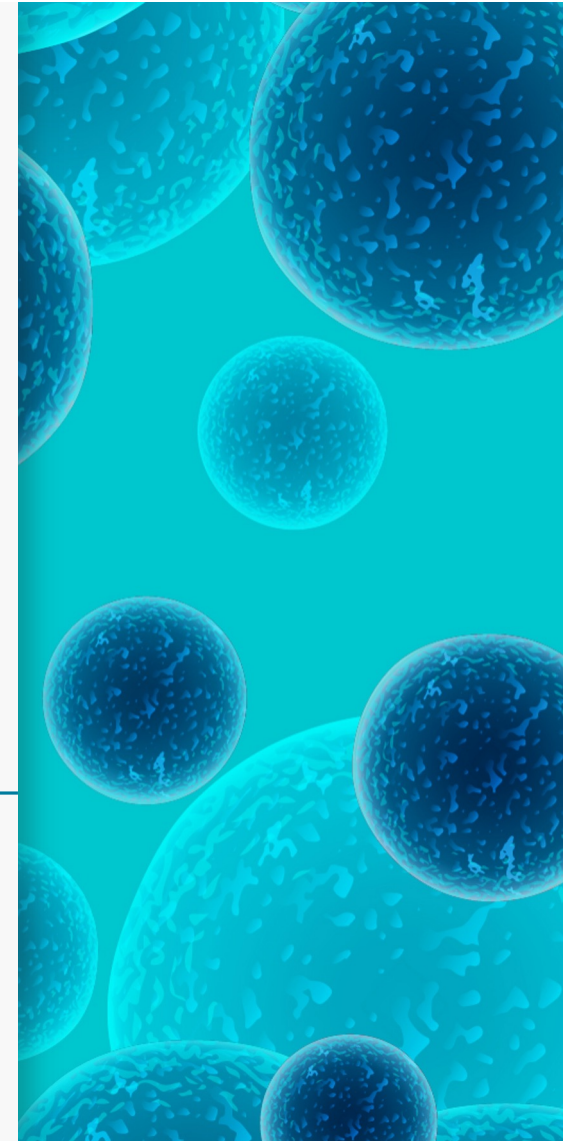


# **OTRS-en-CRISPRi:**

**A novel therapeutic approach in  
Anaplastic Thyroid Cancer treatment**

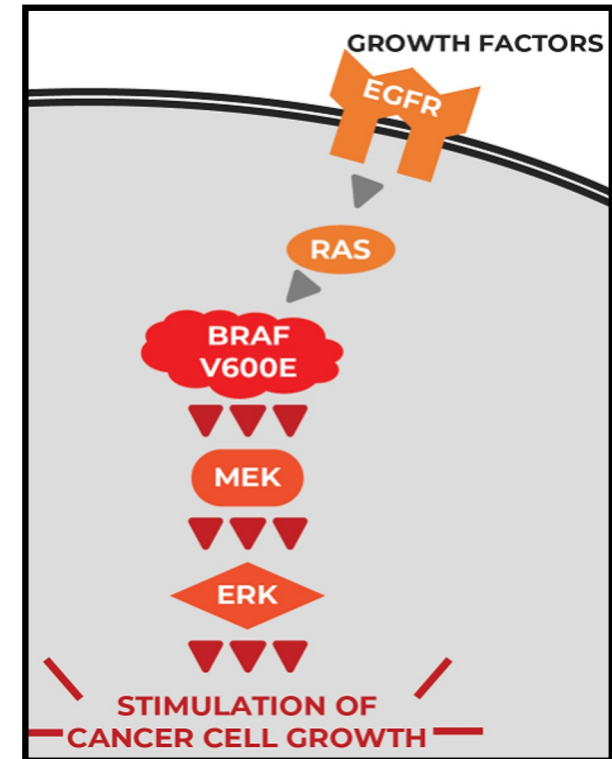
Gabriele Virgilio, Alessandro Belvedere,  
Emanuela Unhe Jeong, Nashon Majaliwa



# Background

**Anaplastic thyroid cancers (ATCs) are highly aggressive tumors and account for 30% of thyroid cancer deaths.**

- The ***BRAF*<sup>V600E</sup> point mutation** is a common early molecular event and is detected in **45%** of ATC cases;
- Cancer cells expressing BRAF V600E are **less responsive to radioactive iodine therapy** due to **downregulation** of the sodium iodide symporter (**NIS**);
- BRAF chemical inhibitors can cause **paradoxical BRAF activation**, leading to the formation of secondary cancers.

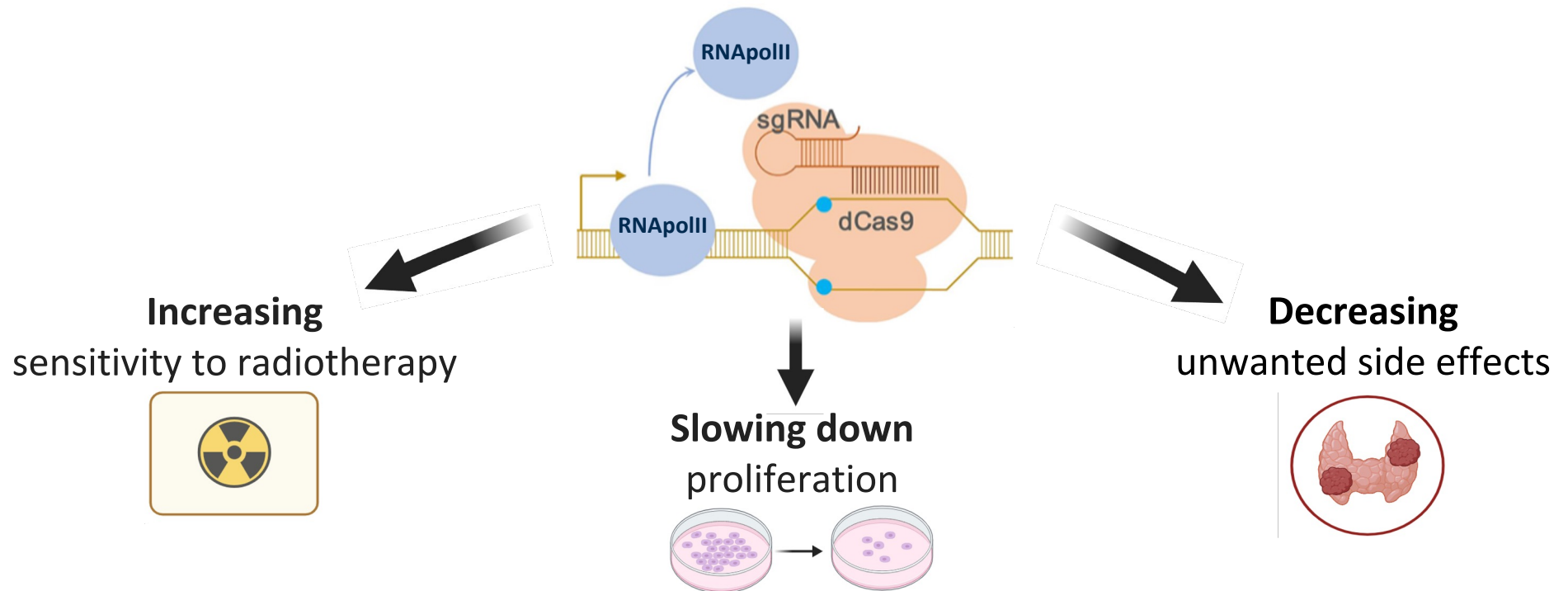


By NCI staff, 2019

**BRAF** is a proto-oncogene involved in the activation of the MAPK pathways, which regulates cell proliferation.

# Aim of the project

Suppress *braf* transcription through  
Off Target Regulatory Sequence-enhanced-CRISPRi (OTRS-en-CRISPRi)



# OTRS-en-CRISPRi: a novel strategy

## Classical CRISPRi

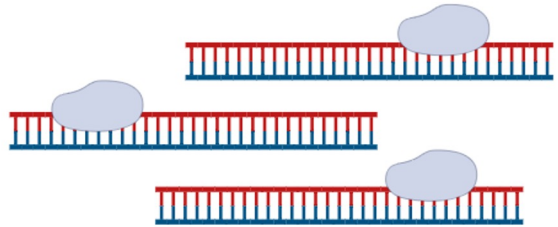
braf promoter  
(target)



Construct

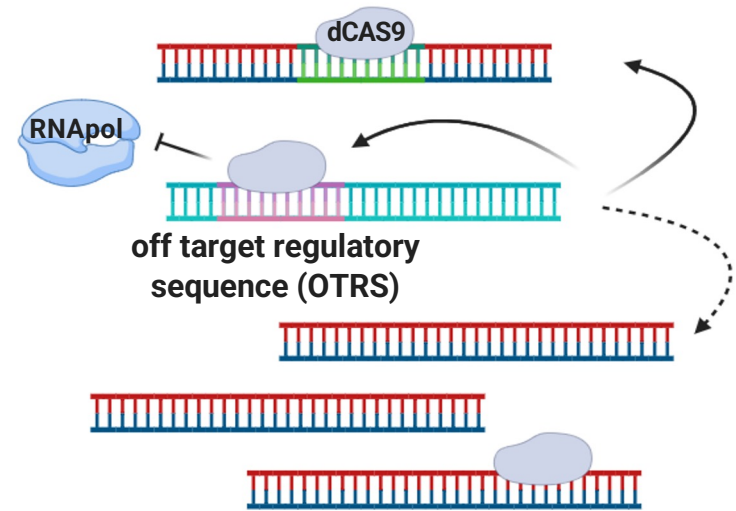


Off-targets



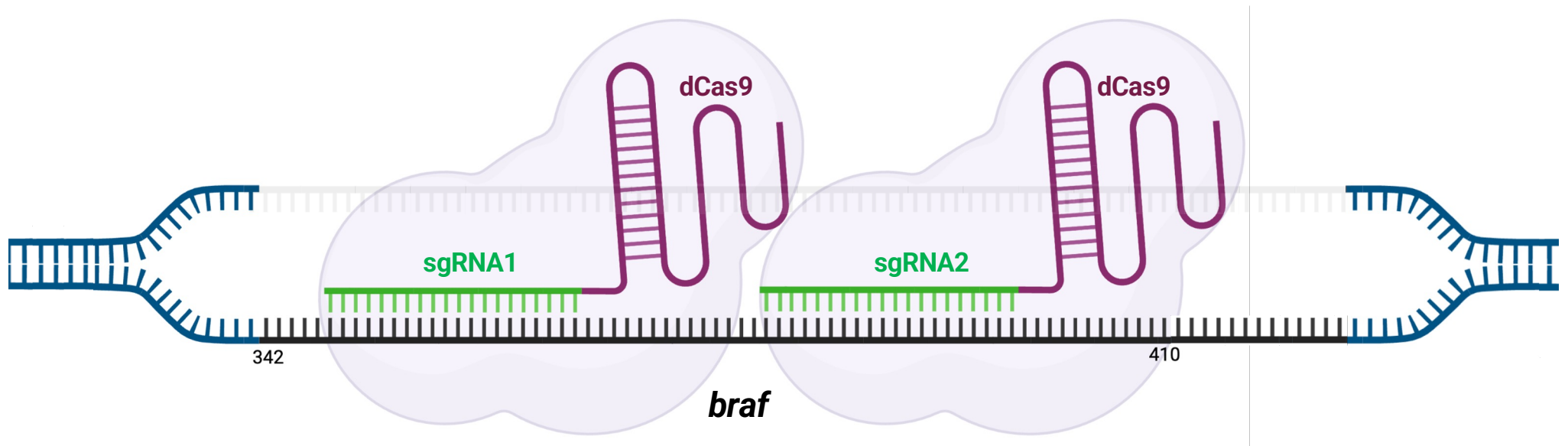
dCAS9 is produced and **binds** to the **target** and **off-target** sites

## OTRS-en-CRISPRi



dCAS9 is produced and **binds** to both the **target** and the **OTRS**, **preventing** further dCas9 transcription and **off target** binding

# sgRNA & OTRS design



**sgRNA1**

CATG**A**CGG**A**GA**A**GGGACACGG

**sgRNA2**

GCCATT**G**TGTGTGTTT**AC**GT

**OTRS1**

found in the PCAT1 gene

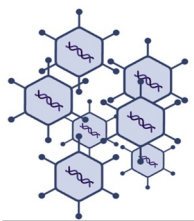
CATG**G**CGG**C**GC**C**GGGACACGG

**OTRS2**

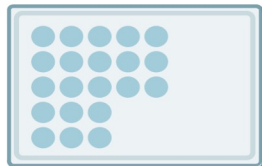
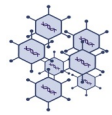
found in the C1GALT1 gene

GCCATT**T**TGTGTGTTT**GG**GT

# *In vitro* experimental plan



Adenovector production



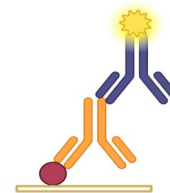
**DAY 0**

- Ad transduction in 8505c cell line
- Start cell count for 144 hours every 24h



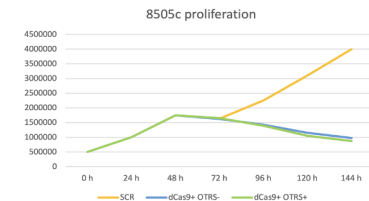
**DAY 2**

Start selection with puromycin



**DAY 4**

- Molecular assays:
- qRT-PCR;
  - Western blot;
  - Immunofluorescence;
  - CHIP;
  - TUNEL assay.



**DAY 6**

End cell count

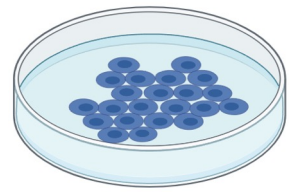
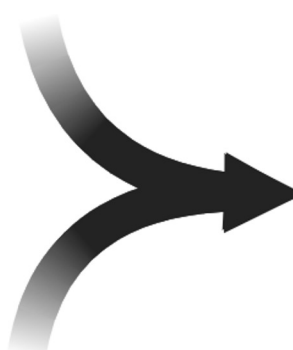
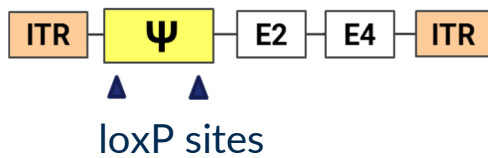


# Adenovector Design & Production

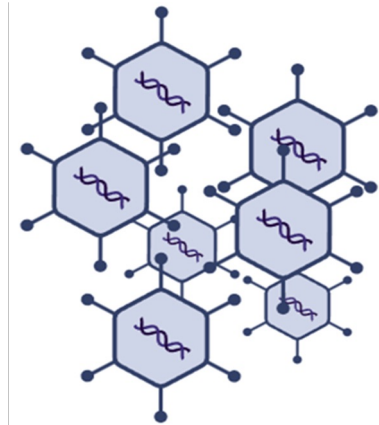
OTRS-en-CRISPRi vector



Helper virus



HEK293T Cre E1+  
cell line cotransfection



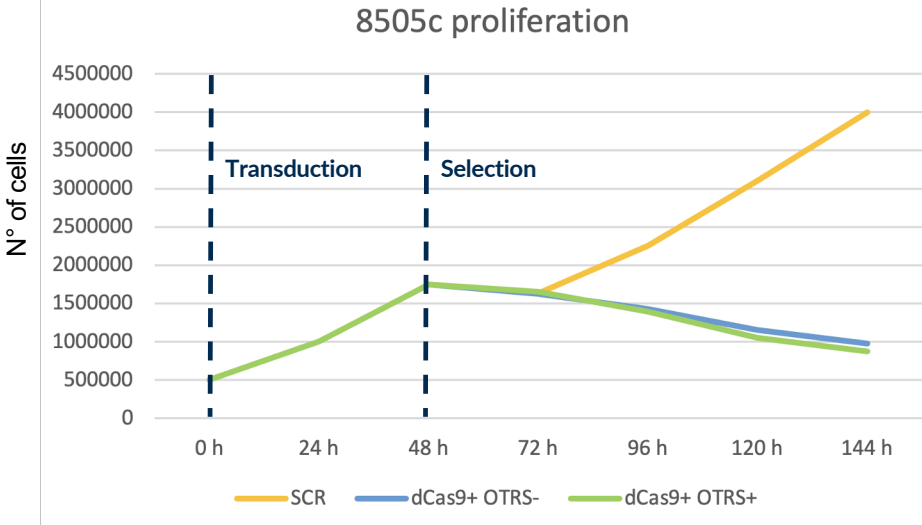
Adenovectors



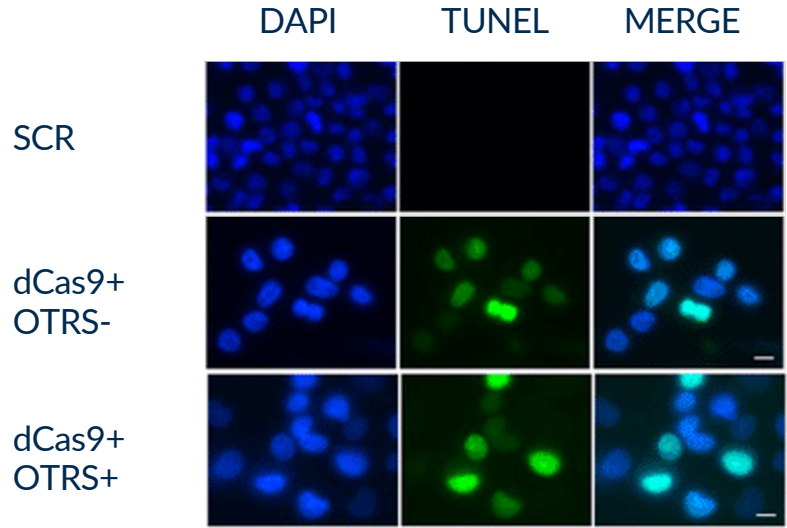


# Cell proliferation assay

Cell count



TUNEL assay



Adapted from N. Zhang et al. 2018

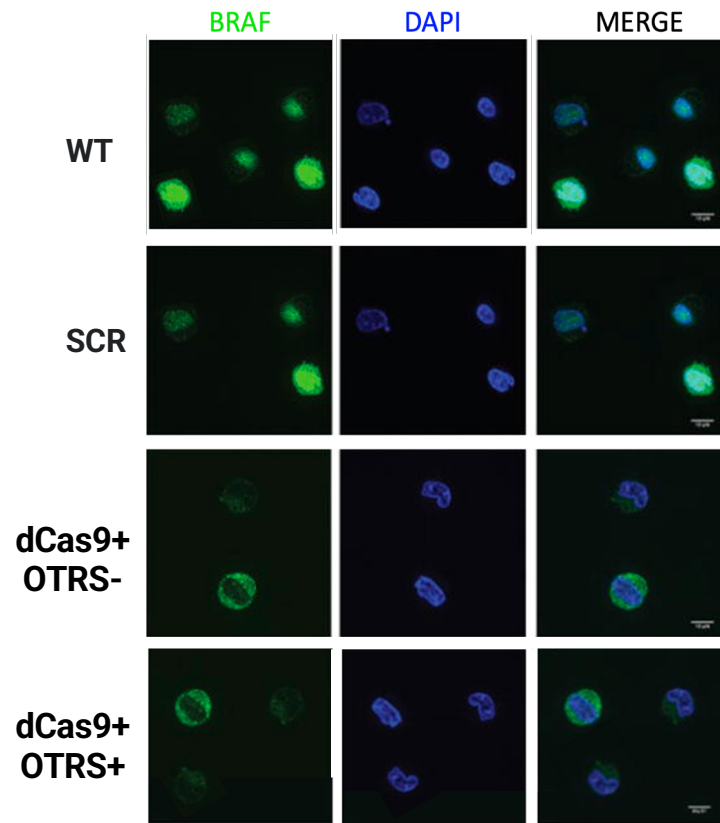
Cell count shows high efficiency of the strategy in the dCas9+OTRS + cells. Cell death in day 4 is analyzed through TUNEL assay that shows higher fluorescence in dCas9+OTRS+ cells.





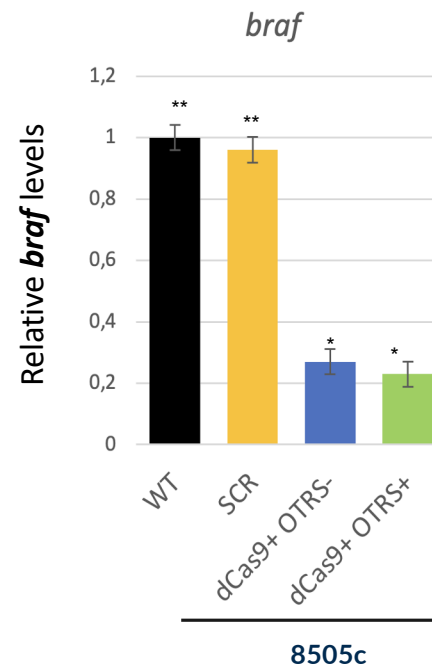
# Is braf expression inhibited in vitro?

## Immunofluorescence

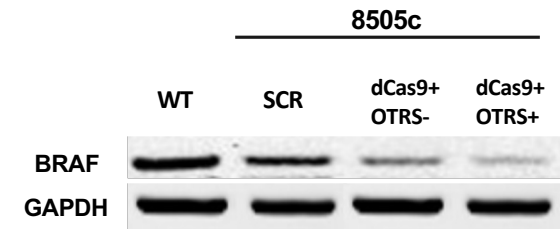


Adapted from Hess G. T. et al. 2016

## qRT-PCR

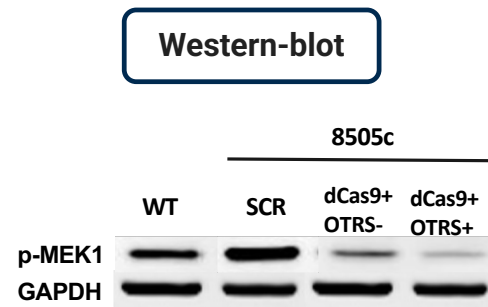
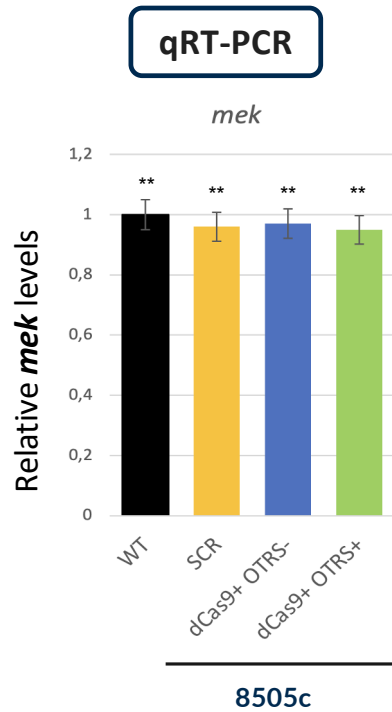


## Western-blot

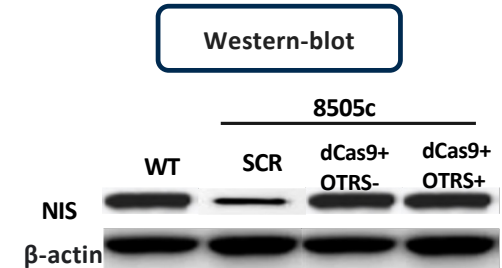
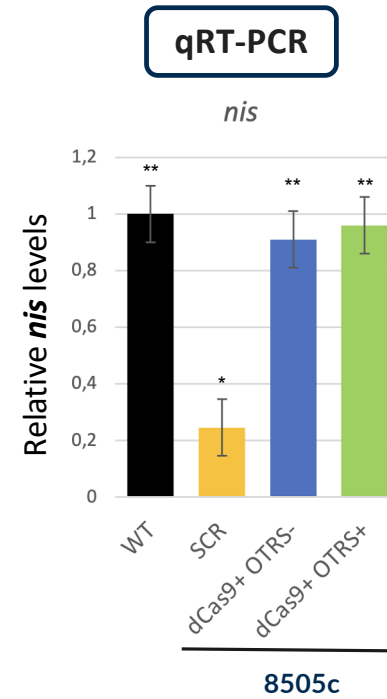


Braf expression is low both in transcription and translation level; the immunofluorescence shows a lower concentration of the protein in the dCas9+OTRS+ cells.

# Effects of braf inhibition



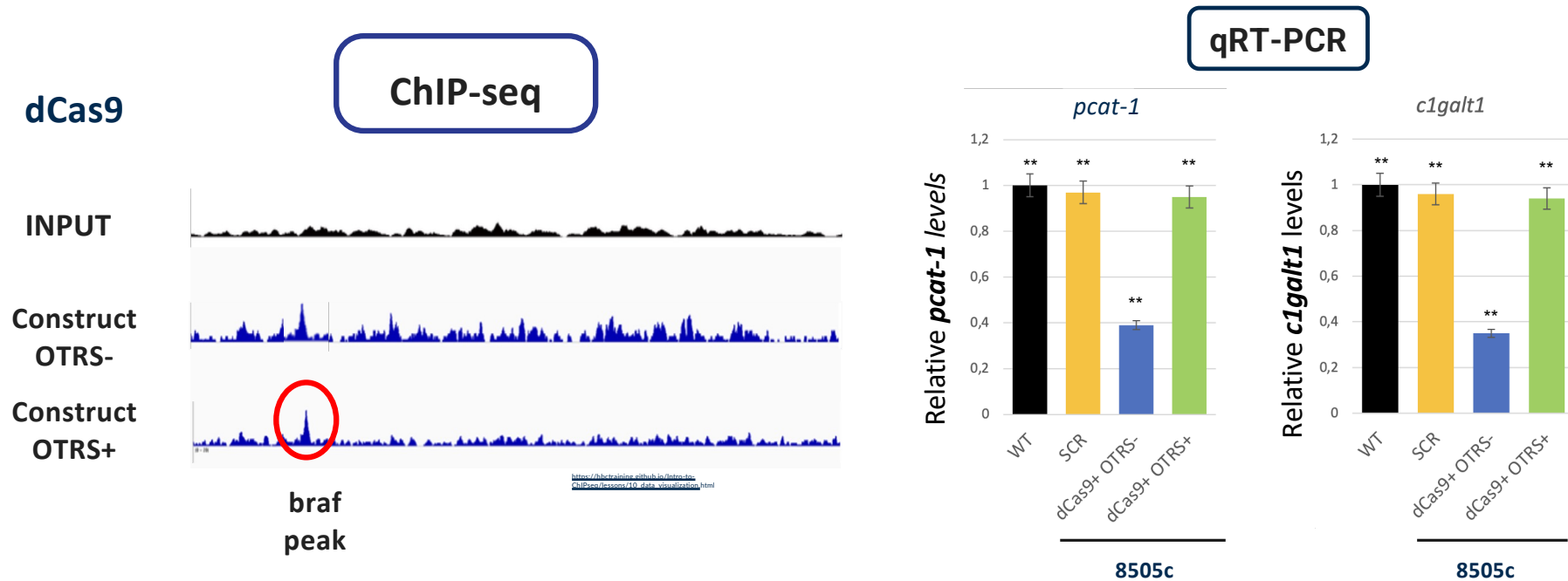
In dCas9+OTRS+ cells braf inhibition doesn't show a direct effect on mek transcription; whereas following braf expression inhibition, there is a decrease in the levels of phosphorylated Mek.



In dCas9+OTRS+ cells NIS expression is restored both in the transcription and translation level.



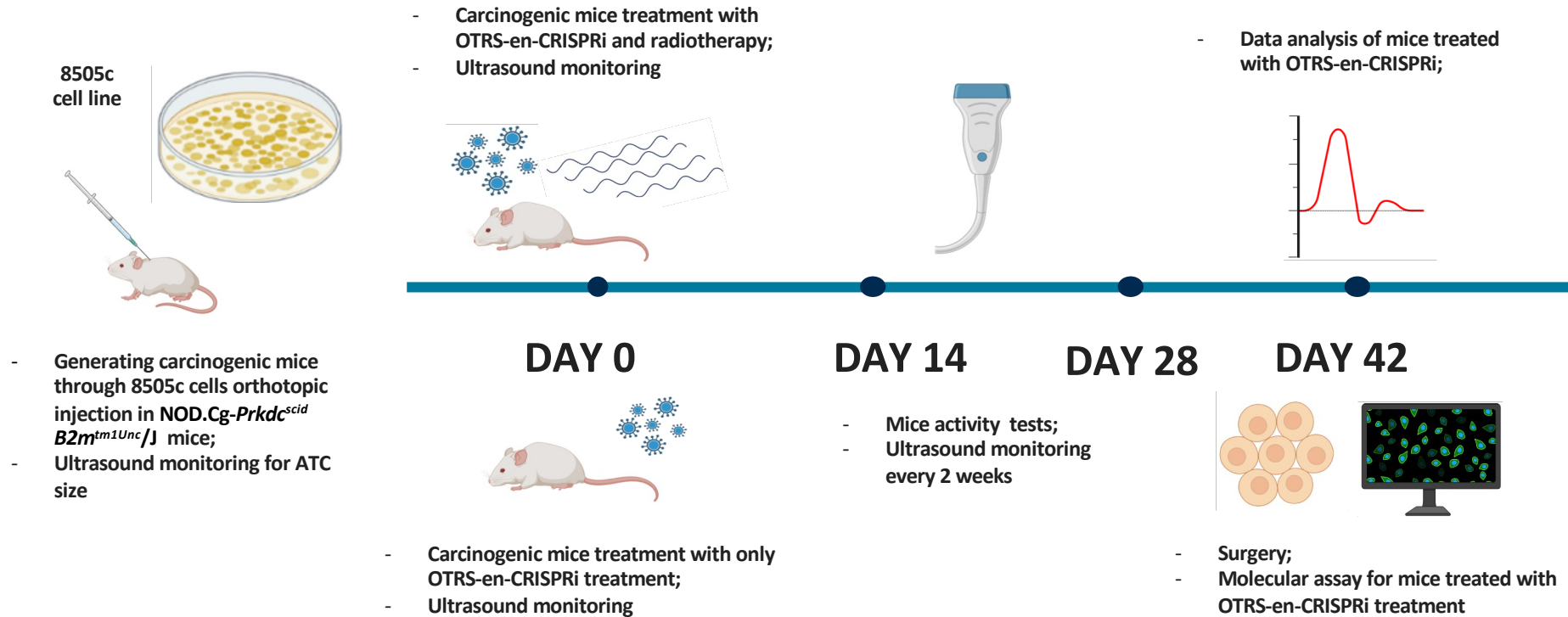
# OTRS-en-CRISPRi advantages



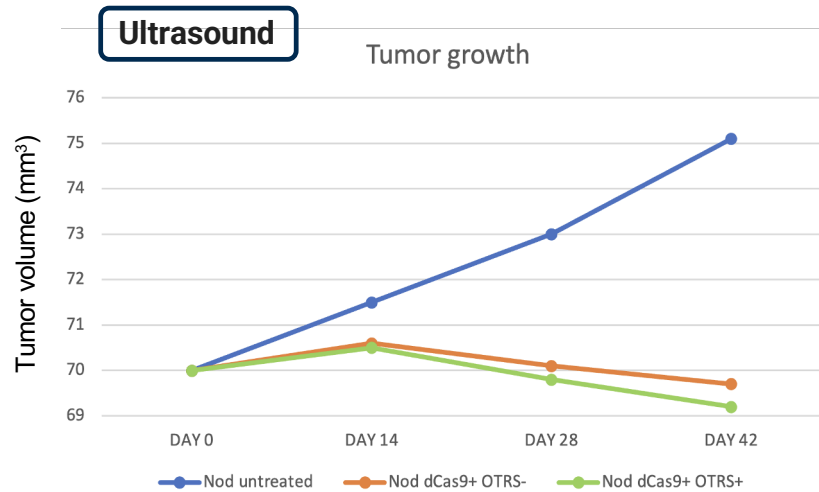
ChIP-seq analysis were done to observe OTRS-enCRISPRi efficacy: we demonstrate that the OTRS sequence allows to restrict the dCas9 binding mainly to the target sequence decreasing the binding to the other off target sites in the genome. Further results have been produced through qRT-PCR to evaluate the level of transcription of the 2 related genes.



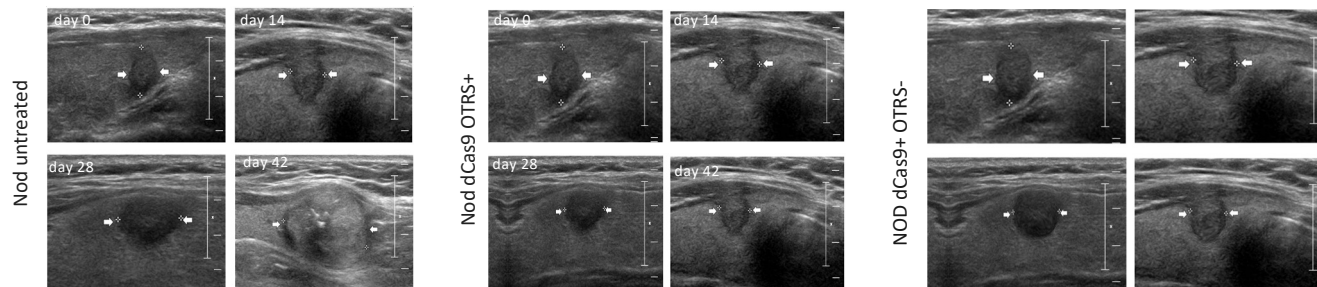
# In vivo experimental plan



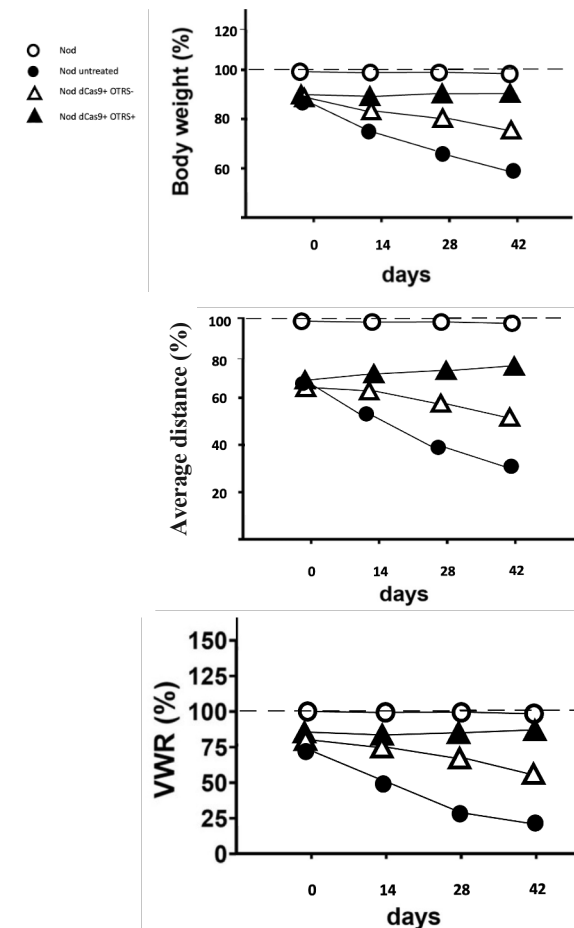
# Monitoring tumor growth and mice activity



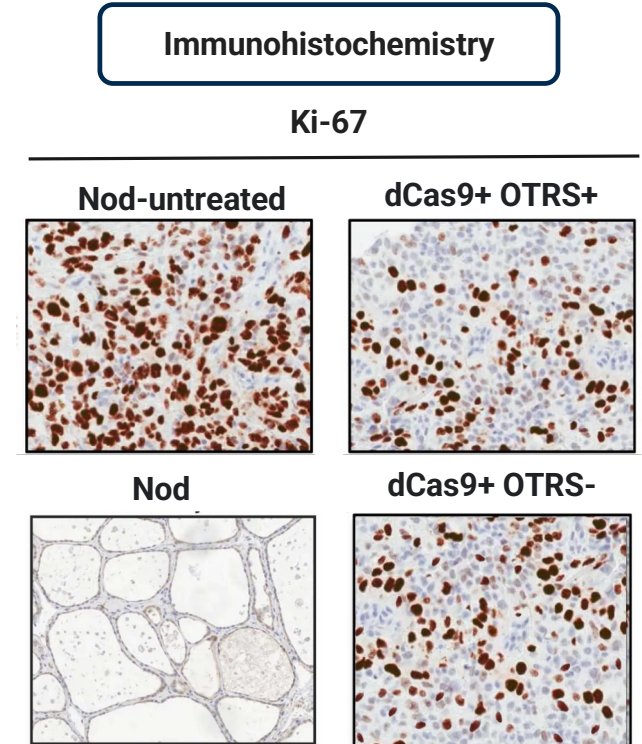
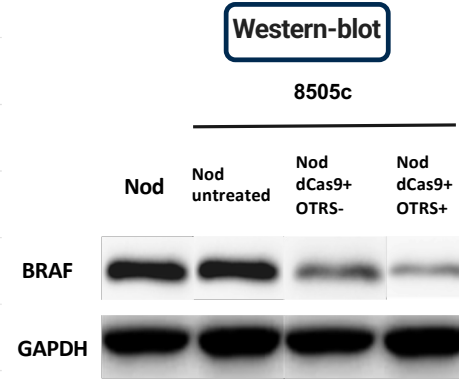
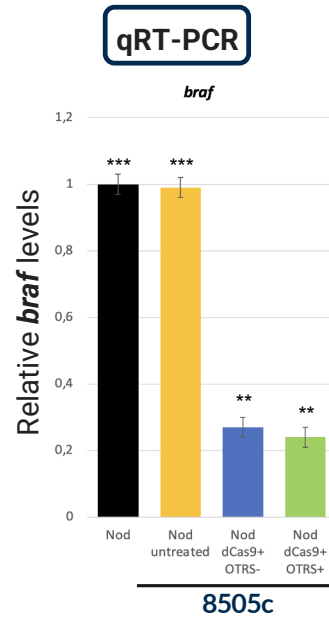
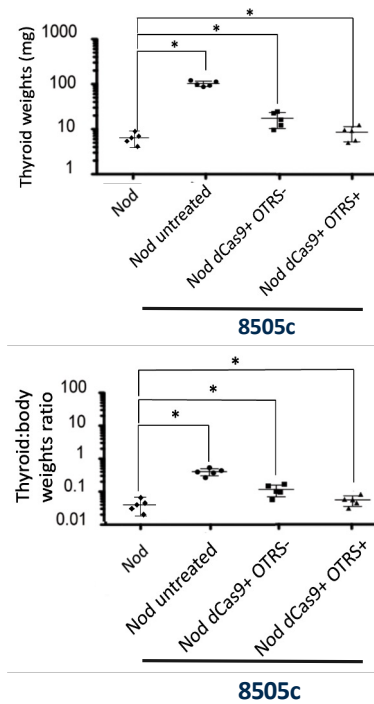
The ultrasound shows significant difference in the level of cell proliferation between Nod untreated and the dCas9+OTRS+ mice: we observe an amelioration of the cancer on the treated mice group after 42 days.



adapted from Baek HJ et al. 2018



# Effects of braf inhibition *in vivo*



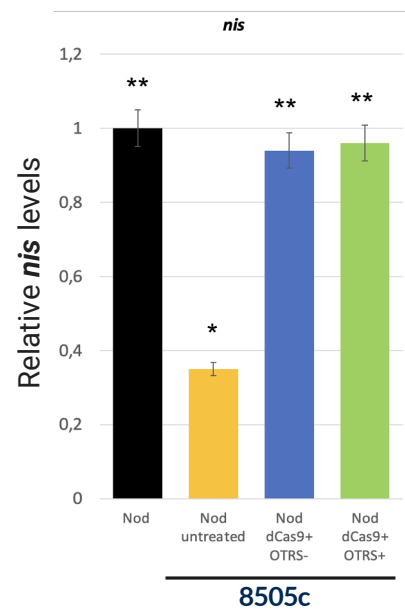
Immunohistochemistry and further molecular assays show decrease of *braf* expression.

adapted from Punsy K. et al. 2016

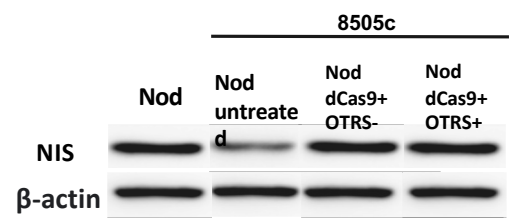


# OTRS-en-CRISPRi and radiotherapy synergistic effect

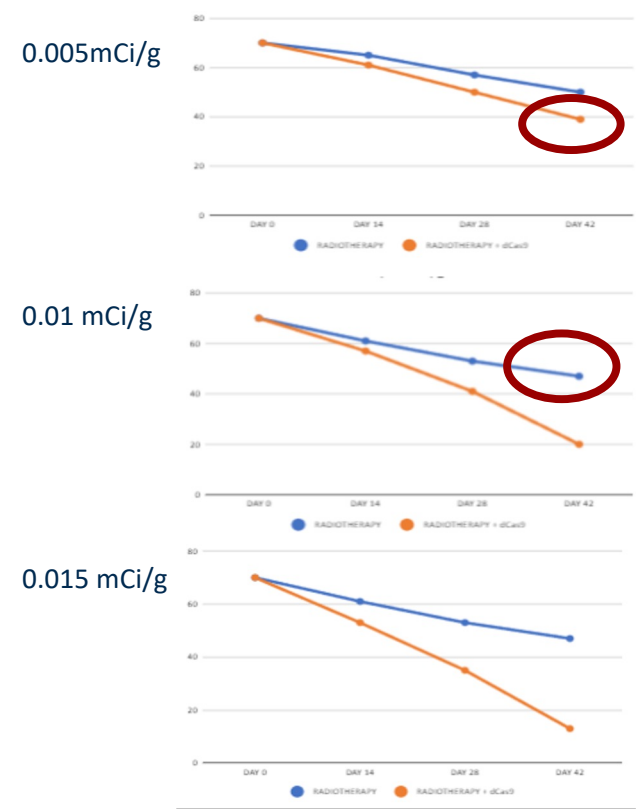
qRT-PCR



Western-blot



The level of expression of sodium iodide symporter (NIS) both in the level of transcription and translation show increase pattern due to braf inhibition. Increase in sensibility to radiotherapy is analyzed through radiotherapy with Iodine-131.



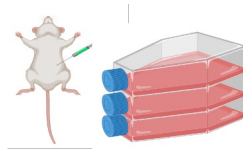


# MATERIALS & BUDGET



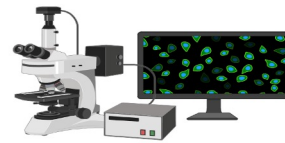
Lab equipment

5000 €



Cell lines and animal models

2827 €



Molecular assays and radiotherapy

10 339 €



Team

19 500 €

**2 YEARS RESEARCH : 188 000 €**



# PITFALLS & SOLUTIONS

- **Adeno Vectors infect both non-replicative and replicative cells.**
- **Cell specificity infection.**
- **Lack of exclusive BRAFV600E transcription inhibition with respect to BRAF.**



- **Could be resolved by using integration incompetent gamma-retroviruses.**
- **Could be resolved through adenovector pseudotyping.**
- **Could be resolved by using more sophisticated tools able to discriminate point mutations.**



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