

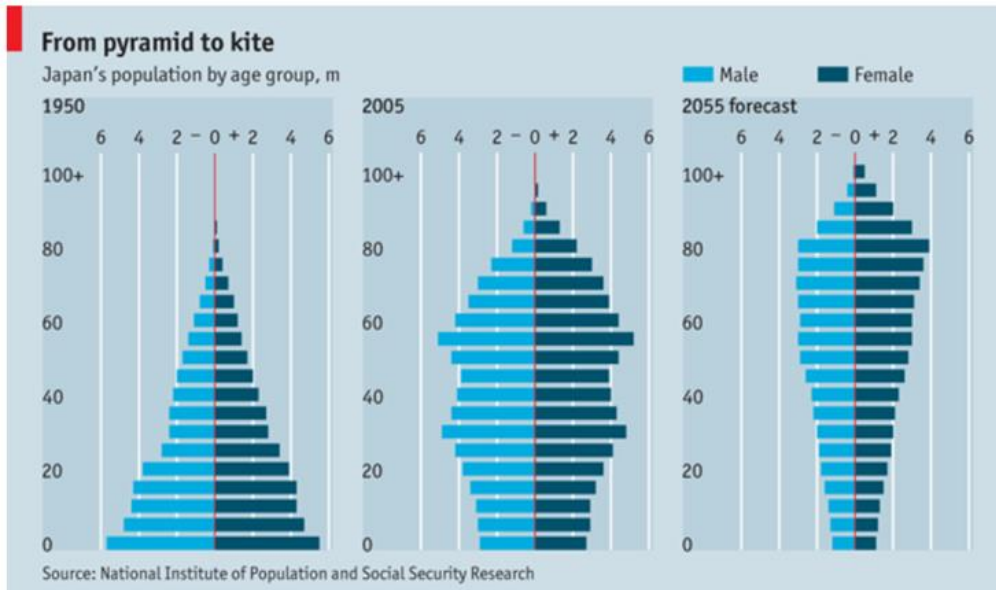
Who Wants To Live Forever?

Killifish as a short lived vertebrate model for aging and disease.

2nd TJC – Johanna Schaffenrath



An aging population



Investigating aging and age related diseases

Supercentenarians

Mutation Accumulation

Antagonistic Pleiotrophy

Programmed Death

Molecular Clock

Why aging?

Investigating age related changes

Neurodegeneration

Cancer

Loss of abilities

Cardiovascular Diseases

Aging models

1 Short-lived invertebrates are convenient for genetic screens, but lack key features of vertebrate biology, such as an internal skeleton or adaptive immune system, that are affected by aging.



5-14 DAYS

Budding yeast (*Saccharomyces cerevisiae*)

A 'mother' yeast cell divides asymmetrically, budding off 'daughters', but the mother can replicate only a limited number of times.

12-18 DAYS

Nematode (*Caenorhabditis elegans*)

Some of the same genes involved in worm senescence control the hibernation-like dauer state.



30-40 DAYS

Fruit fly (*Drosophila melanogaster*)

Flies and vertebrates share some common features, such as a brain, a heart and adult stem cells.



2 Vertebrates with mid-range lifespans are suitable for experimentation, and their biology is closer to that of a human.

3-12 MONTHS

Turquoise killifish (*Nothobranchius furzeri*)

Killifish, vertebrates with a remarkably short lifespan, are garnering attention from researchers studying ageing.

3-5 YEARS

Zebrafish (*Danio rerio*)

Although it is easy to perform genetic modifications on zebrafish, their relatively long lifespan precludes many ageing studies.



2-3 YEARS

Mouse (*Mus musculus*)

Mice share many features with humans, but are expensive to maintain and relatively slow to breed.

5-20 YEARS

Dog (*Canis lupus familiaris*)

Pet canines share their environments, lifestyle habits and many common age-related diseases with people, and so they serve as practical models for healthy-ageing treatments.



3

With longer-lived species, researchers can perform comparative studies.

28-31 YEARS

Naked mole-rat (*Heterocephalus glaber*)

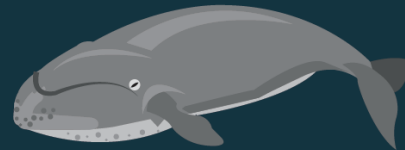
The naked mole-rat resists age-related disease; for example, cancer is extremely rare.



Human (*Homo sapiens*)

Life expectancy varies geographically; at its extremes, it is 50 years in Chad and 90 in Monaco.

-71 YEARS



UP TO -41 YEARS

Brandt's bat (*Myotis brandtii*)

Precise figures are scarce and come from wild animals, but 40 years is the oldest age yet known. Low levels of reproduction and hibernation might contribute to bats' lengthy lives.



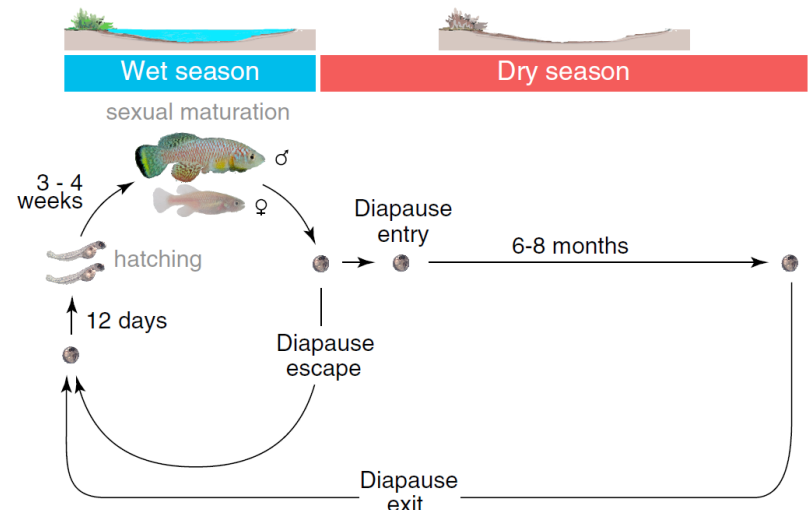
-200 YEARS

Bowhead whale (*Balaena mysticetus*)

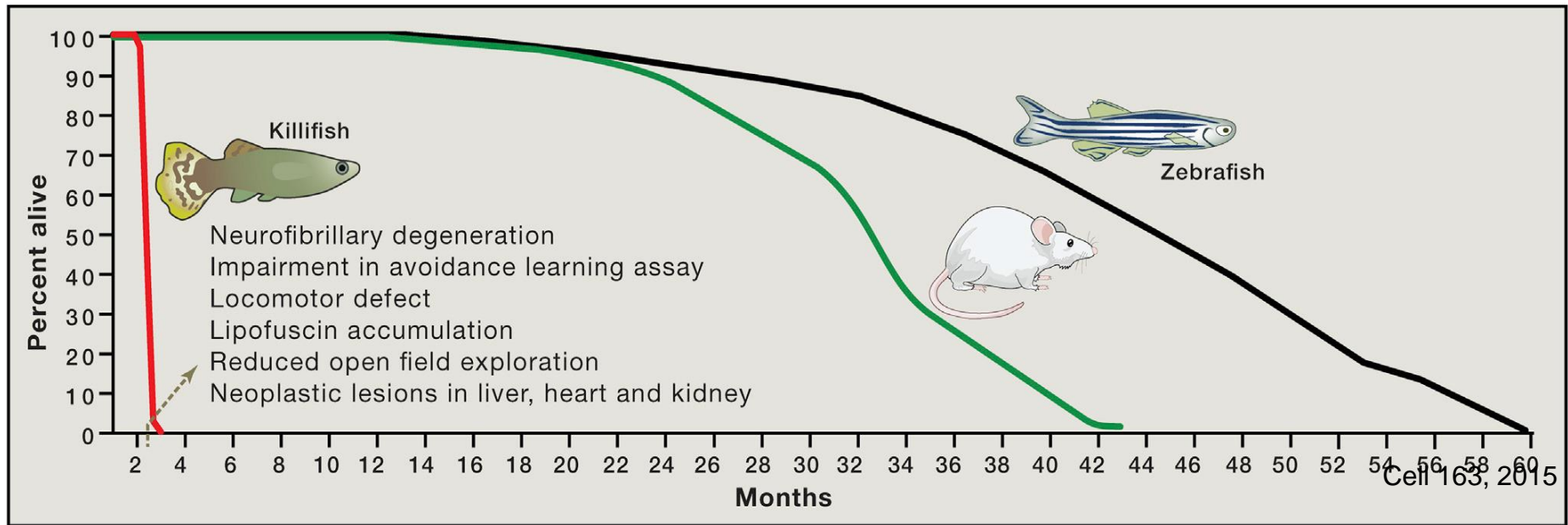
This estimate is based on chemical changes to amino acids in whale eyes, but living whales have also been found toting whaling tools that are more than a century out of date.

The Killifish as a model for aging

- End of 20th century → first Killifish studies
- Development in transient mud pools during wet season - dormant state in dry season - hatching in wet season
- Many different killifish with different lifespans
- Reference genome & CRISPR/Cas9 protocol (2015)



Do Killifish age in a similar way to humans?



GFAP upregulation	Increased apoptosis	Mitochondrial impairments
Lipofuscin accumulation	Spinal curvature	Decreased fin regeneration
Degeneration of neurons	Decreased fecundity	Age-dependent gene expression
Amyloid aggregation	Telomer shortening	
Decreased learning	Neoplastic lesions	

Advantages & Disadvantages

Pros	Cons
Short lifespan	Many parameters influencing lifespan
Vertebrates	Requires good surveillance
High reproductivity	Special facilities required
Fish with range of lifespans	Common techniques not always possible
Similarities with human aging	
Orthologues for genes involved in human dysfunctions	
Similar telomere length	

A Platform for Rapid Exploration of Aging and Diseases in a Naturally Short-Lived Vertebrate

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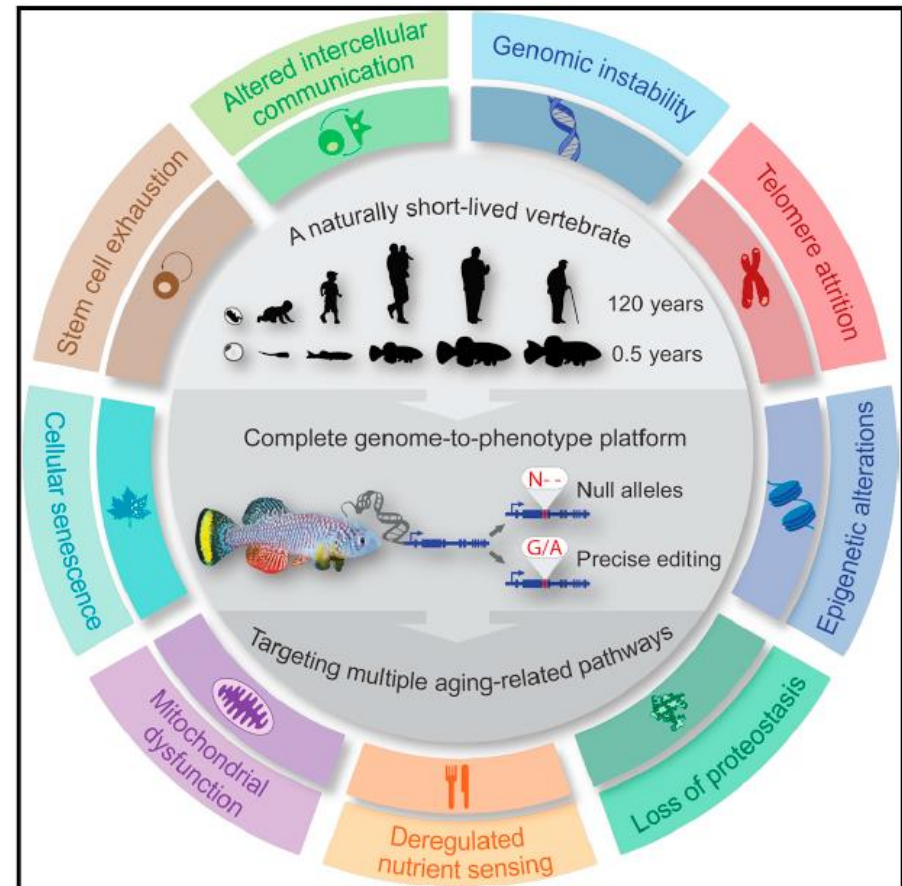
Cell Systems
Article

Longitudinal RNA-Seq Analysis of Vertebrate Aging Identifies Mitochondrial Complex I as a Small-Molecule-Sensitive Modifier of Lifespan

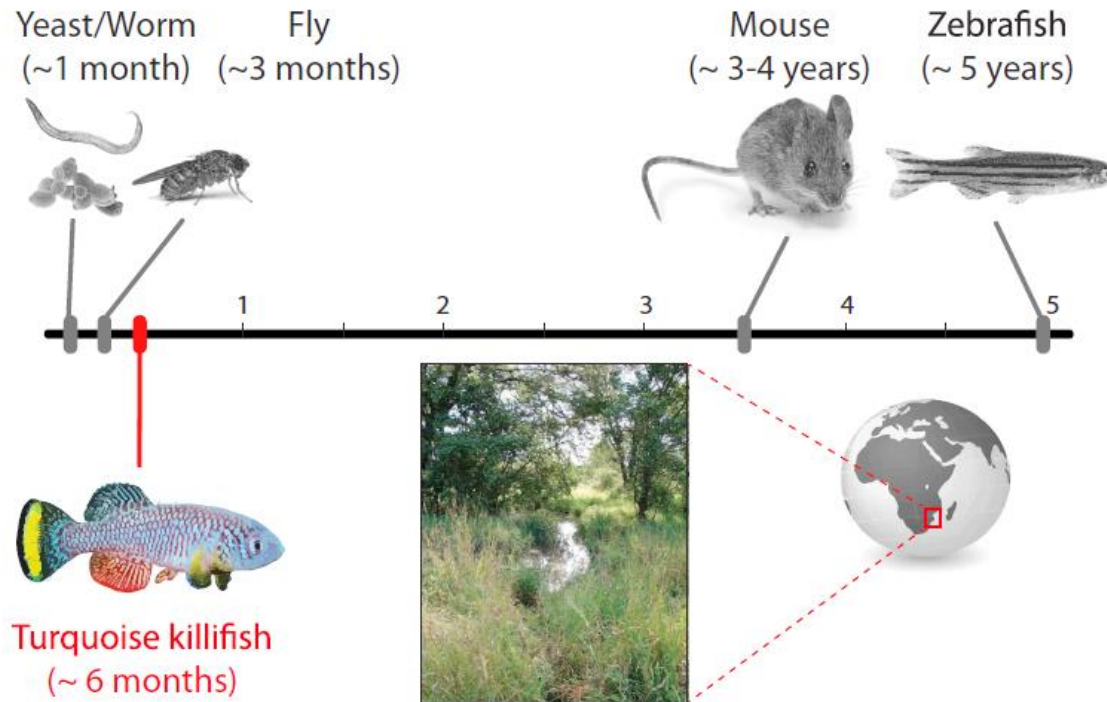
Mario Baumgart,^{1,6} Steffen Priebe,^{2,6} Marco Groth,^{1,6} Nils Hartmann,^{1,6} Uwe Menzel,² Luca Pandolfini,^{3,7} Philipp Koch,¹ Marius Felder,¹ Michael Ristow,⁴ Christoph Englert,^{1,5} Reinhard Guthke,² Matthias Platzer,¹ and Alessandro Cellerino^{1,3,*}

A Platform for Rapid Exploration of Aging and Diseases in a Naturally Short-Lived Vertebrate

- Integrative genomic and genome-editing toolkit
 - De novo assembled genome
 - CRISPR/Cas9
- Mutation of many genes encompassing the hallmarks of aging
 - Establishing stable lines
- Tools to further investigate candidates arising from human genome-wide studies

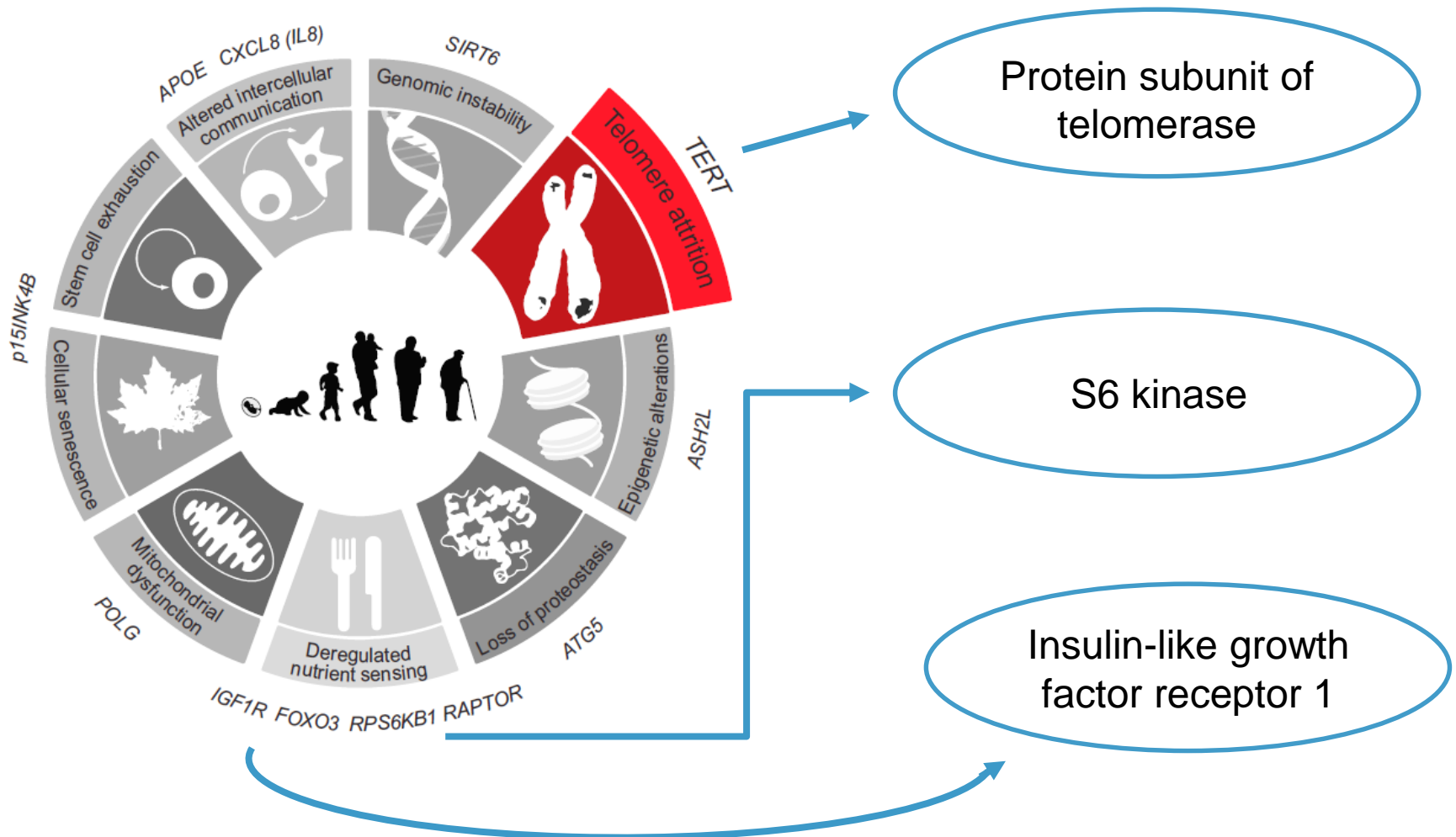


Lifespan of non-vertebrate and vertebrate aging model systems

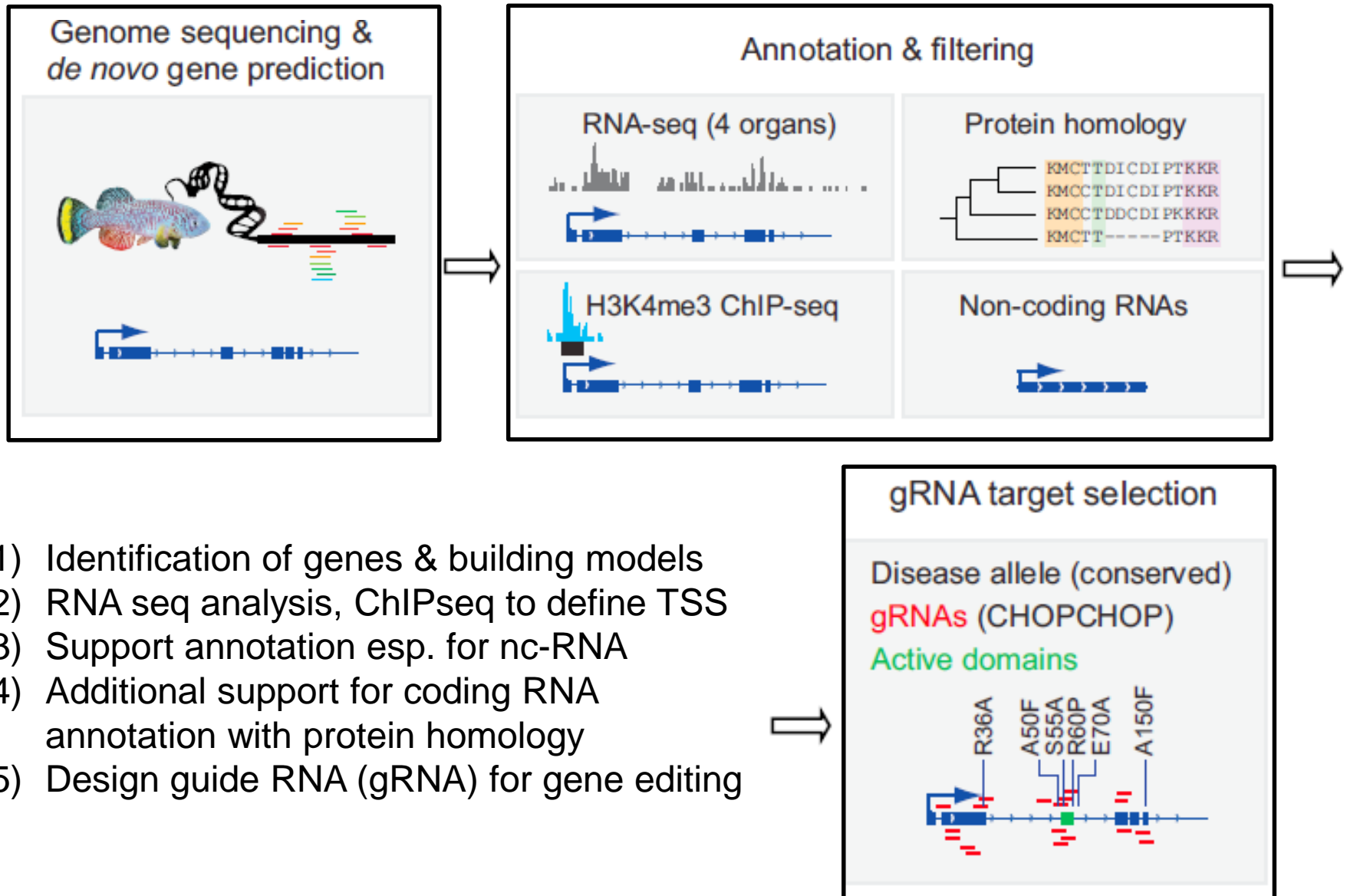


- Compressed life cycle due to brief rainy season in Zimbabwe and Mozambique
- 30-40 d from egg to egg laying adult
- 4-6 mth lifespan in lab conditions

Genes encompassing the 9 hallmarks of vertebrate aging

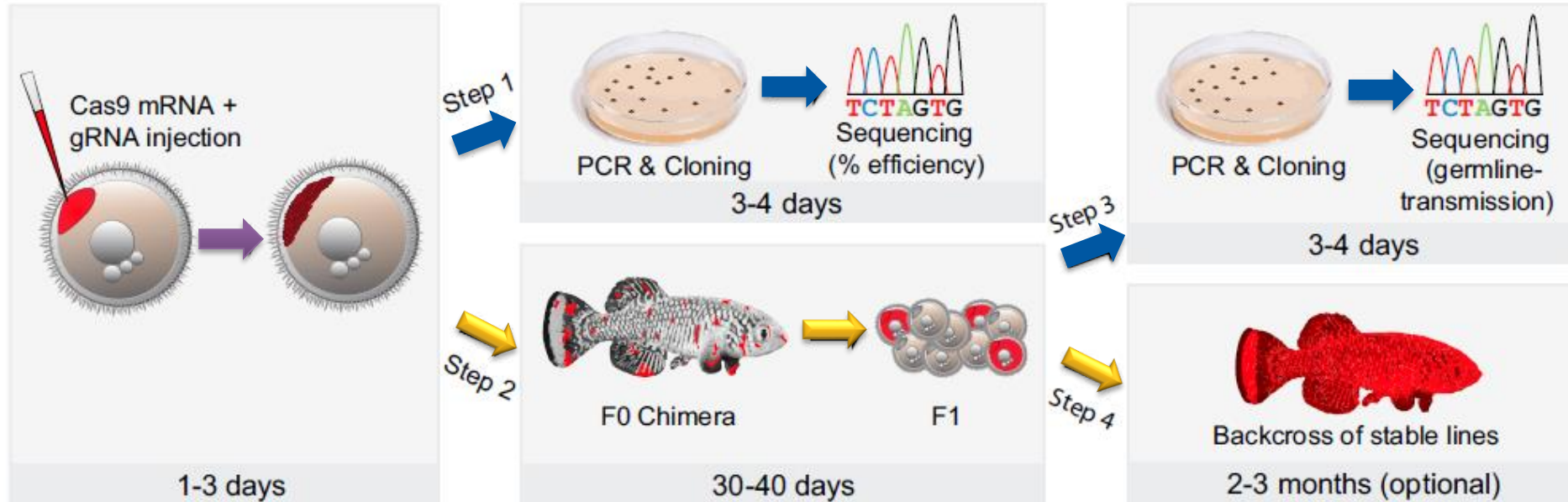


CRISPR/Cas9 gRNAs in a new model organism



- 1) Identification of genes & building models
- 2) RNA seq analysis, ChIPseq to define TSS
- 3) Support annotation esp. for nc-RNA
- 4) Additional support for coding RNA annotation with protein homology
- 5) Design guide RNA (gRNA) for gene editing

CRISPR/Cas9 genome-editing strategy



Generation of 2-5 independent gRNA sequences.

Microinjection into fertilized eggs at single cell stage.

Successful editing used for F0

F0 crossed with WT to generate F1

For QC – cloning and sequencing after 72 hours.

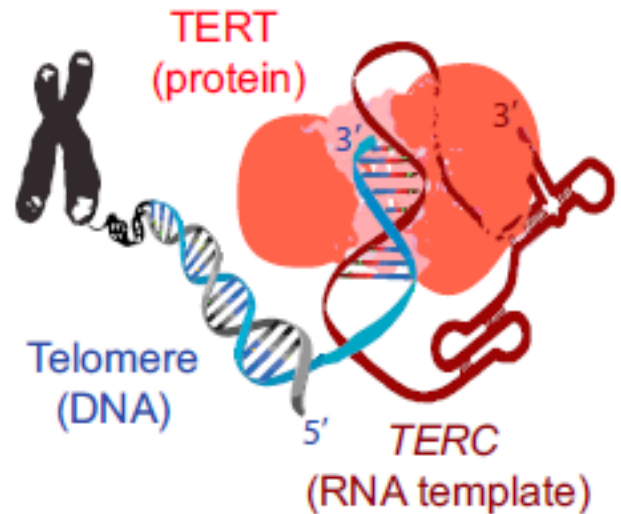
Successful F1 as stable lines

Backcrossed to minimize potential off target editing

Rapid genome editing of TERT

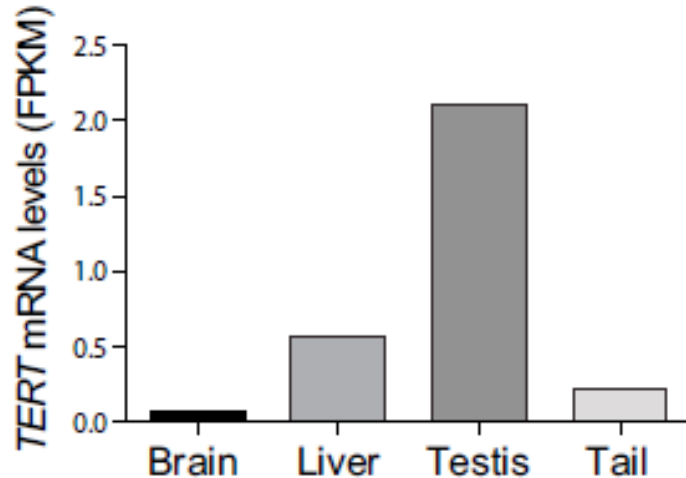
For humans: mutations in TERT cause tissue homeostasis failure like Dyskeratosis congenita

- Premature aging
- Bone marrow failure
- Pulmonary fibrosis
- Reduced fertility
- Cancers



Prediction of TERT sequence in gene model suggests conserved telomerase components in turquoise killifish

TERT expression in different tissues



RNA seq analysis of different tissues:

mRNA expression enriched in testis

Similar to human DKC patients

Successful gene editing

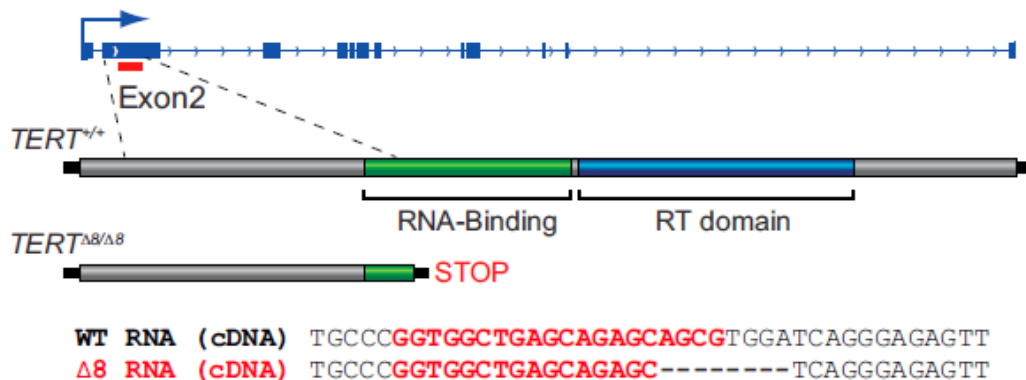
TERT Exon2 - 10% [5/50]

TGCCC	GGTGGCTGAGCAGAGCAGCG	TGGATCAGGGAGAGTT	WT
TGCCC	GGTGGCTGAG-----	-----CAGGGAGAGTT	$\Delta 15$
TGCCC	GGTGGCTGAGCA-----	GTGGATCAGGGAGAGTT	$\Delta 7$
TGCCC	GGTGGCTGAGCAGAGC---	GTGGATCAGGGAGAGTT	$\Delta 3$
TGCCC	GGTGGCTGAGCAGAGC-----	TCAGGGAGAGTT	$\Delta 8$

gRNA, PAM, Stable lines

2 gRNAs targeting regions in *TERT* exon2:

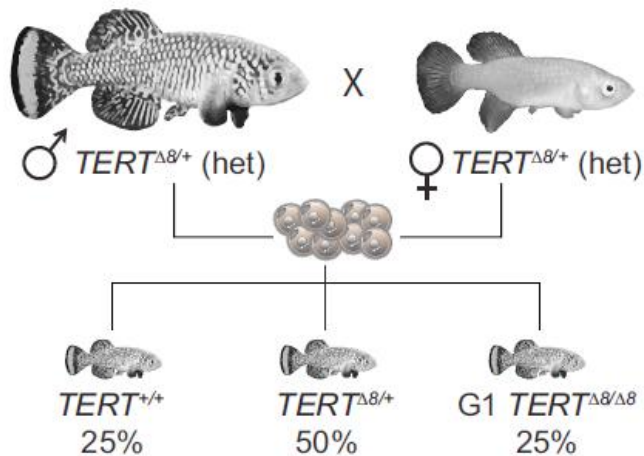
- Injection in embryos
- Raised to sexual maturity
- Crossed with WT
- Stable lines with 2 deletions
- *TERT* premature stopped after 3 or 8 bp



TERT $\Delta 8/\Delta 8$ successfully introduced within 2 months

Generation of stable mutant lines

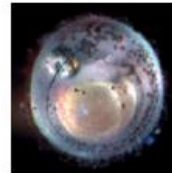
A Design to create first generation (G1) *TERT* mutant fish



Normal Mendelian distribution suggests no embryonic lethality

G1 $TERT^{\Delta 8/\Delta 8}$ fish are outwardly normal

Wild-type ($TERT^{+/+}$)



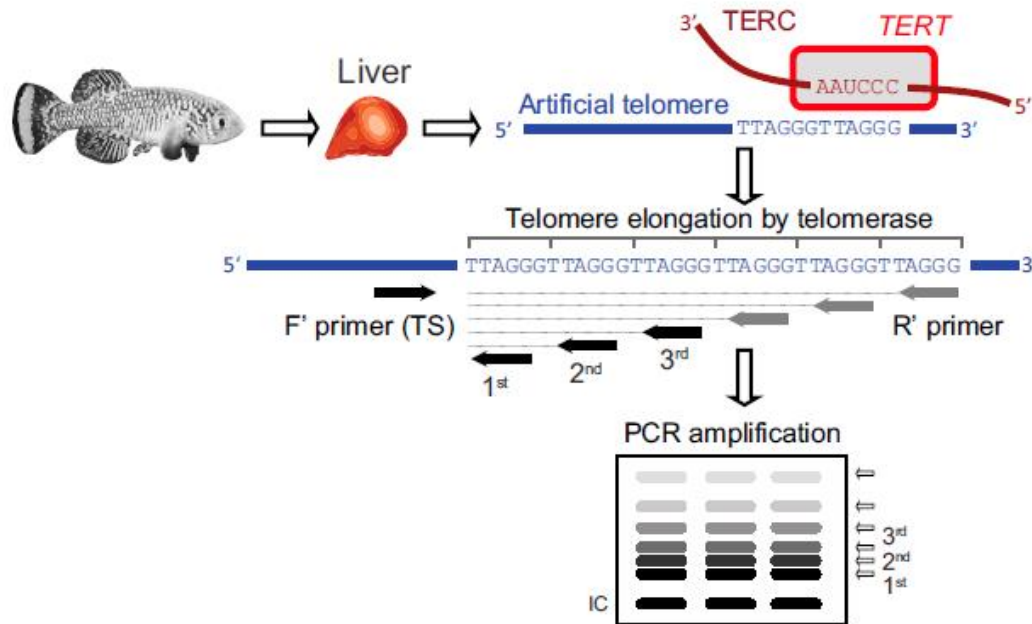
Mutant ($TERT^{\Delta 8/\Delta 8}$)



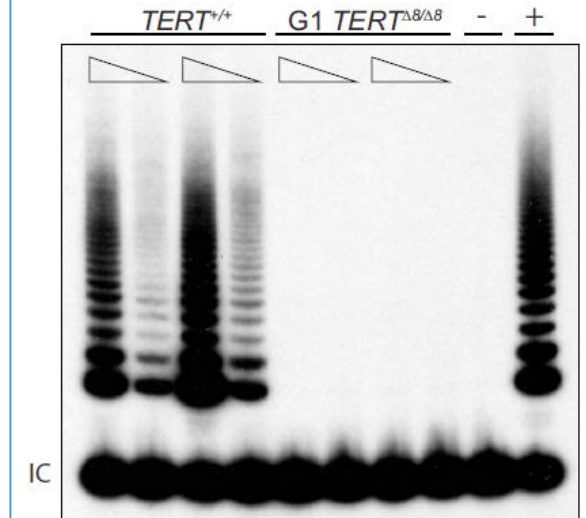
Back-crossing 3 generations to prevent off target mutations & crossing heterozygous fish to generate again homozygous individuals

Telomerase activity loss of TERT $\Delta 8/\Delta 8$

C TRAP assay



D Telomerase activity



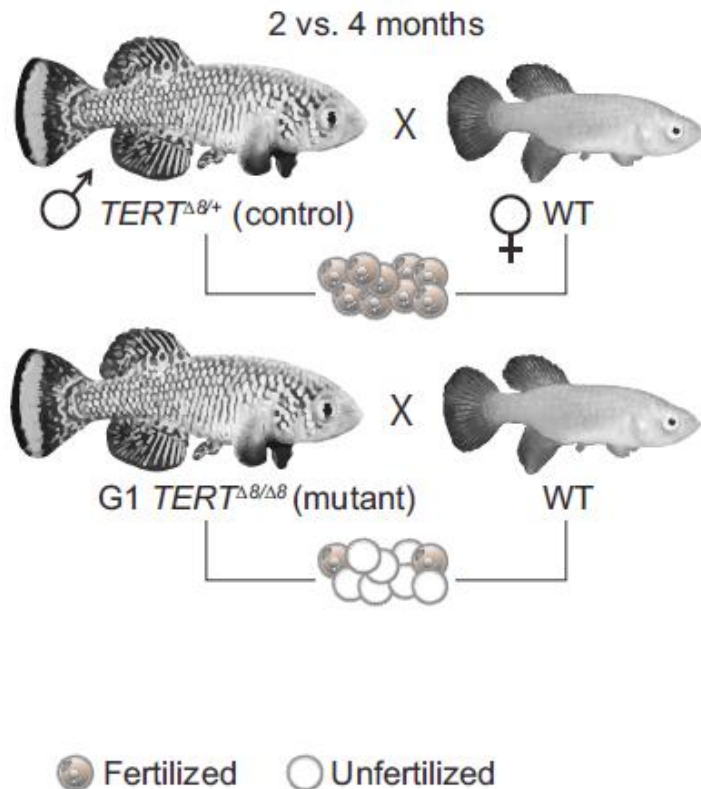
Enzymatic activity of
telomerase

TRAP assay – to check for true loss of function:

Radiolabelled NTs used in PCR amplification to
get autoradiographic products.

Investigation of male fertility in $TERT^{\Delta 8/\Delta 8}$ G1

E Design to test male fertility



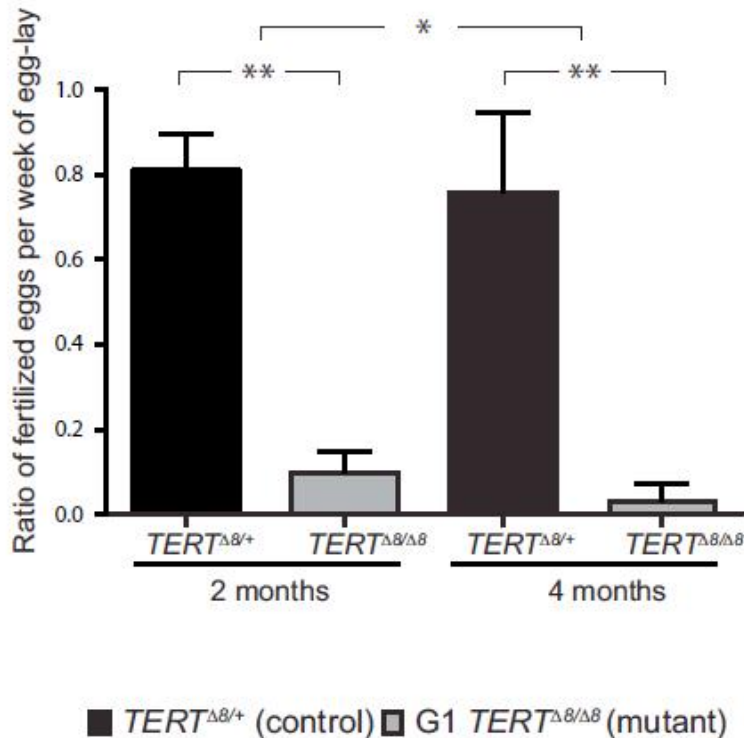
humans with haploinsufficiency for telomerase show failure in Highly proliferative tissue

- Blood
- Skin
- Intestine
- Germline (high TERT)

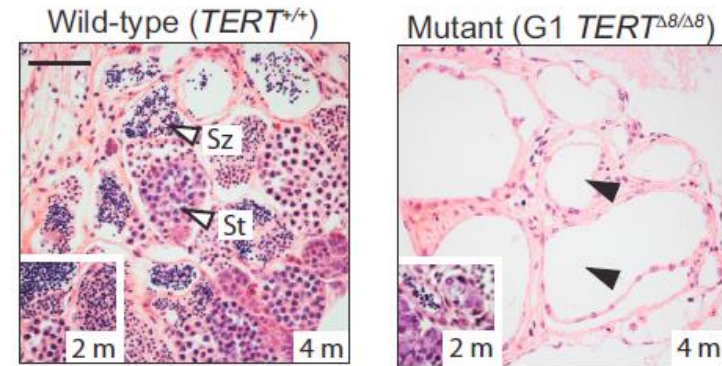
Fertility test of $TERT^{\Delta 8/\Delta 8}$ G1 males compared to $TERT^{\Delta 8/+}$ siblings by crossing to young WT females

Investigation of male fertility in $TERT^{\Delta 8/\Delta 8}$ G1

F Male fertility



G Testis histology

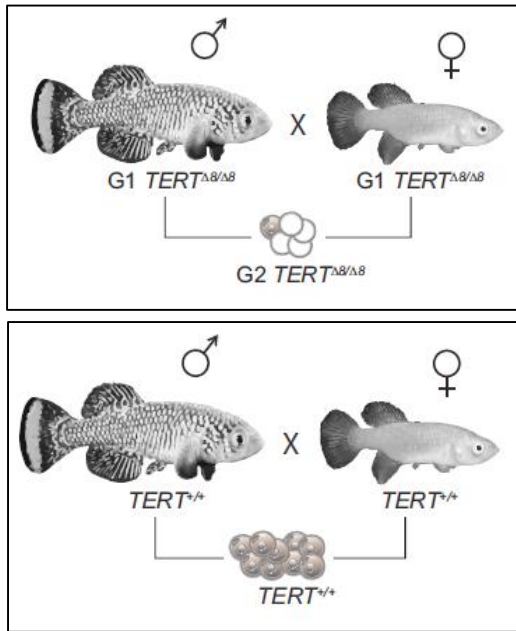


Fertilizing ability strongly decreased in $TERT^{\Delta 8/\Delta 8}$ males

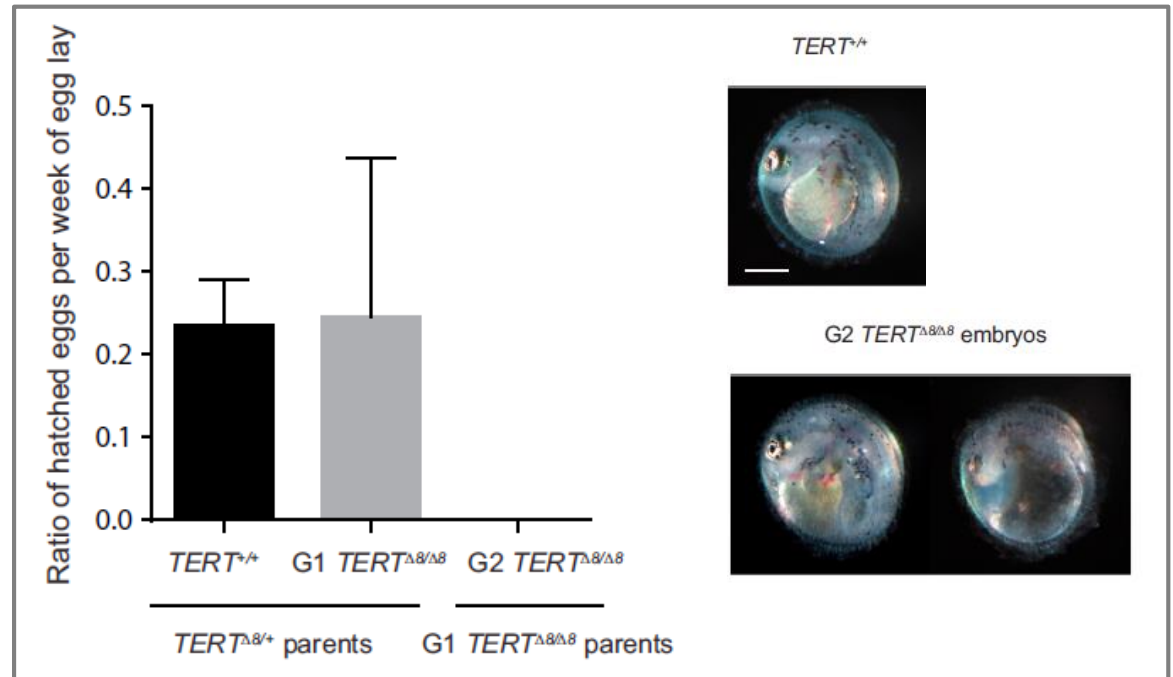
Testes showed atrophy and loss of germ cells (also females affected)

Low proliferative tissue (heart, muscle, liver, kidney) showed no significant defects

Analysis of genetic anticipation



Crossing
G1 $TERT^{\Delta 8/\Delta 8}$ to
generate
G2 $TERT^{\Delta 8/\Delta 8}$
embryos

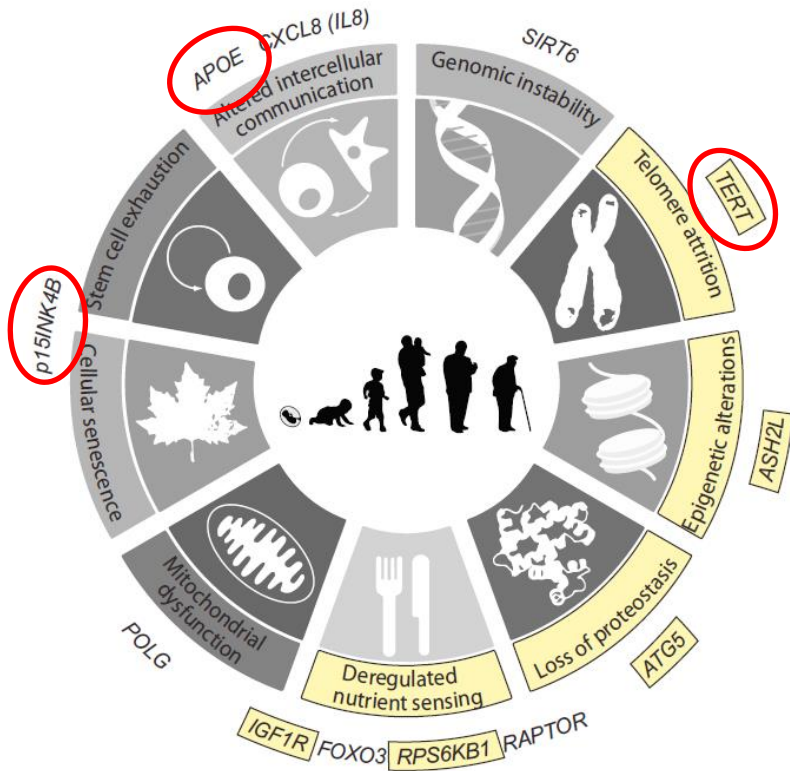


G2 embryos showed gross abnormalities and
died prior to hatching

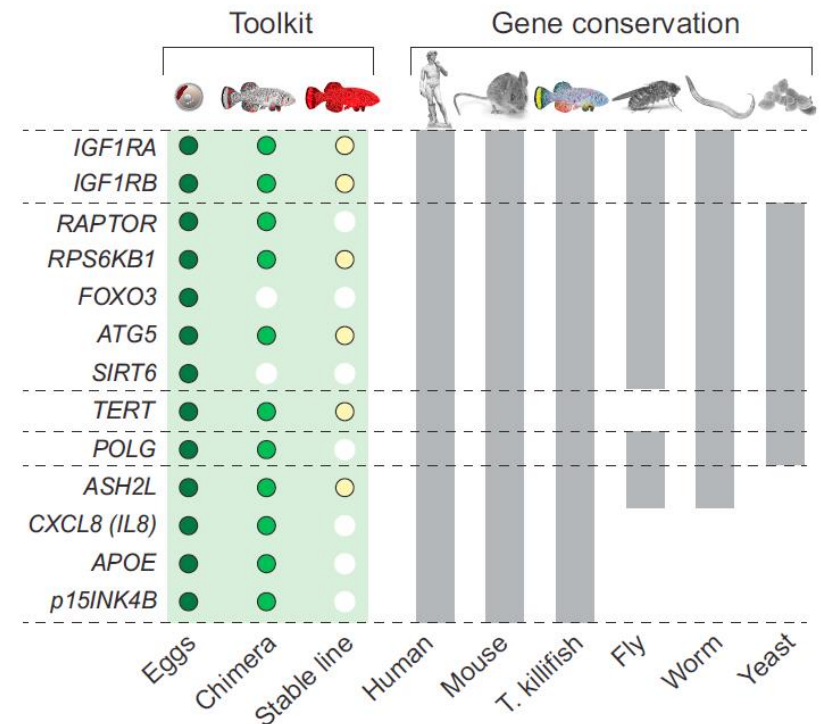
Genetic anticipation = cumulative germline damage;
also occurring in human DKC patients

Toolbox of mutants encompassing the hallmarks of aging

Targeted genes and pathways with stable lines



B Targeted genes and evolutionary conservation



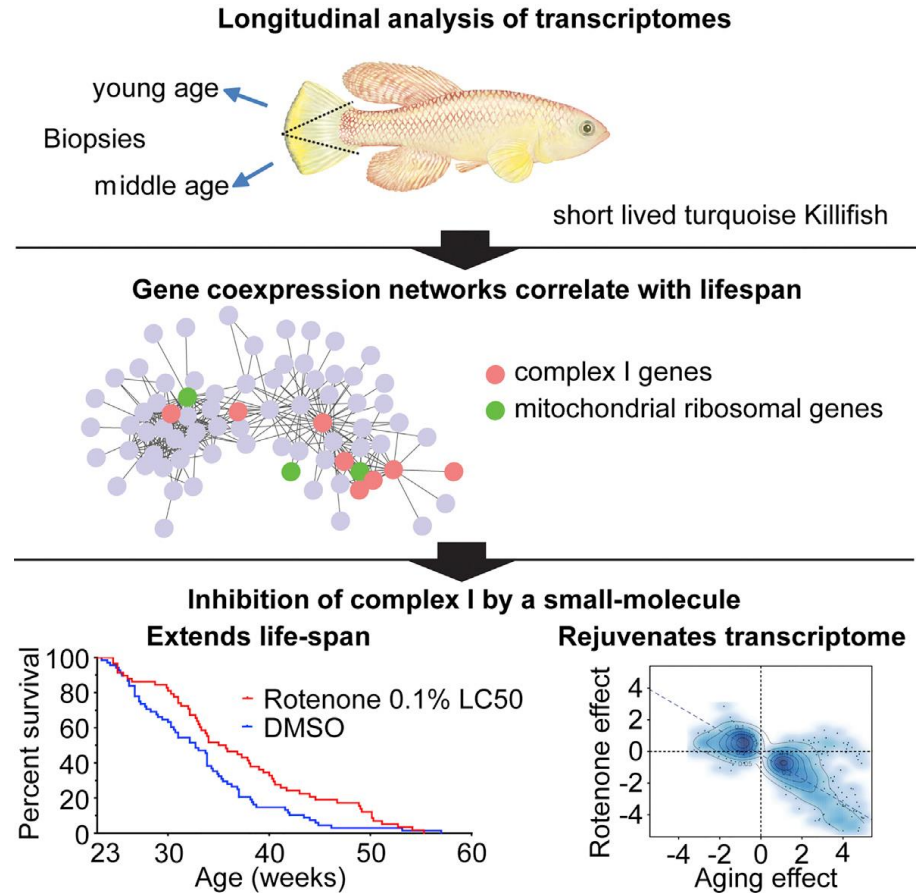
Construction of 5 gRNA for each gene of interest → identification of at least 1 successful gRNA → generation of stable mutant lines → online platform

Conclusions

- Turquoise killifish with
 - Short / compressed lifespan,
 - well characterization &
 - low costsis highly suited for aging reasearch
- Good for exploration of human longevity candidate genes

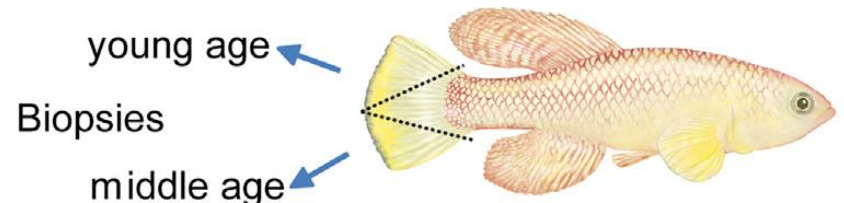
Longitudinal RNA-Seq Analysis of Vertebrate Aging Identifies Mitochondrial Complex I as a Small-Molecule-Sensitive Modifier of Lifespan

- Longitudinal transcriptomics
- Transcriptomics of shorter and longer lived individuals
- Identification of lifespan modulators
- Rejuvenation of the transcriptome



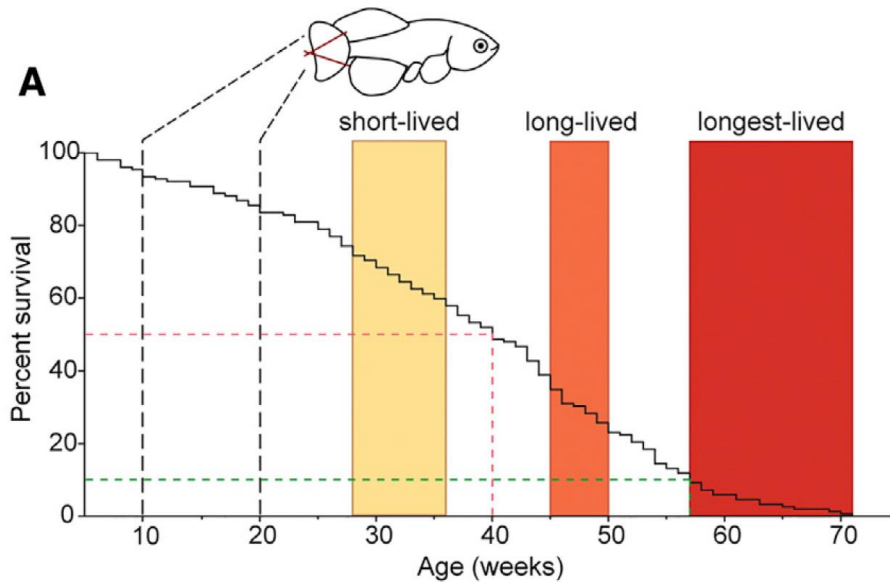
Basic experimental setup

- Different Killifish models available (3-12 month lifespan)
 - Highly inbred strains (e.g. GRZ) with shorter lifespans
 - Strains collected more recently (e.g. MZM0410) with longer lifespans



- Taking 2 fin biopsies at two time points during early adult stage
 - RNA seq analysis
 - Weighted gene co-expression network analysis (WGCNA)

Lifespan analysis of MZM-0410



MZM-0410 shows 42 times more variations than highly inbred strains

Biopsies at 10 & 20 weeks of age

Median lifespan 40 weeks

10% survivorship after 58 weeks

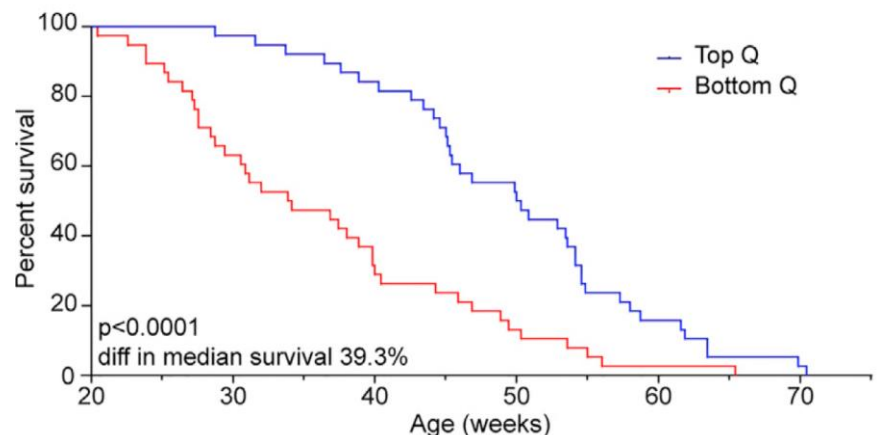
Differences in survival between first and last quartile of increase in body weight

45 individuals for:

Short-lived group (28-36 weeks)

Long-lived group (45-50 weeks)

Longest-lived group (57-71 weeks)



Analysis of 10 & 20 weeks biopsy

Recording of:

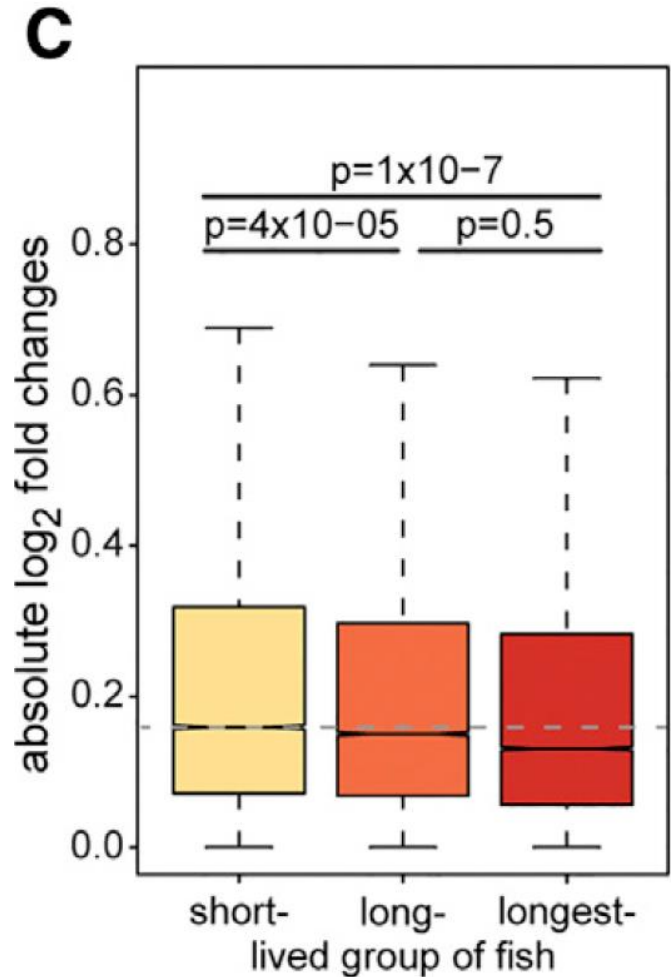
- Age at sexual maturity
- Weight
- Length
- At 10 & 20 weeks each
- Calculated growth rate between biopsies

RNA seq analysis of 23 546 genes

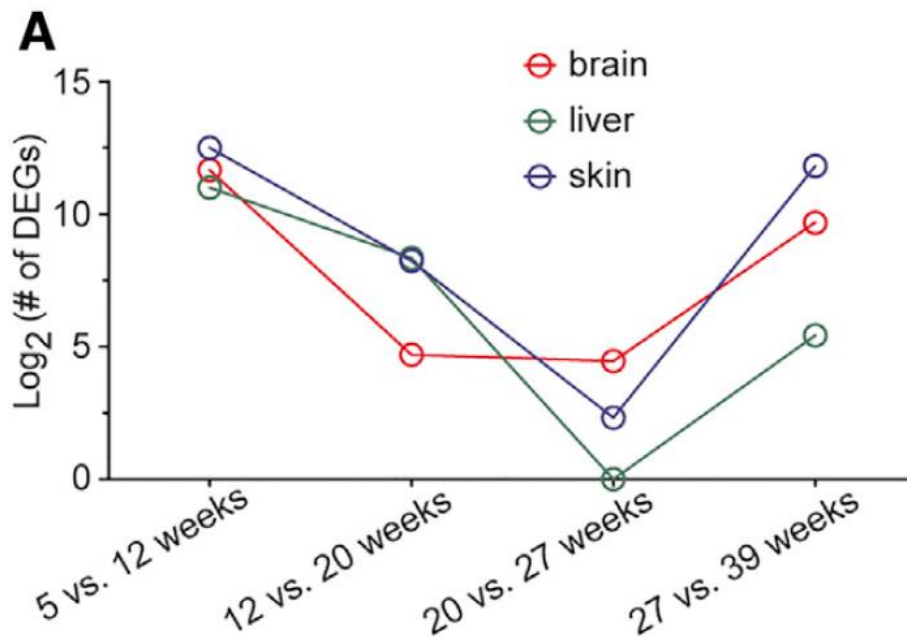
Compared to the *N. furzeri* reference genome

Comparison between both biopsies of the same fish:

- Largest difference in short-lived fish
- Smallest modulations in longest-lived fish



Charakterization of age dependent gene expression

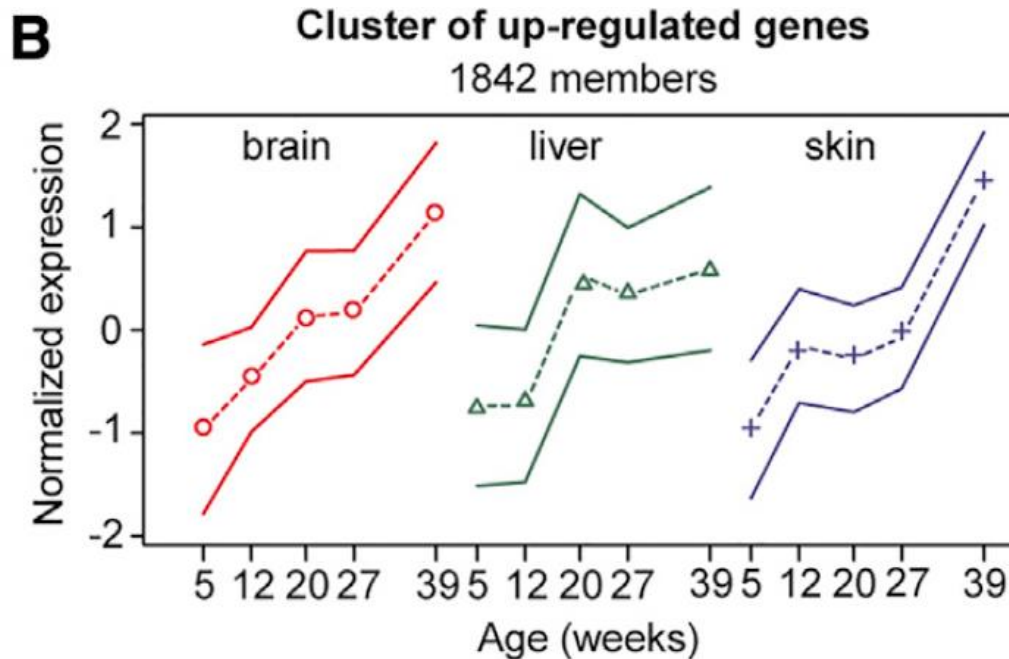


Non genetic factors contributing to age of death are present in early life

Analysis of RNAseq data from 5 age groups of differentially expressed genes between first and last age group

Pairwise comparison of age groups regarding differentially expressed genes

Age dependent regulation across tissues



Analysis of genes differentially expressed in at least 2 tissue types

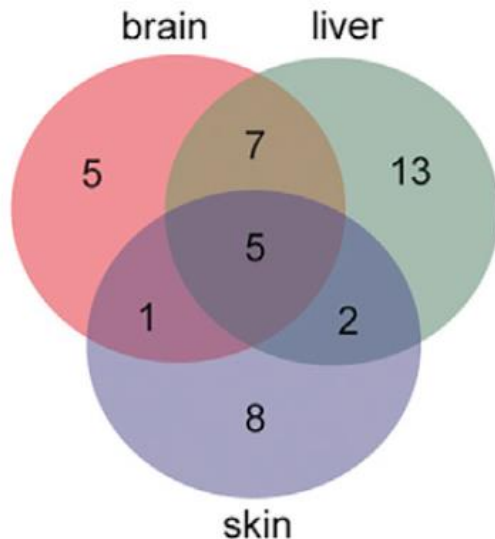


Age dependent regulation seems to be similar across the 3 tissues

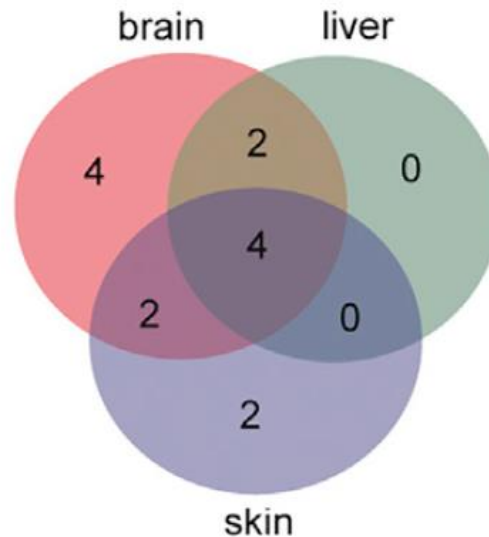
Different KEGG pathways expression in different tissues

C

down-regulated pathways



up-regulated pathways



down-regulated pathways

dre04110 Cell cycle
dre03030 DNA replication
dre00020 Citrate cycle (TCA cycle)
dre03013 RNA transport
dre00310 Lysine degradation

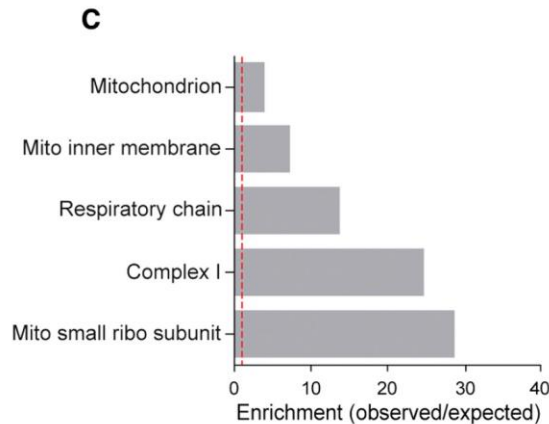
up-regulated pathways

dre03010 Ribosome
dre04060 Cytokine-cytokine receptor interaction
dre04620 Toll-like receptor signaling pathway
dre04630 Jak-STAT signalling pathway

Up- and Down-regulated age associated pathways in different tissues

9 of the KEGG pathways are regulated in all three tissues

Activity of Respiratory Chain Complex I affects lifespan



WGCNA analysis of 936 genes correlated with age of death

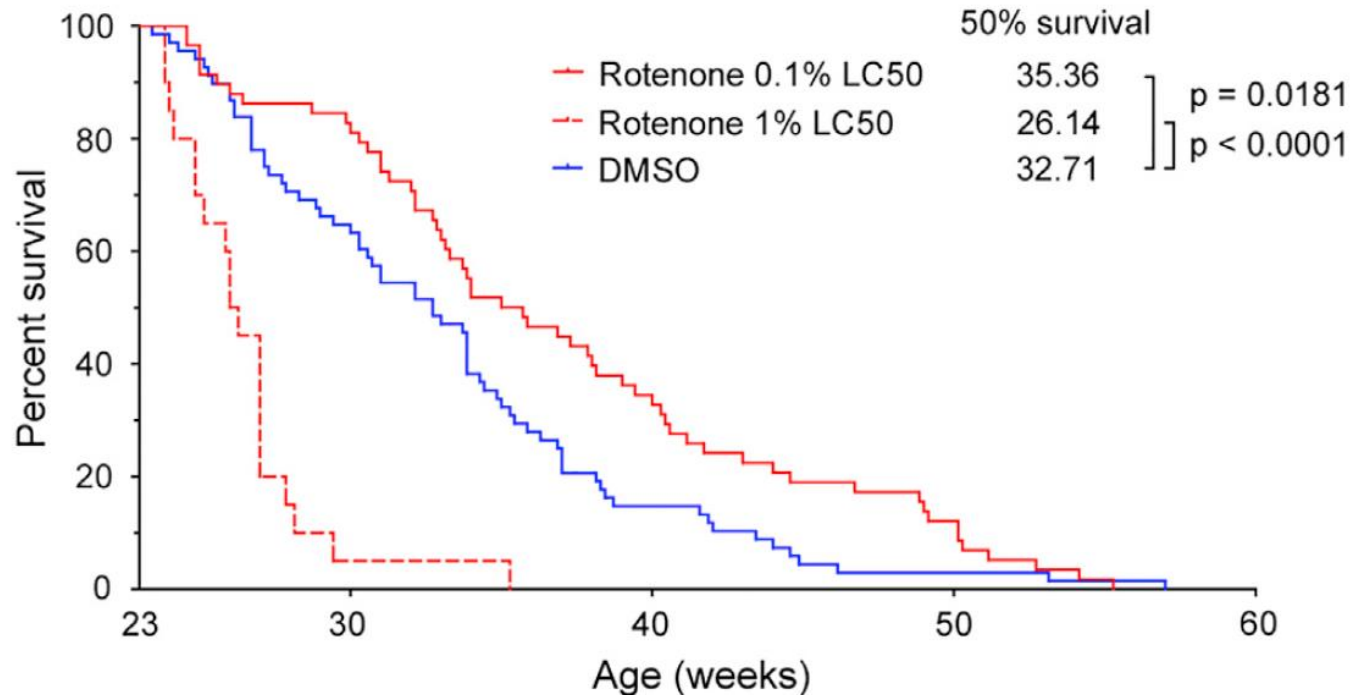
Top enrichment for complex I of respiratory chain and genes coding for parts of complex I

Network analysis showed genes coding for NDUFs & MRPs are highly co-regulated → Consistent with mouse studies

2 experiments for confirmation:

Comparison of skin RNAseq data from highly inbred GRZ strain with MZM-0410 (12 weeks) → higher complex I gene expression in GRZ

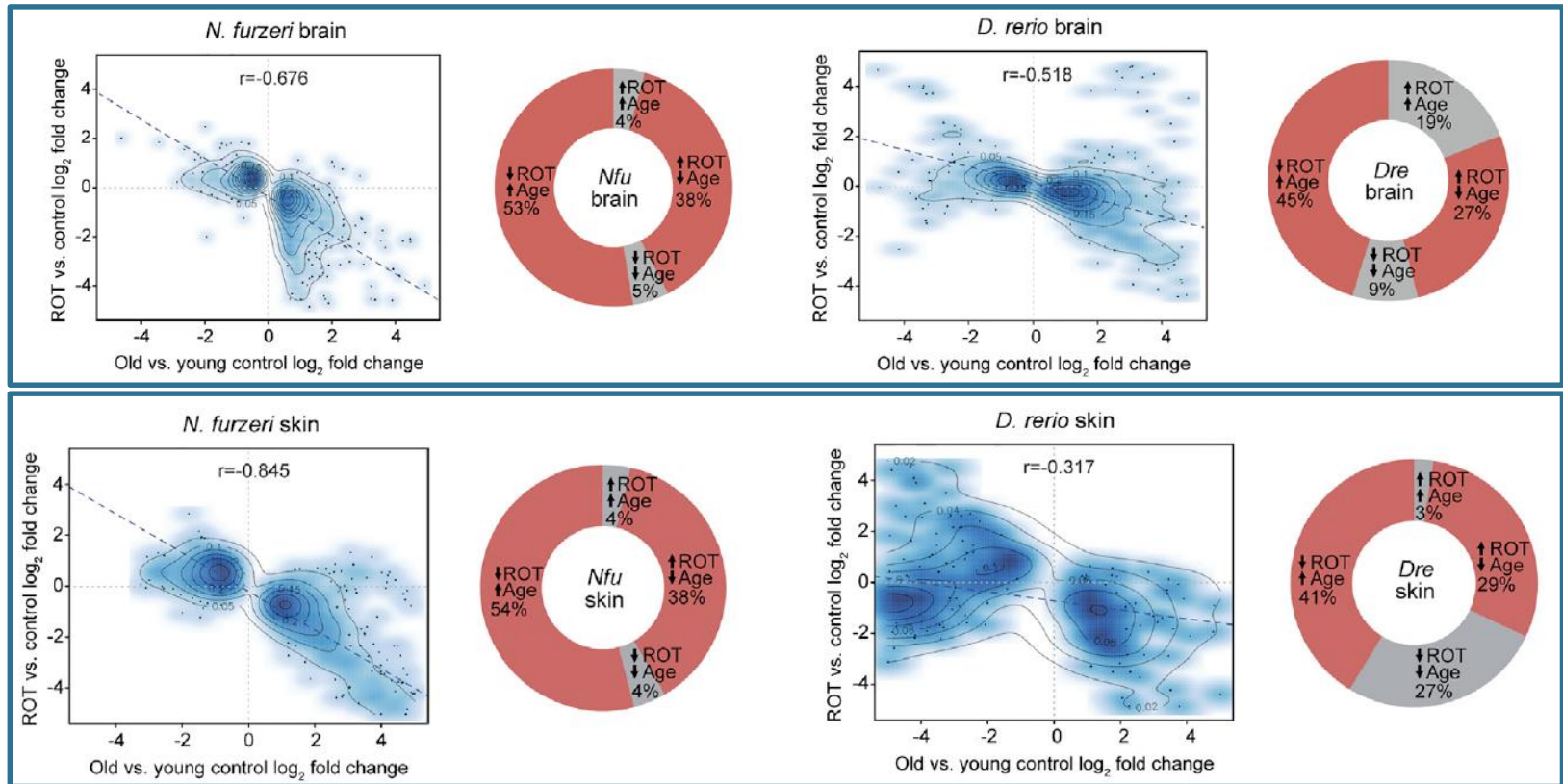
Relevance of Complex I for lifespan determination



Inhibition of Complex I by rotenone treatment from 23 weeks:

- Lower concentration → lifespan extension
- Higher concentration → lifespan shortening

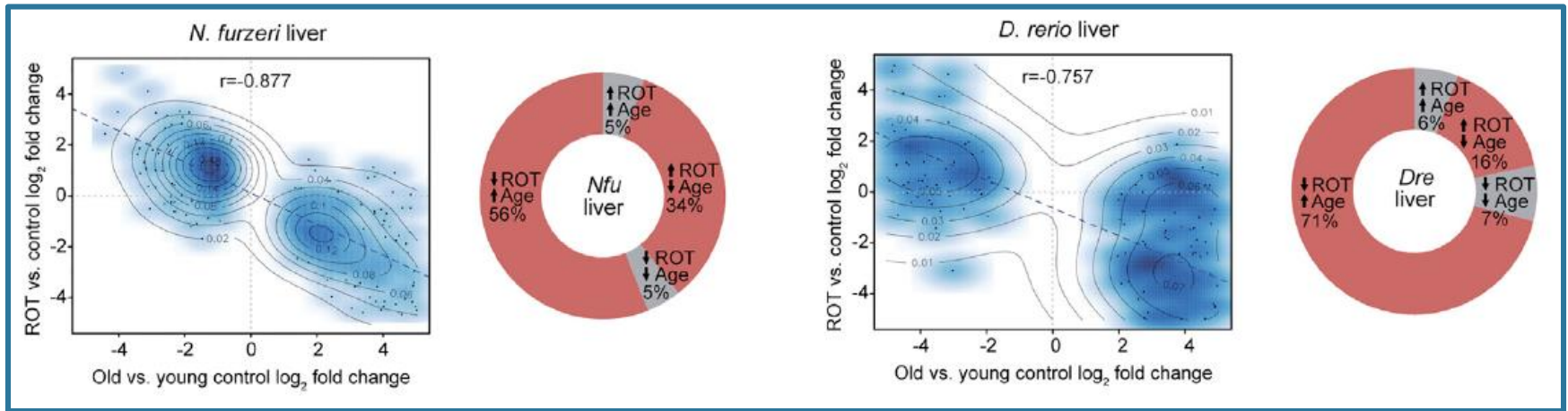
RNAseq analysis of different tissue after ROT treatment



4 weeks treatment with 15pM ROT & vehicle of old (23 w) and young (5 w) killifish

8 weeks treatment with 3.75nM ROT & vehicle of old (36m) and young (12m) zebrafish

RNAseq analysis of different tissue after ROT treatment



In brain, skin & liver the vast majority (~90%) of upregulated genes during aging were downregulated by ROT and vice versa

→ Resulting in a highly significant negative regression

Conclusions

- Data obtained with *N. furzeri* in short time could be confirmed with *D. rerio*
- Data suggest complex I of respiratory chain as potential target for prevention of age-related dysfunctions

Thank You For
Your Attention

