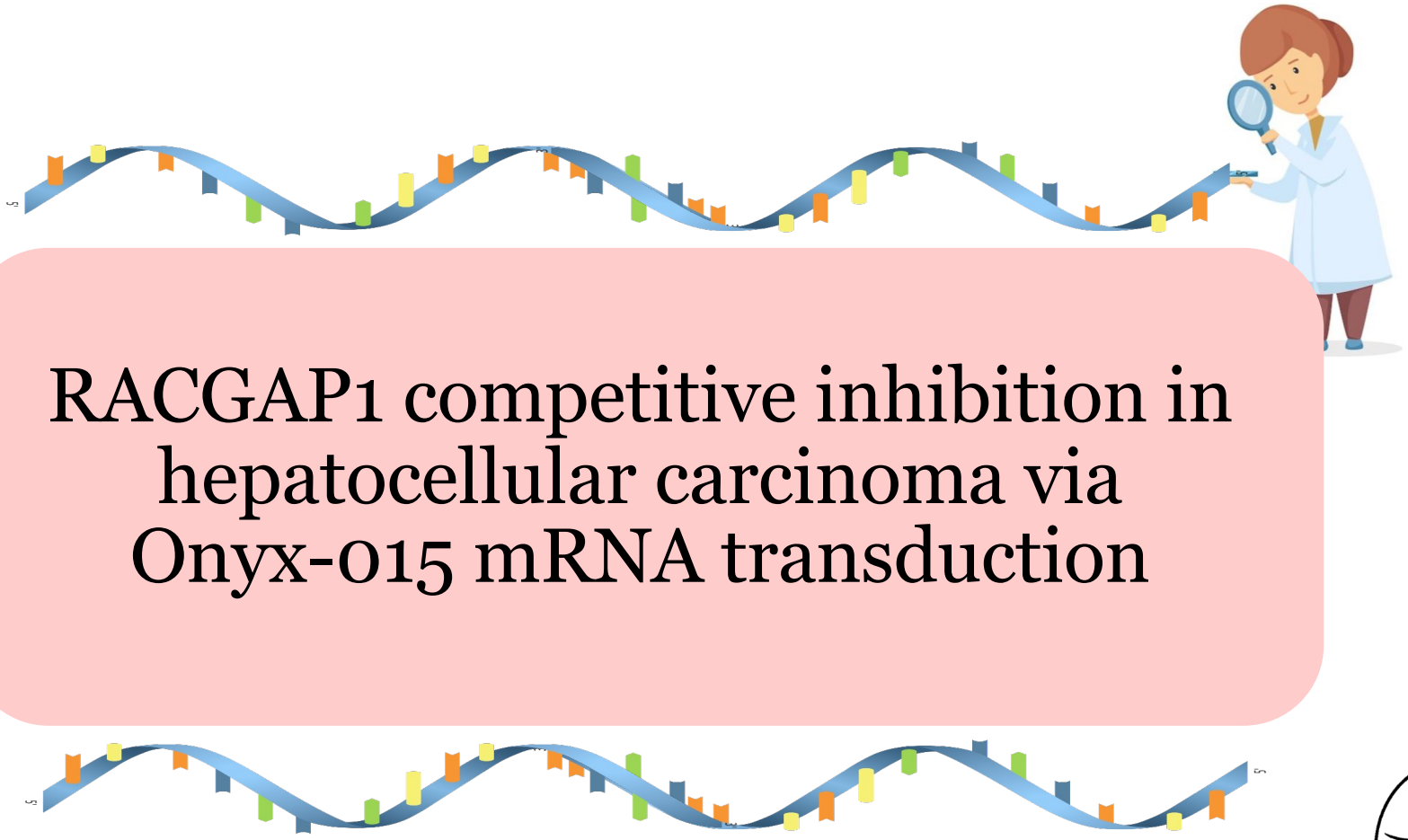


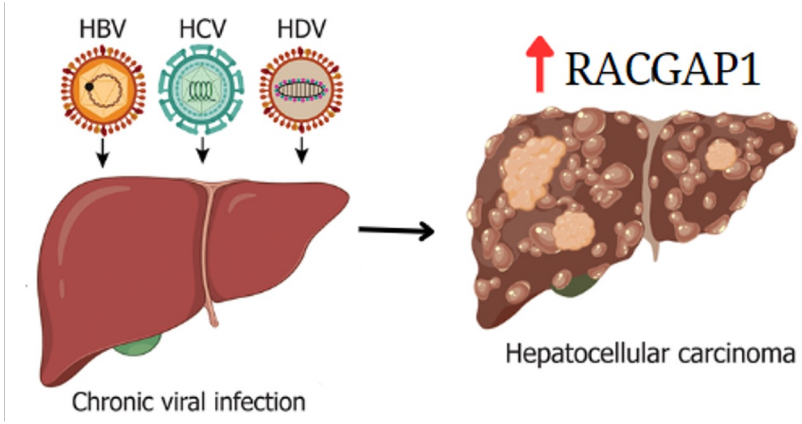
RACGAP1 competitive inhibition in hepatocellular carcinoma via Onyx-015 mRNA transduction

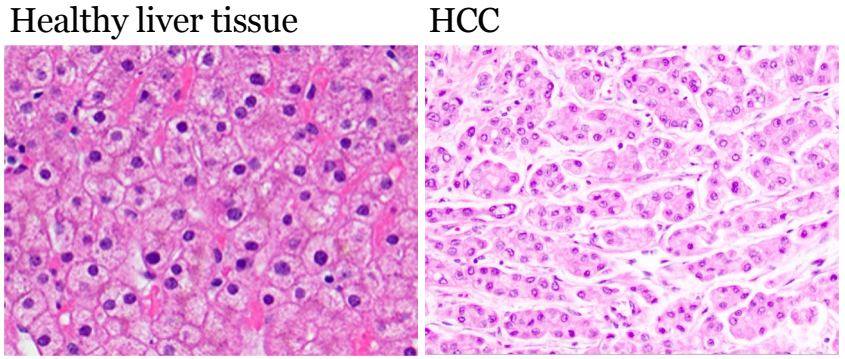
Lavinia Pace
Miriana Santacroce
Ernest Serra

Antonio Duarte
Luigi Fanelli

SAPIENZA
UNIVERSITÀ DI ROMA

Background: Hepatocellular Carcinoma (HCC)



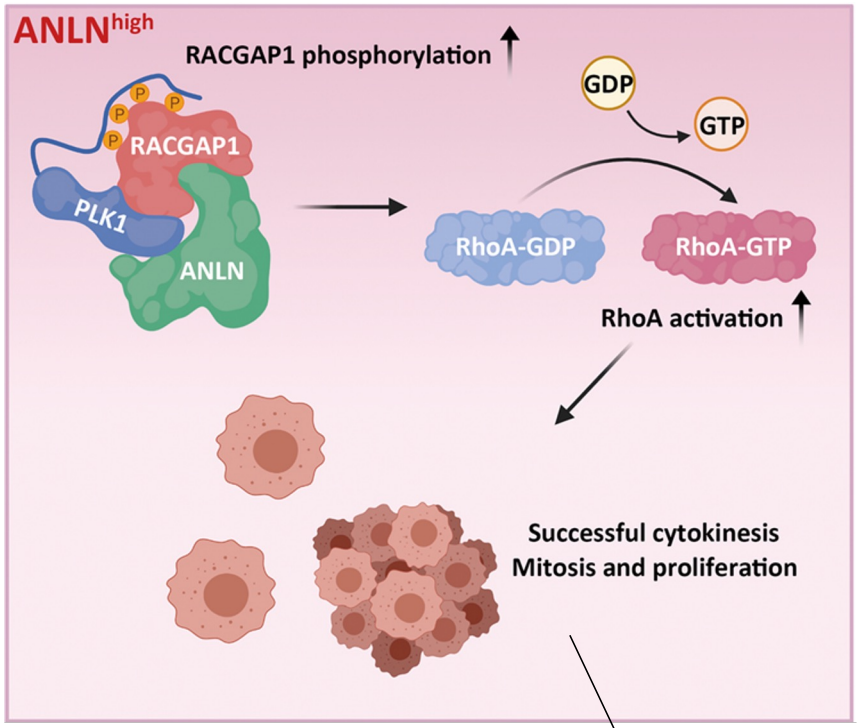


- Liver cancer is the **third most lethal cancer** globally. **Infection by hepatitis B\C** viruses is the main risk factor for HCC development

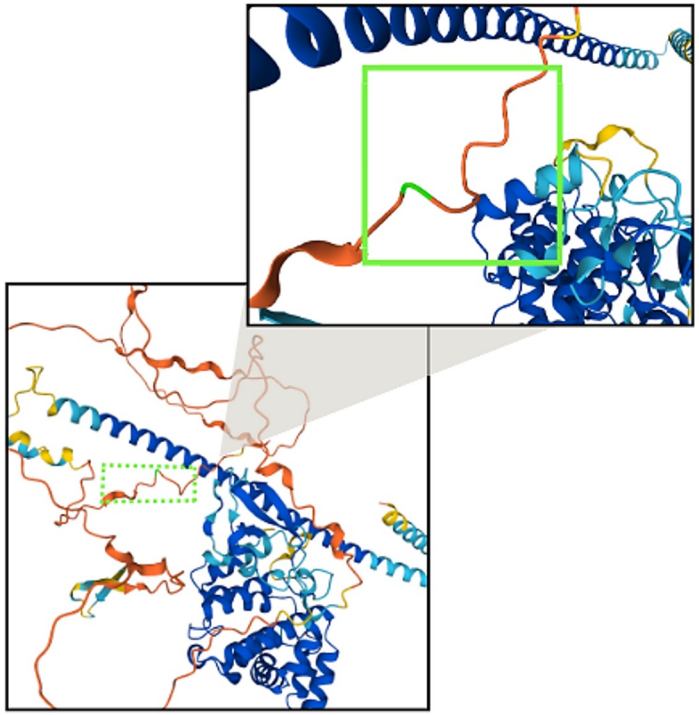
- The median age: > **60** years

- HCC **recurrence** is significantly associated with **RACGAP1 upregulation**: activation of RACGAP1/Rho/ERK signaling axis

Background: RACGAP1 pathway



https://www.nature.com/articles/s41388-022-02274-1#Sec13



RACGAP1 3D structure via Uniprot

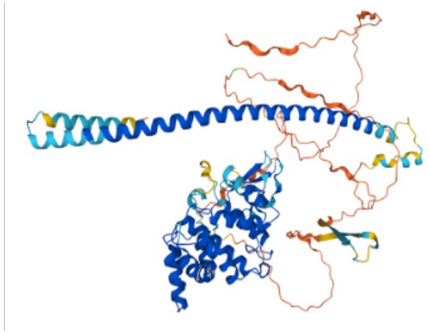
serine 149, 157, 164, 170 phosphorylated by PLK1

Aim of the project

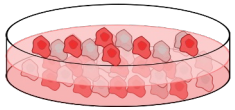
- Induce a **competitive inhibition of RACGAP1** by mutating its phosphorylation sites
- Reduced activation of Rho-A
- Inhibition of self proliferation and decrease in HCC size

How do we do it?

In silico



In vitro



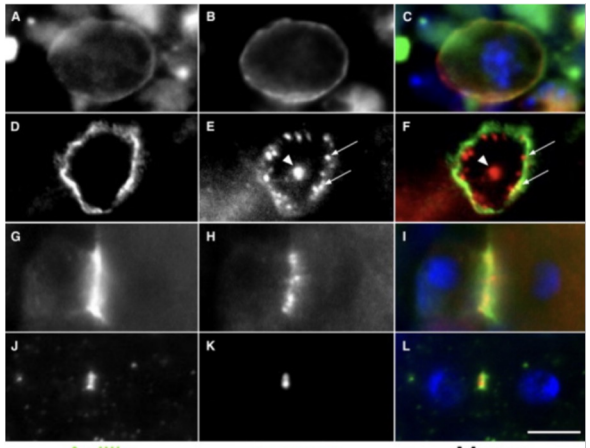
HCCLM3

BALB/c

In vivo

In silico

Amino acid modifications: does the aminoacidic change cause any effects on the protein?



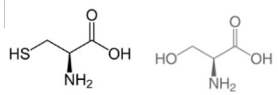
RACGAP1 and ANLN location during cell division
https://www.sciencedirect.com/science/article/pii/S0960982207023354

```
WT          AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE  120
modified    AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE  119
            ************************************************************

WT          QKSALAFLNRGQPSSSNAGNKRLSTIDESGSILSDISFDKTDESLDWDSSLVKTFKLKKR  180
modified    QKSALAFLNRGQPSSSNAGNKRLSTIDECGSILSDICFDKTDECLDWDSCLVKTFKLKKR  179
            ****************************.*******.******.******.**********

WT          EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP  240
modified    EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP  239
            ************************************************************
```

MSA, ClustalW from ebi
https://www.ebi.ac.uk



Cysteine Serine

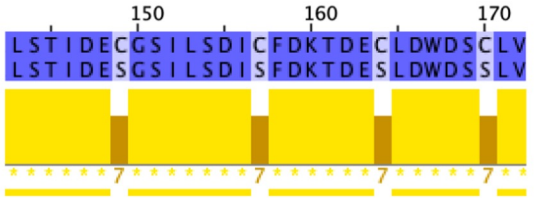
Intracellular proteins: Aminoacid effect changes on serine

Neutral → Cys (o) Asp (o) Glu (o) Lys (o) Gly (o) His (o) Asn (o) Pro (o)
Gln (o) Arg (o) Ala (o) Thr (o)

http://www.russelllab.org/aas/Ser.html

RACGAP1_HUMAN_MODIFIED_SEQUENCE
sp|Q9H0H5|RGAP1_HUMAN

Conservation

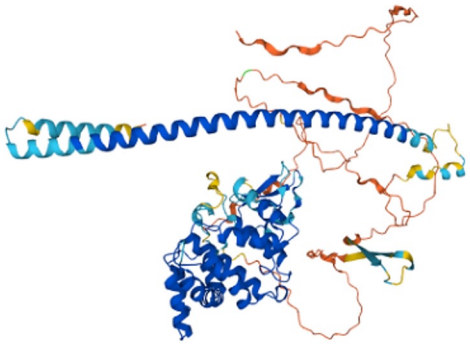


Number of conserved physico-chemical properties /10
https://www.jalview.org/help/html/calculations/conservation.html

In silico

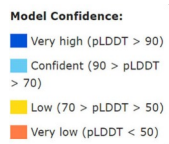
Structural predictions

A WT RACGAP1



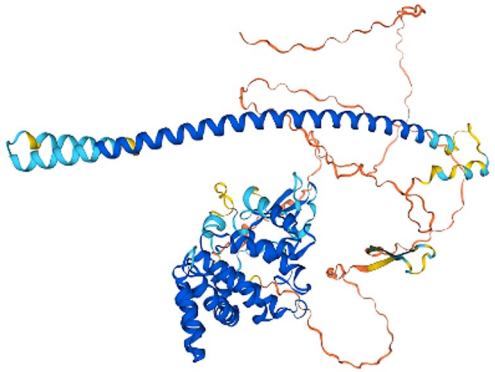
Structure prediction via alphaphold

https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer



https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer

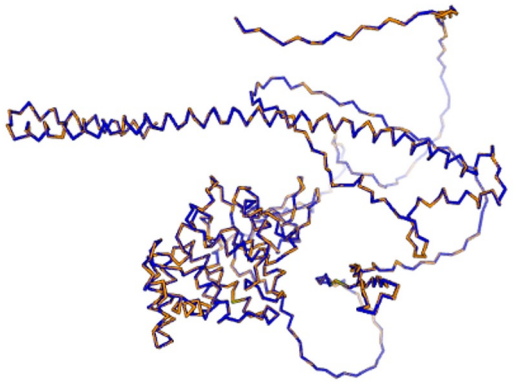
B RACGAP1(UGU)



Structure prediction via Swissprot

https://swissmodel.expasy.org/interactive/Xk9YBQ/models/

C WT RACGAP1/RACGAP1(UGU)



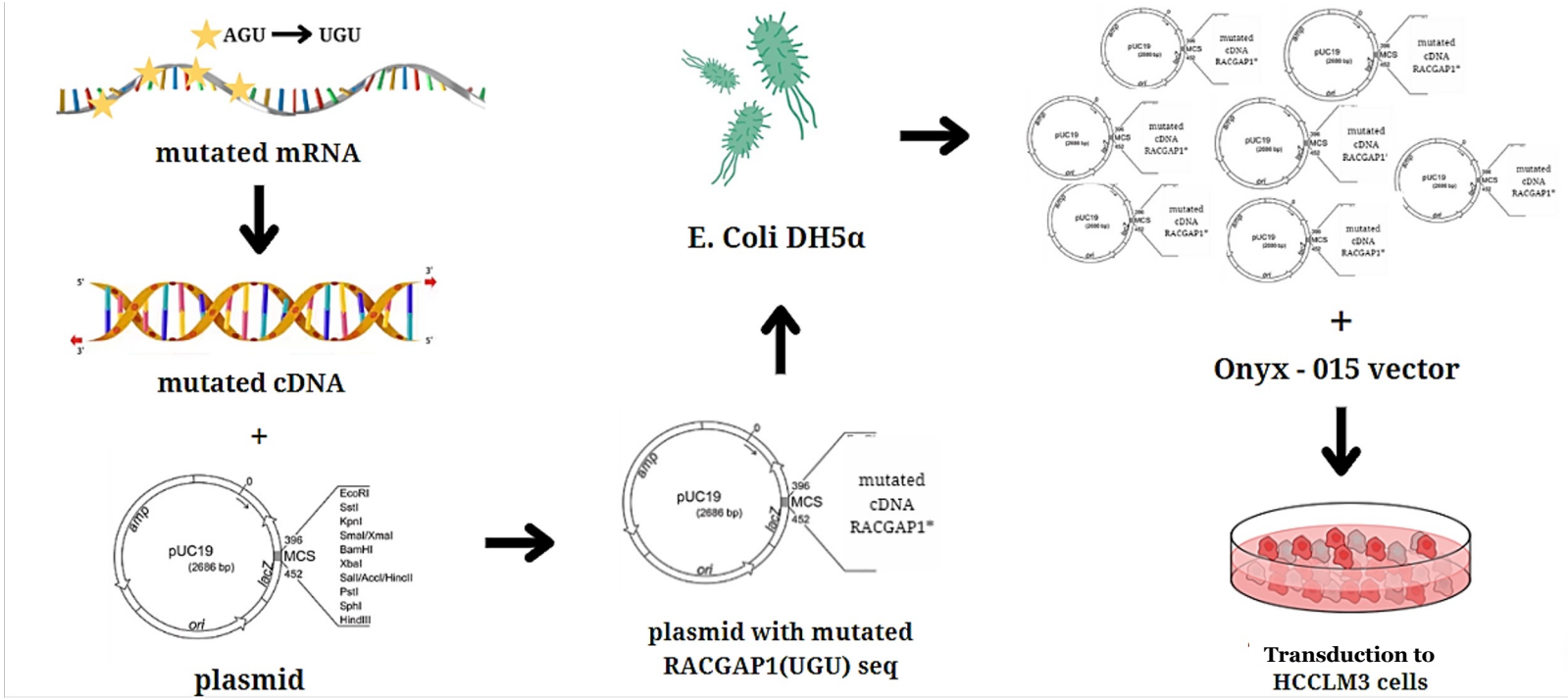
3D structure superposition via DALI

Legend: **Structure conservation**
Dark blue regions are structurally aligned
http://ekhidna2.biocenter.helsinki.fi/dali/DaliTutorial.pdf

Z-score=46.8
Significant similarities' have a Z-score above 2
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2639270/

In vitro

Cloning and transduction of RACGAP1(UGU) mRNA



In vitro

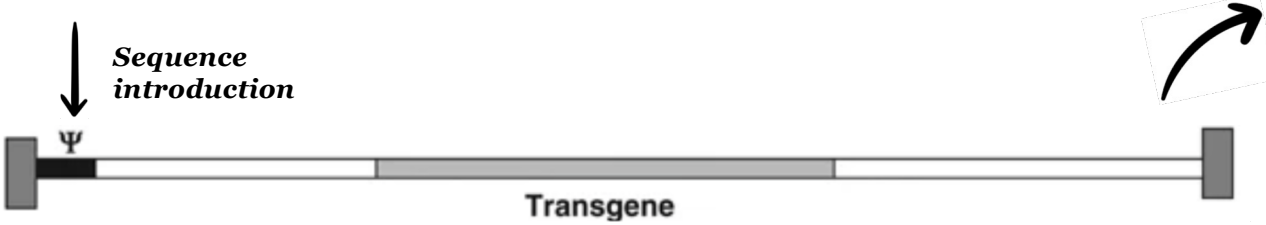
AF-20 Mab specific targeting for HCC

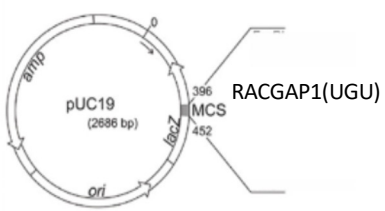
https://www.gastrojournal.org/article/S0016-5085(04)01616-6/fulltext?referrer=https%3A%2F%2Fpubmed.ncbi.nlm.nih.gov%2F

Also specific for HHCLM3 cell line

https://onlinelibrary.wiley.com/doi/10.1002/smll.201702037

How is the vector designed?





Introduction of RACGAP1(UGU)

```
WT          AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE  120
modified    AETERSALDVKLKHARNQVDVEIKRRQRAEADCEKLERQIQLIREMLMCDTSGSIQLSEE  119
            ************************************************************

WT          QKSALAFLNRGQPSSSNAGNKRLSTIDESGSILSDISFDKTDESLDWDSSLVKTFKLKKR  180
modified    QKSALAFLNRGQPSSSNAGNKRLSTIDECGSILSDICFDKTDECLDWDSCLVKTFKLKKR  179
            ***************************.*******.******.*****.**********

WT          EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP  240
modified    EKRRSTSRQFVDGPPGPVKKTRSIGSAVDQGNESIVAKTTVTVPNDGGPIEAVSTIETVP  239
            ************************************************************
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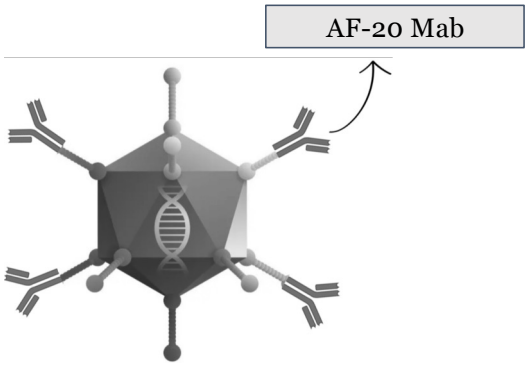
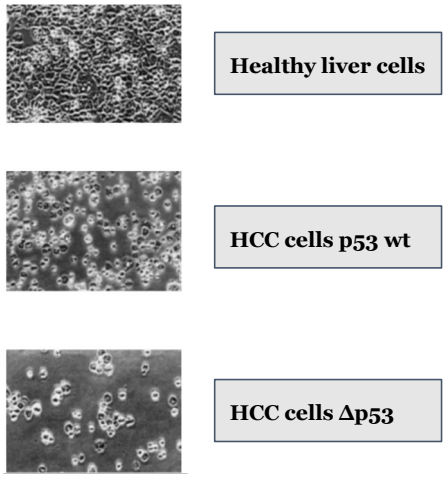




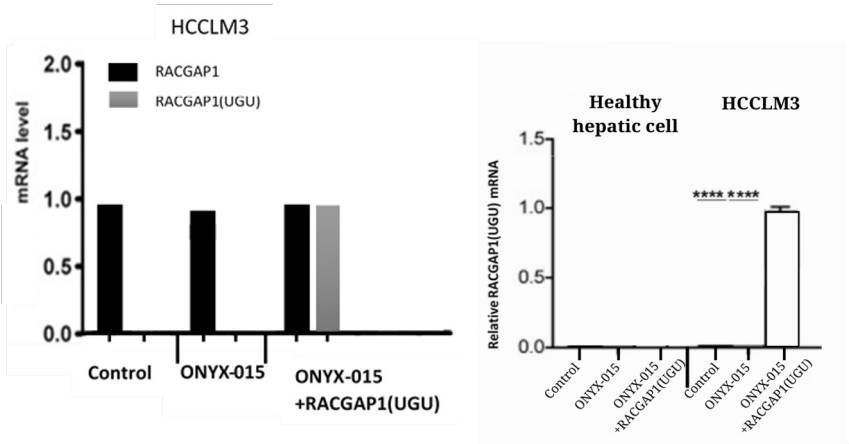
ONYX-015 + RACGAP1(UGU) effect on different cells

https://images.app.goo.gl/86RRRzDvhZAAyWtd6

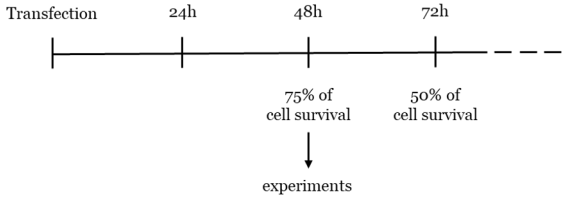
In vitro

Is RACGAP1(UGU) expressed?

1.

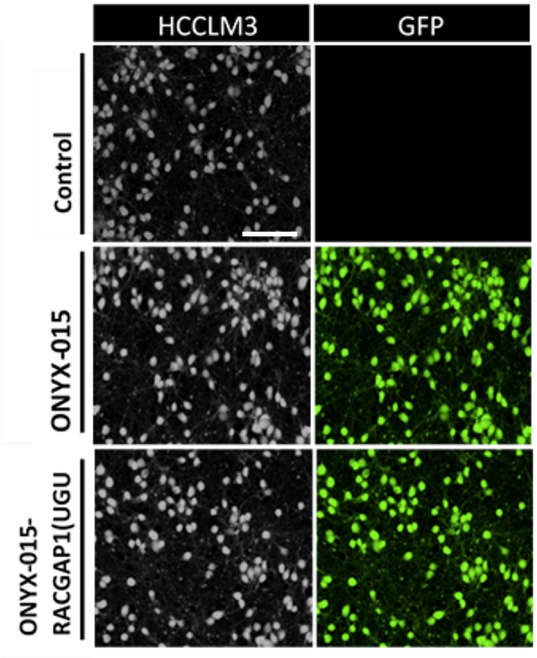


RT-PCR - Expression of RACGAP1 and RACGAP1(UGU) in HCCLM3 cells. Expression of RACGAP1(UGU) in healthy and tumoral cell



Experiments carried out after 48h using onyx-015
https://www.sciencedirect.com/science/article/pii/S153561080400337X#FIG5
https://academic.oup.com/jcem/article/87/6/2525/2846729#59549491

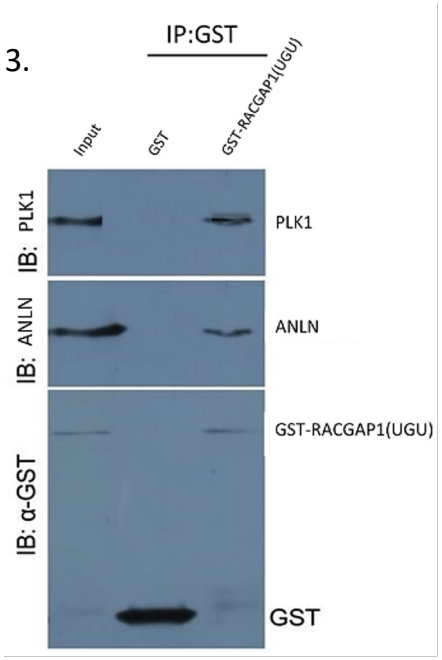
2.



Immunofluorescence assay-GFP expression in HCCLM3 treated with ONYX-15-GFP and ONYX-15-RACGAP1(UGU)-GFP

Does the RACGAP1(UGU)-ANLN-PLK1 complex form?

3.

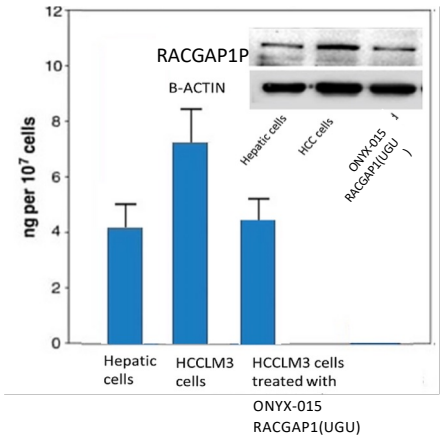


Pull down- RACGAP1(UGU)-ANLN-PLK1 complex formation

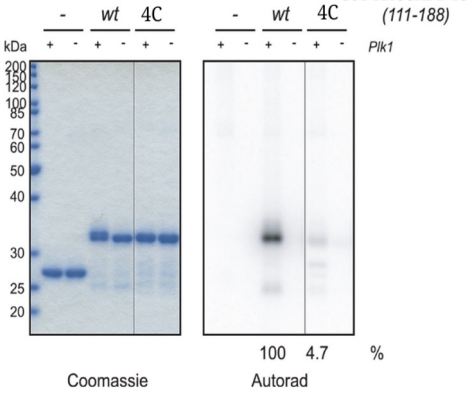
In vitro

Is RACGAP1(UGU) phosphorylated?

1.



2.

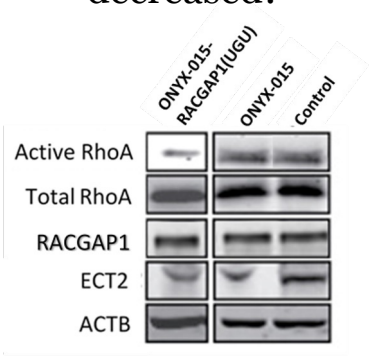


Phosphorylation assay - ELISA and Western Blot - Normal levels of RACGAP1P in healthy cells, elevetated levels of RACGAP1P in HCCLM3, reduced levels of RACGAP1P in treated HCCLM3

GST(-), GST-RACGAP1-111/188 (Wt), and GST-RACGAP1-4C-111/188 (4C) were incubated with Plk1 and [γ-32P] ATP. Relative incorporation of 32P to GST-RACGAP1 was quantified. (111-188=peptide from amino acid 111 to 188)

Is RohA activity decreased?

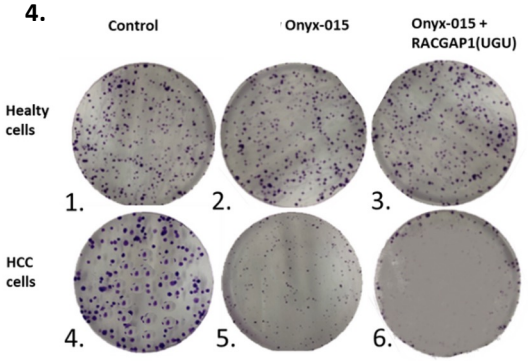
3.



Western blot - Detection of RhoA activity and also ECT2 and RACGAP1 expression in HCCLM3 after trasfection of RACGAP1(UGU)
Adapted from (Yang et al., 2018)

What happens to the cells?

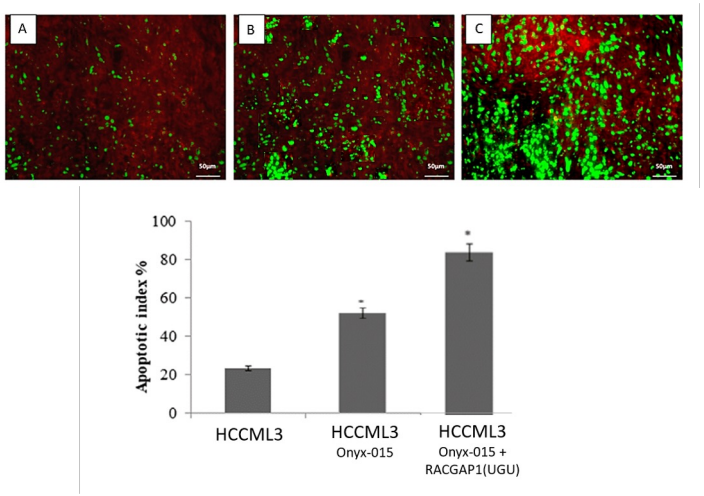
4.



Clonogenic assay:
1. Healthy Hepatic cells,
2. Healthy Hepatic cells with transduction of empty Onyx-015,
3. Healthy Hepatic cells with transduction of RACGAP1(UGU) mutated protein,
4. Hepatocarcinoma HCCLM3 cells,
5. Hepatocarcinoma HCCLM3 with transduction of empty Onyx-015,
6. Hepatocarcinoma HCCLM3 with transduction of RACGAP1(UGU) mutated protein,

In vitro

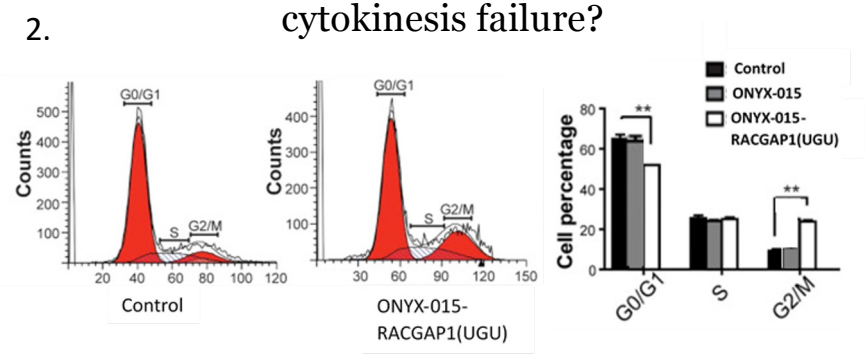
1. Does RACGAP1(UGU) cause apoptosis?



TUNEL assay - **A.** HCCLM3 non treated and no apoptotic cells are detected. **B.** HCCLM3 treated with emptyvector, no apoptotic cells are detected **C.** HCCLM3 treated with the mutated RACGAP1(UGU), incresead levels of apoptotic cells

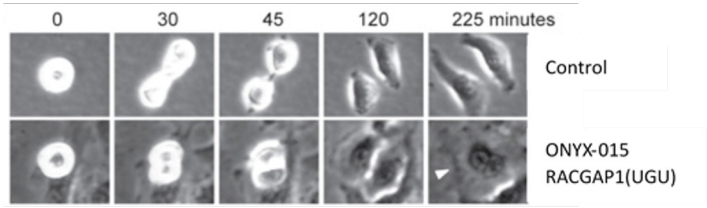
Adapted from
https://www.researchgate.net/publication/335679404_In_Vivo_Anti-Tumor_Effects_of_Citral_on_4T1_Breast_Cancer_Cells_via_Induction_of_Apoptosis_and_Downregulation_of_Aldehyde_Dehydrogenase_Activity

2. Does RACGAP1(UGU) cause cytokinesis failure?



Cell cycle analysis - **1.** Cell count in different cell cycle phases, RACGAP1(UGU) vs control HCCLM3 cells.
2. Cell percentage in different cell cycle pahses, RACGAP1(UGU) vs control HCCLM3 cells.

3.



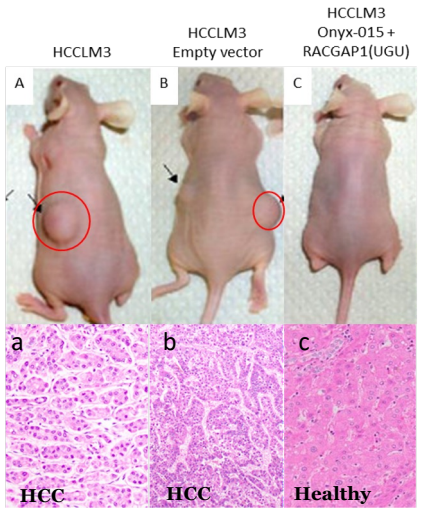
Cytokinesis analysis - Selected frames from time-lapse imaging of ONYX-015-RACGAP1(UGU) and control HCCLM3 cells

Adapted from Adapted from (Yang et al., 2018)

In vivo

Is there a change in the tumor mass?

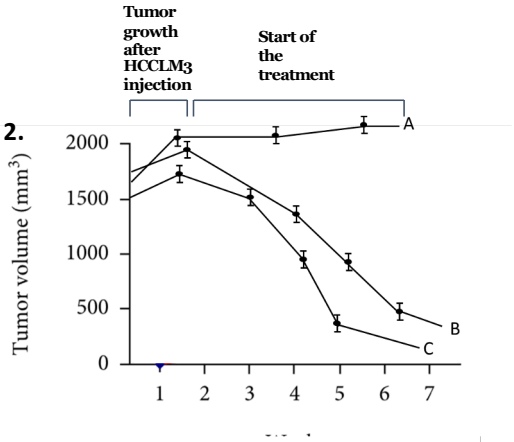
1.



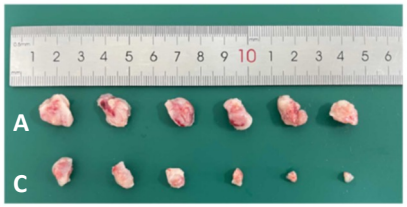
Tumor detection in BALB/c mouse injected with **A.** HCCLM3 cells; **B.** HCCLM3 cells + empty vector; and **C.** HCCLM3 cells + onyx-015 + RACGAP(UGU) and **relative histological samples.**

Adapted from https://bmccancer.biomedcentral.com/articles/10.1186/1471-2407-11-425/figures/7

2.



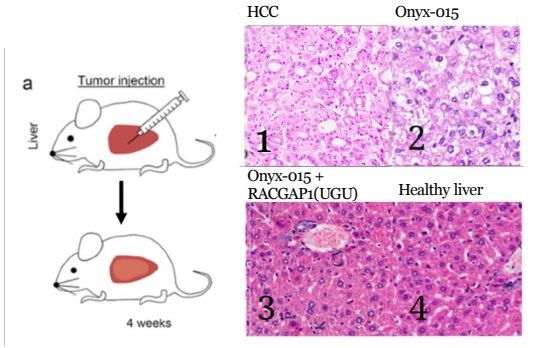
3.



2.Analysis of tumor size on BALB/c mice injected with
A. HCCLM3
B. HCCLM3 treated with empty vector
C. HCCLM3 treated with onyx-015 + RACGAP(UGU)

3.Representative images of tumors removed from BALB/c mice from samples A and C

4.



HCC histological samples from C57BL/6 mice
1. HCC tissue
2. Tissue sample injected with empty vector
3. Tissue sample injected with RACGAP1(UGU) vector
4. Healthy liver tissue

Adapted from https://pubmed.ncbi.nlm.nih.gov/15649325/

https://www.hindawi.com/journals/omcl/2022/3034150/

Project budget

Cloning and transduction

mRNA → $5 640 ($10/RNA base)
plasmid → $94
vector for in vitro and vivo → $2670
sequencing → $75
Total → $8479

In vitro

- reverse transcription → $490
- cell line→ $700
- plasmids→ $800
- qPCR → $800
- co-ChIP → $700
- Elisa → $700
- Western blot → $700
- clonogenic assay→ $200
- TUNEL→ $600
- cell cycle analysis→ $350
- **Total→ $6040**

In vivo

4 BALB/c nude mouse per group x3 (36 tot) → $2160
4 C57BL/6 mouse x3 (36 tot)→ $720
Mice maintenance → $10 000
Total → $2880

Salaries

3 PhD → $60 000/year
2 post DOC → $ 50 000/year
Total → $110 000/year

Total for three years of work
$357 399

Pitfalls and solutions

What is the problem?	How do we solve it?
Low specificity of infection	Enhancement of cancer cell targeting
Overcoming already existing siRNA therapies	siRNAs can have off-targets and higher immunogenicity, while our protein endogenously exist in human
Possibility of introducing the RACGAP1(UGU) like a drug	More specificity towards the target with our therapy, more efficiency with the ONYX-015 coupled therapy

References

Chen, J., Li, Z., Jia, X., Song, W., Wu, H., Zhu, H., Xuan, Z., Du, Y., Zhu, X., Song, G., Dong, H., Bian, S., Wang, S., Zhao, Y., Xie, H., Zheng, S., Song, P., 2022. Targeting anillin inhibits tumorigenesis and tumor growth in hepatocellular carcinoma via impairing cytokinesis fidelity. Oncogene 41, 3118–3130. https://doi.org/10.1038/s41388-022-02274-1

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RACGAP1 - Rac GTPase-activating protein 1 - Homo sapiens (Human) | Feature viewer | UniProtKB | UniProt [WWW Document], n.d. URL https://www.uniprot.org/uniprotkb/Q9H0H5/feature-viewer (accessed 11.29.23).

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