected?

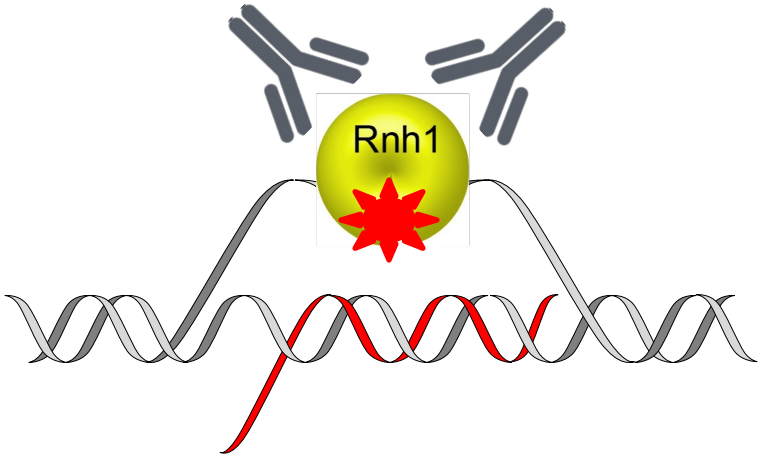
All R-loop detection to date is based on two different methods

2. Catalytic dead RNase H1

-This reagent can recognize an R-loop but does not degrade it and it then gets „stuck“ on the R-loop

-an antibody against RNase H1 can then pull down the complex

-less non-specific binding compared to S9.6
-reagent has to be made
-get stabilization of R-loops



Used for R-ChIP

Antibody to Rnh1 pulls down associated sequences which are subsequently quantified by either qPCR or sequencing

Brian Luke
b.luke@imb-mainz.de

All methods are variations of S9.6 and cat dead RNase H1 precipitations

Table 2. R-Loop-Mapping Technologies

Method Name	Fragmentation Method	Detection Method	Molecule Sequenced	Advantages	Disadvantages	Primary Reference
DRIP-seq	Restriction digest	S9.6	dsDNA	Robust signal, widely adopted, easy to set up	Low resolution, no strand specificity, not *in situ*	Ginno et al., 2012
DRIVE-seq	Restriction digest	Catalytically inactive RNase H	dsDNA	Provides independent verification of some DRIP-seq results	Low enrichment, low resolution, no strand specificity, reagent not commercially available, not *in situ*	Ginno et al., 2012
S9.6-ChIP-seq	Sonication after cross-linking	S9.6	dsDNA	May overcome bias and resolution issues in DRIP-seq	Not strand specific, cross-linking could affect results	El Hage et al., 2014
S1-DRIP-seq	Sonication	S9.6	dsDNA	Higher resolution than DRIP-seq	Not strand specific, not *in situ*	Wahba et al., 2016
DRIPc-seq	Restriction digest	S9.6	RNA	Strand specific, high resolution	Not *in situ*, requires lengthier sample preparation, S9.6 may recognize dsRNA	Sanz et al., 2016
RDIP-seq	Sonication	S9.6	RNA	Strand specific, high resolution	Not *in situ*, lengthier preparation, S9.6 recognizes dsRNA	Nadel et al., 2015
ssDRIP-seq	Sonication	S9.6	ssDNA	Strand specific, easy compared to other strand-specific techniques	Not *in situ*, low resolution	Xu et al., 2017
Bis-DRIP-seq	Restriction digest	S9.6	dsDNA with bisulfite conversions	Strand specific, provides additional control to ensure S9.6 signal arises from an R-loop *in situ*	Requires many replicates	Dumelie and Jaffrey, 2017
R-ChIP-seq	Sonication	Catalytically inactive RNase H	ssDNA	Strand specific, *in situ* capture	Cell line must be engineered to express catalytically inactive RNase H construct, inactive RNase H may alter hybrid dynamics	Chen et al., 2017

Crossley et al, 2019, Mol Cell

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

RNA-DNA hybrids

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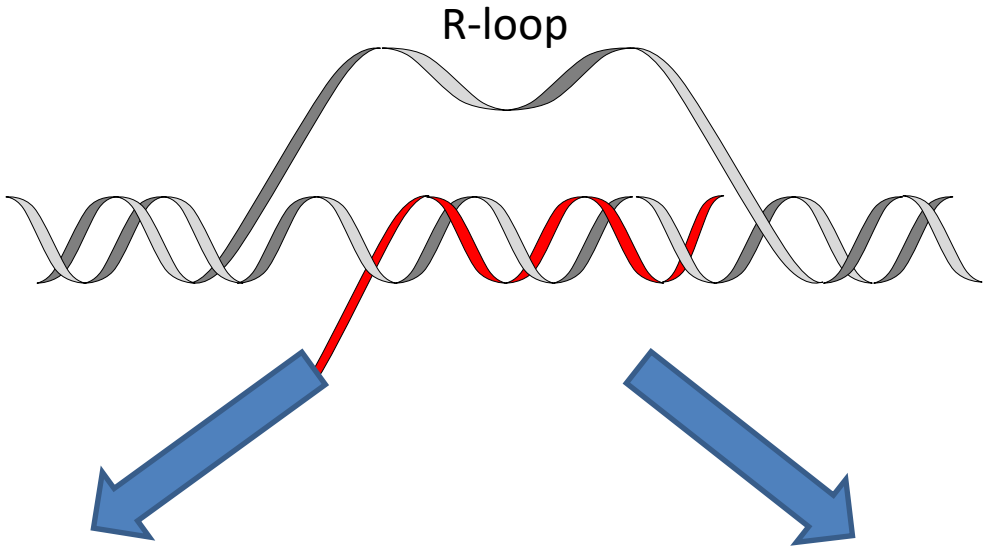
4. Functional R-loops
5. R-loops at telomeres and the timing of RNA-DNA hybrid removal by RNase H
6. R-loops as regulators of DNA methylation

Are R-loops all bad? – could be used to find a needle in the haystack



- Replication stress
- DNA damage
- Genome instability
- disease

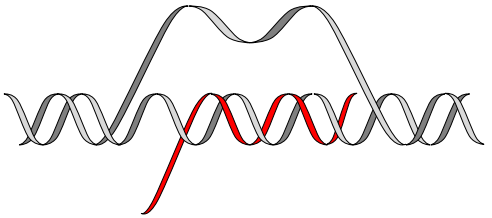
- Ideal way to find a specific sequence
- Gene regulation
- Damaged DNA

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

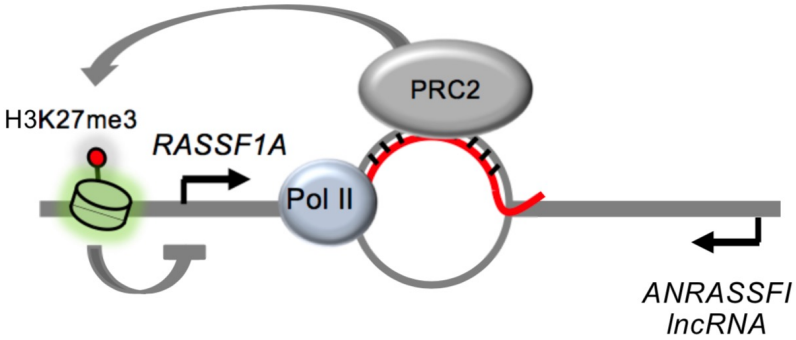
R-loops as transcriptional regulators

-R-loops are frequently found in **promoter regions**
-specifically promoter regions with **CpG islands**
-frequently associated with a strong **GC skew**
-R-loops can exert different activities at different promoters
-frequently associated with decreased DNA methylation



A few examples

Repressive histone modifiers

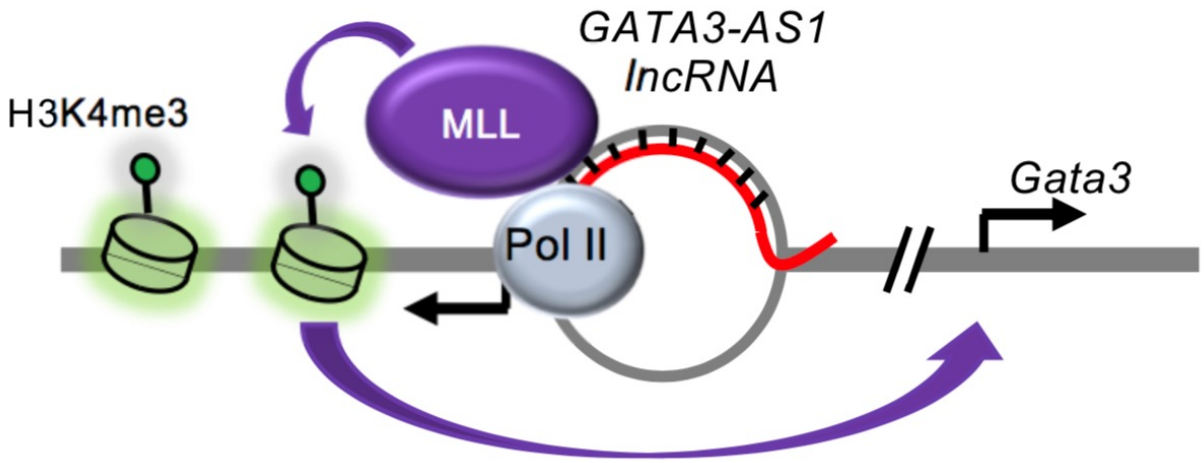


-RASSF1A is a tumor suppressor
-expression of antisense RNA (ANRASSF1) forms an R-loop which recruits the polycomb repressive complex 2
-this suppresses transcription and increased cell proliferation

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

Activating histone modifiers

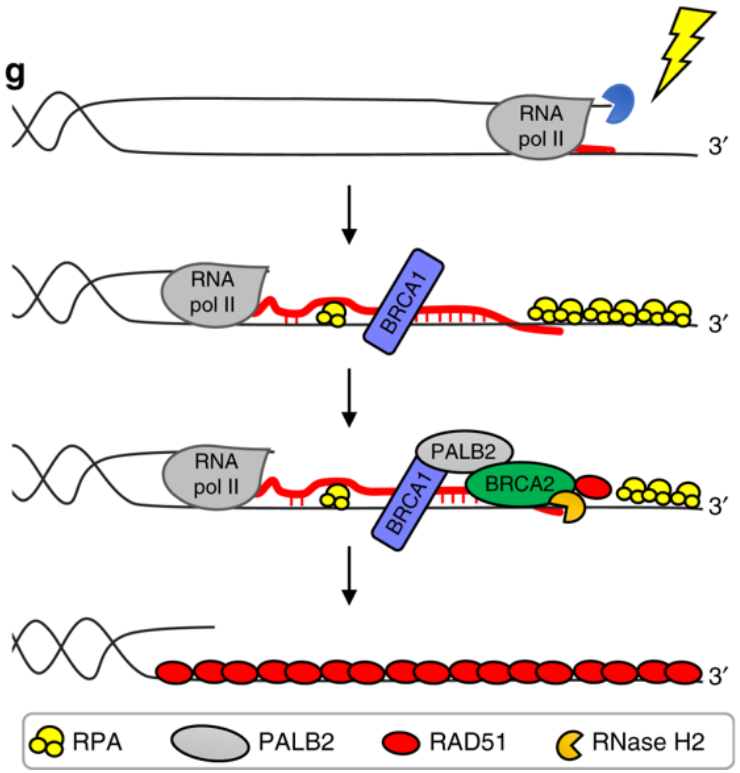


-an anti-sense R-loop formed in the Gata3 promoter leads to the recruitment of MLL and promtes transcription
-this RNA can also be expressed in trans and will activate Gata3 by the same mechanism
-therefore regulatory R-loops can act *in trans*....but do they?

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

R-loops promote DNA repair



D'Alessandro et al, 2018, Nat. Comm

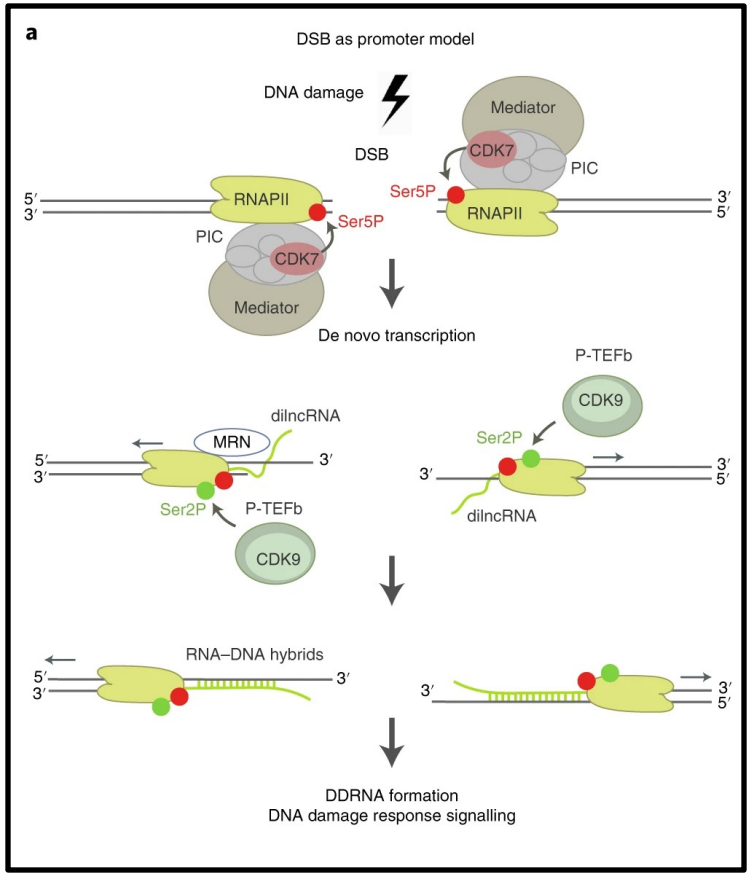
-at a break the 3`end is recognized by RNAPII
-the RNA forms a hybrid which gets recognized by BRCA1 and eventually BRCA2
-the hybrid is then degraded by RNase H2 and then Rad51 is loaded
-this then drives HR
-there are other theories regarding how hybrids work to repair DNA

Brian Luke
b.luke@imb-mainz.de

Institute of Molecular Biology

Why are hybrids at Double strand breaks? – 2 predominant models

1. DSB as a promoter



Marnef and Legube, 2021

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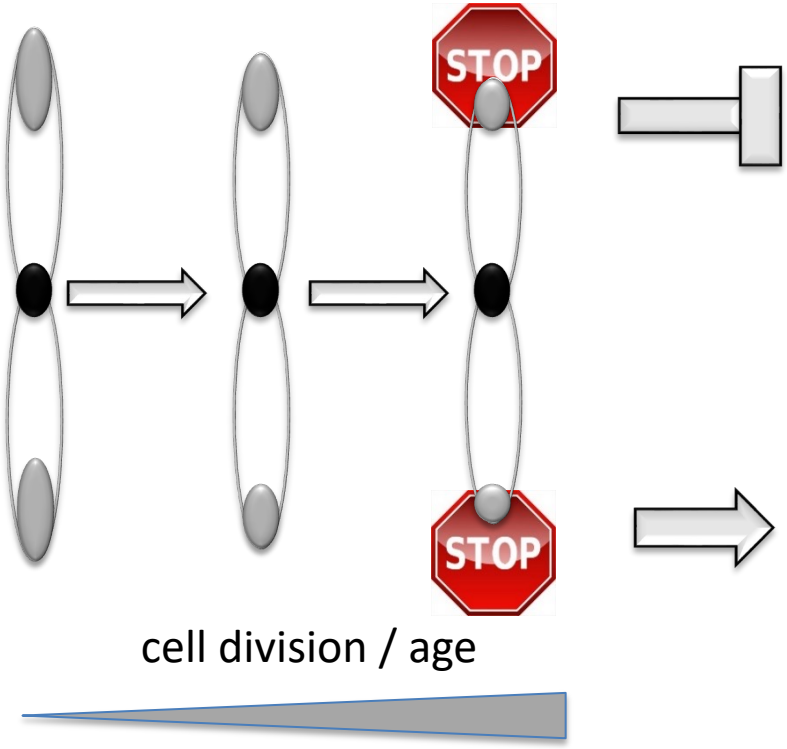
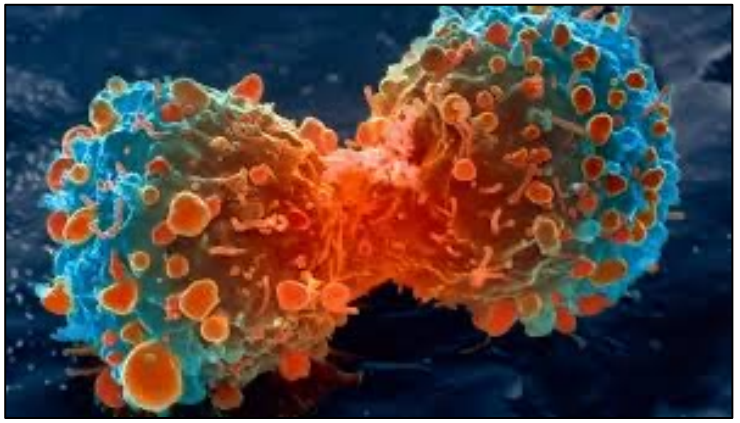
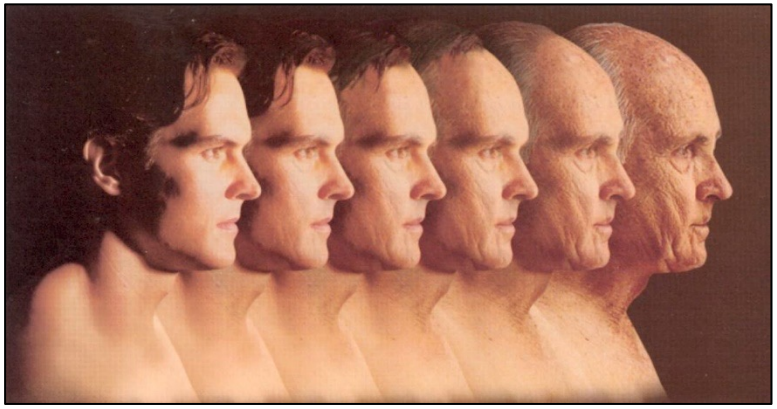
The double-edge sword of telomere shortening

Checkpoint arrest
replicative senescence
(tumor suppressive)



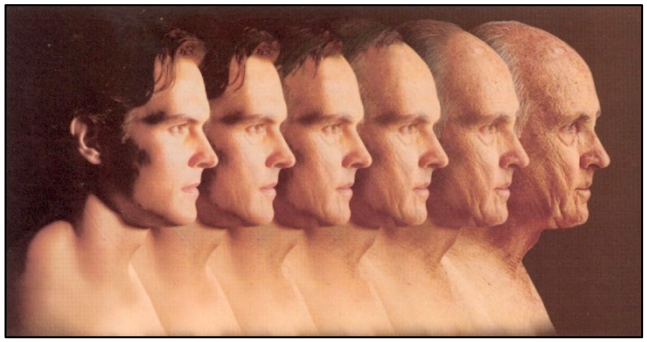
Important in long-lived organisms



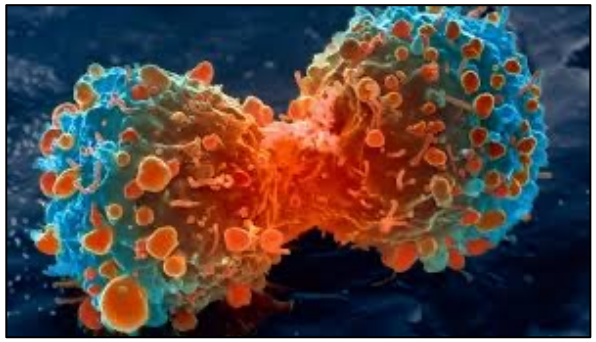


Telomere length must be balanced to prevent disease

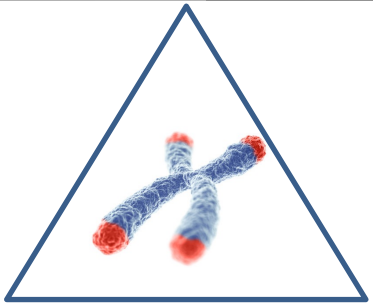
Short telomeres
Aging



Telomere maintenance
Cancer

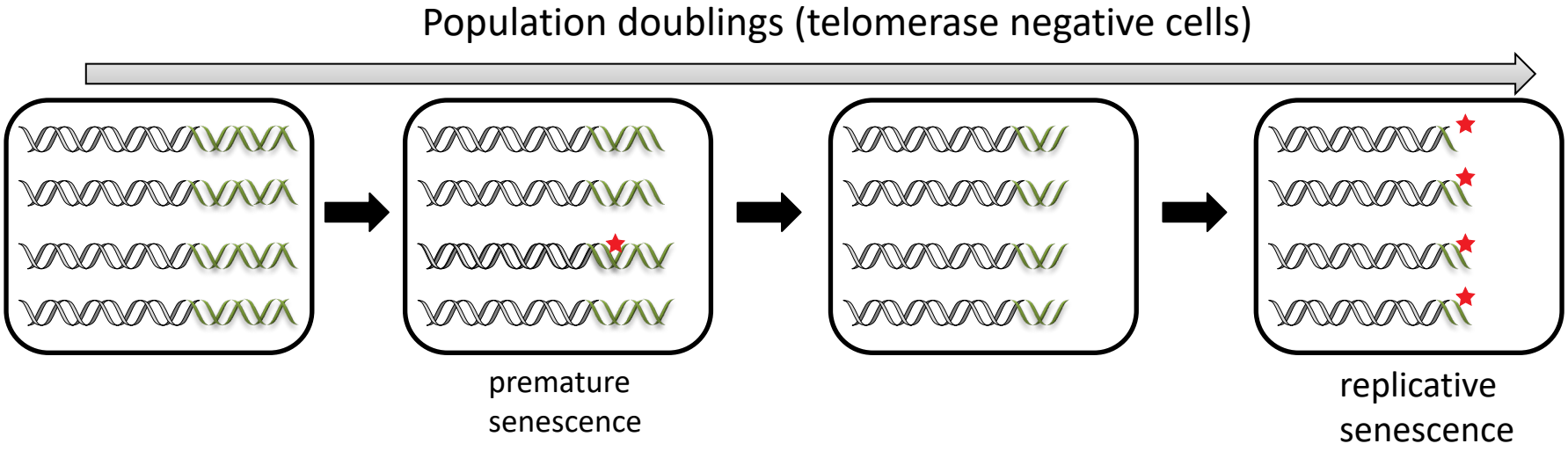


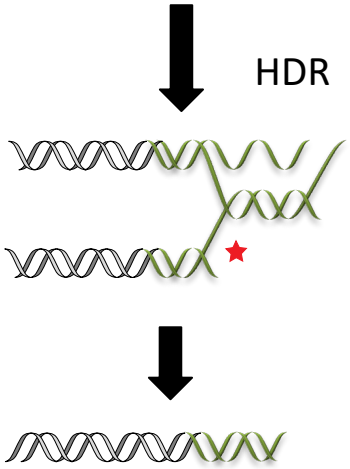
Dyskeratosis Congenita
Blooms, Werners Syndromes
Idiopathic Pulmonary Fibrosis
Aplastic Anemia

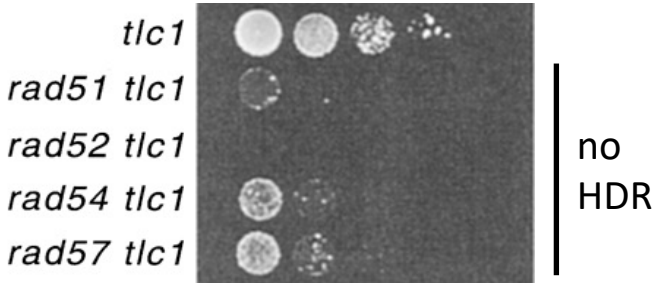


How can the telomere shortening process be regulated to ensure a non-pathogenic balance is achieved?

Recombination ensures that premature senescence is avoided







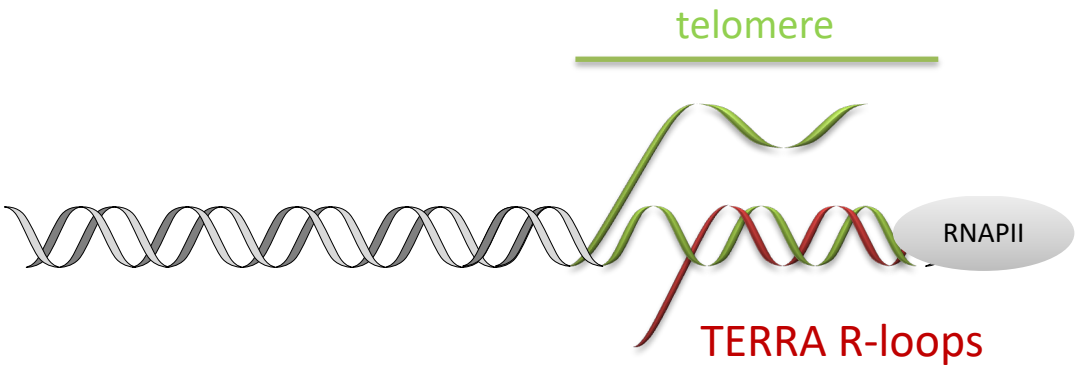
Le et al, 1999

Why does only the short telomere get extended and not the others?

Brian Luke
b.luke@imb-mainz.de

imb Institute of Molecular Biology

Telomeres are transcribed into TERRA molecules



TERRA

Azzalin et at, 2007, Science
Luke et al, 2008, Mol Cell

Balk and Maicher et al, 2013, NSMB
Pfeiffer et al, 2013 EMBO J
Arora et at, 2014, Nat Comm

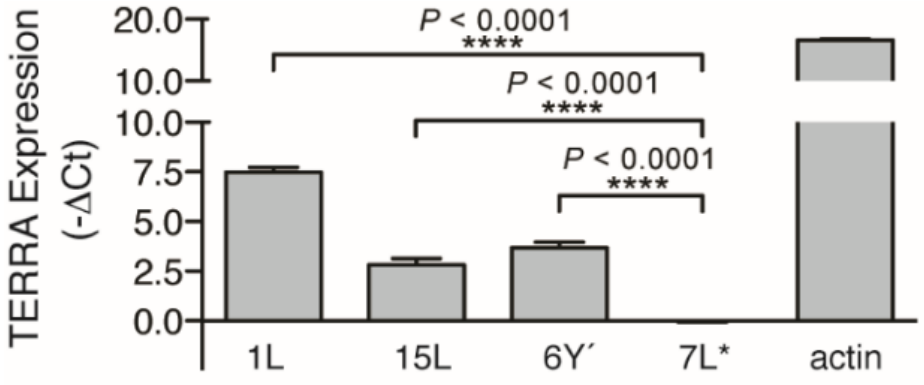
1. Why are telomeres transcribed?

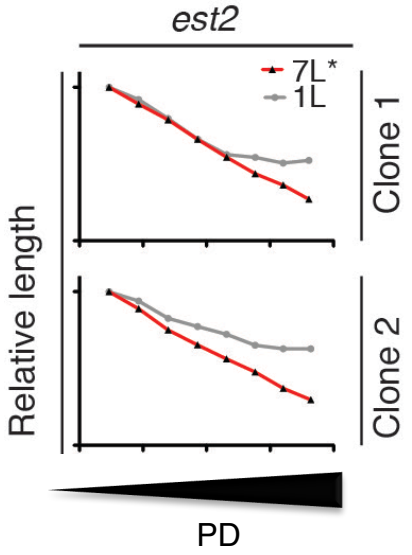
2. Do the **R-loops** have any physiological relevance?

Brian Luke
b.luke@imb-mainz.de

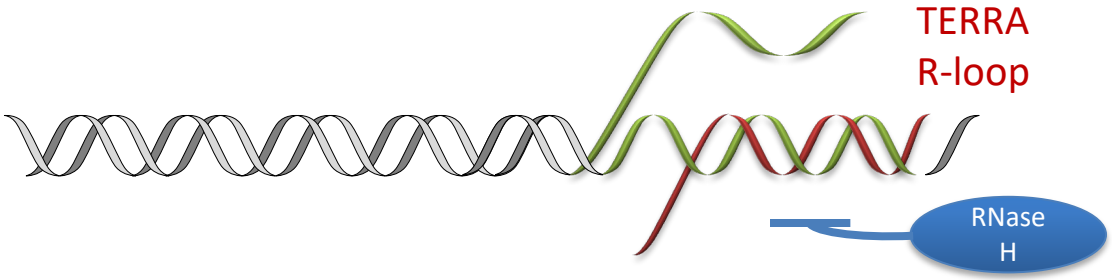
imb Institute of Molecular Biology

TERRA-less telomeres do not recombine

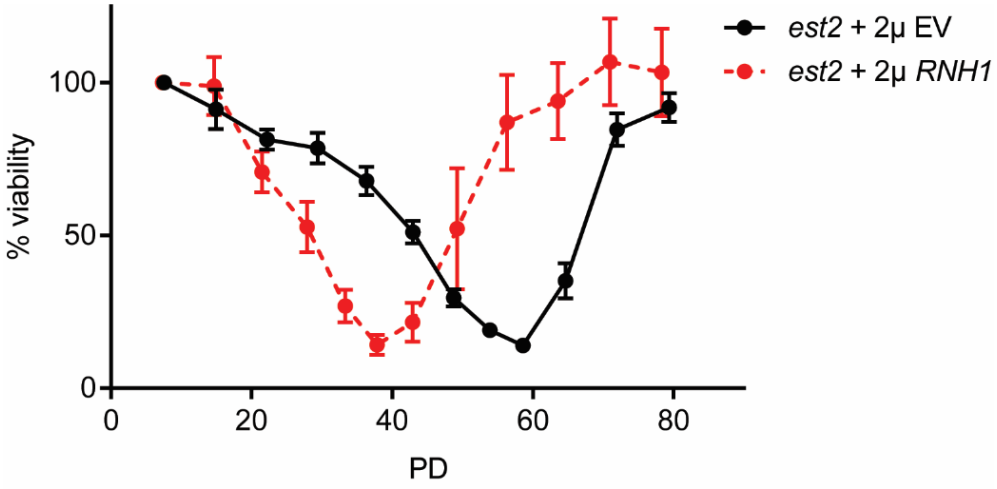




R-loop levels can influence rates of senescence

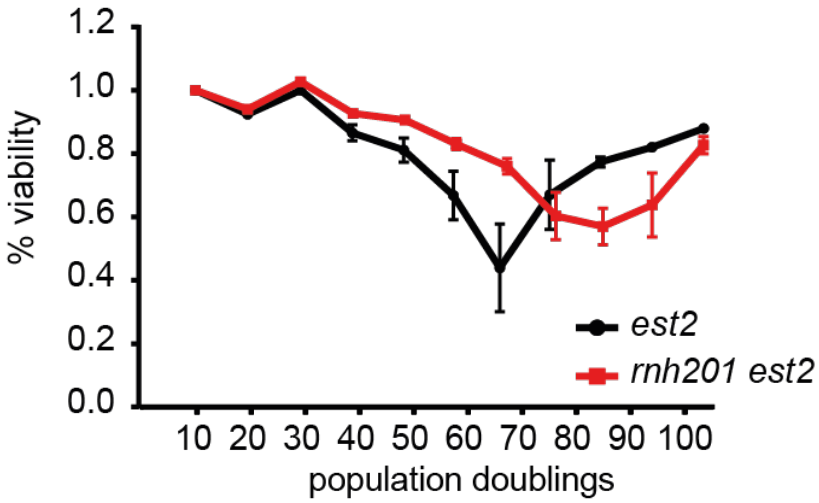


R-loops = fast senescence



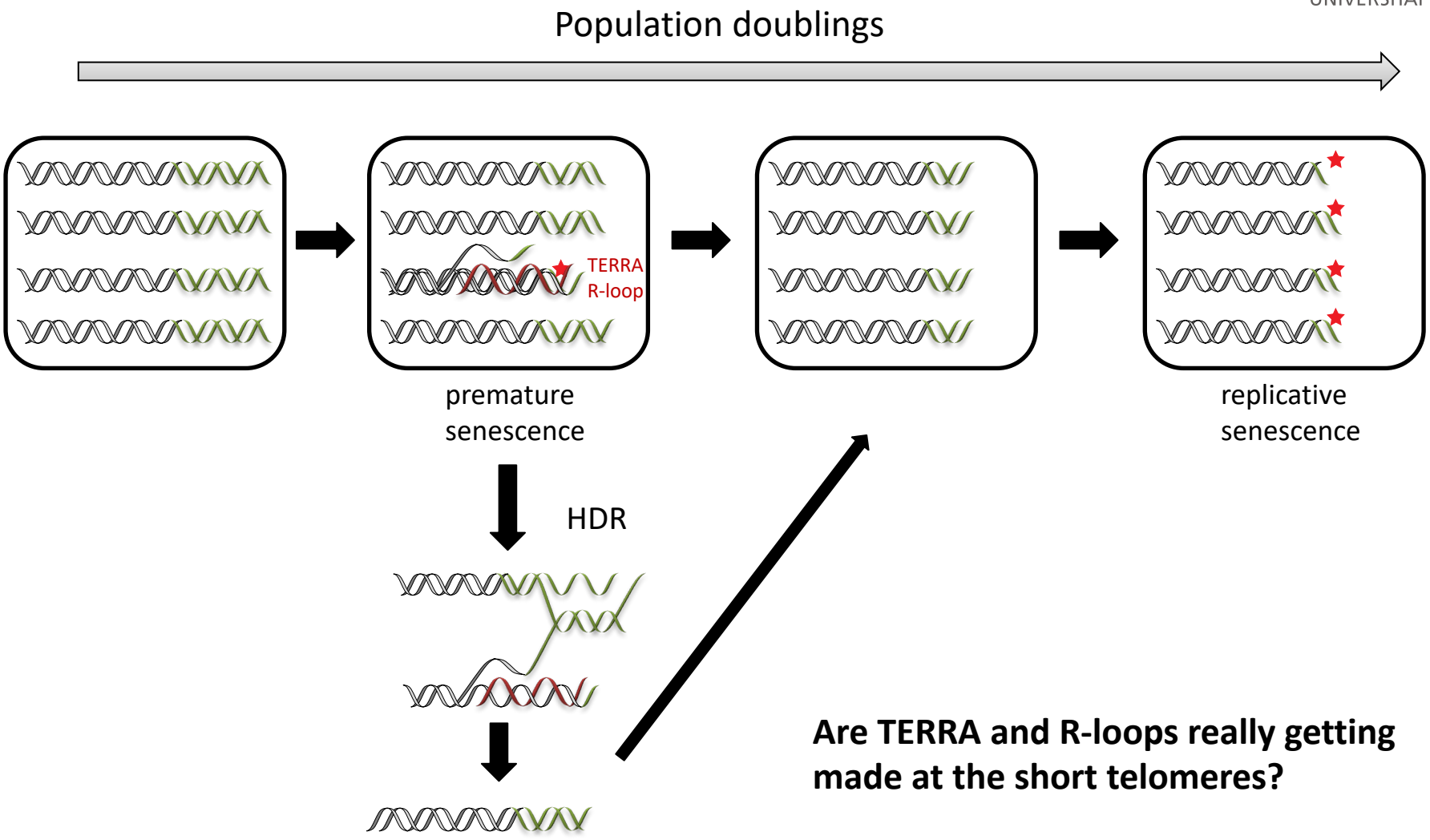
Balk and Maicher et al, 2013, *Nat Struc Mol Biol*

R-loops = slow senescence



Graf et al, 2017, *Cell*

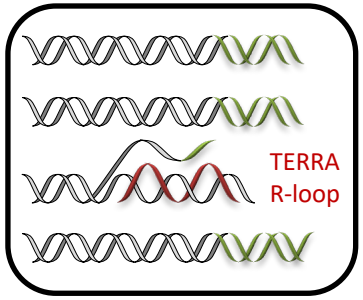
R-loops may promote elongation of the shortest telomeres



Are TERRA and R-loops really getting made at the short telomeres?

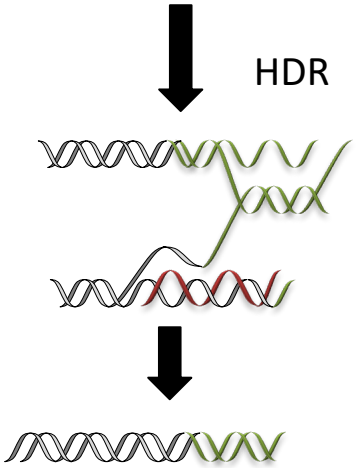
TERRA and R-loops accumulate at the critically short telomeres

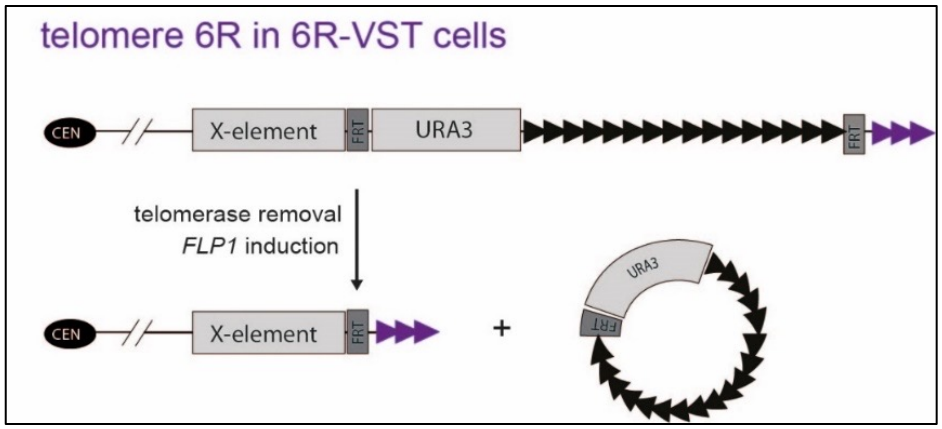
TERRA at short telomeres?

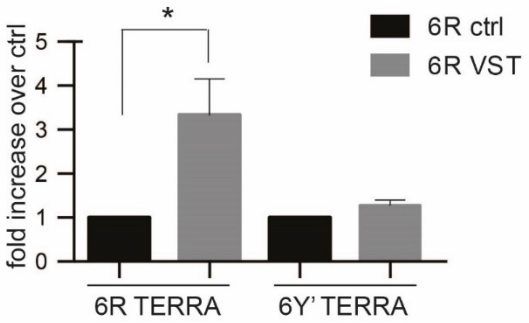


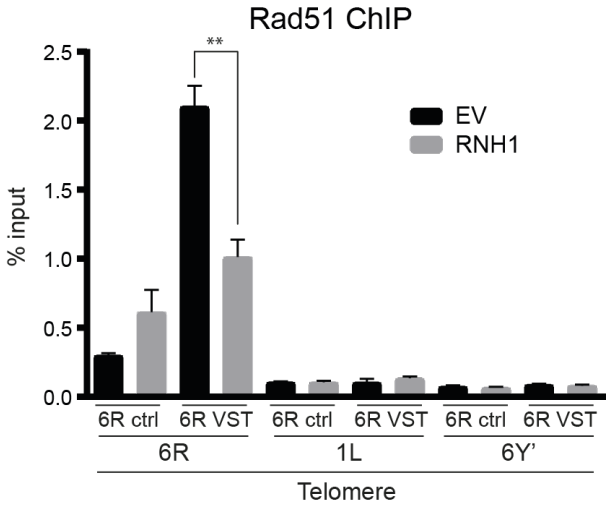
premature senescence

HDR



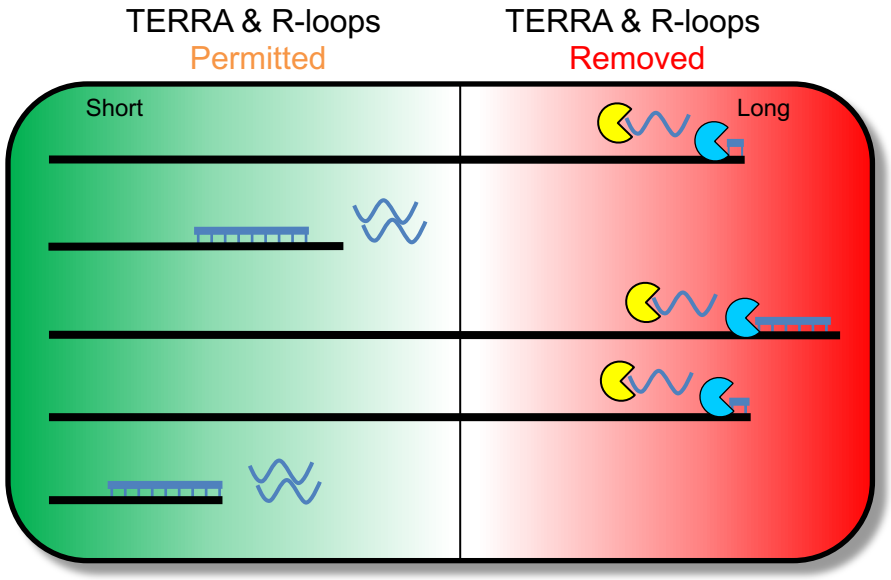




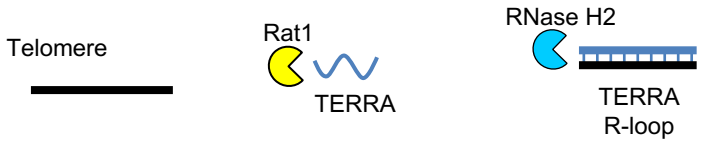


Why is TERRA at short telomeres?

TERRA and R-loops are preferentially removed at long telomeres

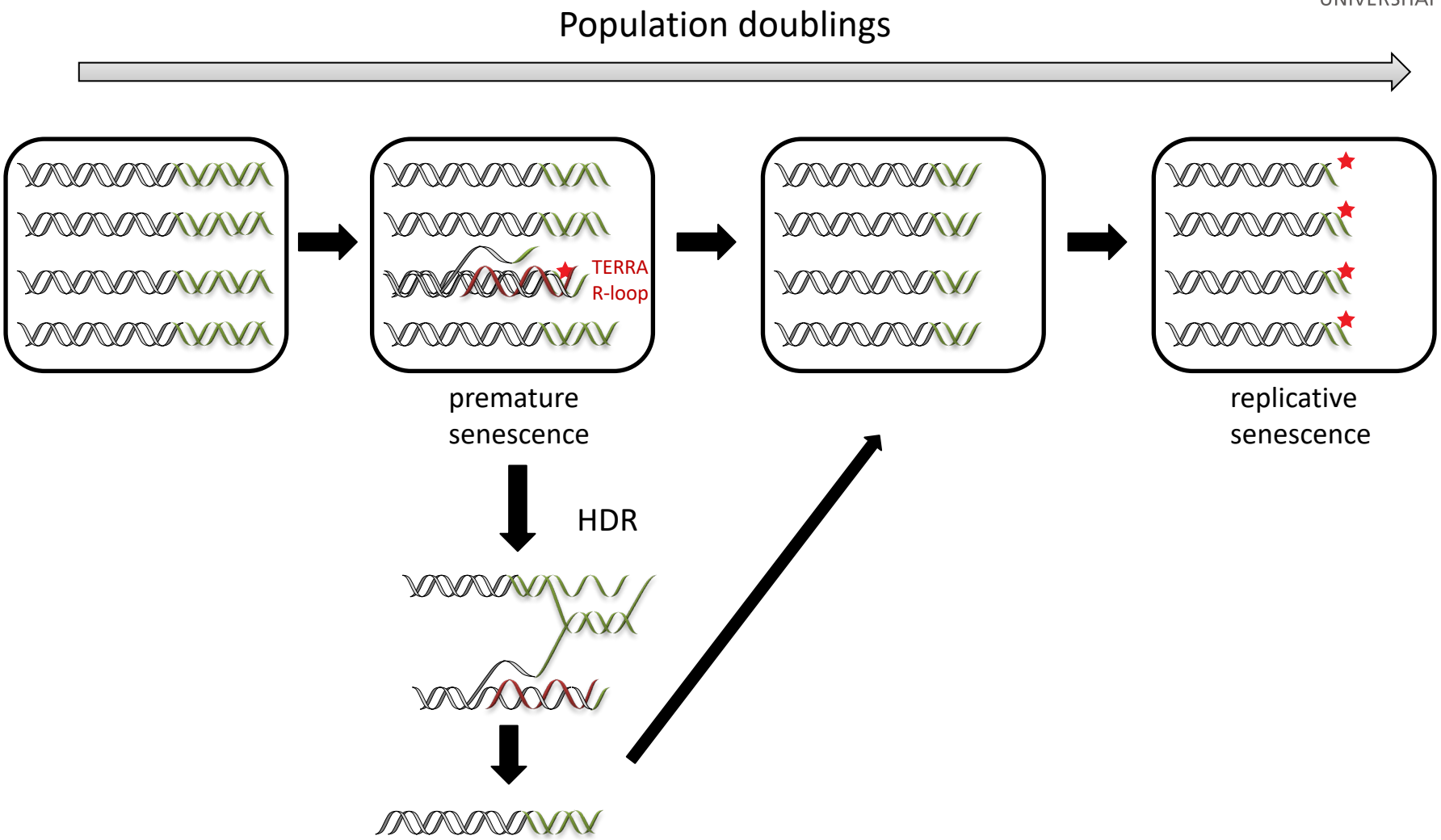


Graf et al, 2017, Cell



So why does this drive recombination?

R-loops promote elongation of the shortest telomeres



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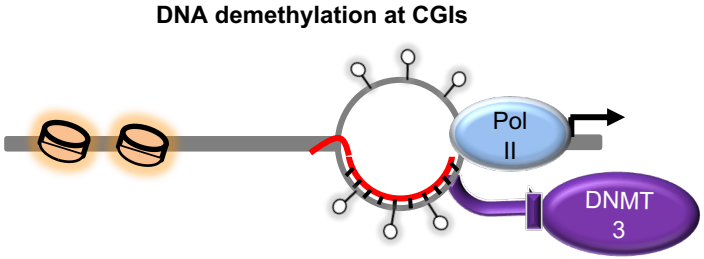
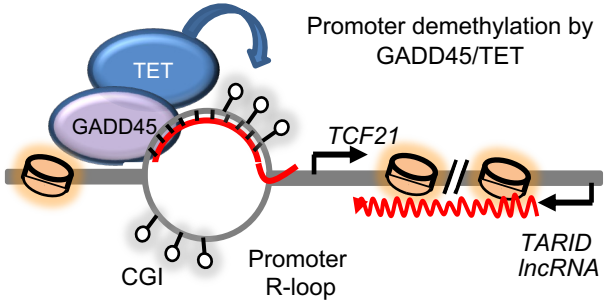
R-loops affect the local methylation state via 2 means

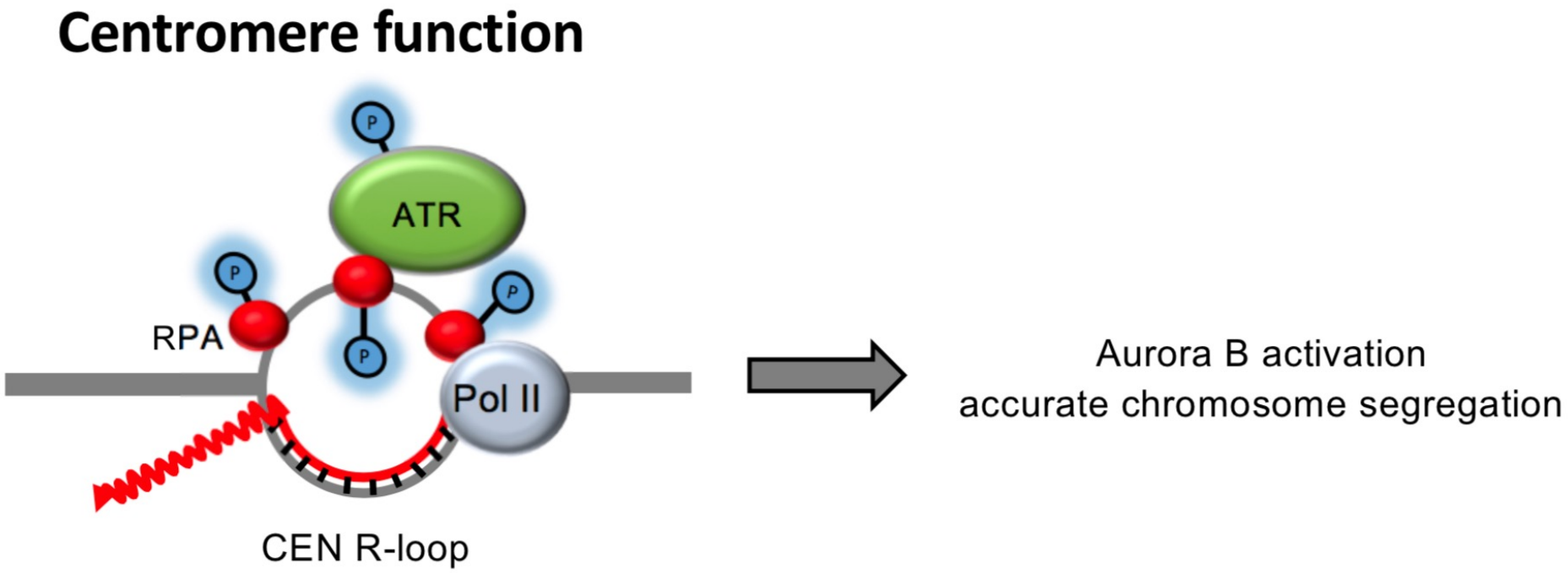
- R-loops at CGIs prevent the association of DNA methyltransferases resulting in hypomethylation of promoters (Ginno et al, 2012, Grunseich et al, 2018)



- The GADD45 proteins binds directly to R-loops and recruits the TET DNA demethylases
- Approximately 4% of TET1 bidning sites at CGI promoters may be R-loop dependent
- This has been characterized extensively at the TCF21 locus whereby an anti-sense RNA (TARID) forms an R-loop



RNA-DNA Hybrid are also critical for centromere function



Brian Luke
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Institute of Molecular Biology