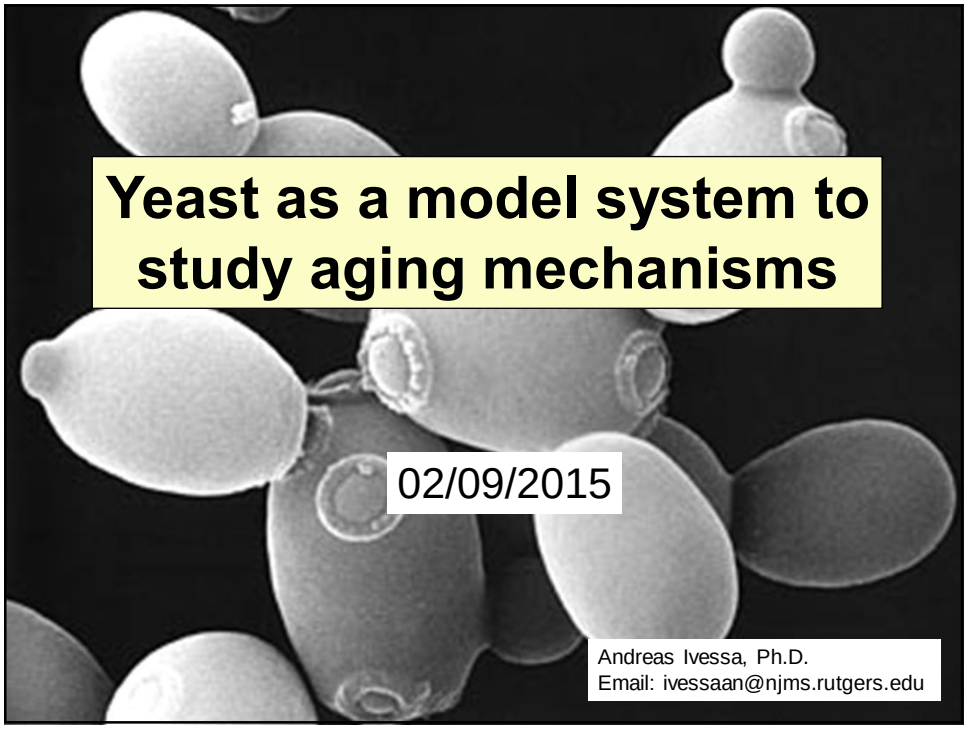


Budding yeast as a model system to study aging:

Has multiple features of higher eukaryotic model organisms

organelles: nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, vacuoles (lysosomes)

chromosomes containing telomeres (physical ends) and centromeres

multiple processes are similar (mitosis, meiosis)

multiple metabolic and signal transduction pathways are similar

Budding yeast as a model system to study aging:

advantages:

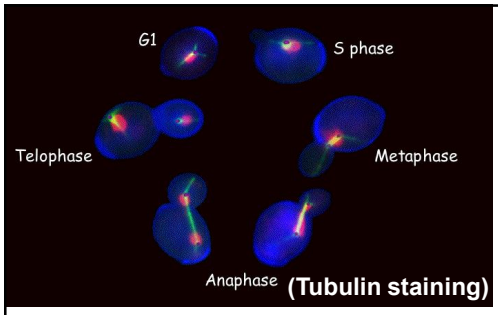
- "fast" cell division (~ 110 minutes)
- in-expensive growth media
- convenient growth conditions
- non-pathogenic, so can be handled with few precautions
- highly versatile DNA transformation system
- can be maintained in stable haploid and diploid states that facilitate genetic analyses
- novel techniques (2-hybrid, Yeast Artificial Chromosomes (YACs)) make yeast valuable for studies of many organisms.
- rather small genome size (~1/100th of mammals):
  haploid: 16 chromosomes (12 Mb)
  many genes present as single copy

disadvantage:

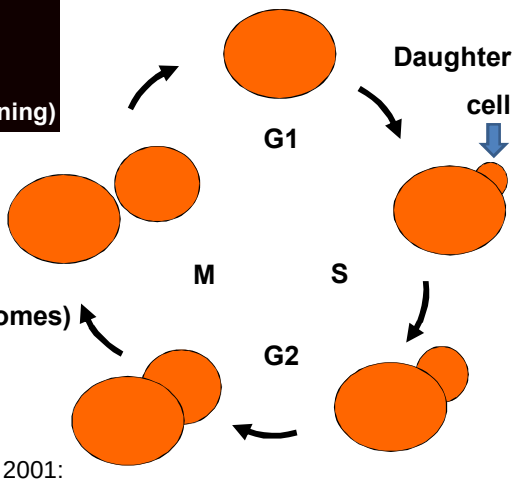
- cell differentiation processes (like in higher eukaryotic systems (flies, worms)) can almost not be studied
  Yeast is mostly present as a unicellular organism.

Examples of how studies using yeast can reveal how human cells work

- Cell cycle studies by Lee Hartwell
  (2001 Nobel Prize for Physiology or Medicine)
  - Mitotic spindle assembly depends on the completion of DNA synthesis
  - The concept of START (transition from G1 to S phase)
  - Identification of cdc28, the first cyclin-dependent kinase
  - Other crucial concepts critical for cancer, etc.
- Dissection of the secretory pathway by Randy Schekman
  (2013 Nobel Prize for Physiology or Medicine)
  [together with Jim Rothman and Tom Suedhof]
  - Identified dozens of complementation groups involved in protein transport throughout the secretory pathway (Endoplasmic reticulum, Golgi Apparatus, Plasma membrane, Lysosomes, …)
- Many other studies of conserved processes such as DNA synthesis, transcription, translation, cell signaling, …



Budding yeast as a model system to study the cell cycle



Budding yeast:

Asymmetric cell division

S-phase (DNA replication)

M-phase (separation of chromosomes)

G1, G2: gap-phases

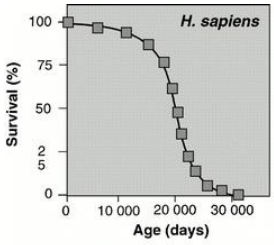
(prepare for S or M phases)

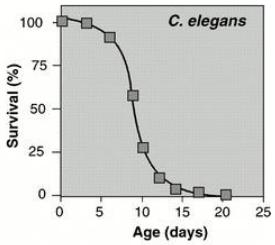
Nobel prize for Physiology or Medicine 2001:
Lee H. Hartwell
(Key regulators for the cell cycle)

"Aging" is characterized as a functional decline of living units (cells, tissues, whole organisms).

Is it random or does it follow a specific program?

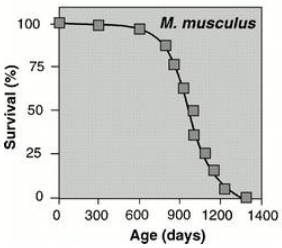
Humans

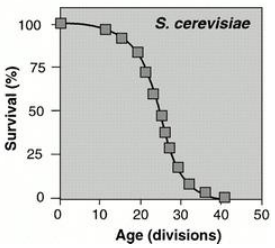




Worms

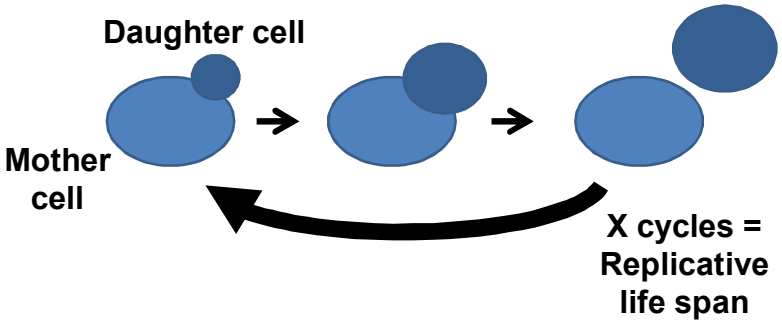
Mice





Budding yeast
Baker's yeast

Replicative aging of budding yeast cells



Replicative life span is defined as the number of daughter cells which a budding yeast mother cell can generate.

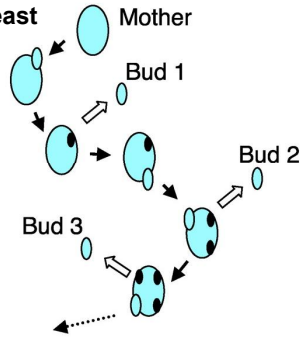
In most mammalian cell types mother and daughter cells cannot be distinguished.

***In contrast*, in budding yeast the daughter cell is gradually emerging from the mother cell allowing to determine the number of daughter cells produced by one mother cell.**

Replicative life span versus chronological life span

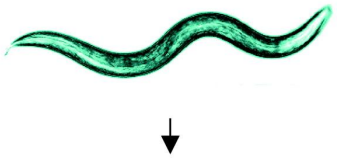
Aging in the yeast *S. cerevisiae* is the number of buds a mother cell produces

Budding yeast
Baker's yeast



REPLICATIVE AGING of 1 MOTHER CELL

Aging in the worm *C. elegans* is the number of days a worm lives

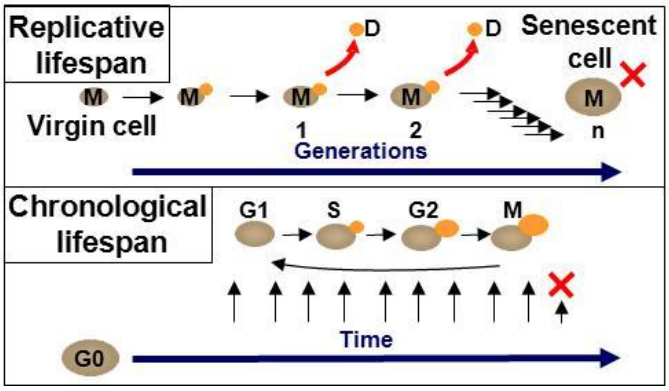


Prod individuals daily to check for the ability to move any part of the body

CHRONOLOGICAL AGING of 959 MOTHER CELL

S Hekimi, L Guarente Science 2003;299:1351-1354

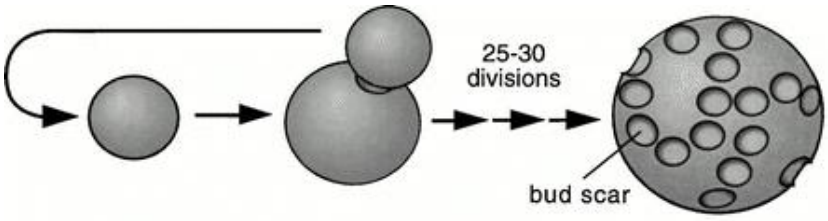
Replicative versus chronological lifespan using budding (baker's) yeast as a model system



Yeast **replicative lifespan** is thought to be comparable to aging phenomena observed in asymmetrically dividing cells of higher eukaryotes, such as **stem cells**.

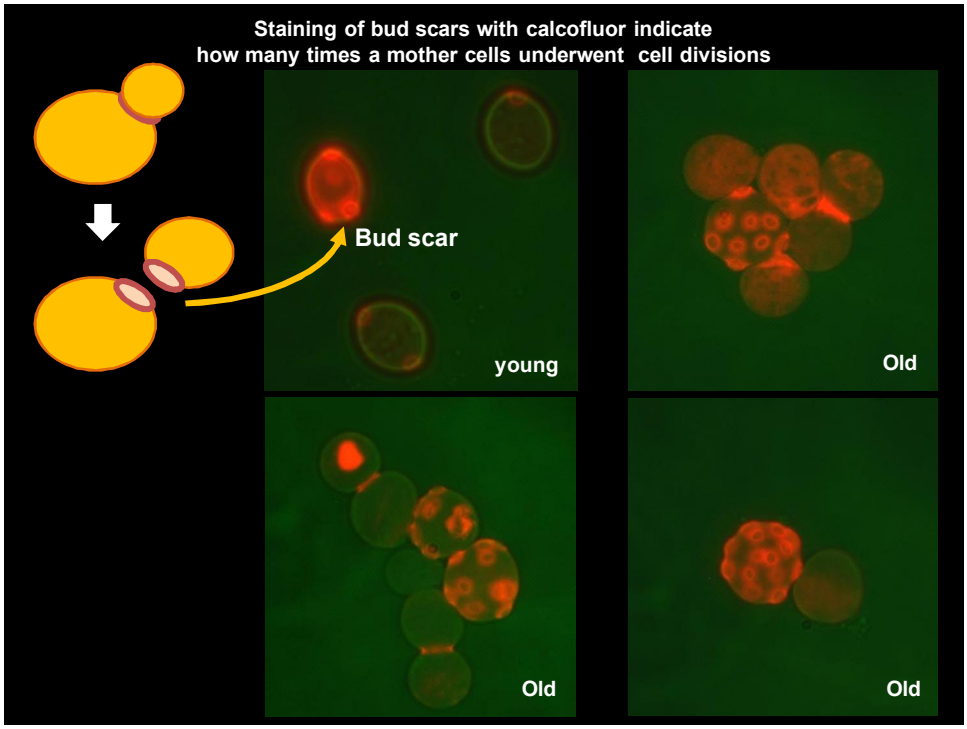
Yeast **chronological aging** is similar to aging of non-dividing higher eukaryotic cells such as **end-differentiated neurons or cardiac cells**.

Phenotypic changes during the replicative aging process of budding yeast



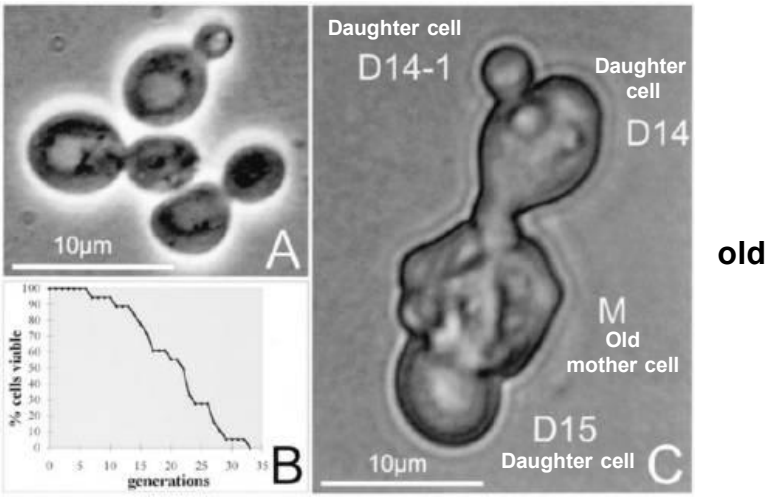
Size:	small	large
Cell surface:	smooth	wrinkled
Cell division:	1.5 hr / asymmetrical	2 – 3 hrs / symmetrical
Daughters:	full life span	reduced life span
Nucleolus:	intact	fragmented

Nucleolus: Part of the nucleus that contains the ribosomal DNA genes.



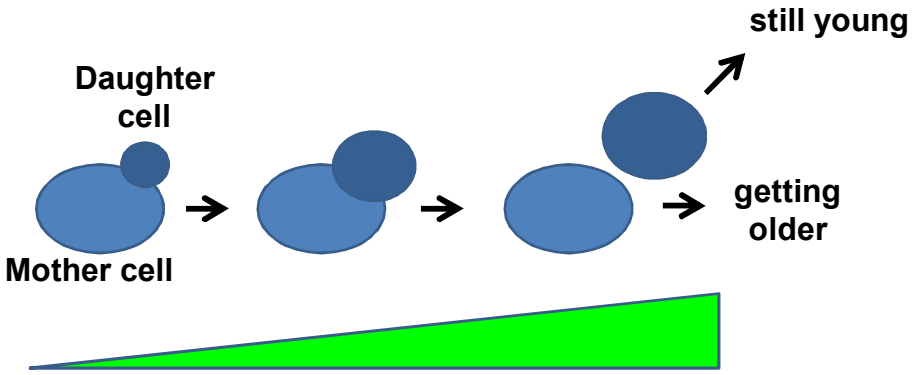
Phenotypic changes during the aging process of budding yeast

young



old

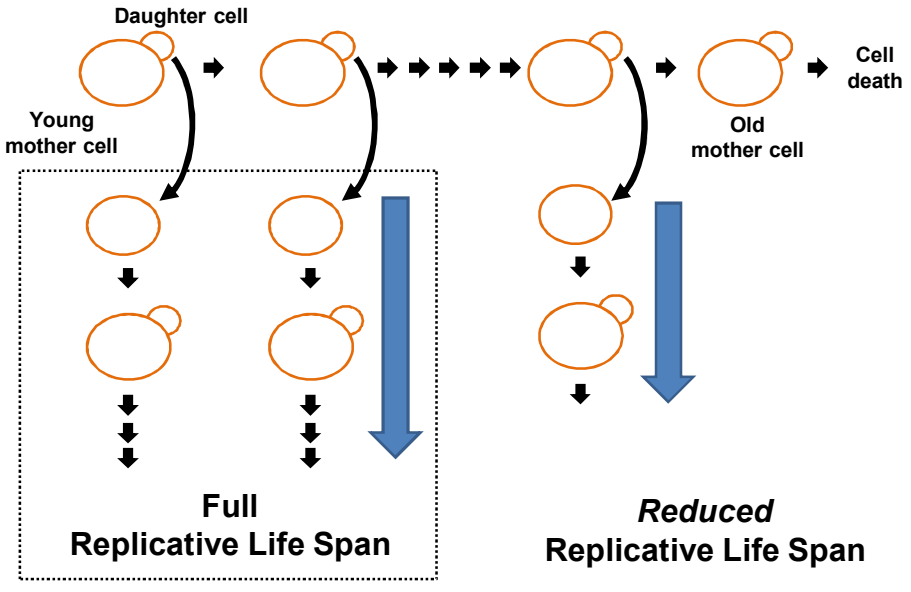
The "aging clock"



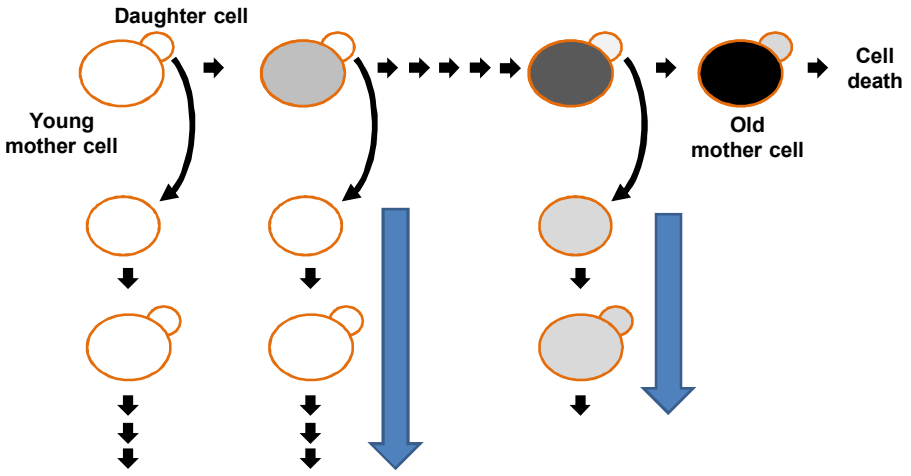
Accumulation of a senescence factor in mother cells?

Is there an intrinsic factor(s) which determine how many times a mother cell can generate daughter cells?

Whereas mother cell is continuously aging, the daughter cells deriving from the mother cell have a full replicative life span



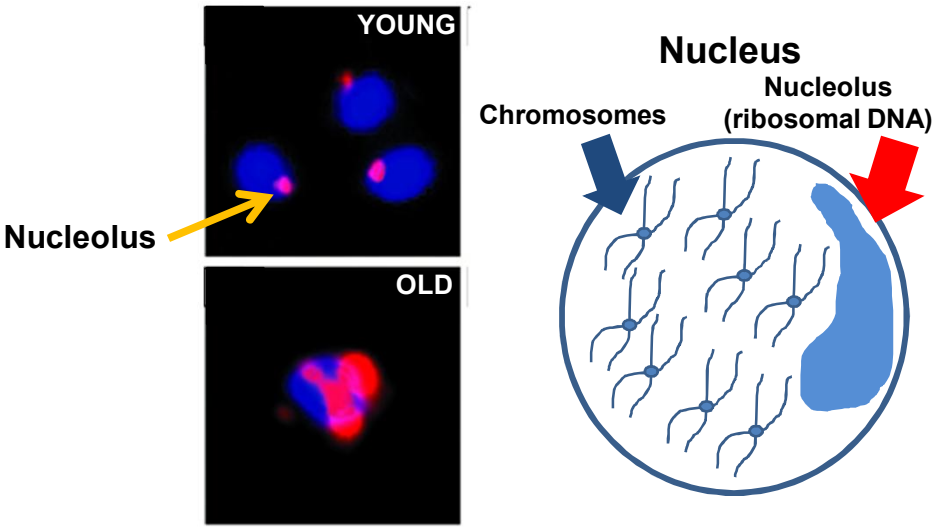
Accumulation of a senescence factor in the mother cell, but not in the daughter cell, during replicative aging in yeast



Full Replicative Life Span

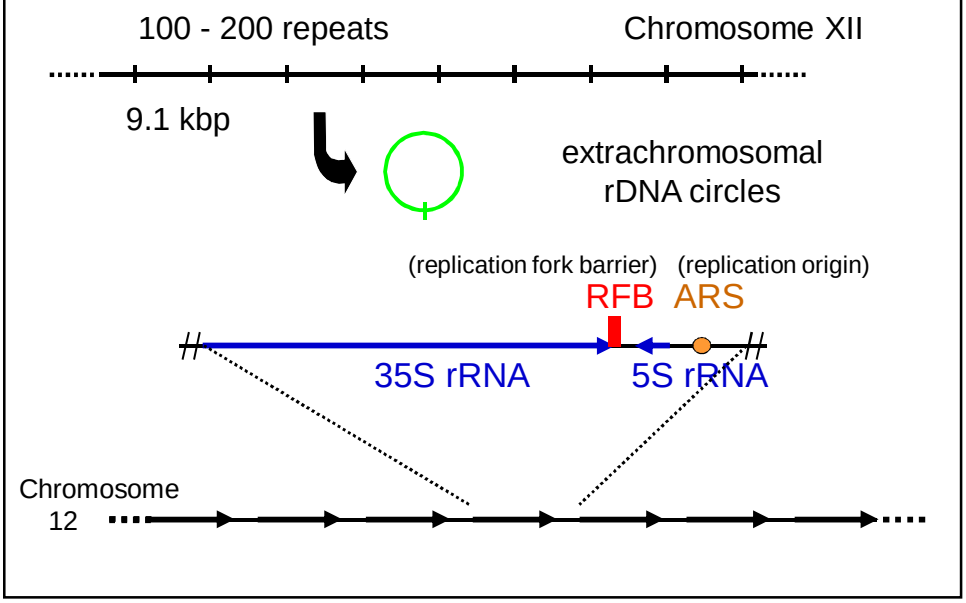
***Reduced* Replicative Life Span**

Fragmented nucleolus (ribosomal DNA) in old cells

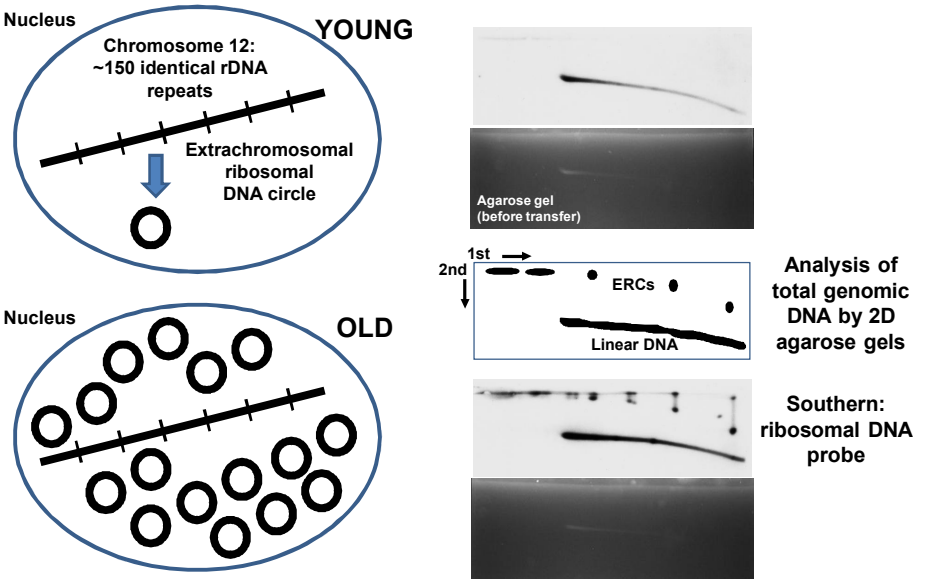


Cells were stained with anti-Nop1p (red, nucleolus) and DAPI (blue, DNA in nucleus)

Ribosomal DNA in yeast

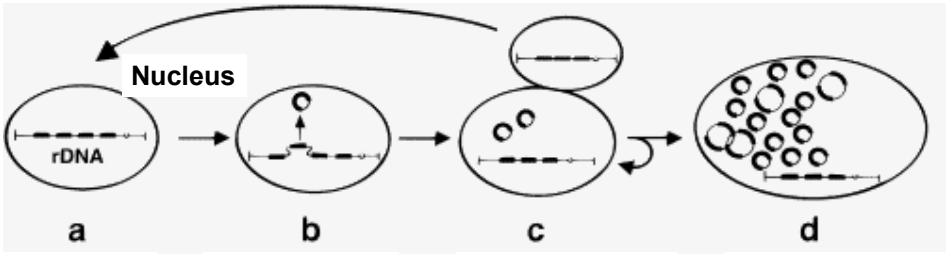


Extrachromosomal ribosomal DNA circles (ERCs) accumulate in a mother-cell specific manner



Sinclair and Guarente *Cell* 1997

Model of yeast aging due to the accumulation of *E*xtrachromosomal *R*ibosomal DNA *C*ircles (ERCs)



Young cell | **Excision/ inheritance of ERC** | **Replication Recombination *Asymmetrical* segregation** | **Old cell Nucleolar fragmentation Cell death**

(A) As young mother cells divide, the initiating event of the aging process is the generation of an ERC by homologous recombination between repeats within the rDNA array on chromosome XII.

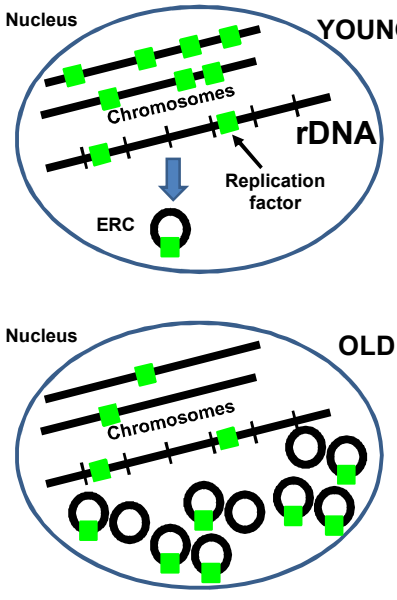
(B) Most daughter cells do not inherit ERCs and youth is temporarily restored. The excised rDNA repeats are rapidly regenerated by amplifying existing repeats. Daughters from very old mothers can inherit ERCs due to the break down in the asymmetry of inheritance.

(C) ERCs have a high probability of replicating during S phase and are maintained almost exclusively within mother cells.

(D) ERCs accumulate exponentially in mother cells resulting in fragmented nucleoli, cessation of cell division, and cellular senescence.

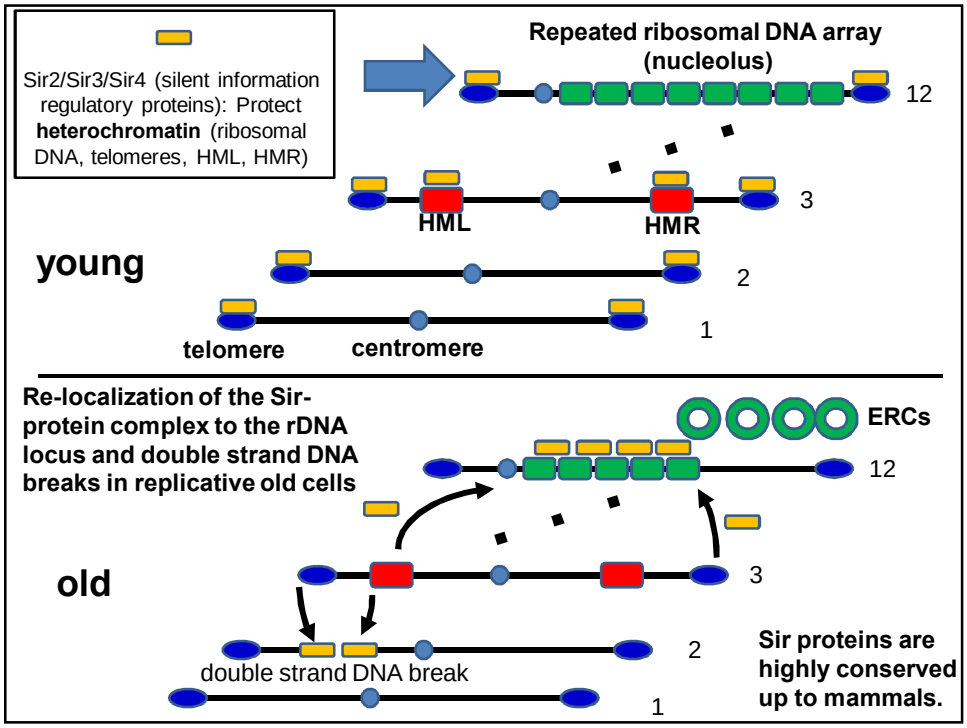
Sinclair and Guarente *Cell* 1997

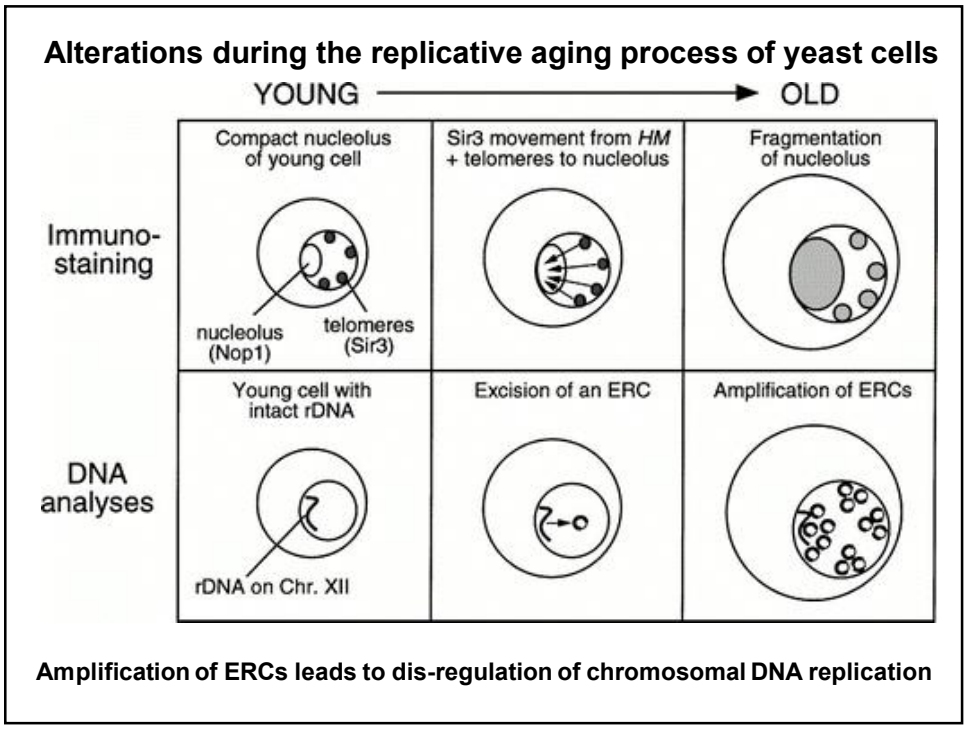
Extrachromosomal ribosomal DNA circles (ERCs) may titrate essential replication factors



Each extrachromosomal ribosomal DNA circle contains a replication origin which may titrate essential replication factors

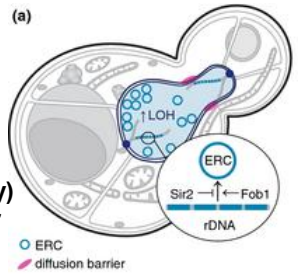
Failure in regulation of chromosomal DNA replication which may lead to non-replicated DNA

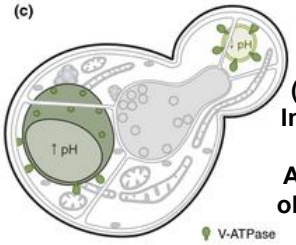

Sir2/Sir3/Sir4 (silent information regulatory proteins): Protect heterochromatin (ribosomal DNA, telomeres, HML, HMR)
Repeated ribosomal DNA array (nucleolus)
12
HML
HMR
3
young
2
1
telomere
centromere
Re-localization of the Sir-protein complex to the rDNA locus and double strand DNA breaks in replicative old cells
ERCs
12
old
3
2
double strand DNA break
1
Sir proteins are highly conserved up to mammals.


Alterations during the replicative aging process of yeast cells
YOUNG
OLD
Immuno-staining
DNA analyses
Compact nucleolus of young cell
nucleolus (Nop1)
telomeres (Sir3)
Sir3 movement from HM + telomeres to nucleolus
Fragmentation of nucleolus
Young cell with intact rDNA
rDNA on Chr. XII
Excision of an ERC
Amplification of ERCs
Amplification of ERCs leads to dis-regulation of chromosomal DNA replication

Budding yeast as a model organism to study the effects of age

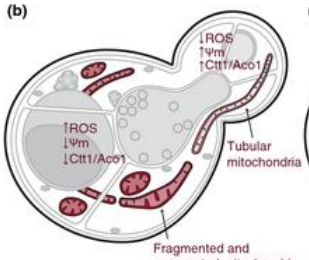
Nucleus: Increase of ERCs, LOH (Loss-of-Heterozygosity) in old mother cells

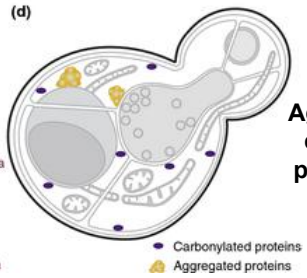




Vacuoles (Lysosomes): Increase in size and Acidification in old mother cells

Mitochondria: Fragmentation Aggregation Increased ROS in old mother cells

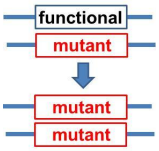




Cytoplasm: Aggregated and carbonylated proteins in old mother cells

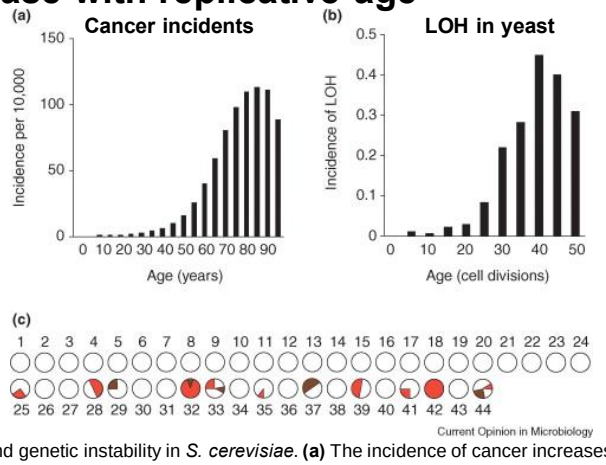
FEMS Microbiology Reviews
Volume 38, Issue 2, pages 300-325, 26 FEB 2014 DOI: 10.1111/1574-6976.12060
http://onlinelibrary.wiley.com/doi/10.1111/1574-6976.12060/full#fmr12060-fig-0004

LOH: Loss-of-Heterozygosity events increase with replicative age



Loss of the function of a tumor suppressor gene: increases numbers of cancer incidents

Science. 2003 Sep 26;301(5641):1908-11.
An age-induced switch to a hyper-recombinational state.
McMurray MA, Gottschling DE.



A function of age: human cancer and genetic instability in *S. cerevisiae*. **(a)** The incidence of cancer increases dramatically with age in humans. Shown are probabilities of developing cancer by 5-year interval from 1998ï 2000 for all types of cancer. **(b)** The incidence of LOH in diploid budding yeast cells increases with similar kinetics during replicative aging. Shown are probabilities of producing a daughter cell that gives rise to a colony containing an LOH event, grouped by 5-division intervals for a cohort of 39 wild-type diploid mother cells. **(c)** Detection of age-induced LOH events by pedigree analysis. Each circle represents the colony produced by successive daughters of a single mother cell; the succession is labeled with numbers. Colored shapes represent sectors of cells having experienced LOH, where red and brown designate LOH at different loci.

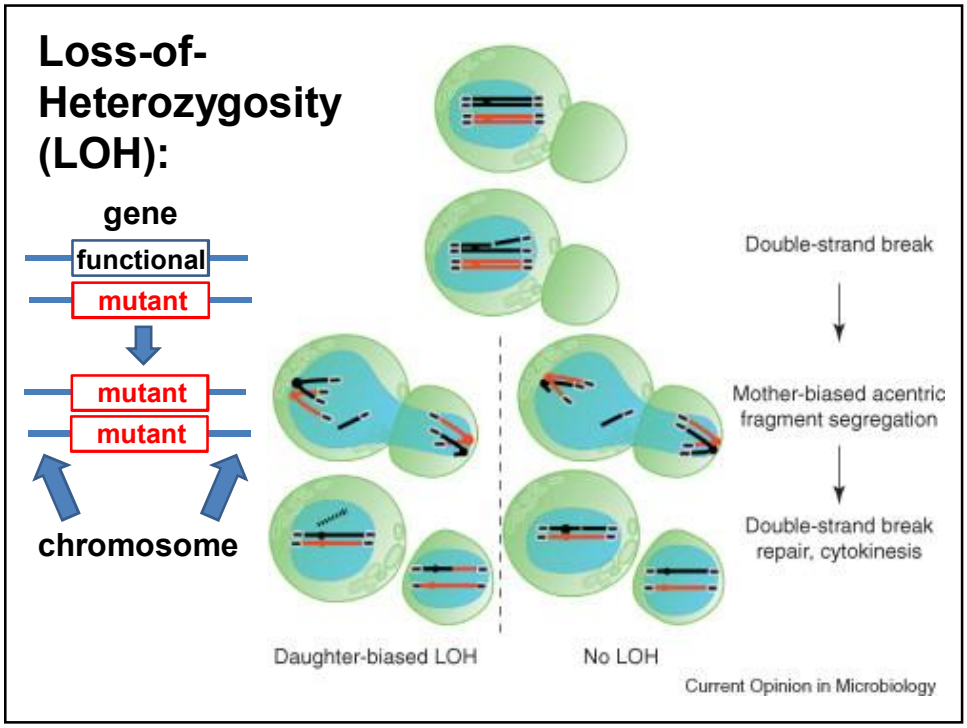


Figure legend to previous slide:

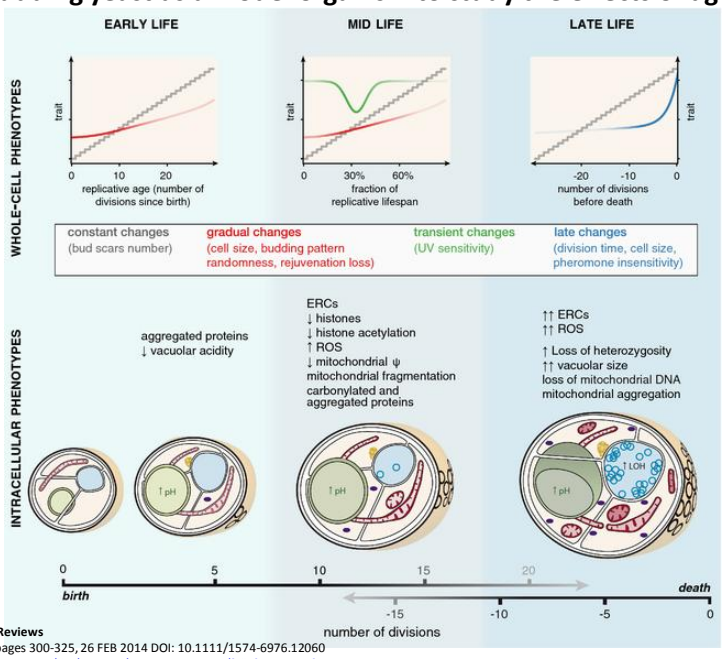
A model for asymmetric age-induced LOH. A diploid mother yeast cell is depicted with two homologous chromosomes (red and black ð centromeres are filled circles) contained within the nucleus (blue). The cell wall is shown in green and bud scars are depicted.

Top, a mother cell after DNA replication with duplicated chromosomes. A double strand DNA break (DSB) in one sister chromatid of the black chromosome is followed by mitosis without repair, resulting in two potential outcomes: on the left, the broken centromere-containing chromosome fragment segregates to the daughter; on the right, it segregates to the mother. In both cases, the acentric chromosome fragment remains in the mother cell after cytokinesis.

On the left, the two fragments of the broken chromosome are separated by mitosis, and repair of the broken centromere-containing fragment occurs by break-induced replication (BIR), resulting in duplication from the homologous chromosome of all sequences centromere-distal to the break. The acentric fragment remaining in the mother cell is shown to be degraded (dashed), but could have other fates.

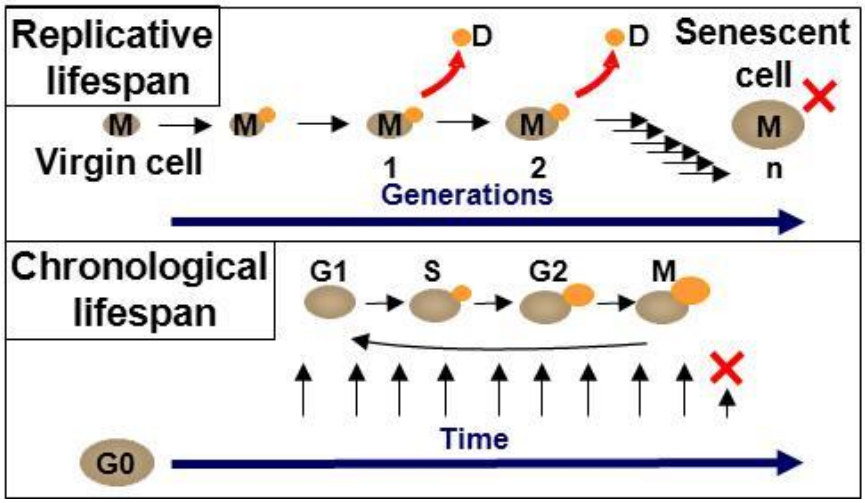
On the right, where the mother inherits both fragments, DSB repair by non-homologous end-joining or local gene conversion without crossing over preserves both alleles at distal loci (signified by the small red segment in the mother's otherwise black chromosome). Note that, before DNA replication, gene conversion accompanied by crossing over would not cause LOH.

Budding yeast as a model organism to study the effects of age

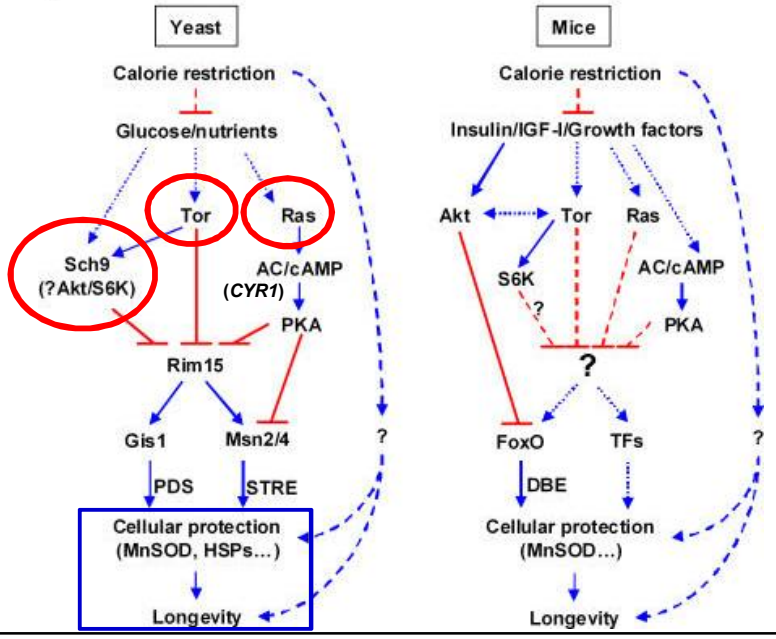


FEMS Microbiology Reviews
Volume 38, Issue 2, pages 300-325, 26 FEB 2014 DOI: 10.1111/1574-6976.12060
http://onlinelibrary.wiley.com/doi/10.1111/1574-6976.12060/full#fmr12060-fig-0005

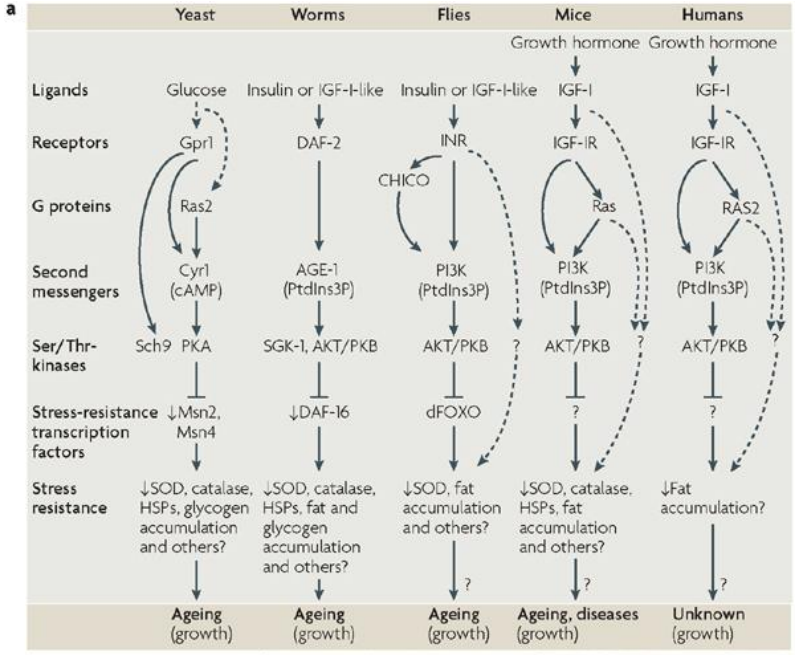
Replicative versus chronological lifespan



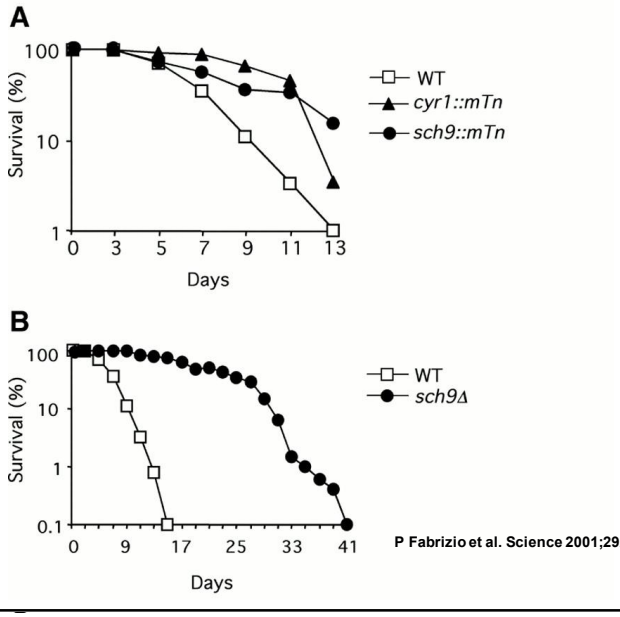
The Ras, Tor, and Sch9 pathways reduce the response to oxidative stress and shorten the lifespan



Conserved signalling transduction pathways

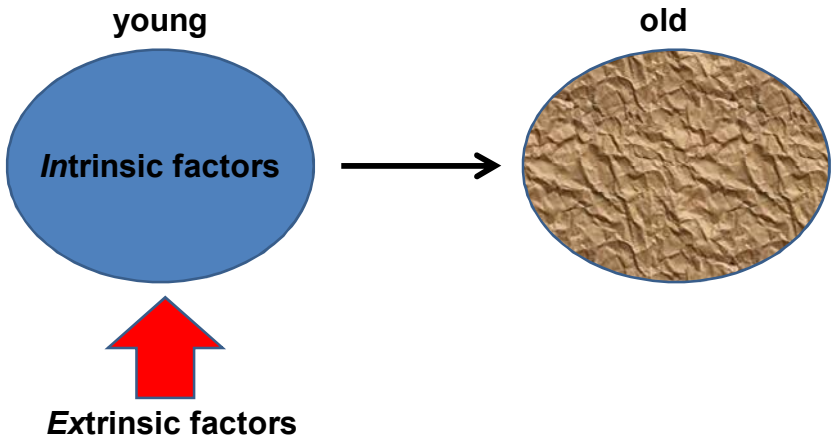


Mutations in CYR1 (adenylate cyclase) and in SCH9 increase the *chronological* life-span of budding yeast.



P Fabrizio et al. Science 2001;292:288-290

Impact on aging:



Experiments on dietary restriction (DR) and genetic or chemical alteration of nutrient-sensing pathways have been performed on a range of model organisms.

	Life-span increase		Beneficial health effects	
	Dietary restriction	Mutations/ drugs	Dietary restriction	Mutations/ drugs
Yeast	3-fold	10-fold (with starvation/ DR)	Extended reproductive period	Extended reproductive period, decreased DNA damage/mutations
Worms	2- to 3-fold	10-fold	Resistance to misexpressed toxic proteins	Extended motility Resistance to mis-expressed toxic proteins and germ-line cancer
Flies	2-fold	60–70%	None reported	Resistance to bacterial infection, extended ability to fly
Mice	30–50%	30–50% (~100% in combination with DR)	Protection against cancer, diabetes, atherosclerosis,cardio-myopathy, autoimmune, kidney, and respiratory diseases; reduced neurodegeneration	Reduced tumor incidence; protection against age-dependent cognitive decline, cardio-myopathy, fatty liver and renal lesions. Extended insulin sensitivity
Monkeys	Trend noted	Not tested	Prevention of obesity; protection against diabetes, cancer, and cardiovascular disease	Not tested
Humans	Not determined	Not determined (GHR-deficient subjects reach old age)	Prevention of obesity, diabetes, hypertension Reduced risk factors for cancer and cardiovascular disease	Possible reduction in cancer and diabetes

L Fontana et al. Science 2010;328:321-326

Premature aging: Werner's syndrome

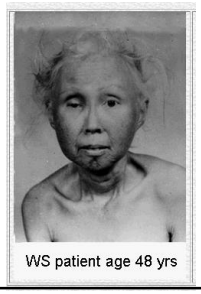
Mutations in *WRN* result in Werner's syndrome, a disease with symptoms resembling premature aging.

Patients with Werner's syndrome display many symptoms of old age including graying and loss of hair, osteoporosis, cataracts, atherosclerosis, loss of skin elasticity, and a propensity for certain cancers.

Cells isolated from patients with Werner's syndrome divide approximately half as many times in culture as those from normal individuals.

The *WRN* gene encodes a DNA helicase which is proposed to detect chromosomal DNA damage.

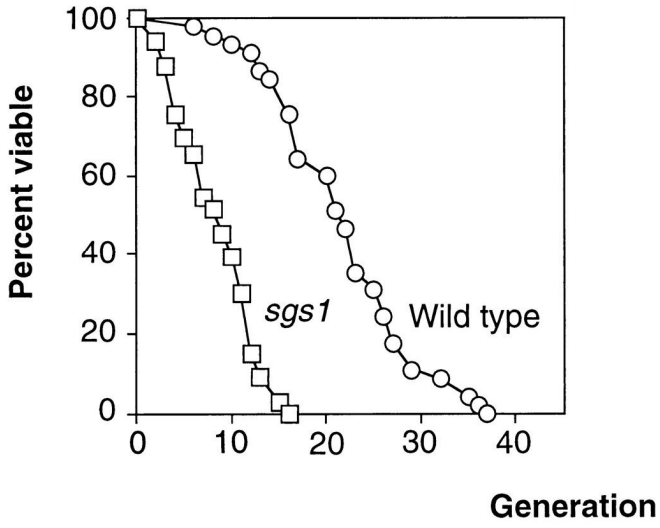
WS patient age 15 yrs



WS patient age 48 yrs

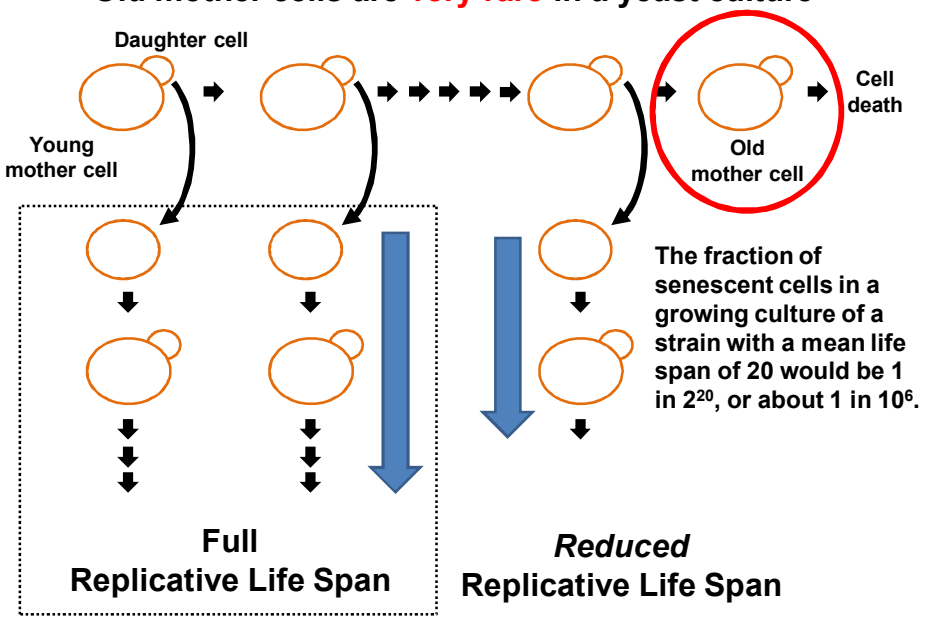
Image from http://www.pathology.washington.edu/research/werner

Yeast mutants of the Werner's syndrome homolog *sgs1* cells have a short life-span.



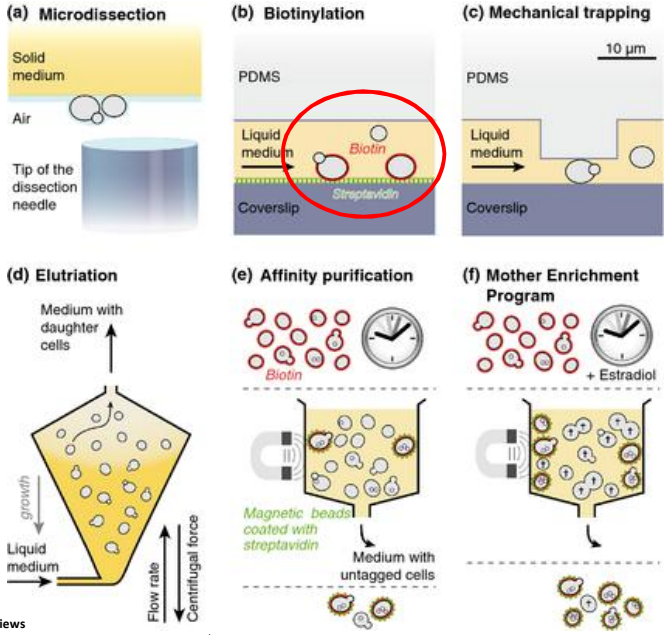
D A Sinclair et al. Science 1997;277:1313-1316

Purification of old cells: Old mother cells are very rare in a yeast culture



The fraction of senescent cells in a growing culture of a strain with a mean life span of 20 would be 1 in 2^{20}, or about 1 in 10^6.

Enrichment of replicative old budding yeast to study the effects of age



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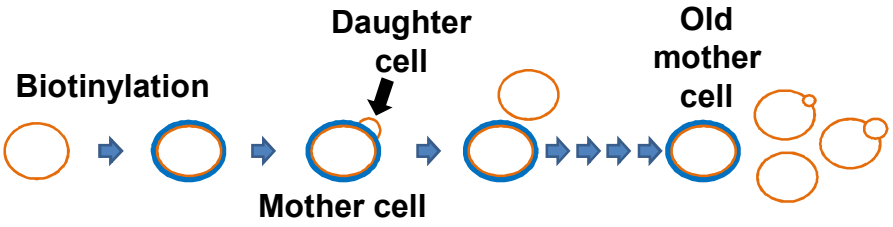
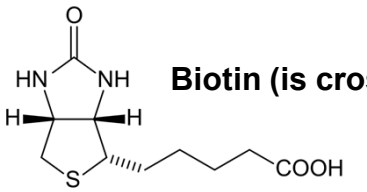
http://onlinelibrary.wiley.com/doi/10.1111/1574-6976.12060/full#fmr12060-fig-0002

The cell wall is formed *de novo*

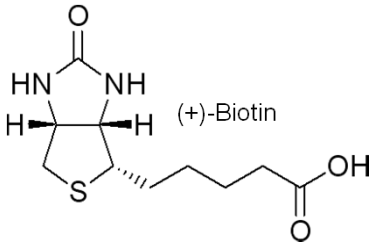




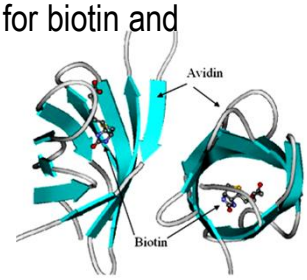
Biotin (is cross-linked to the cell wall)
Smeal *et al.* Cell 1996

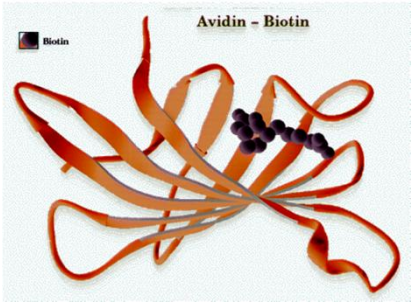
Avidin (streptavidin) binds to biotin.

Avidin/streptavidin. Affinity proteins for biotin and biotinylated biomolecules

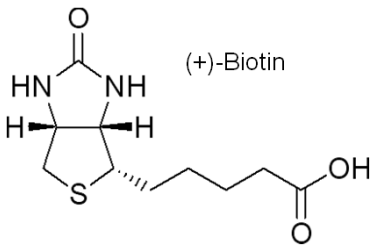


(+)-Biotin





Avidin/streptavidin. Affinity proteins for biotin and biotinylated biomolecules

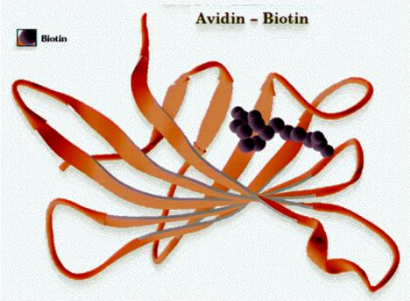


(+)-Biotin

❶ Biotin, also known as vitamin H, is a small molecule (MW 244.3) that is present in tiny amounts in all living cells.
❷ The valeric acid side chain of the biotin molecule can be derivatized in order to incorporate various reactive groups that are used to attach biotin to other molecules. Once biotin is attached to a molecule, the molecule can be affinity purified using an immobilized version of any biotin-binding protein. Alternatively, a biotinylated molecule can be immobilized through interaction with a biotin-binding protein, then used to affinity purify other molecules that specifically interact with it.

❸Many manufacturers offer biotin-labeled antibodies and a number of other biotinylated molecules, as well as a broad selection of biotinylation reagents to label any protein.
❹Some applications in which the avidin-biotin interaction has been used include **ELISA; immunohistochemical staining; Western, Northern and Southern blotting; immunoprecipitation; cell-surface labeling; affinity purification; and fluorescence-activated cell sorting (FACS).**

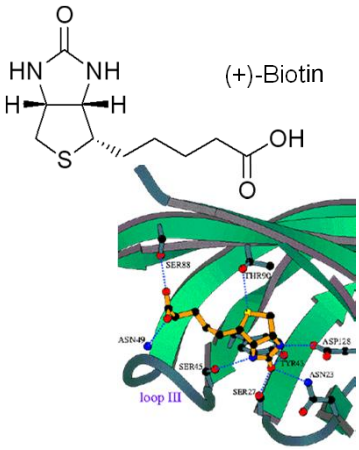
Avidin/streptavidin. Affinity proteins for biotin and biotinylated biomolecules



Biotin-Binding Proteins
Avidin

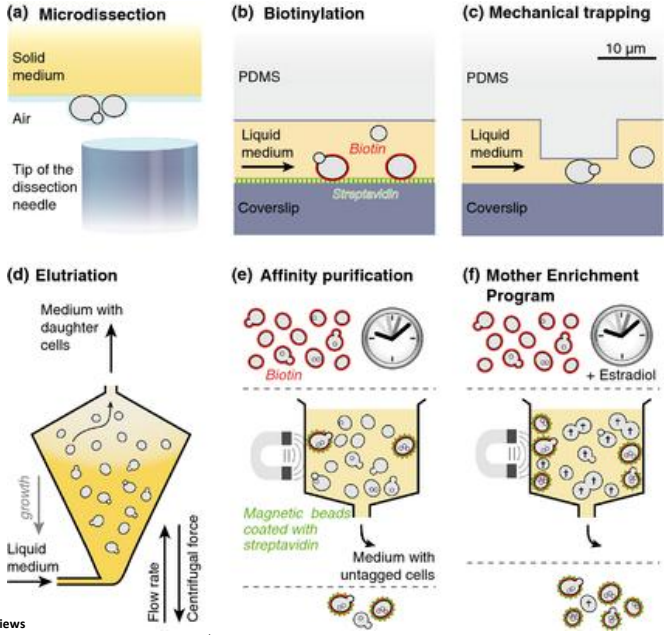
❶The extraordinary affinity of **avidin** for biotin allows biotin-containing molecules in a complex mixture to be discretely bound with avidin.
❷Avidin is a glycoprotein found in the egg white and tissues of birds, reptiles and amphibia.
❸It contains four identical subunits having a combined mass of 67,000-68,000 daltons. Each subunit consists of 128 amino acids and binds one molecule of biotin.
❹The extent of glycosylation on avidin is very high; carbohydrate accounts for about 10% of the total mass of the tetramer.
❺Avidin has a basic isoelectric point (pI) of 10-10.5 and is stable over a wide range of pH and temperature.
❻Extensive chemical modification has little effect on the activity of avidin, making it especially useful for protein purification.
❼Because of its carbohydrate content and basic pI, avidin has relatively high nonspecific binding properties.

Avidin/streptavidin. Affinity proteins for biotin and biotinylated biomolecules



❶The avidin-biotin complex is the strongest known non-covalent interaction (Ka = 10^{15} M^{-1}) between a protein and ligand.
❷The bond formation between biotin and avidin is very rapid, and once formed, is unaffected by **extremes of pH, temperature, organic solvents and other denaturing agents**.
❸These features of avidin ï features that are shared by streptavidin and NeutrAvidin Protein ï make immobilized forms of the biotin-binding protein particularly useful for purifying or immobilizing biotin-labeled proteins or other molecules.
❹However, the strength of the interaction and its resistance to dissociation make it difficult to elute bound proteins from an immobilized support.
❺ **Harsh, denaturing conditions (8 M guanidine•HCl, pH 1.5 or boiling in SDS-sample loading buffer) are required to efficiently dissociate avidin:biotin complexes**.

Enrichment of replicative old budding yeast to study the effects of age



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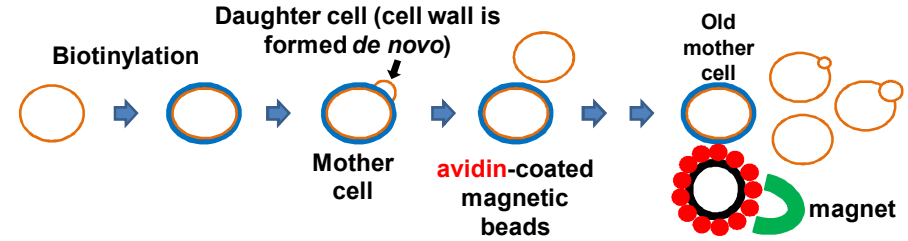
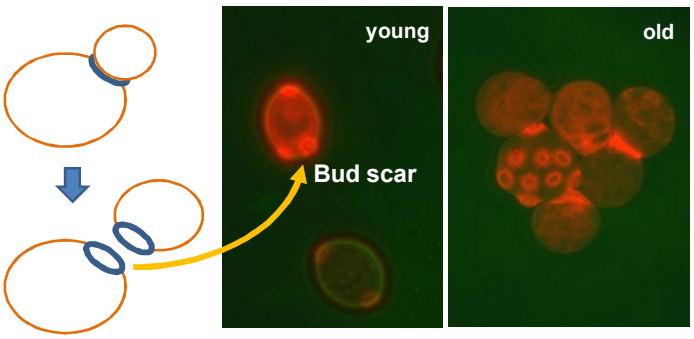
http://onlinelibrary.wiley.com/doi/10.1111/1574-6976.12060/full#fmr12060-fig-0002

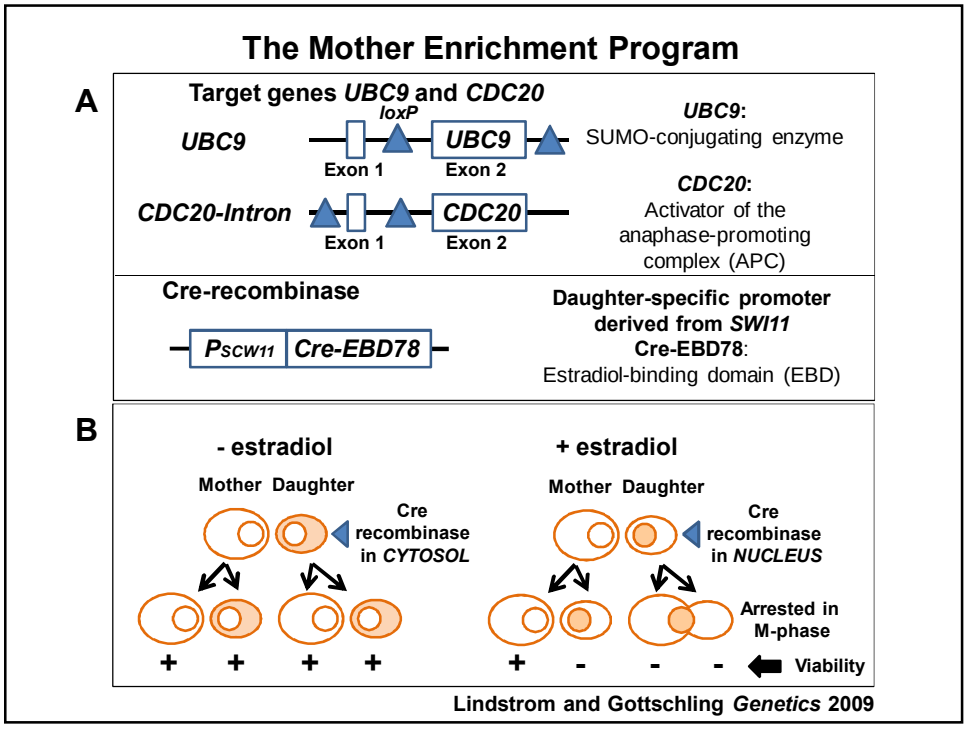
Biotinylation of cells and purification of old cells with avidin-coated magnetic beads

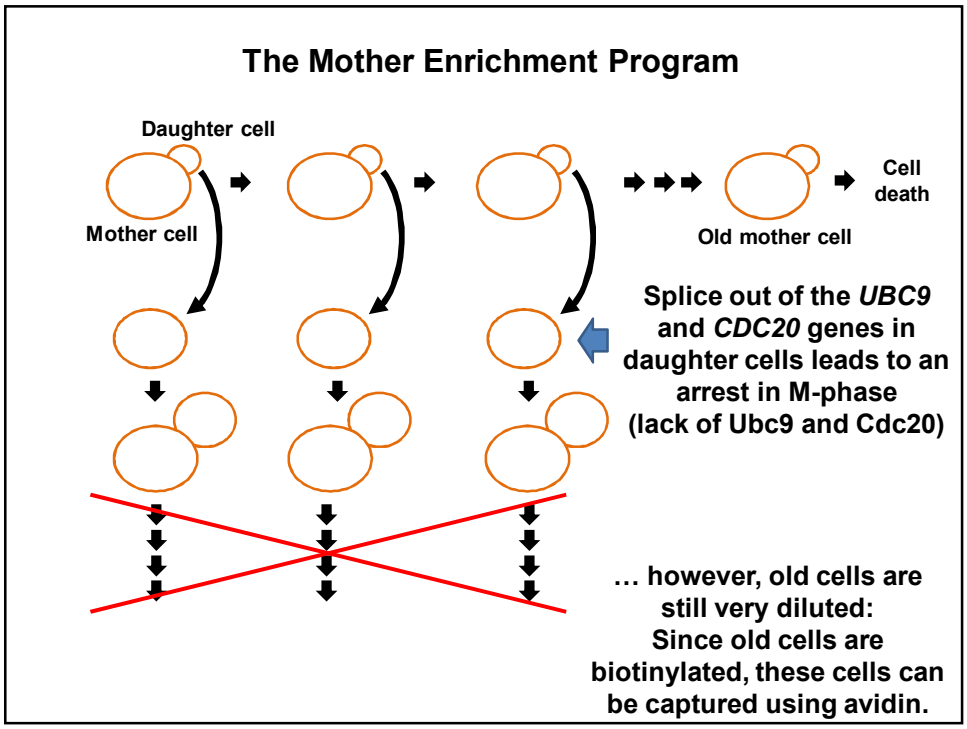




Cells stained with calcofluor, which indicates the sites where daughter cells budded off

Smeal et al. *Cell* 1996


The Mother Enrichment Program
A
Target genes UBC9 and CDC20
loxP
UBC9
UBC9
Exon 1
Exon 2
CDC20-Intron
CDC20
Exon 1
Exon 2
UBC9:
SUMO-conjugating enzyme
CDC20:
Activator of the anaphase-promoting complex (APC)
Cre-recombinase
PSCW11
Cre-EBD78
Daughter-specific promoter derived from SWI11
Cre-EBD78:
Estradiol-binding domain (EBD)
B
- estradiol
Mother Daughter
Cre recombinase in CYTOSOL
+ + + +
+ estradiol
Mother Daughter
Cre recombinase in NUCLEUS
Arrested in M-phase
+ - - -
Viability
Lindstrom and Gottschling Genetics 2009


The Mother Enrichment Program
Daughter cell
Mother cell
Old mother cell
Cell death
Splice out of the UBC9 and CDC20 genes in daughter cells leads to an arrest in M-phase (lack of Ubc9 and Cdc20)
… however, old cells are still very diluted: Since old cells are biotinylated, these cells can be captured using avidin.

Projects:

Various forms of DNA damage accumulate during aging: DNA strand breaks, loss of bases, bulky adducts (inter-strand crosslinks). (Burgess et al. Current Opinion in Cell Biology 2012; Garinis et al. Nature Cell Biology 2008)

Is chromosomal DNA equally affected by DNA damage during aging or are there chromosomal sites which are preferentially affected? (e.g. Fragile sites)

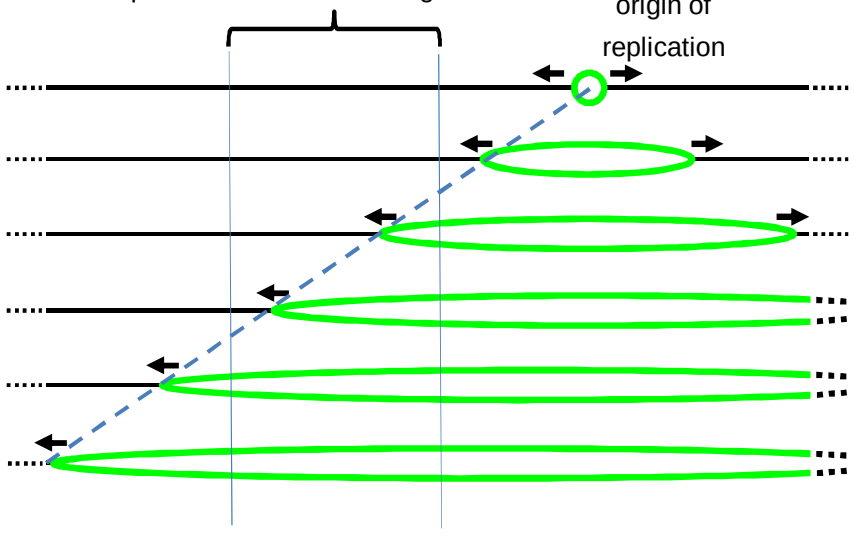
Initiation of chromosomal DNA replication is highly regulated: In yeast there are about 400 replication origins. Locations and timing of activation of these origins during the cell cycle is precisely known. (Raghumaran *Science* 2001)

Is DNA replication changing during aging?

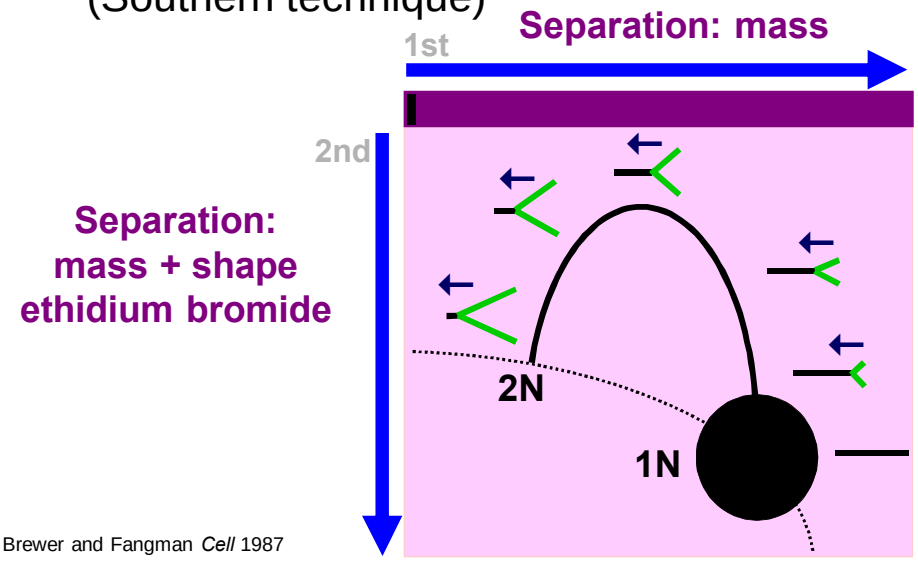
Analyses of DNA replication intermediates in yeast have been studied extensively in YOUNG cells but not in OLD cells, because it is difficult to obtain sufficient numbers of replicative old yeast cells.

Study of DNA replication

How does DNA replication occur in a particular location in the genome ?

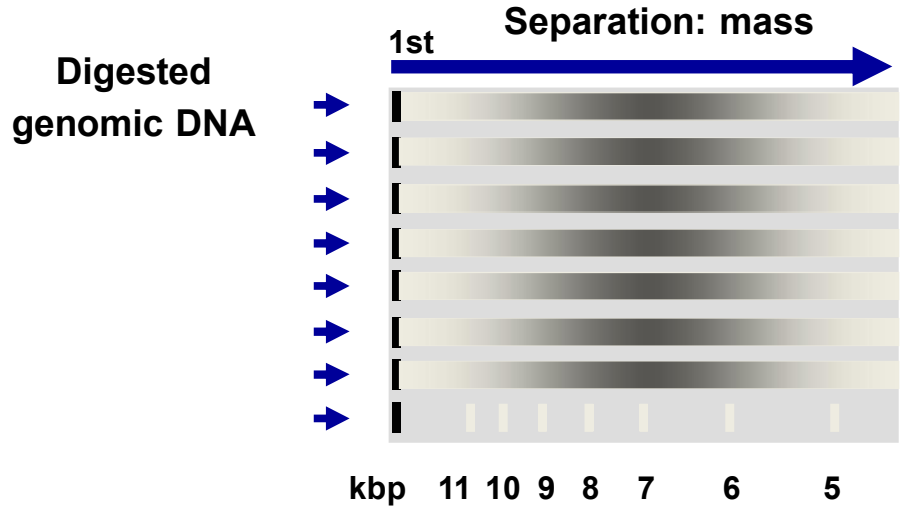


Study of replication intermediates by two-dimensional agarose gels (Southern technique)



Brewer and Fangman *Cell* 1987

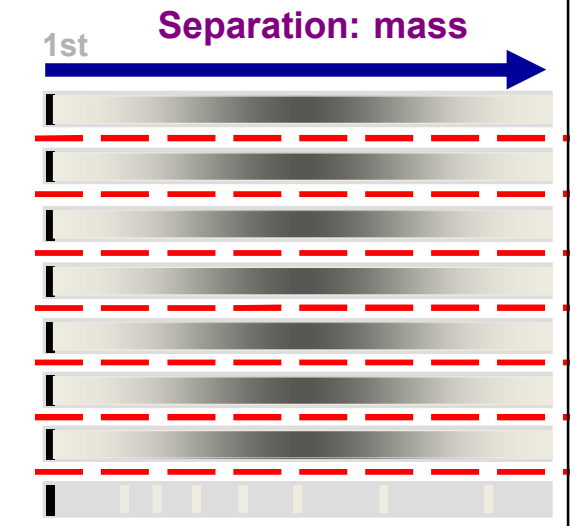
Study of replication intermediates by two-dimensional agarose gels



Brewer and Fangman *Cell* 1987

Study of replication intermediates by two-dimensional agarose gels

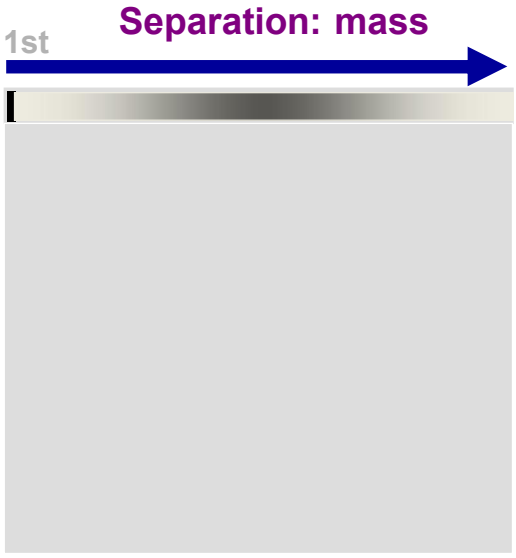
Digested genomic DNA



Brewer and Fangman *Cell* 1987

Study of replication intermediates by two-dimensional agarose gels

Digested genomic DNA

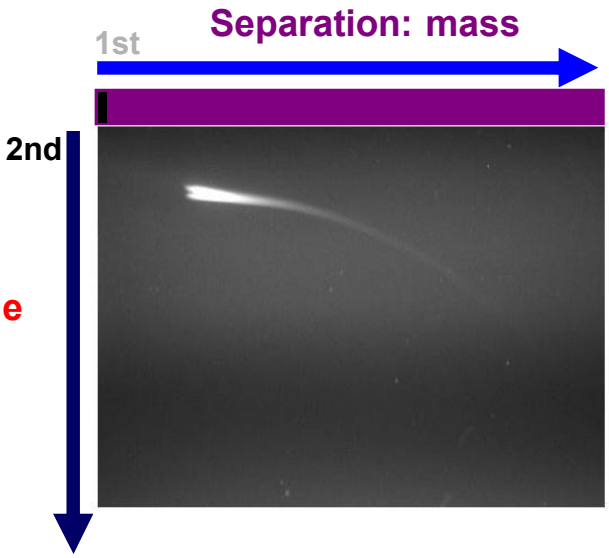


Brewer and Fangman *Cell* 1987

Study of replication intermediates by two-dimensional agarose gels

Digested genomic DNA

Separation: mass + shape ethidium bromide



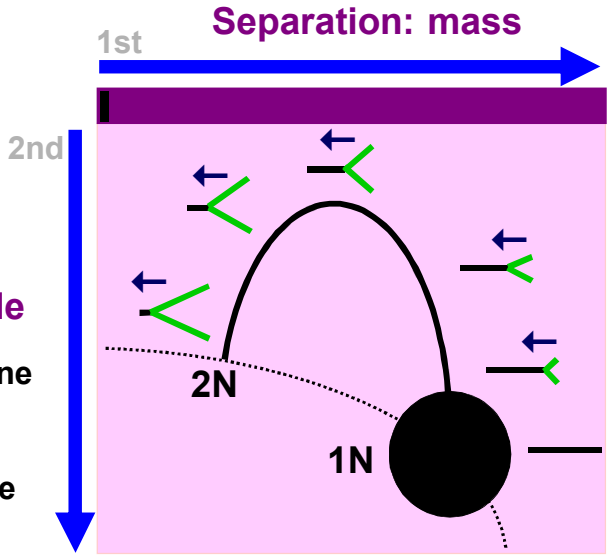
Brewer and Fangman *Cell* 1987

Study of replication intermediates by two-dimensional agarose gels

Digested genomic DNA

Separation: mass + shape ethidium bromide

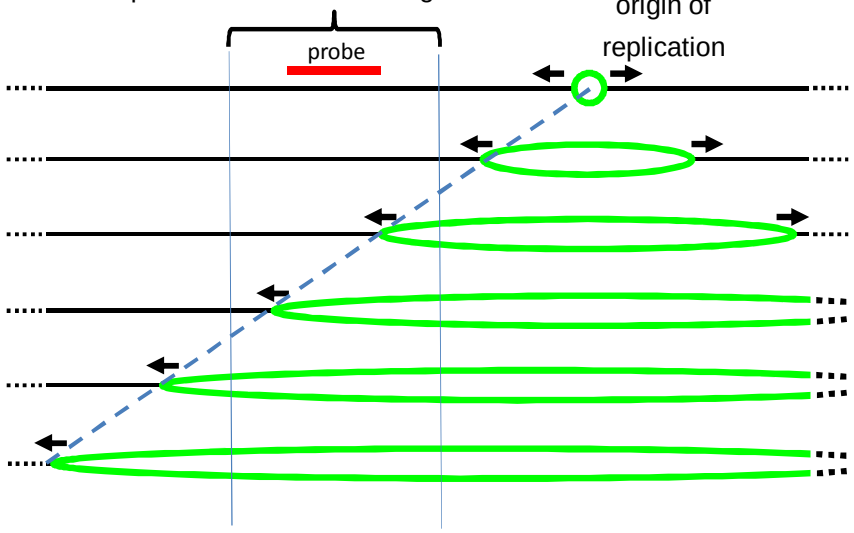
transfer to membrane and hybridize with specific DNA probe

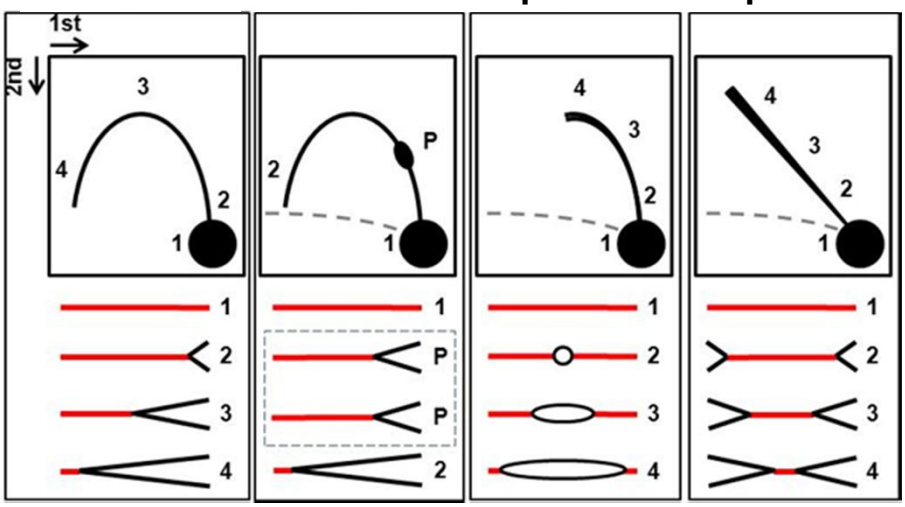


Brewer and Fangman *Cell* 1987

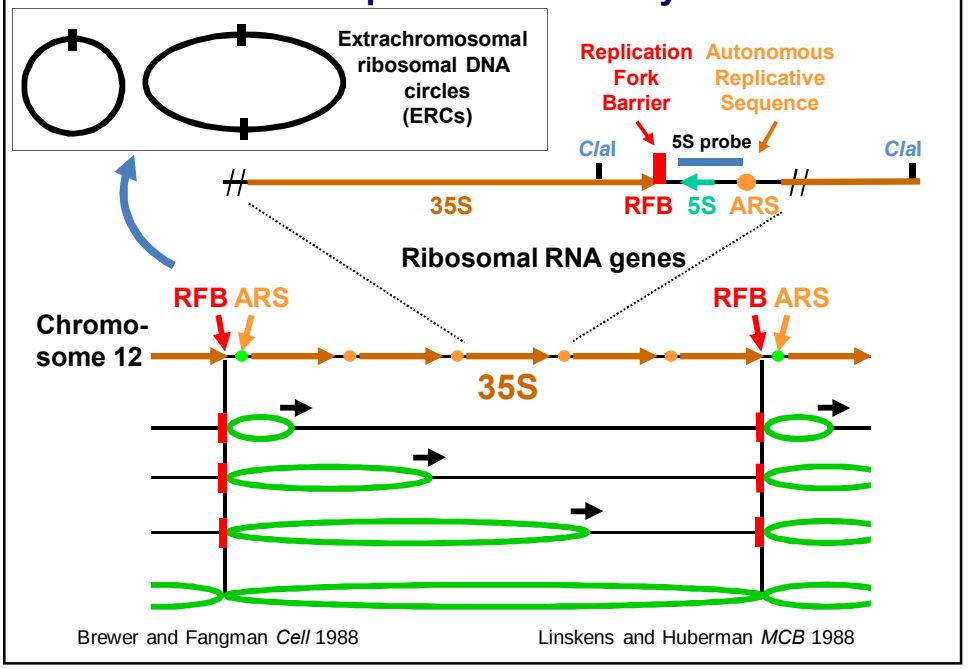
Study of DNA replication

How does DNA replication occur in a particular location in the genome ?





Ribosomal DNA replication is mostly unidirectional

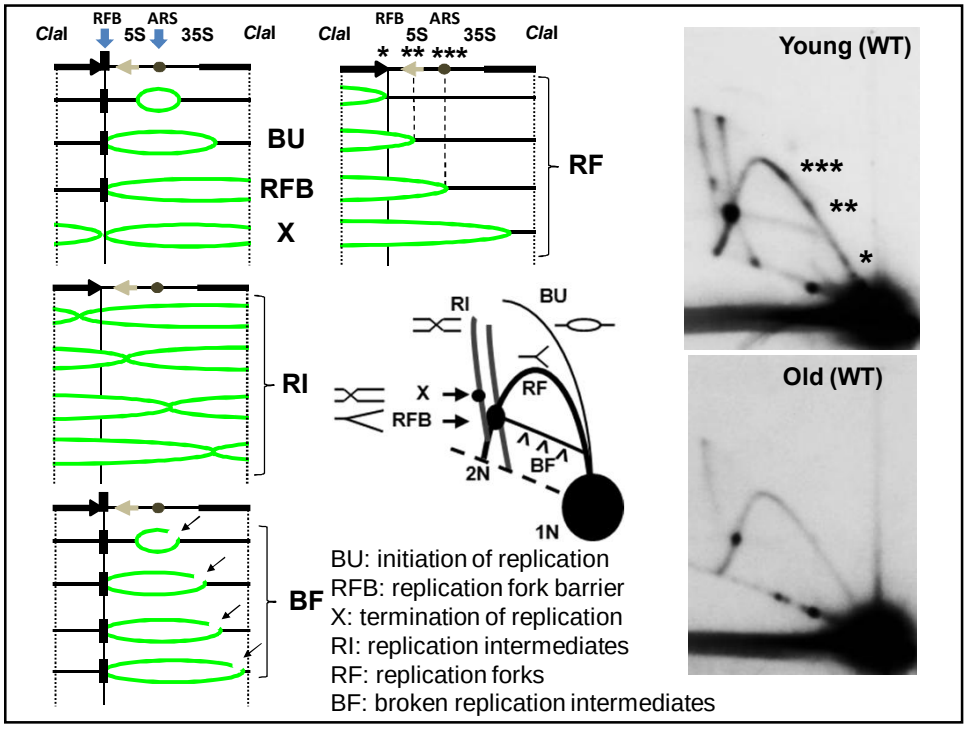


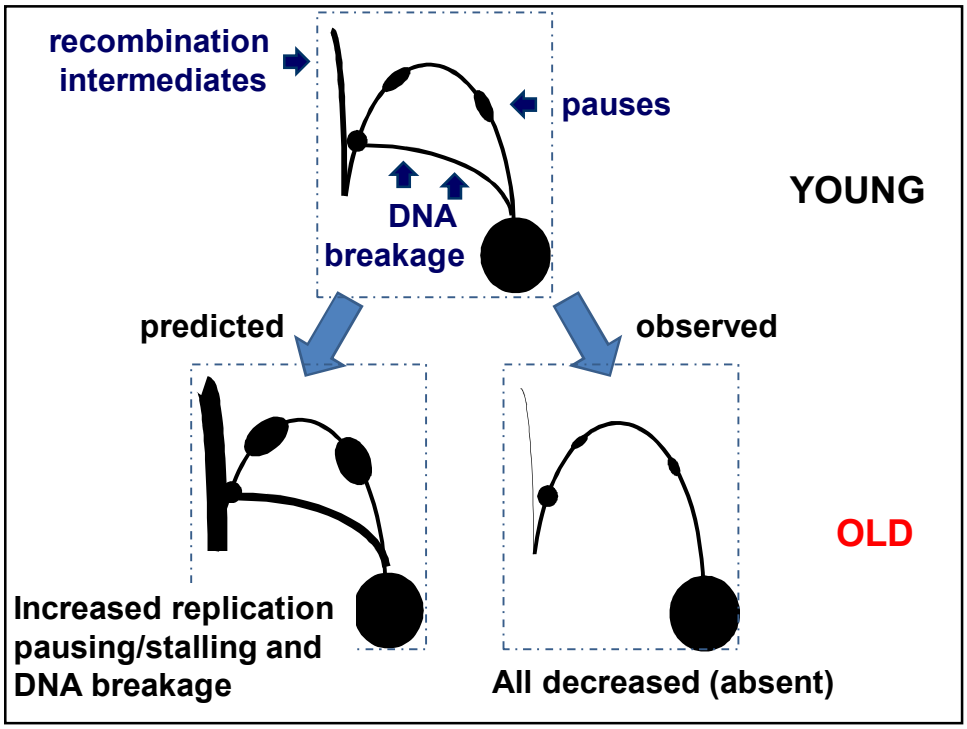
Brewer and Fangman *Cell* 1988 Linskens and Huberman *MCB* 1988

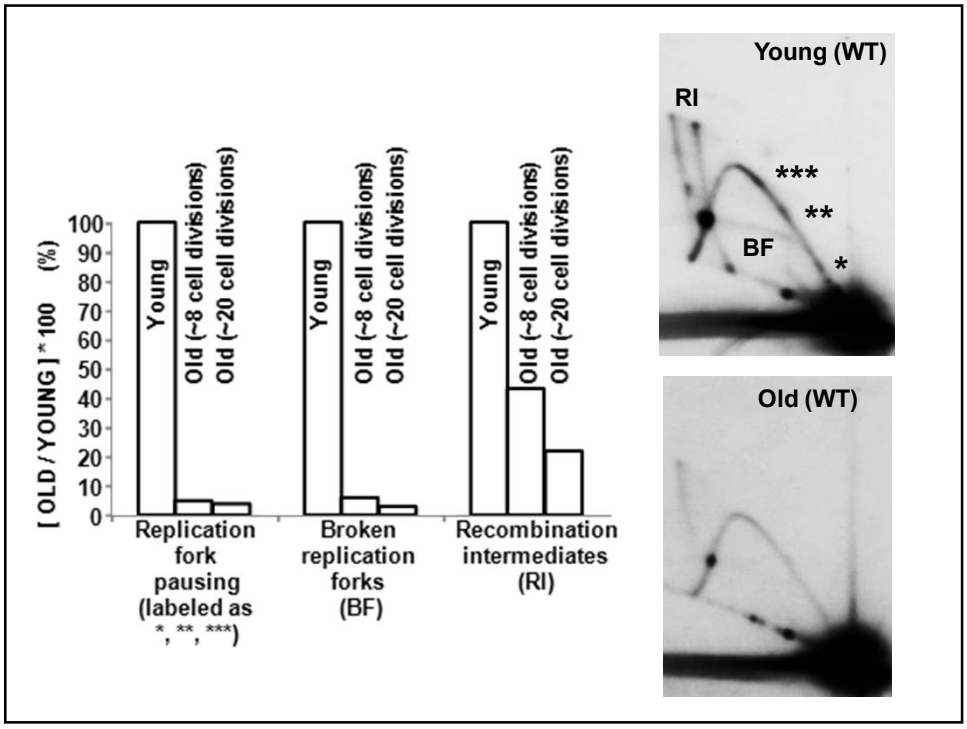
Predictions:

We propose that various forms of DNA damage will accumulate during aging:

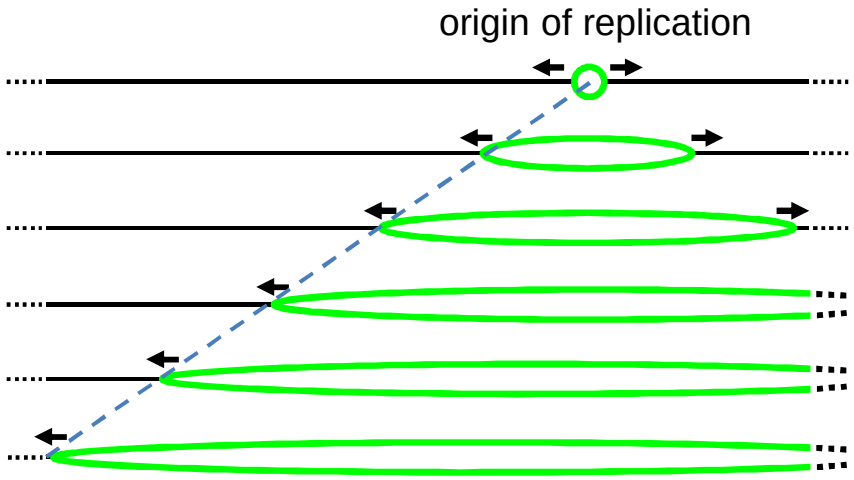
- ❑ **Increased slowing of replication movement (pausing)**
- ❑ **Increase frequency in DNA strand breaks**
- ❑ **Increase in DNA exchange (recombination) which is indicative of increased DNA damage**


ClaI
RFB
ARS
5S
35S
ClaI
ClaI
RFB
ARS
5S
35S
ClaI
* ** ***
BU
RFB
X
RF
RI
BF
RI
BU
X
RFB
RF
BF
2N
1N
Young (WT)
***
**
*
Old (WT)
BU: initiation of replication
RFB: replication fork barrier
X: termination of replication
RI: replication intermediates
RF: replication forks
BF: broken replication intermediates

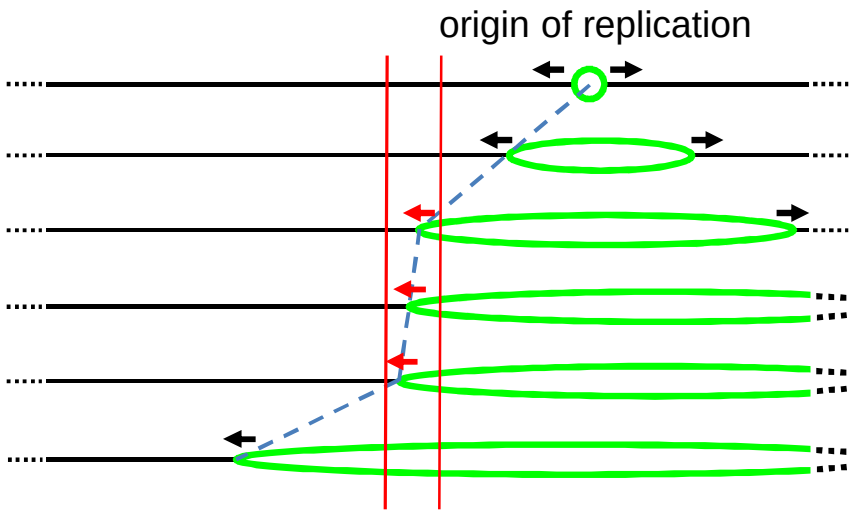

recombination intermediates
pauses
DNA breakage
YOUNG
predicted
observed
OLD
Increased replication pausing/stalling and DNA breakage
All decreased (absent)



DNA replication does not always occur with the same speed é



é but slows down at some sites.

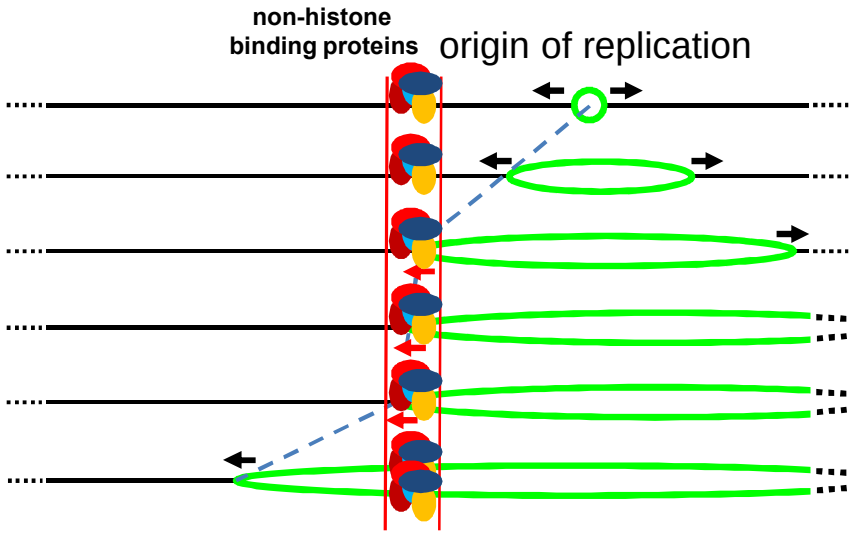


Examples of DNA "replication slow zones"

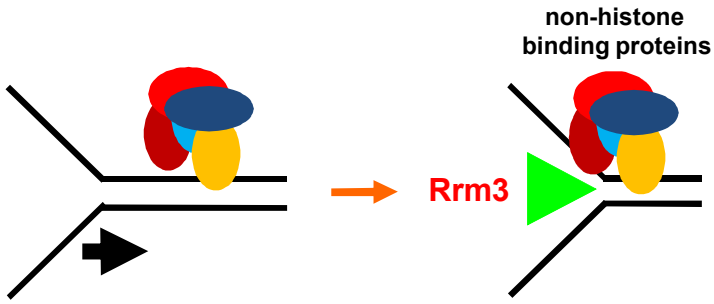
- Å centromeres
- Å transfer RNA genes
- Å telomeres
- Å silent replication origins
- Å ribosomal DNA array (5S rRNA gene, replication fork barrier)

Pausing, stalling, breakage ➡ ñfragile sitesò

These sites are covered mostly by
<u>non-histone binding proteins</u>

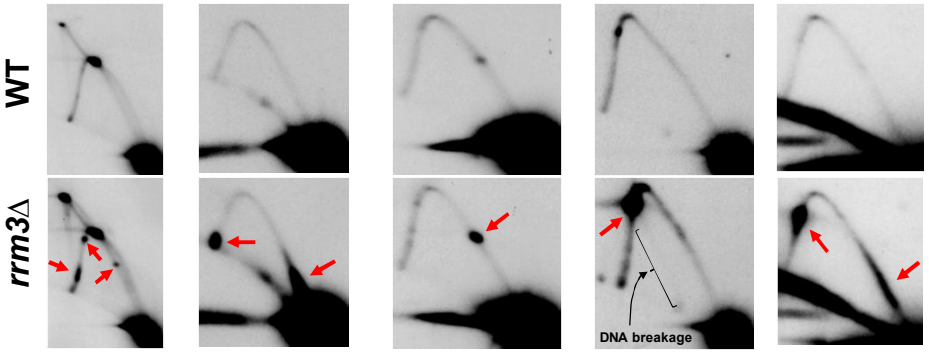


The Rrm3 helicase



The Rrm3 DNA helicase facilitates replication past chromosomal sites covered with certain non-histone binding proteins

The Rrm3p DNA helicase is needed for normal DNA replication at over 1000 sites in the yeast genome

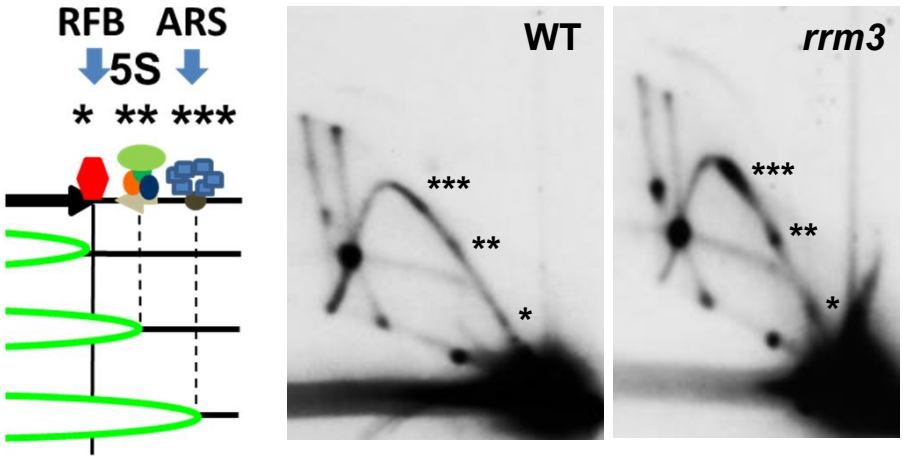


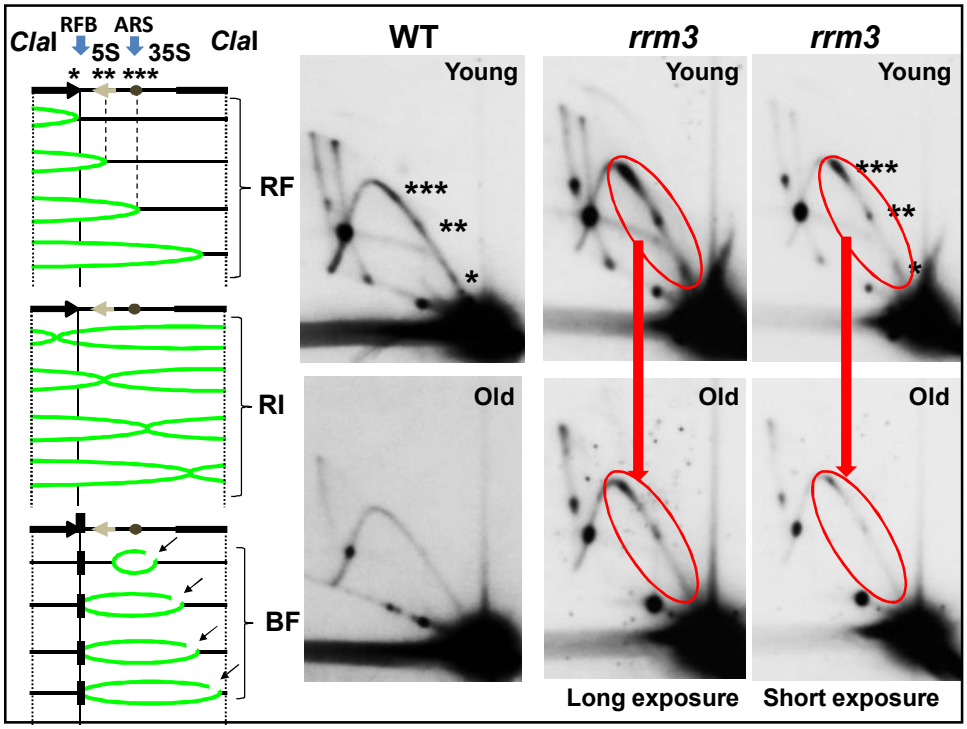
rDNA	telomeres, subtelomeric regions	centromeres	tRNA	inactive replication origins
600	**32 (+ 120)**	**16**	**>230**	**>100**

Pausing often accompanied by DNA breakage

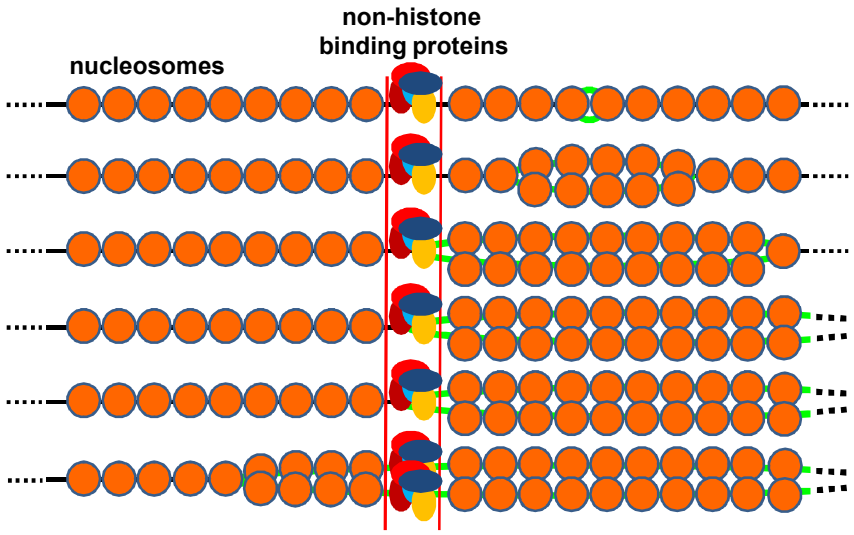
Ivessa *et al.* *Cell* 2000, *G&D* 2001, *Mol. Cell* 2003)

Non-histone protein complexes attached to ribosomal DNA are causing replication pausing

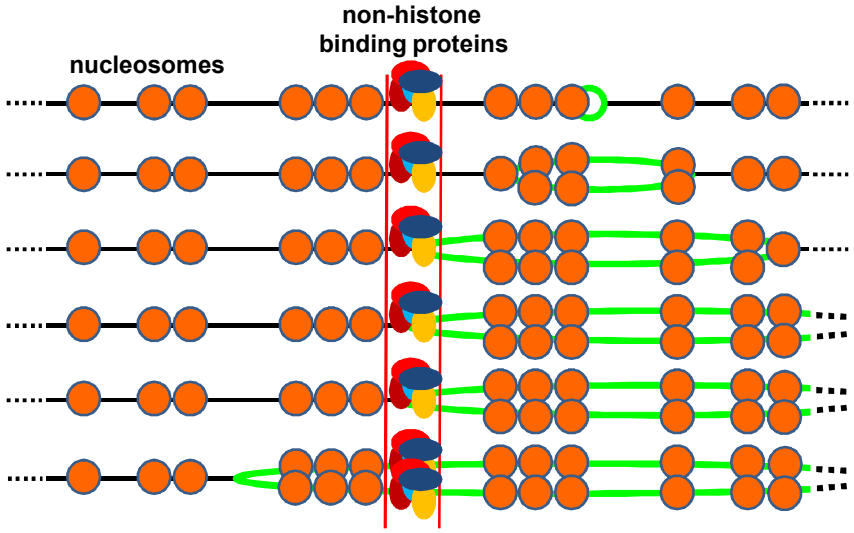




The genome of **YOUNG** cells is packed with histones (nucleosomes):

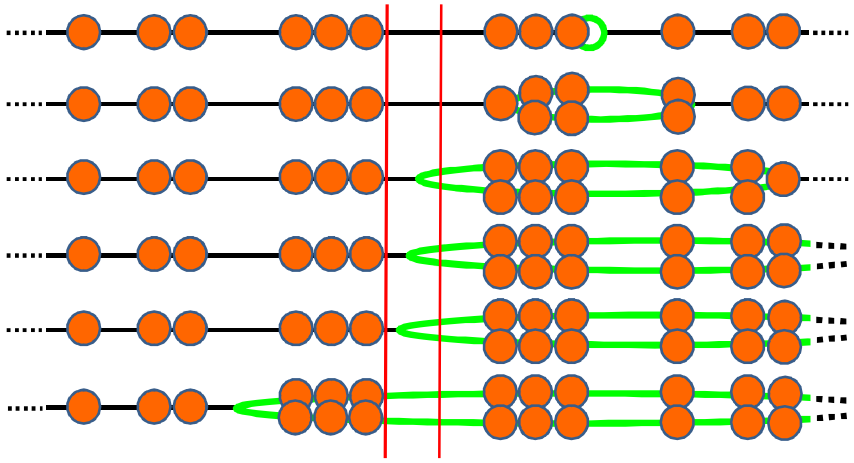


The genome of **OLD** cells loses histones (nucleosomes):

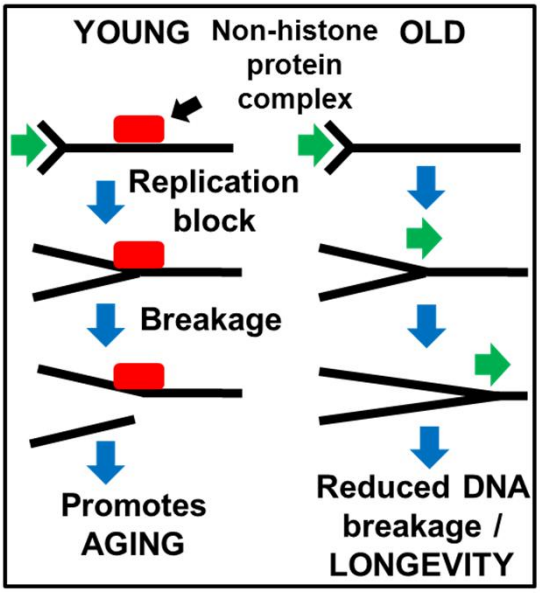


Feser é Tyler *MolCell* 2010; O Sullivan é Karlseder *Nat Struc Mol Biol* 2010; Hu é Tyler *G&D* 2014

Interpretation of our results:
The genome of OLD cells *appears* to lose also (certain) **non-histone protein complexes**:

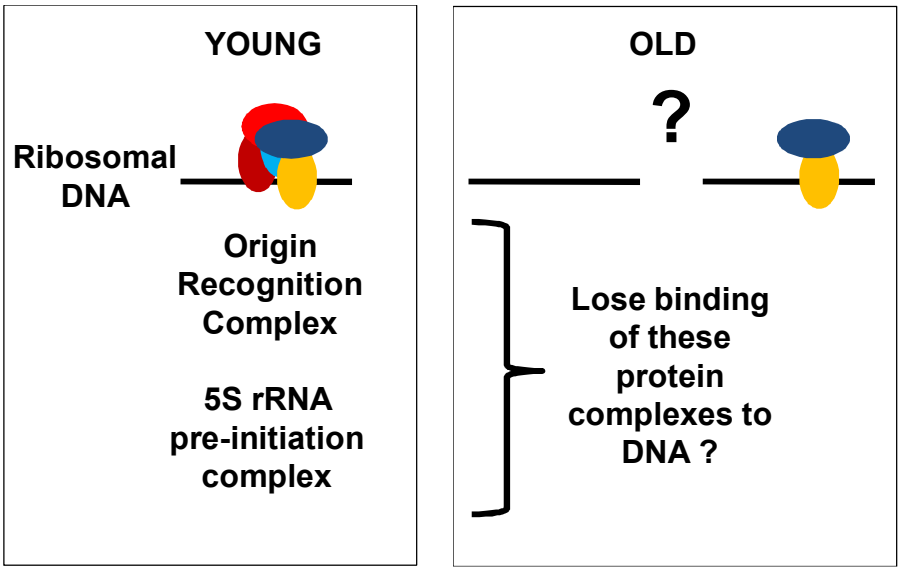


Working Hypothesis

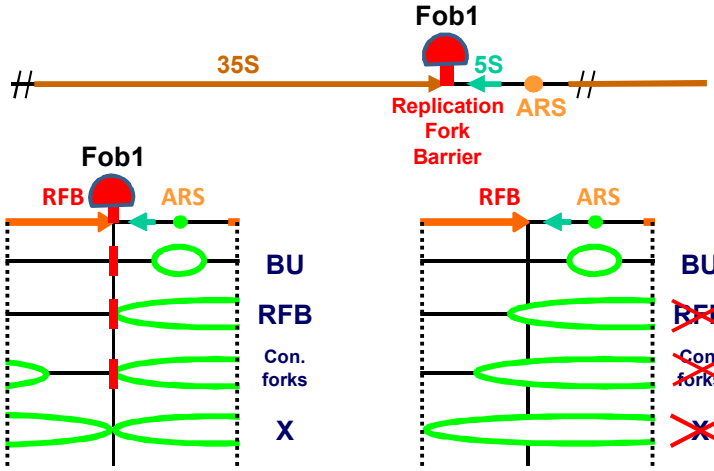


Future studies

Chromatin immuno precipitation experiments:



The fork blocking protein (Fob1p) binds to the replication fork barrier site

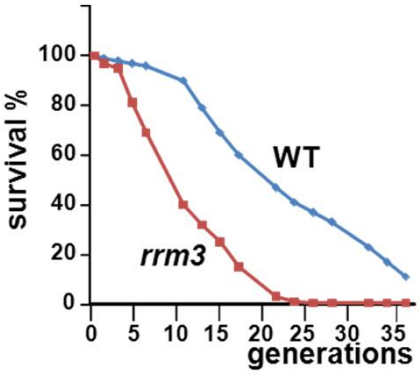


-- reduced breakage at the RFB site
-- reduced numbers of ERCs
-- longer replicative life span
(Defossez et al. *Mol. Cell* 1999)

Loss of pausing / stalling at other fragile sites in the genome of old cells:

- centromeres
- transfer RNA genes
- telomeres
- silent replication origins

Cells lacking the DNA helicase Rrm3p have a short replicative lifespan



Extension of the replicative lifespan in a strain moderately overexpressing the helicase Rrm3p?