



**Department of Science**  
*PhD Program in Molecular, Cellular, and  
Environmental Biology*  
*Cycle XLI*

# **Targeting Replication Fork Stability as a Therapeutic Vulnerability in ALT Tumors**

**PhD:**

Giulia Capitani

**Tutor:**

Prof. Antonella Sgura

# Human Telomeres: Structure and Function

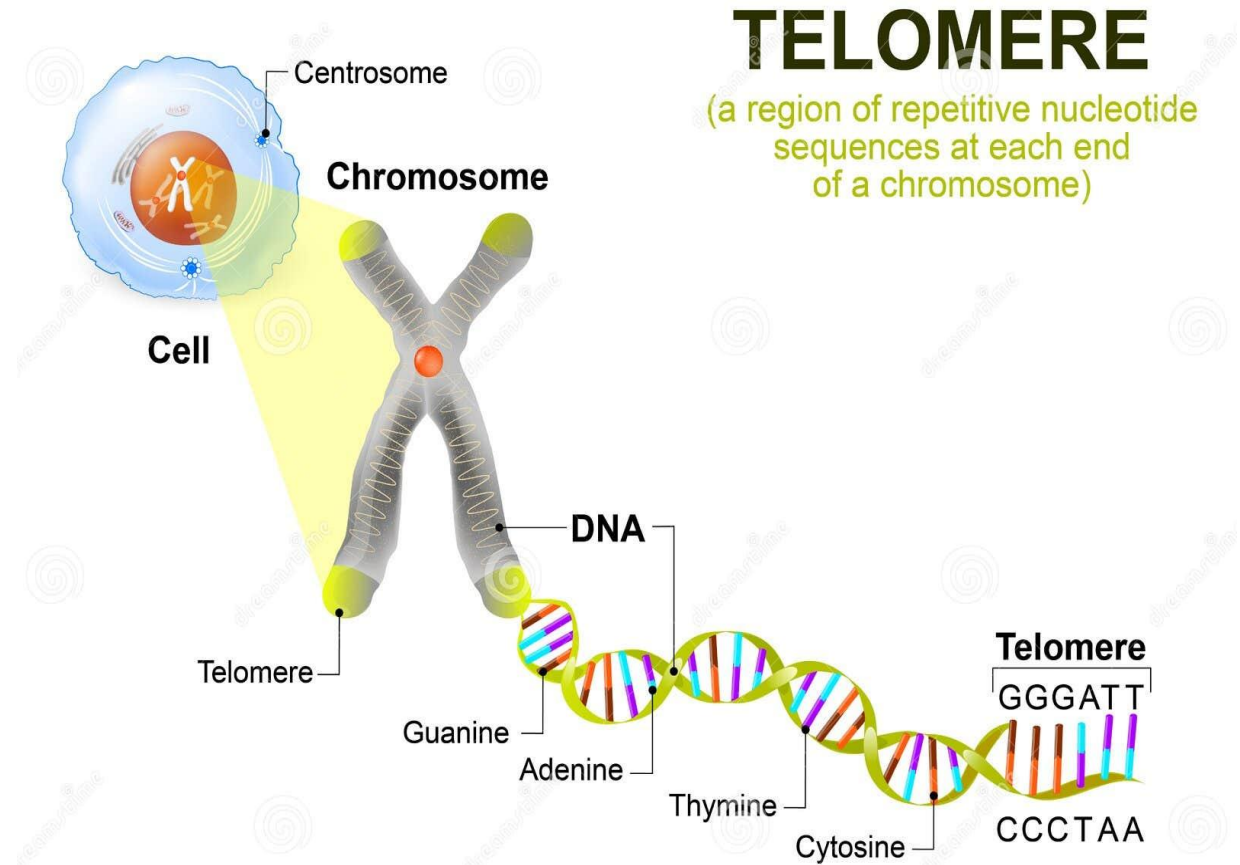
**Composition:** Human telomeres consist of repetitive DNA sequences of (TTAGGG) $n$  located at the terminal ends of each linear chromosome.

**Length:** In humans, these sequences typically span between 10 and 15 kilobases (kb).

**Structure:** Telomeric DNA does not remain as a simple linear string; it folds back on itself to form a specialized structure called a **T-loop** (Telomeric loop).

**Chromatin State:** They are condensed as heterochromatin.

**Protective Barriers:** They protect coding DNA from attrition through structural barriers



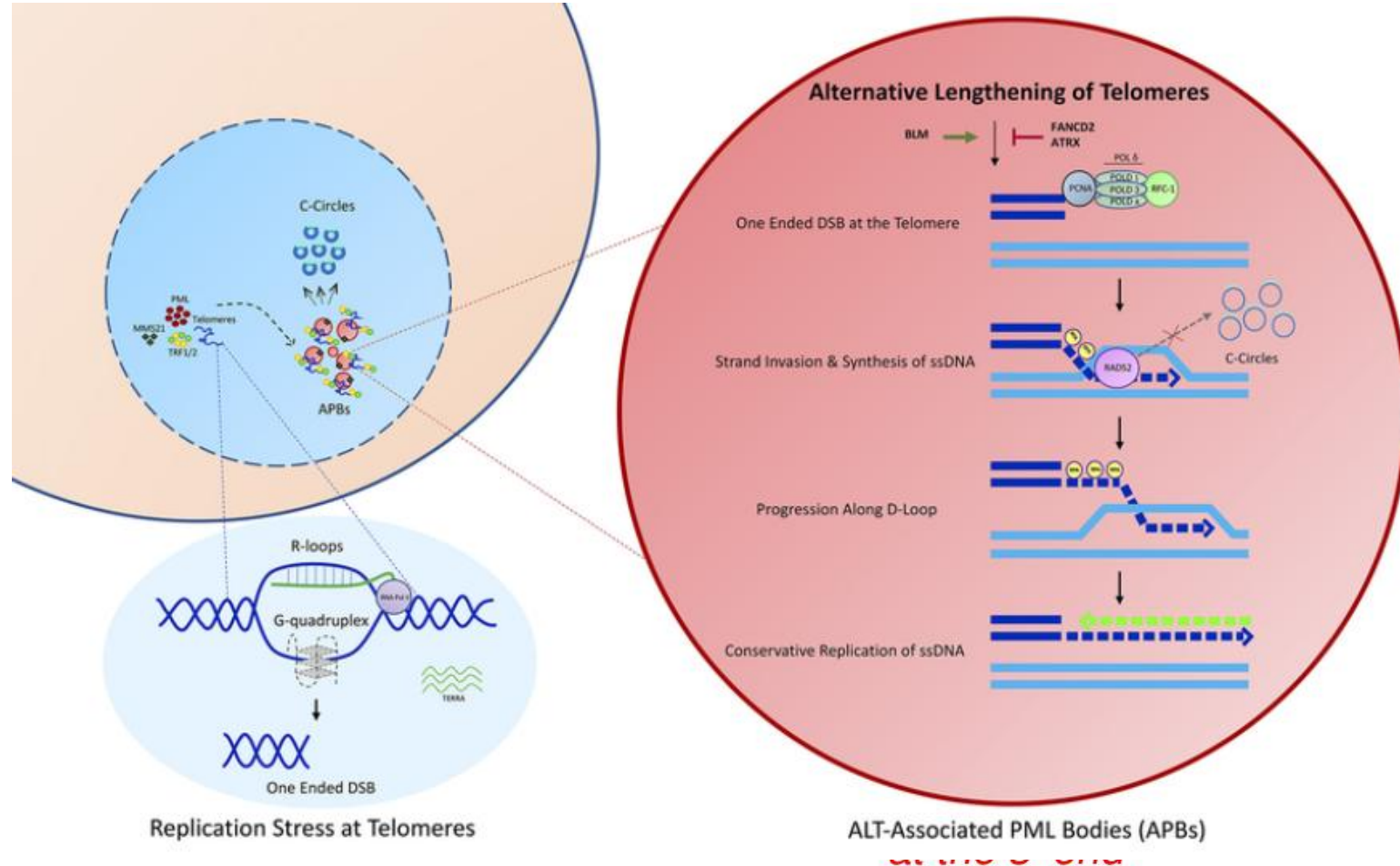
# The *END REPLICATION PROBLEM*

## Normal cells:

- Without Telomerase → Hayflick limit.
- With Telomerase

## Cancer cells:

- Reactivation hTERT
- **Alternative Lengthening of Telomeres (ALT)** → mechanism based on **Homologous recombination**. ALT-positive cells use telomeric repeats as templates to "copy and paste" sequences from one chromosome to another.



# BIR (Break Induced Replication)

Loss of Chromatin remodelers  
or Helicases

Leads to **unresolved  
secondary structure.**

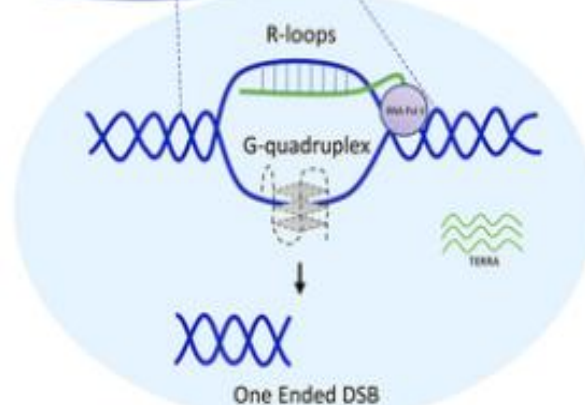
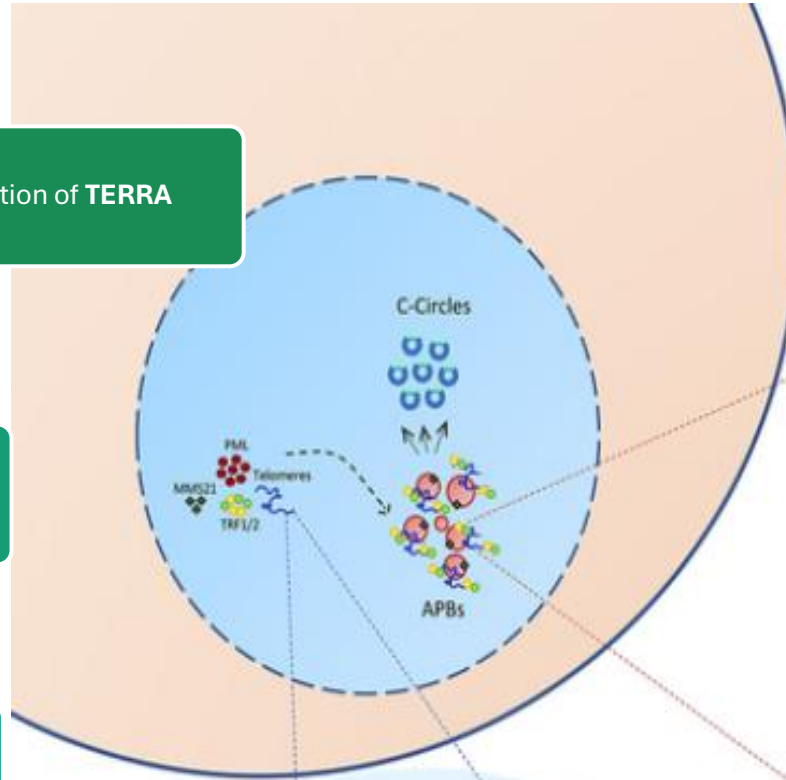
Transcription of **TERRA**

**Stalled Fork and DSB**

Assembly of **APBs (ALT-  
associated PML Bodies )**

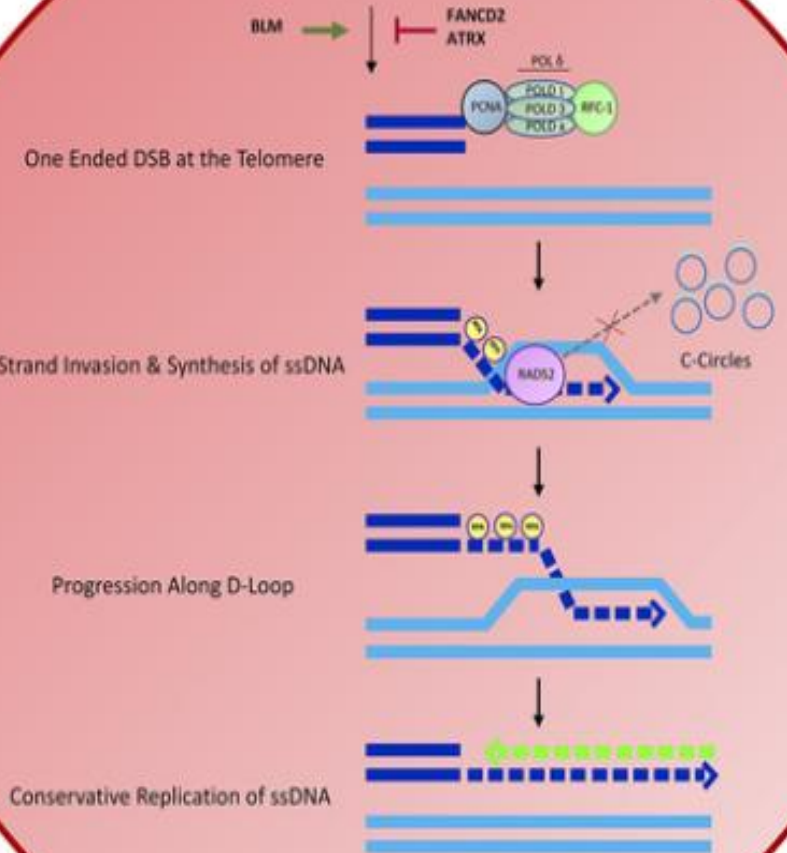
**Homologous Recombination  
(HR)**

Complex recombination  
intermediates are formed →  
**C-Circles**



Replication Stress at Telomeres

## Alternative Lengthening of Telomeres



ALT-Associated PML Bodies (APBs)

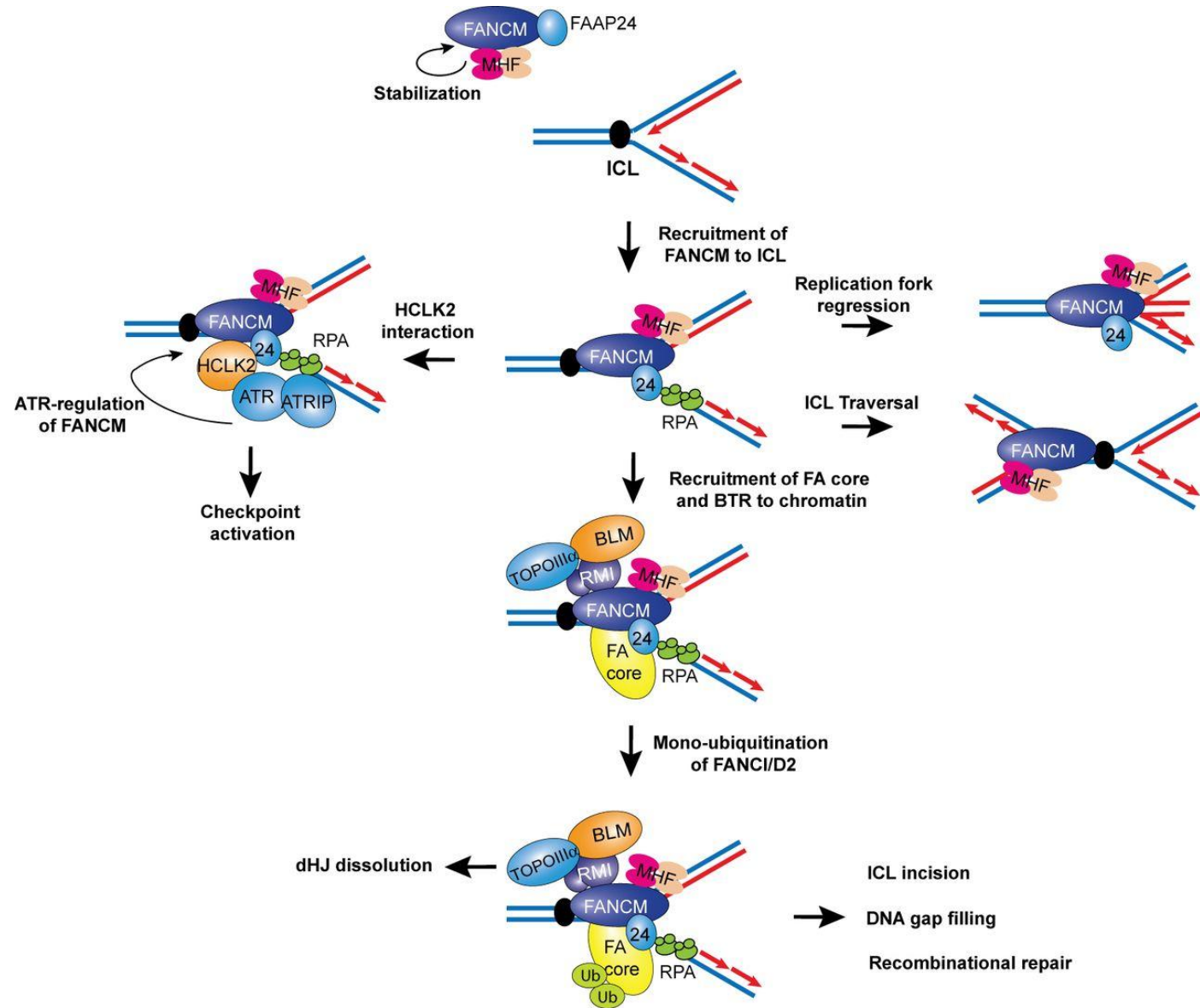
# ALT Mechanism

This ssDNA is immediately coated by the **RPA** protein, which recruits the **ATR** kinase

**ATR** then activates **FA complex** to prevent "fork collapse," a catastrophic event where the replication machinery physically breaks apart

**FANCM** functions as a master sensor and engine for repair. It is anchored to the damage site.

Therefore, **FANCM must maintain replication stress** at a level high enough to allow **BIR**, but not so high as to cause **genomic collapse**.

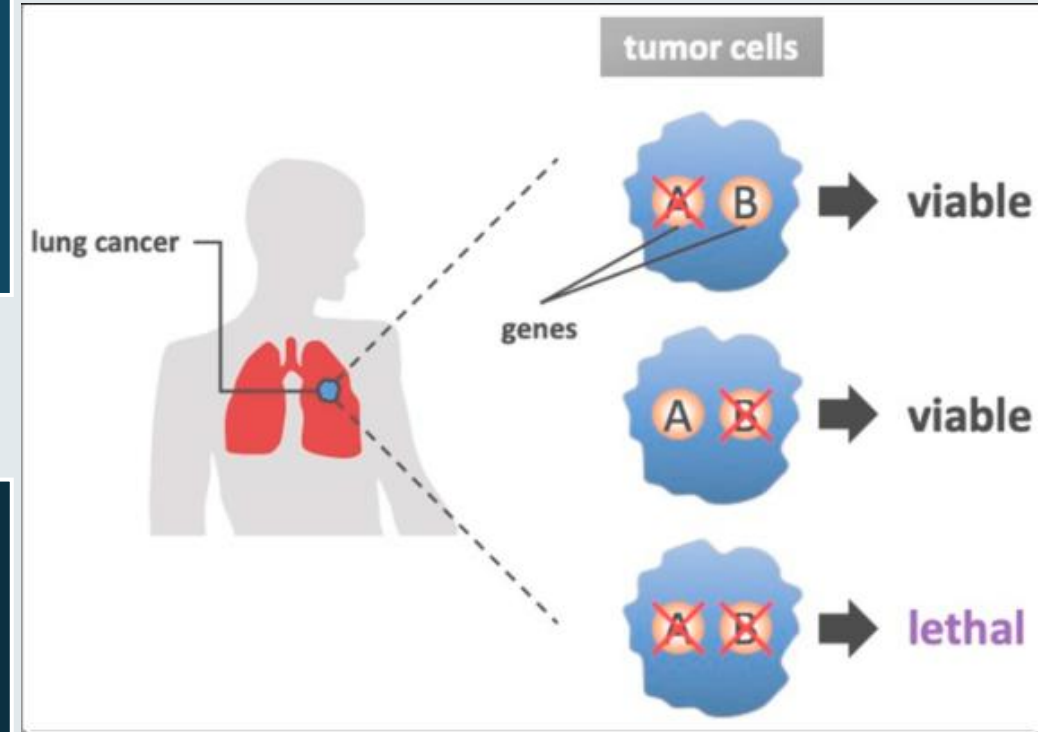


# Synthetic lethality

**Synthetic lethality** occurs when the **simultaneous loss of two genes leads to cell death**, whereas the loss of either gene alone does not. Since cancer cells have already lost one gene due to a mutation



We can use targeted drugs to inhibit its genetic 'partner.'  
This creates a highly selective trap: we kill the tumor while sparing healthy cells.

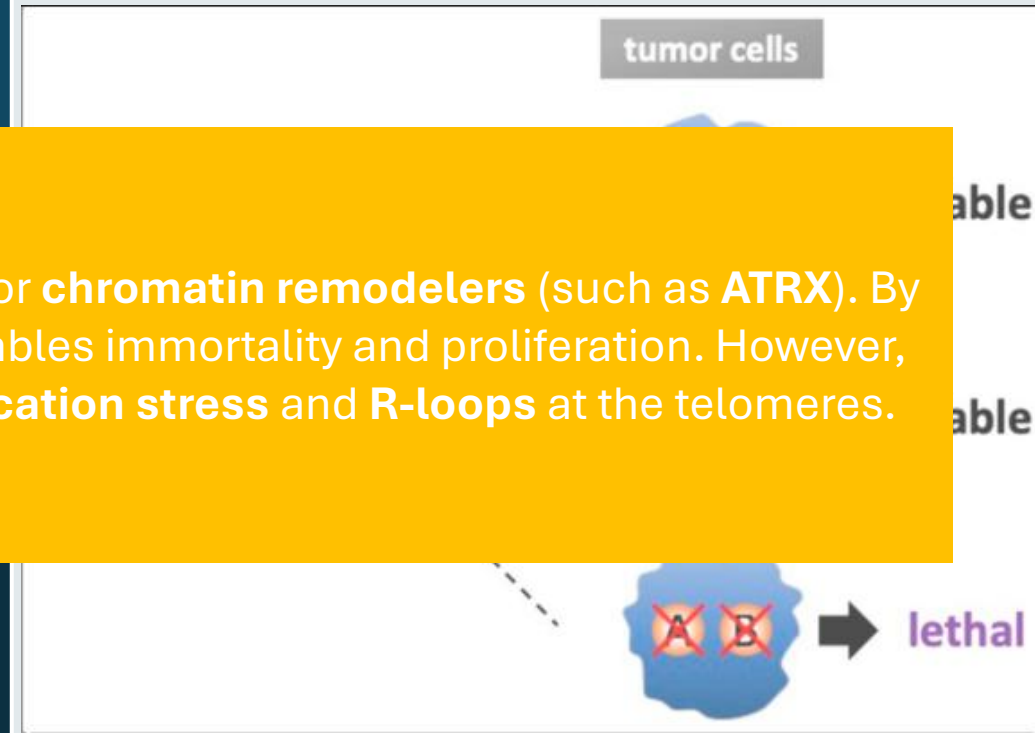


# Synthetic lethality

**Synthetic lethality** occurs when the **simultaneous loss of two genes leads to cell death**, whereas the

In **ALT cancer cells**, the primary 'defect' is the loss of a **helicase** or **chromatin remodelers** (such as **ATRX**). By itself, this condition is not lethal to the cell; on the contrary, it enables immortality and proliferation. However, this creates a significant vulnerability: a massive burden of **replication stress** and **R-loops** at the telomeres.

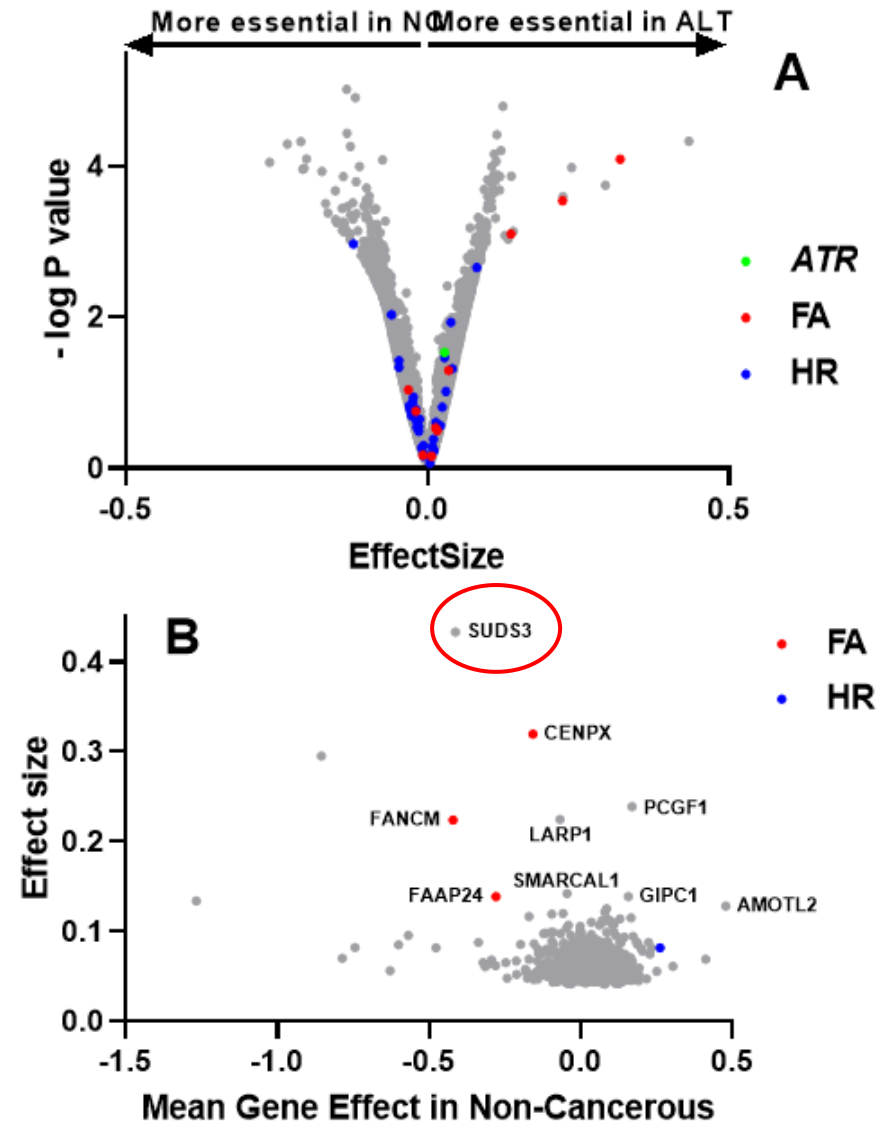
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By analyzing genomic databases, in our laboratory, gene essentiality was compared between ALT-positive cells and normal (non-cancerous) cells.

In **Graph A**, we utilized a **volcano plot** to visualize the differential essentiality of genes. Points shifted to the right (positive Effect Size) represent genes that are **more essential for ALT cells** than for normal cells

**Graph B** highlights specific candidates for **synthetic lethality**. We plotted the effect size in ALT cells against the mean gene effect in non-cancerous models. Genes such as **FANCM**, **FAAP24**, and **CENPX** (all members of the FA complex) show high essentiality in ALT cells while having a minimal effect on normal cell viability. However, another gene, not part of the FA complex but of the Sln3a complex, called **SUD3S**, was singled out for its large effect on ALT but minimal effect on normal cells.



# AIM

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To induce the silencing of **FANCM**, **ATR**, **SUDS3**, **FAAP24**, **MHF1**, and **MHF2** to selectively exploit **replication fork stalling** as a therapeutic vulnerability

## EXPERIMENTAL MODEL

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### Cancer cells (ALT +)



- **U2OS** osteosarcoma
- **SAOS-2** osteosarcoma
- **CAL-78** chondroblastoma
- **RH4** Alveolar rhabdomyosarcoma
- **SK-LU-1** lung adenocarcinoma

### Normal cells



- **HFFF2** human fibroblasts
- **HaCaT** human keratinocytes
- **HUVEC** human endothelial cells

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## EXPERIMENTAL MODEL

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### Cancer cells

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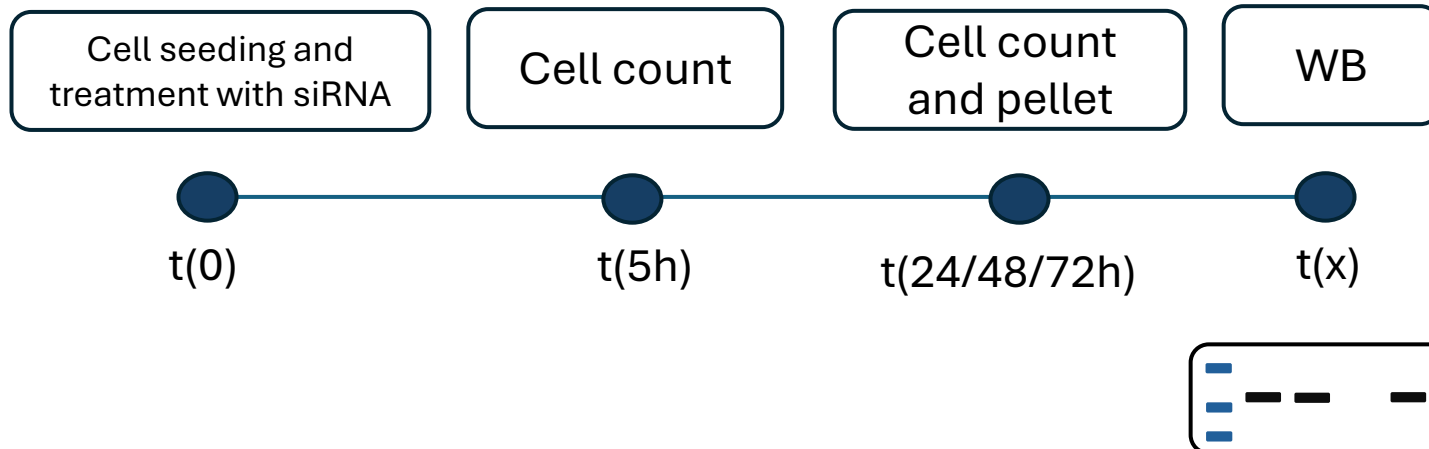
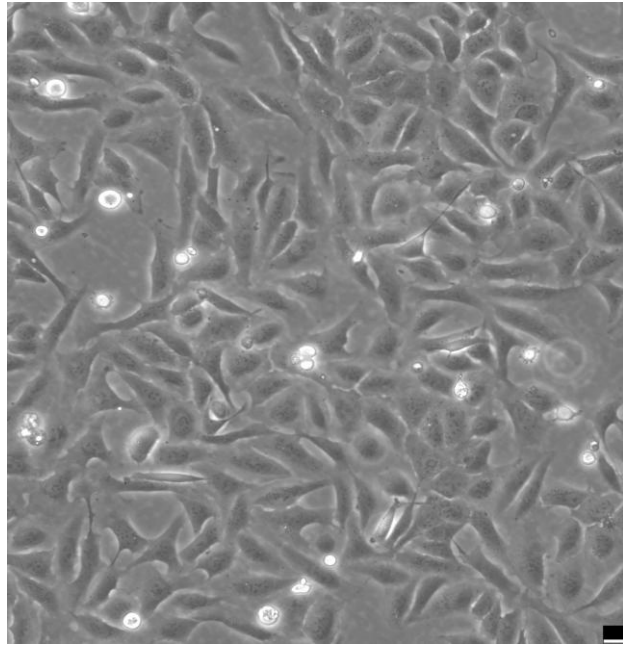
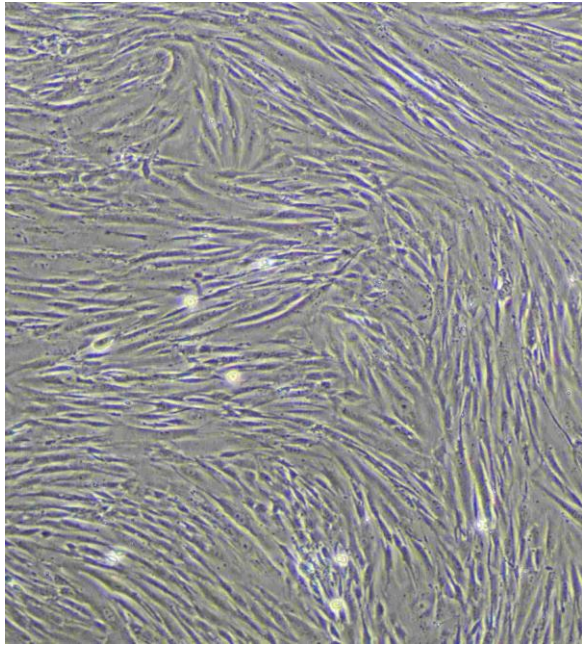
This approach allows us to **evaluate the effects of gene silencing across various genetic backgrounds** of ALT-positive cells and different healthy tissue.

Our goal is to **identify genes** whose deficiency induces **specific lethality** in the **ALT pathway**, sparing healthy tissue. This comparison is **crucial** for defining a safe therapeutic window for **future treatments**.

### Normal cells

- **HaCaT** human keratinocytes
- **HUVEC** human endothelial cells

# First Year



## 1 → Optimization and Validation

I will optimize the **siRNA transfection efficiency** by performing a time-course analysis (24h, 48h or 72h). To ensure the genes are effectively silenced, the **protein knockdown will be confirmed via Western Blot**. This step is crucial to determine the peak of protein depletion before measuring any biological effects

## 2 → Cell Proliferation Assay

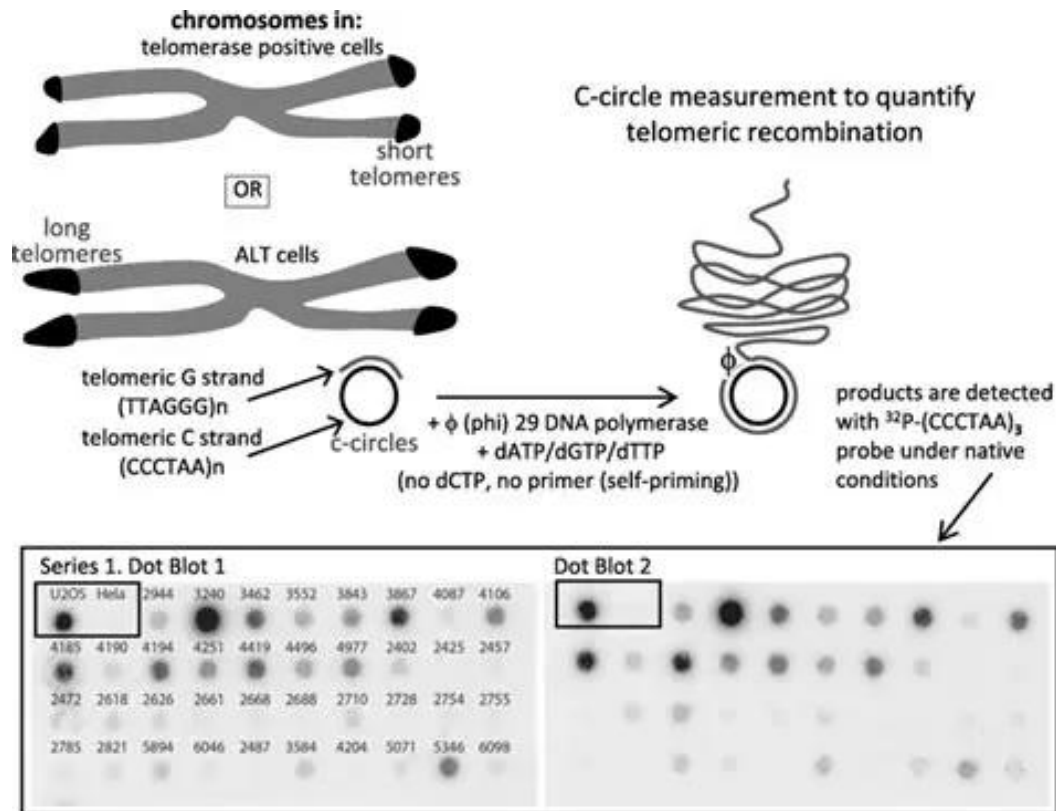
Once the protocol is optimized, I will evaluate the impact of gene silencing on cell growth. Cells will be transfected and counted at two key time points:

- **5 hours post-transfection:** This serves as our **baseline** to verify initial seeding efficiency and cell health after the procedure.
- **24/48/72 hours post-transfection:** to evaluate siRNA effect on cell growth.

## 3 → Data Analysis

The data will be processed to measure the "**lethality**." All counts will be **normalized to the T0 baseline** and expressed as a **fold change** in cell number. By comparing our results to a **non-targeting control (siSCR)**, we can identify which genes selectively reduce the growth of ALT-positive cells while sparing normal cells.

# Second Year



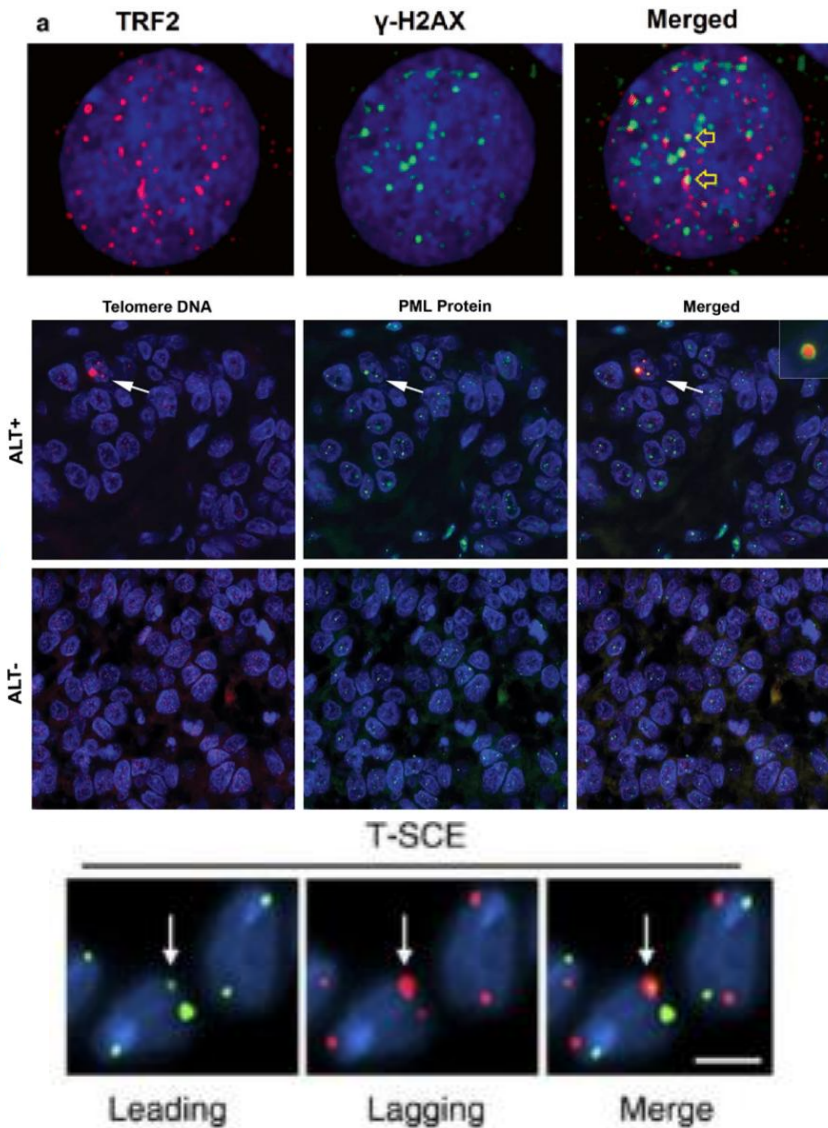
## 1 → Completion and Selection

- **Finish the Screen:** I will complete the cell proliferation assays on all cell lines.
- **Select the "Top Hits":** Based on results, I will choose the genes that show the strongest **synthetic lethality** (high death in ALT cells, low toxicity in normal cells).

## 2 → Non-Radioactive C-circle Assay

- **The Method:** I will set up a **non-radioactive protocol** for the **C-circle** assay. Instead of radioactive isotopes, we will use chemiluminescent probe.
- **How it works:**
  1. The process begins by extracting genomic DNA from cells.
  2. DNA is incubated with the **Phi29 polymerase** enzyme and **DIG-labeled oligonucleotides**. The enzyme **uses C-circles as templates to synthesize** long, repetitive **strands** with a process called **Rolling Circle Amplification (RCA)**.
  3. Amplified DNA is UV-fixed to a nylon membrane detected via **AP-conjugated antibodies**. The resulting **chemiluminescent signal intensity** quantifies the C-circle concentration.
- **The Goal:** To assess whether **silencing our target genes causes a modulation of C-circle levels**, which means telomeres are disintegrating. Recent studies in the literature indicate that **an excessive increase in these C-circle is correlated with a breakdown of the ALT mechanism**.

# Third Year



## 3 → Study of the modulation of other ALT markers after treatment

We will use

**Immunofluorescence (IF)** to visualize:

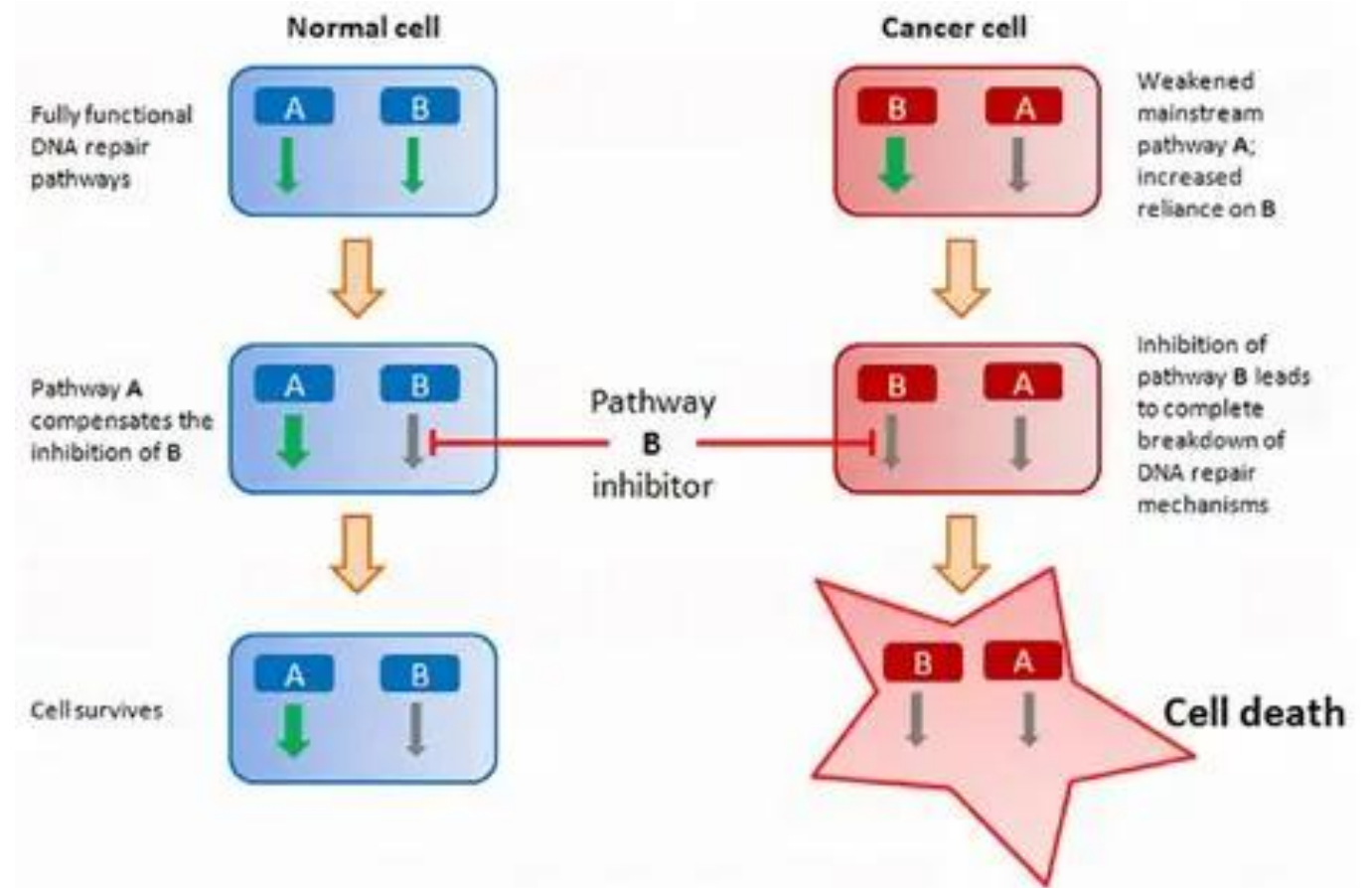
- **TIF Assay (Telomere dysfunction-Induced Foci):** To measure that DSBs (marker:  **$\gamma$ H2AX**) occur specifically at telomeres (marker: **TRF2**).
- **APBs Assay:** To observe how the repair machinery clusters (marker: **PML protein**) around the telomeres (marker: **TRF2**).

**CO-FISH** to analyze sister chromatid exchange after treatment.

**Q-FISH** to analyze alterations in telomere length after treatment.

# In conclusion

To conclude, my research aims to exploit the dependence of ALT cells on the **FA complex**. By comparing normal cells with ALT-positive tumors, I want to identify genes that, when silenced, kill only the cancer cells. This approach, known as **synthetic lethality**, could lead to new treatments that effectively eliminate tumors while **sparing healthy tissues**.



**Thank you for your attention**

# FA complex proteins functions:

## FANCM:

- **Fork Reversal:** Uses ATPase power to convert stalled forks into "chicken foot" structures for protection.
- **HR Dissociation:** Dissociates D-loops to prevent hyper-recombination and telomere collapse.

## FAAP24:

- **Targeting:** Specifically recognizes ss-dsDNA junctions at stalled replication forks.
- **Recruitment:** Acts to anchor FANCM to damaged telomeric sites.

## MHF1/2 (CENPX/S):

- **Binding Enhancer:** increases FANCM's affinity for DNA.
- **Activity Catalyst:** Boosts the efficiency of FANCM-mediated fork reversal.
- **Structural Stability:** Stabilizes the entire complex on branched DNA structures.

# Sin3a complex proteins functions:

## **Sin3A:**

- **Structural Platform:** Acts as the central scaffold for the assembly of the Sin3/HDAC co-repressor complex.
- **Protein Recruitment:** Bridges sequence-specific DNA binding proteins with chromatin-modifying enzymes like HDACs.

## **HDAC1 & HDAC2:**

- **Histone Deacetylation:** Removes acetyl groups from histones, promoting a condensed chromatin.
- **Transcriptional Control:** Regulates the expression of genes involved in DNA repair and cell cycle progression.

## **SUDS3:**

- **Complex Stability:** Essential for the structural integrity and full enzymatic activity of the Sin3-HDAC complex.
- **Functional Bridge:** Connects the Sin3A scaffold with the catalytic HDAC subunits to ensure proper chromatin targeting.

# First Year

**Collection and analysis of scientific literature**

**Cellular culture**

**Establishment of siRNA transfection protocols:** evaluation of 48h vs. 72h incubation periods.

**Cell Proliferation Assay**

**Western Blot**

# Second Year

**Finish the Screen and Select the "Top Hits"**

**Establishment of Non-Radioactive C-circle Assay protocols**

# Third Year

**Immunofluorescence (IF) for TIF and APB**

**Q-FISH**

**CO-FISH**

# ALT Mechanism

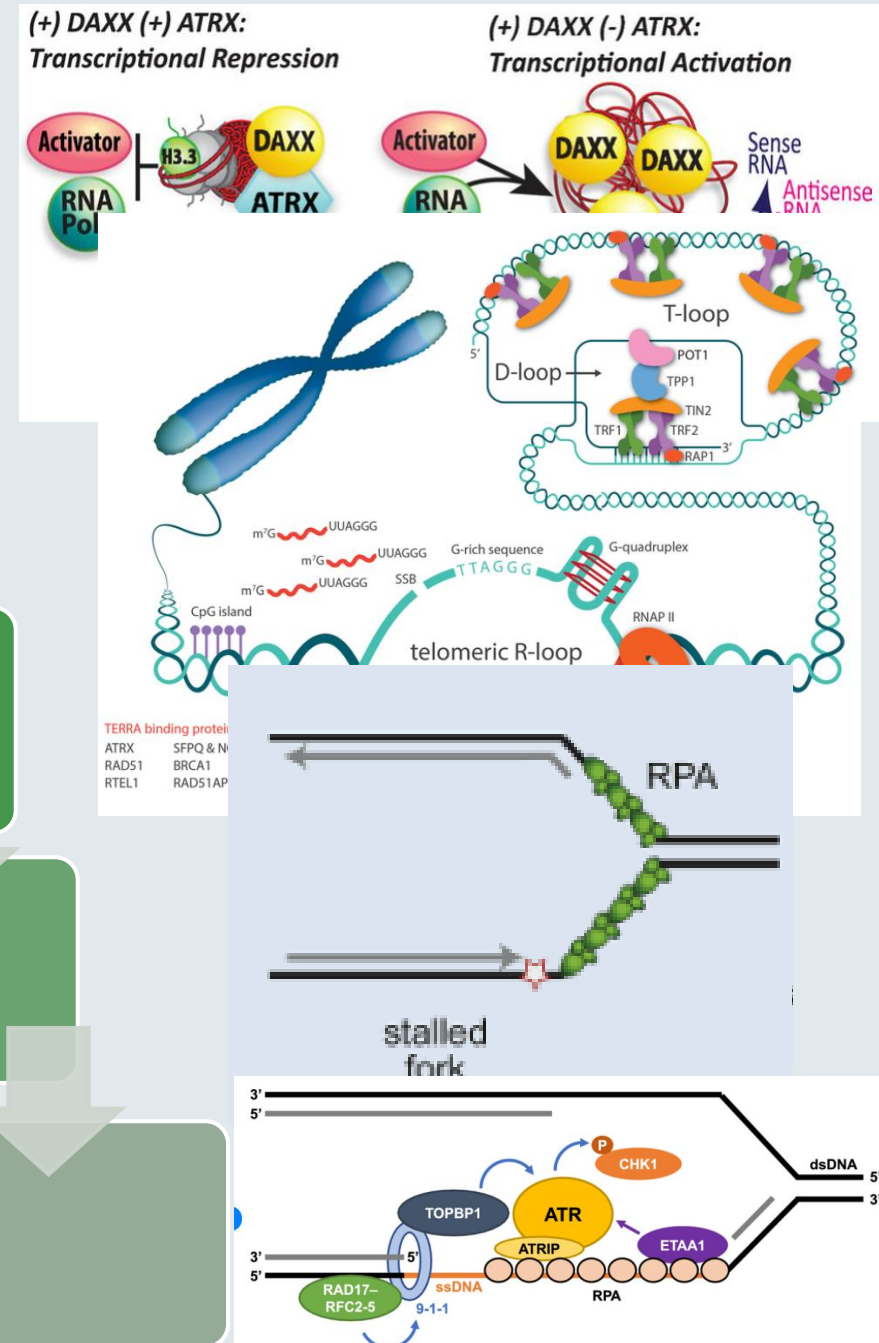
The activation of ALT is closely linked to the loss of **ATRX** or **DAXX**. These proteins normally manage chromatin assembly

Without them, telomeres become unstable and produce high levels of **TERRA** (a long non-coding RNA).

TERRA can invade the DNA to form **R-loops** (RNA:DNA hybrids).

These **R-loops** act as physical barriers that stop DNA polymerase, causing the **replication fork to stall**.

This ssDNA is coated by the **RPA** protein, which recruits the **ATR** kinase.



# ALT Mechanism

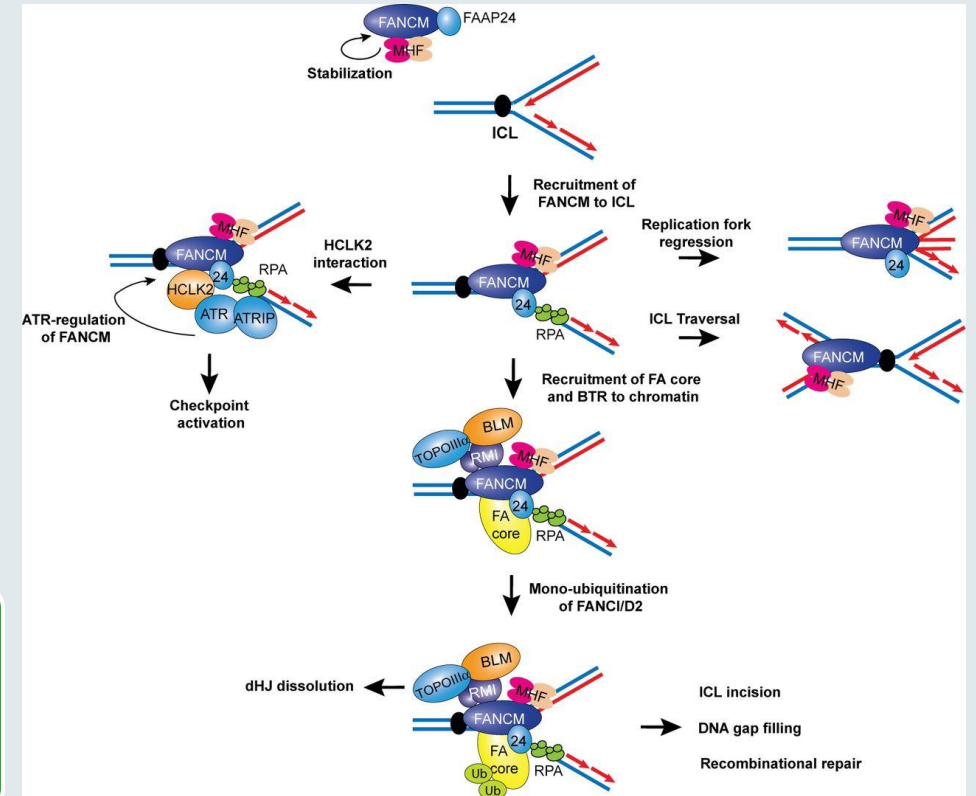
This ssDNA is immediately coated by the **RPA** protein, which recruits the **ATR** kinase

ATR then activates and coordinates **FANCM** to prevent "fork collapse," a catastrophic event where the replication machinery physically breaks apart

**FANCM** functions as a master sensor and engine for repair. It is anchored to the damage site by its partner **FAAP24**, while the **MHF1/MHF2** complex wraps around the DNA to boost FANCM's power to remodel the fork. Furthermore, FANCM interacts with the **Sin3A** complex through **SUDS3** to modulate the local chromatin environment.

By keeping the DNA in a hypoacetylated state, **SUDS3** prevents unwanted **transcription near the stalled fork**, ensuring that no new RNA polymerases collide with the stalled machinery.

Therefore, **FANCM** must maintain replication stress at a level high enough to allow **BIR (Break-Induced Replication)**, but not so high as to cause **genomic collapse**.



- The amplified DNA is loaded onto a nylon membrane and fixed using UV light. After, detect DIG-labeled oligonucleotides with an antibody conjugated to the enzyme alkaline phosphatase which catalyzes a chemiluminescent reaction. We then **measure the signal intensity** of each dot **to quantify the results**. A stronger fluorescent signal means that more C-circles were present in the initial extract.