Exercise: Replicative immortality

1) For memorization:

a) Which of the following characteristics can distinguish senescent cells from normal terminally differentiated cells:

A. Cell flattening and increase in size
B. Failure to re-enter the cell cycle in response to any growth factors
C. Shortened telomeres
D. Expression of a lysosomal β-galactosidase
E. Decrease of heterochromatin and repressive histone marks
F. A distinct senescence-associated secretory phenotype

Answer: All except E.
Explanation: Senescent cells are characterized by an increase rather than a decrease of heterochromatin and repressive histone marks. Induction of this heterochromatin is mediated at least in part by hypophosphorylated RB1.

2) Comprehension of concept (important):

a) Telomere erosion can either induce senescence *or* a "crisis". What determines these alternative fates?
This decision depends on p53 status: In premalignant tumor cells that have only lost RB1 or p16INK4a but not p53, progressive telomere shortening beyond a critical threshold activates p53 through the DNA damage response pathway to induce cellular senescence. By contrast, cells that have inactivated p53 (e.g. by mutation or by loss of ATM or of the MDM2 inhibitor ARF) continue to proliferate until telomere erosion provokes chromosome end fusions.

b) How can you distinguish which of these two states premalignant cells have entered?

Answer: The difference is that senescent cells survive for months, whereas crisis will lead to death except in rare cells (approx. 1 in 10^7) that manage to stabilize their telomeres again and become immortalized.

c) What mechanism kills premalignant cells during crisis?

Answer: Breakage of fused chromosomes during mitosis can release DNA fragments into the cytosol. They activate the cGAS-STING pathway (cyclic GMP-AMP synthase–stimulator of interferon genes), eventually leading to cell death by autophagy.

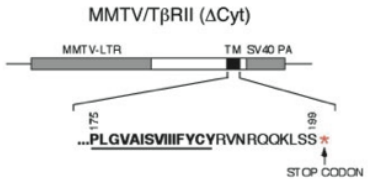
In tumor cells that evade autophagic death, repeated chromosome-breakage-fusion cycles lead to major chromosome imbalance (aneuploidy) and thereby can also provoke death by apoptosis (despite the absence of p53).

d) How can tumor cells escape from crisis and become immortalized?
Answer: On average, 1 in 10^7 cells will stabilize their telomeres. In up to 85% of tumors, this process relies on hTERT reactivation (promoter mutations). Some tumors (<15%) can maintain telomeres independently of hTERT by a recombination-based mechanism called ALT (alternative lengthening of telomeres).

e) How do breakage-fusion-bridge cycles increase the aggressiveness of malignant cancer cells?

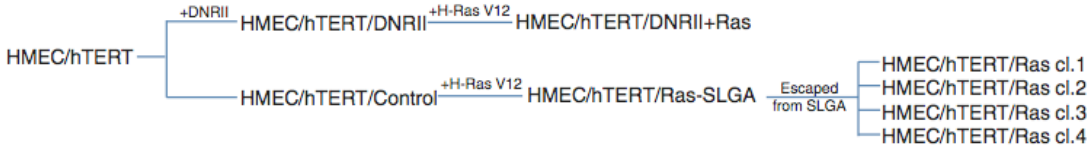
By increasing genetic instability, which is called an "Enabling Characteristic" because by increasing the frequency of mutations, it accelerates the emergence of cancer hallmarks.

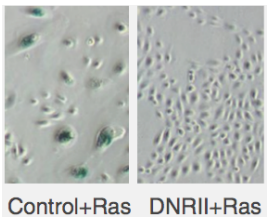
3) Application: Role of TGF-β induced senescence in breast cancer. In the exercise last week, we saw that autocrine TGF-β signaling in healthy mammary glands inhibits the proliferation of hormone receptor-positive cells to thereby prevent excessive duct tissue growth and side branching induced by estrogen and progesterone. To test TGF-β functions in mouse models of breast cancer, its activity was blocked by administering a C-terminally truncated mutant type II TGF-β receptor that cannot signal because it lacks the kinase domain, but which still binds ligand via the extracellular domain and thus acts as a "ligand trap". Genetically, this construct functions as a "dominant negative" mutant by competing with wild-type receptors for ligand binding:



Truncated TβR2 (Δcyt)
(dominant negative)

As a model of human breast cancer, human mammary epithelial cells (HMECs) from breast resections were immortalized by hTERT overexpression (S. Lin et al. 2013 Mol Biol Cell). To **test a role of autocrine TGF-β signaling in oncogene-induced senescence**, the resulting cells were then sequentially transfected with dominant negative mutant type II receptor (DNRII) or with control vector, and with oncogenic H-RasG12V:





The authors found that DNRII inhibited the induction of acidic β-galactosidase (blue staining in panels shown to the left). Interestingly, after selection for anchorage-independent growth in soft agar, a few subclones escaped from a senescence-like growth arrest (SLGA) even in the absence of DNRII (top panel).

a) Based on these results, what mechanism could possibly explain how clones 1 to 4 escaped from senescence?

Since senescence of the hTERT-expressing HMECs was suppressed by DNRII, it is likely mediated by autocrine TGF-β signaling. Accordingly, the most likely explanation how

clones 1 to 4 escaped from senescence is that these clones spontaneously attenuated TGF-β production or signal transduction.

Potential causes of TGF-β signal inhibition could include, for example, impaired TGF-β receptor I or II expression or function, or LOF mutations in SMAD2, 3, or 4.

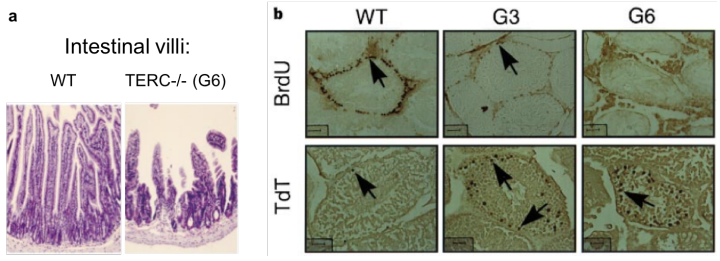
Comment: Confirming a defect in TGF-β signaling, the authors found that cl.4 became partly resistant to inhibition of cell proliferation by exogenously added TGF-β (not shown here).

b) What Western blots or immunostainings would you perform on clones 1 to 4 to test your hypothesis?

To directly test whether TGF-β signaling is impaired, one could monitor Smad2,3 phosphorylation (by anti-pSmad2,3 Western blot) and nuclear translocation (by immunostaining, or by Western blot analysis of nuclear vs cytoplasmic extracts).

In addition, analysis of endogenous TGF-β target genes of senescence programs (p21, or Id1) could be informative to see whether clones lost p21 and/or gained Id1 expression.

4) Data interpretation: Mutant mice lacking the TERC subunit of telomerase unexpectedly showed no defects until after several generations of inbreeding between TERC-/- males and females. By contrast, after 3 to 6 generations of such inbreeding they became infertile, showing high rates of apoptosis in testis, combined with intestinal atrophy, and impaired bone marrow function:



TERC-/- phenotypes. a) Histological sections through WT or 6th generation TERC-/- intestines. **b)** Testes of WT versus TERC-/- males treated with BrdU (top) or after Terminal deoxynucleotidyl transferase (TdT) labelling of apoptotic cells (bottom).

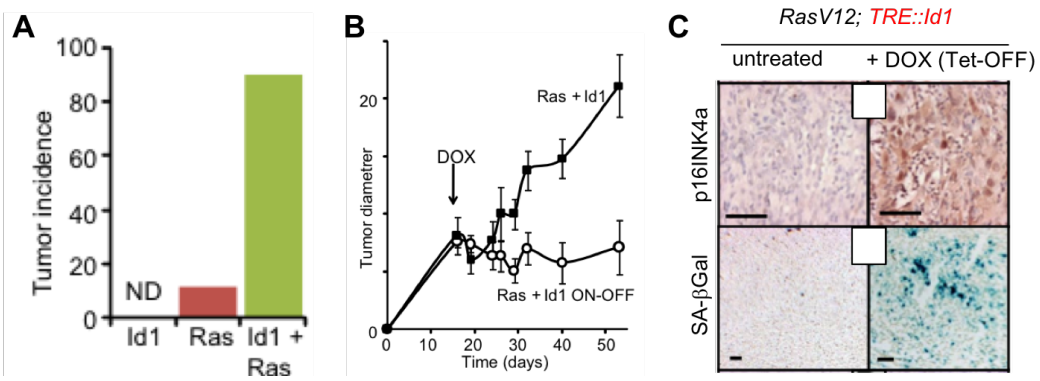
i. What do tissues affected by the deletion of *TERC* have in common?
They all depend on telomerase for their renewal by stem cells during tissue homeostasis.

ii. Mice have longer telomeres than humans. What mechanism(s) could explain how mutant intestines became atrophied, and why only after TERC was lacking in the germline for several generations?
Atrophy is due to the differentiation and subsequent depletion of intestinal stem cells. The late generation onset of these phenotypes led to the discovery that telomeres are 5-10 times longer in mice than in humans (due to a point mutation in the DNA helicase RTEL1, as shown recently by Smoom et al. 2023, Nat. Commun.). Although not discussed in our class, this can explain why it took several generations before telomeres in Terc-/- mice became shortened below the critical threshold required to trigger chromosome end-to-end fusions.

iii. Did the dead cells (stained by TdT labelling, black arrows) undergo replicative senescence prior to their death? Why or why not?

Since progressive telomere shortening normally will induce permanent cell cycle arrest by cellular senescence *without killing* the senescent cells, the observed increase of apoptosis in Terc-/- testes indicates that the cells which died likely died before entering senescence.

5) Data interpretation: To assess its role in breast cancer, an Id1 transgene (TRE::Id1) was introduced in H-RasG12V mouse mammary epithelial cells (MMECs) that were then grafted into mouse mammary glands to monitor tumor formation. In mammary glands of mice treated without the tetracycline analog doxycycline, MMEC grafts expressing only Id1 or H-RasV12 alone very rarely formed tumors compared to grafts expressing both transgenes (**A-B**). Moreover, tumors that received DOX to switch off the TRE::Id1 transgene stained positive for p16Ink4a and SA-βGal expression (**C**).



How would you explain that oncogenic Ras alone was unable to efficiently induce tumorigenesis?

G12V mutant Ras alone is not sufficient to reliably induce tumor formation because it triggers oncogene-induced cellular senescence. Co-expression of Id1 efficiently overcomes this barrier (as shown in panel C).