

Gs α and G-coupled receptor diseases: GH-secreting pituitary adenoma



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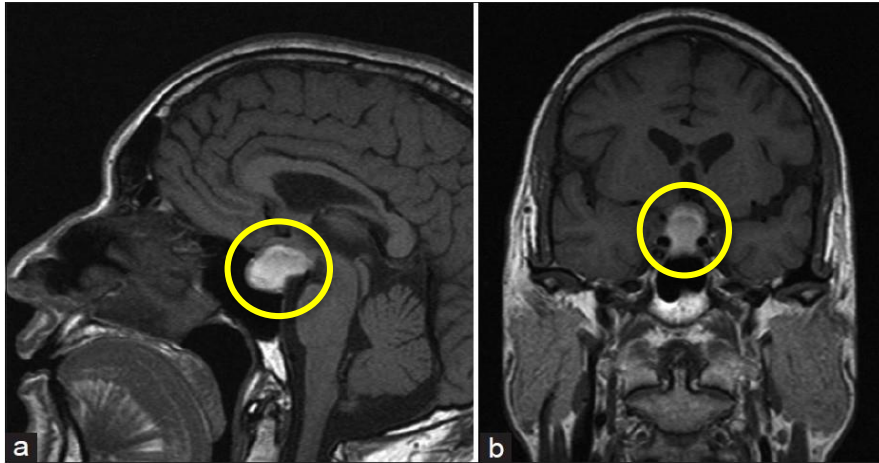
A.A. 2015/2016

Students:

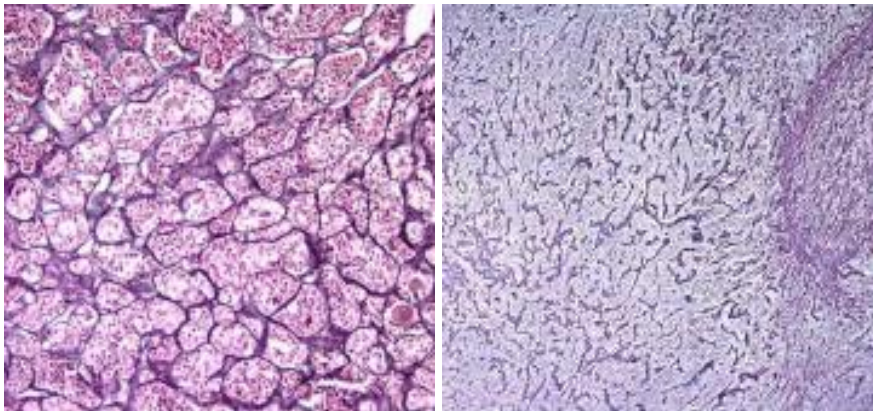
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GH-secreting pituitary adenoma

OMIM: 300393



Adapted from MN Swamy et al., 2013



Adapted from MB S Lopes, 2015

Clinical features:

- hyperplasia → increased intracranial pressure;
- GH upsecretion;
- GHRH receptor overexpression;

Current treatments:

- in order to reduce GH secretion
 - somatostatin analogues
 - GH-receptor antagonist
- in order to reduce tumoral mass
 - radiotherapy
 - surgical procedure

Associated endocrine syndromes:

- Acromegaly

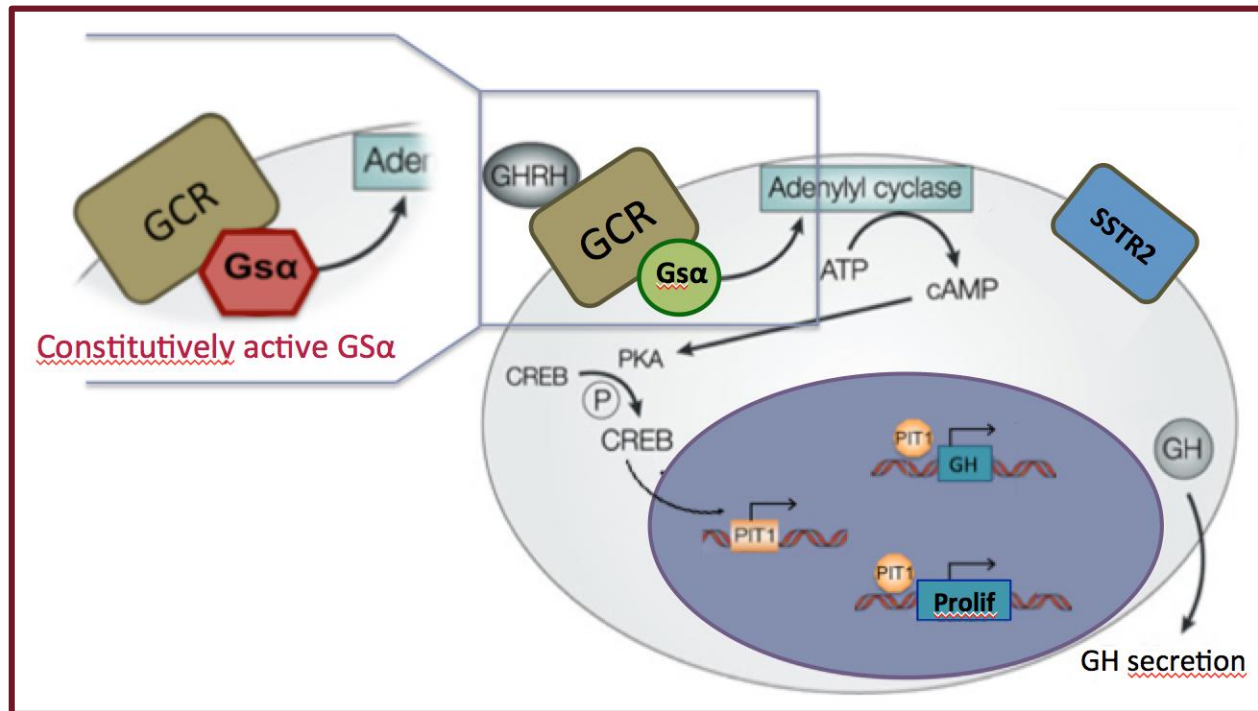
Molecular bases of the disease

In 40% of cases, GH secreting adenoma is caused by an **activating mutation in the stimulatory G-protein α subunit**:

- Arg201 → **Ser201**
- Gln227 → **His227 or Lys227**

Adenoma pituitary cell

Wild-type pituitary cell



Adapted from S L Asa & S Ezzahr, Nature Reviews, 2002

Mutant Gs α is constitutively active and leads to hyperproliferation and GH upsecretion.

Objective and experimental plan

Reduce **Proliferation** and **GH SECRETION** by overexpression of a specific deacetylase (**Sirt1**) using a modified 3rd generation Adenoviral Vector.

- Modify adenoviral fibers with **FWKT** peptide, somatostatin binding domain that recognizes **SSTR2** receptors, overexpressed in adenoma cells;
- Insert the **SIRT1** gene under the control of **GHRHR promoter**, specifically active in pituitary cells;
- **miRNA detargeting**: off target in wild type pituitary cells.
- Directly inject adenoviral particle in tumoral mass (**stereotactic injection**);



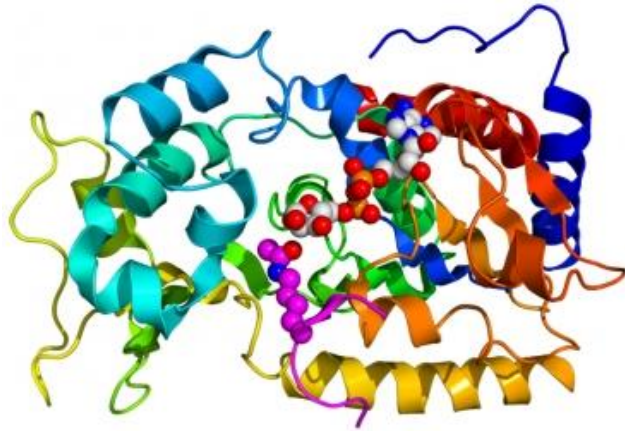
Sirt1 specific overexpression **only** in GH-secreting adenoma cells.



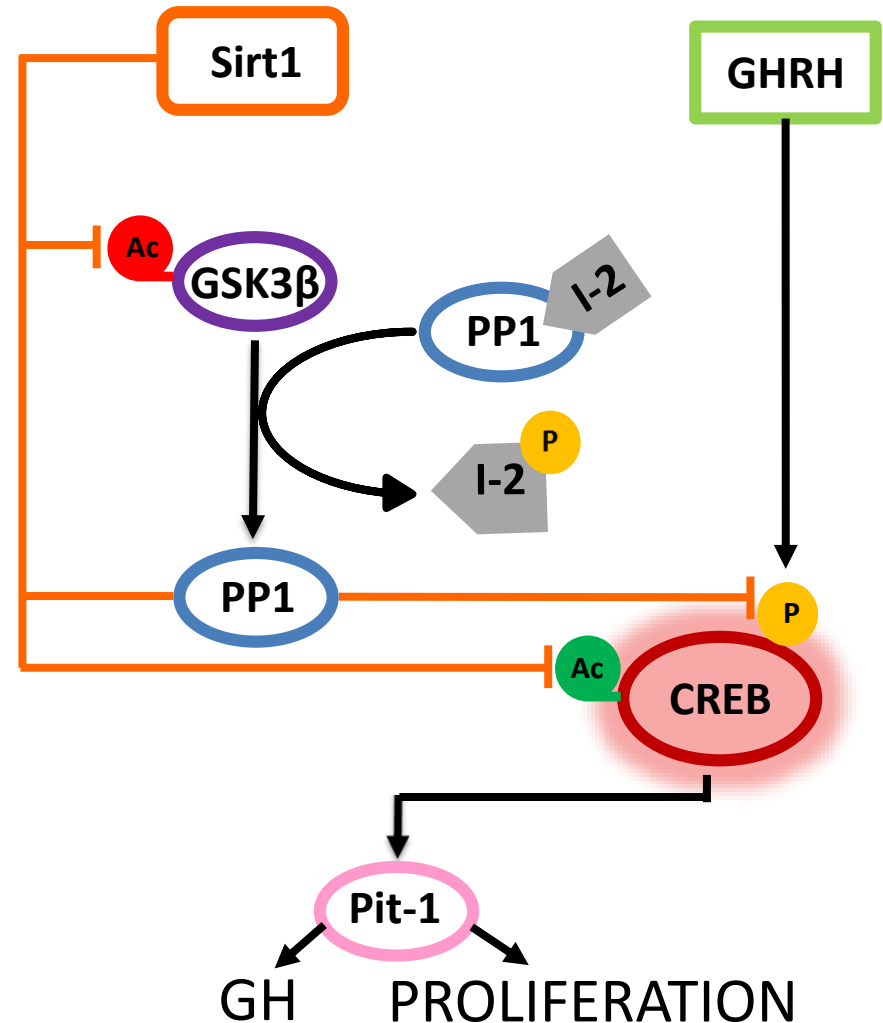
Sirt1 and GH secretion pathway

Sirt1 targets CREB activity at different levels:

- GSK3 β deacetylation \rightarrow activation;
- PP1 activation;
- CREB direct deacetylation \rightarrow inactivation

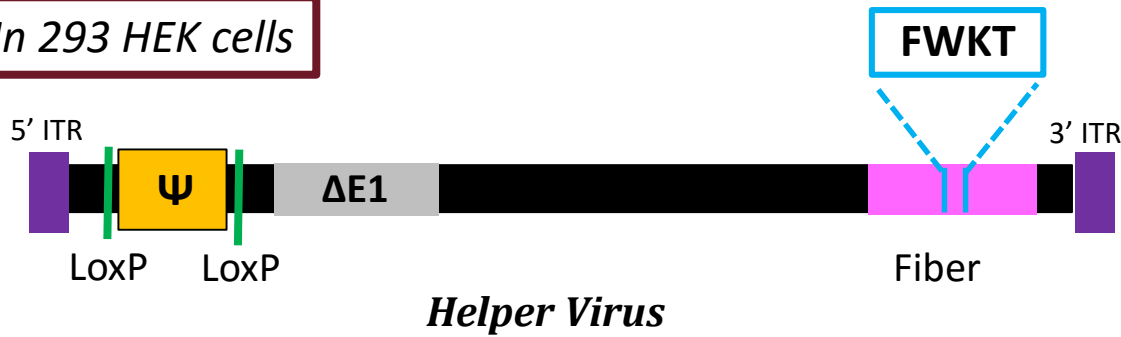


- NAD⁺ binding domain
- Helical module
- Zn²⁺ binding domain
- pseudo-substrate peptide (T,tail)
- C-terminal regulatory segment (CTR)
- Predicted, unstructured regions (U)



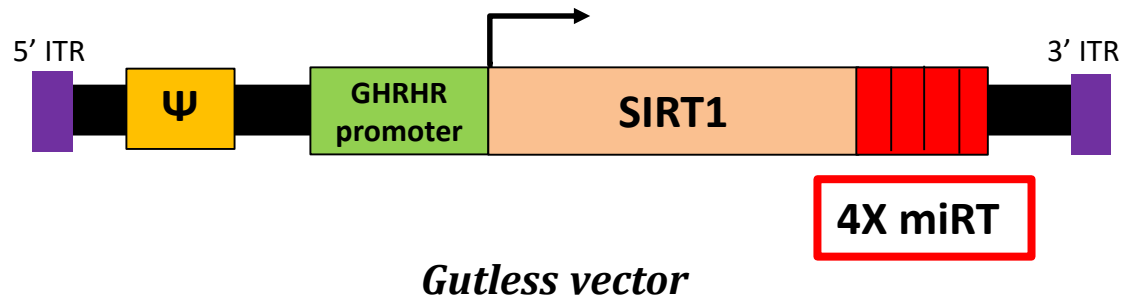
Creating Adenoviral Vector

In 293 HEK cells



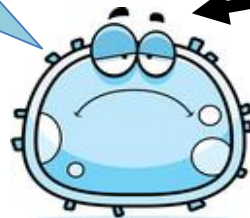
Somatostatin binding domain cloned within HI LOOP of fiber gene

M Essand, Gene Therapy, 2011



“Next generation gene therapy”

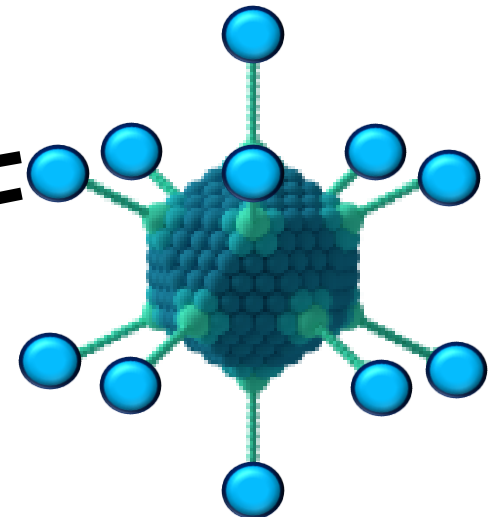
SSTR2 receptor



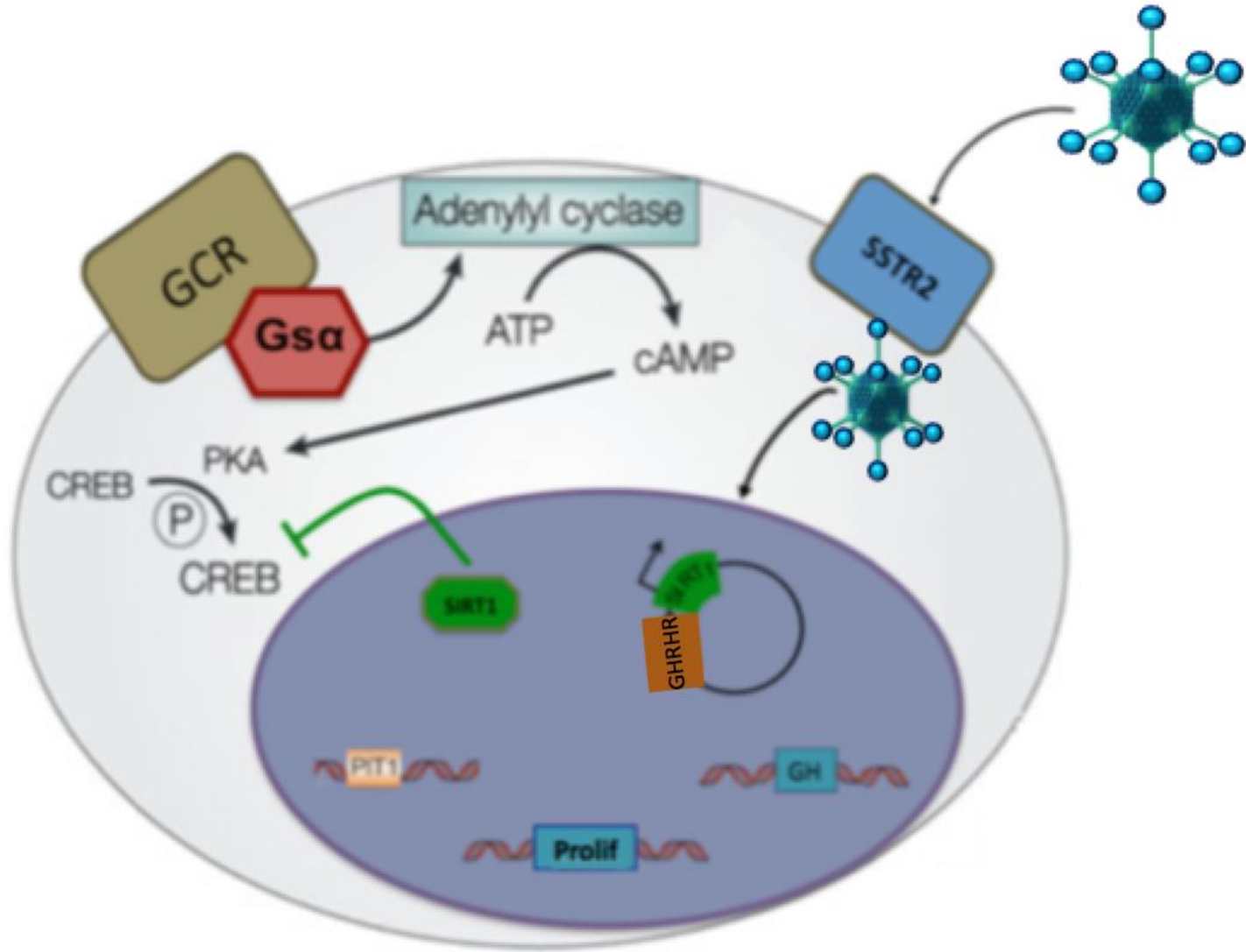
adenoma cells



wild type cells



Viral strategy

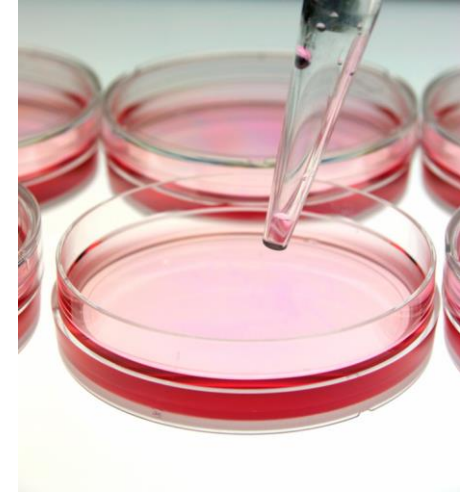


SIRT1 targets active-CREB to prevent the expression of GH and proliferation genes

Cell lines and mouse model

Cell lines:

- **GH-producing pituitary adenoma GH3gsp cells** (*Rattus Norvegicus*)
gsp mutation: Q227L
(M F Scannlon et al., *Molecular and Cellular Endocrinology*, 1997)
- **Wild type primary pituitary cells** (*Rattus Norvegicus*)
- **Primary GH-producing pituitary adenoma cells** (*Homo Sapiens*)
gsp mutation: R201S or G227H or Q227L



Mouse model:

- **Nude rat** (*Rattus Norvegicus*): stereotactic injection of the GH secreting **GH3 cell line** in pituitary gland leads to the formation of tumor.

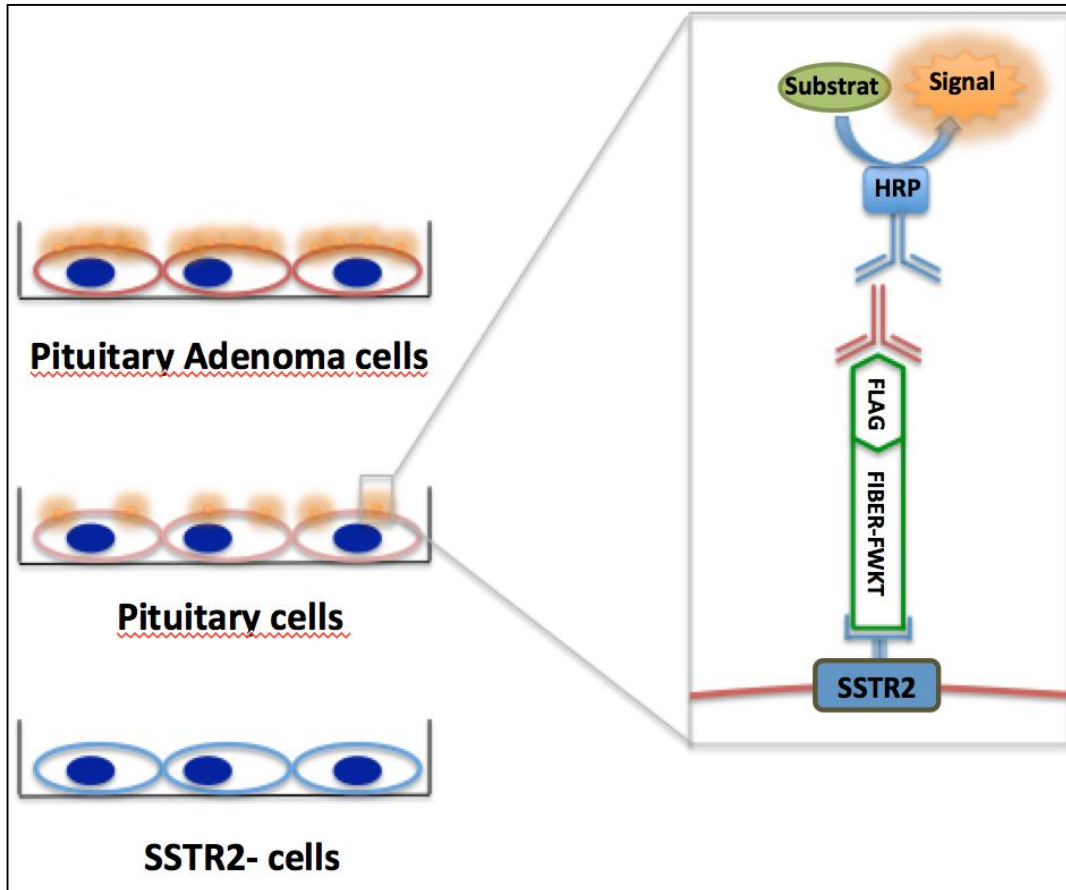
(Timsit J et al., *JCI*, 1990)



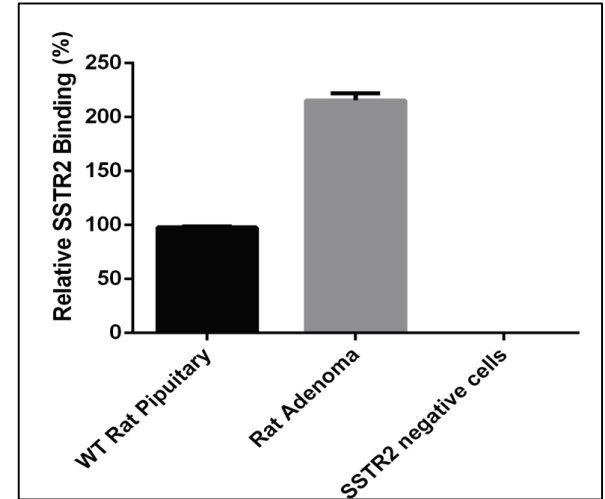
In vitro experiments

Modified Adenoviral Vector-FWKT binds more efficiently adenoma cells

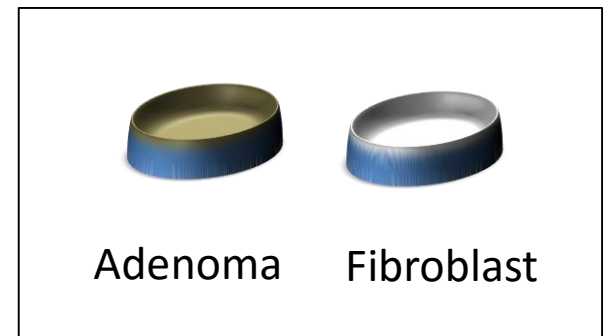
ELISA procedure



ELISA (rat cells)



ELISA (human cells)



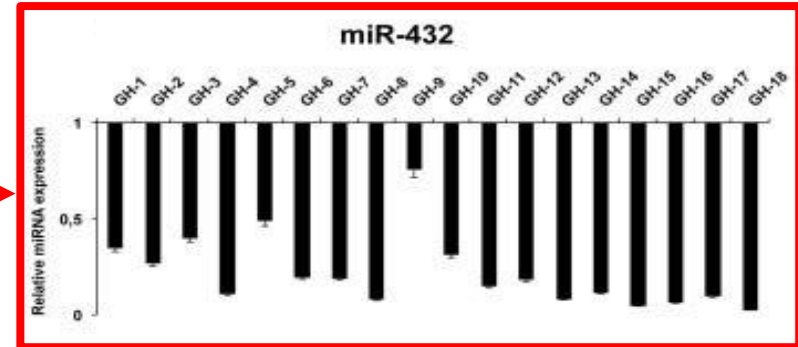
miRNA detargeting

The expression of exogenously introduced Sirt1 gene tagged with artificial miRNA target sequences (miRT) can be negatively regulated by endogenous miRNAs highly expressed only in wilde type pituitary cells.

miRNA in human pituitary adenoma

miRNA	Upregulated or downregulated	Target genes	Tumor type
let-7	Downregulated	<i>HMGA2</i>	PRL, ACTH FSH/LH
miR-23b	Downregulated	<i>HMGA2</i>	GH, NFA FSH/LH
miR-26a	Upregulated	<i>PRKCD</i>	ACTH
miR-26b	Upregulated	<i>PTEN</i>	GH
miR-34b	Downregulated	<i>HMGA1, HMGA2</i>	GH
miR-107	Upregulated	<i>AIP</i>	GH, NFA
miR-128	Downregulated	<i>BMI1</i>	GH
miR-128a	Upregulated	<i>Wee1</i>	NFA
miR-326	Downregulated	<i>HMGA2, E2F1</i>	GH
miR-432	Downregulated	<i>HMGA2</i>	GH
miR-516a-3p	Upregulated	<i>Wee1</i>	NFA
miR-548c-3p	Downregulated	<i>HMGA1, HMGA2</i>	GH
miR-570	Downregulated	<i>HMGA2</i>	GH
miR-603	Downregulated	<i>E2F1</i>	GH

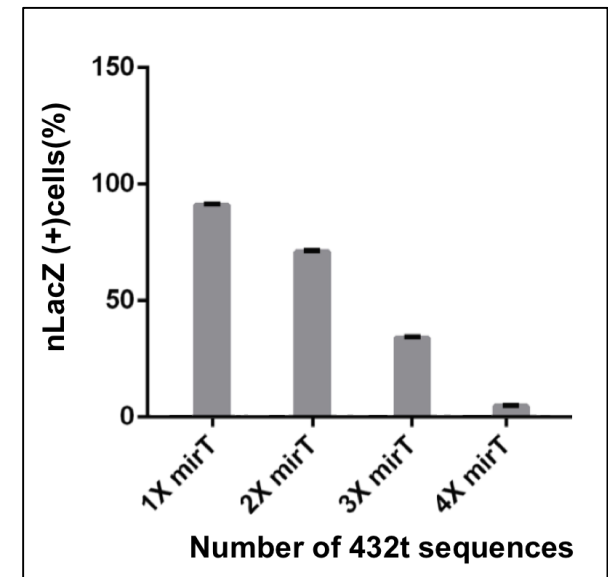
Degli Uberti EC et al., J Cell Physiol, 2007



A Fusco et al., JCEM, 2012

4X miR-432 target sequences determine the entire depletion of protein in rat pituitary wild type cells.

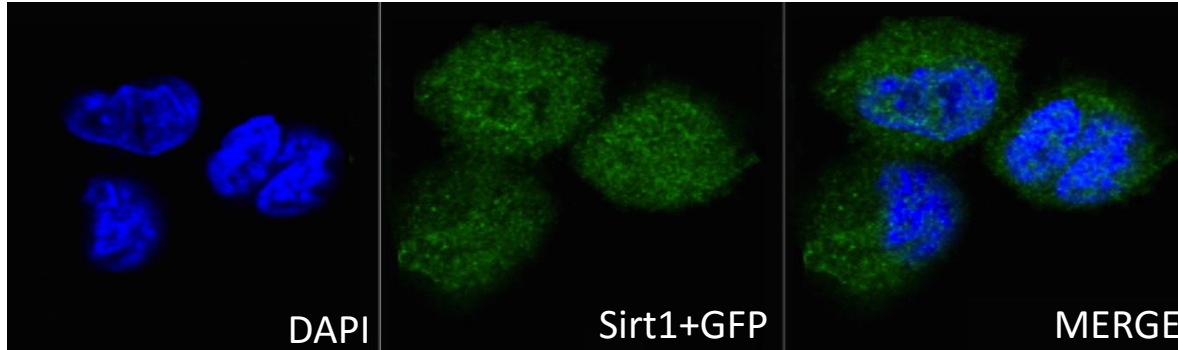
LacZ assay



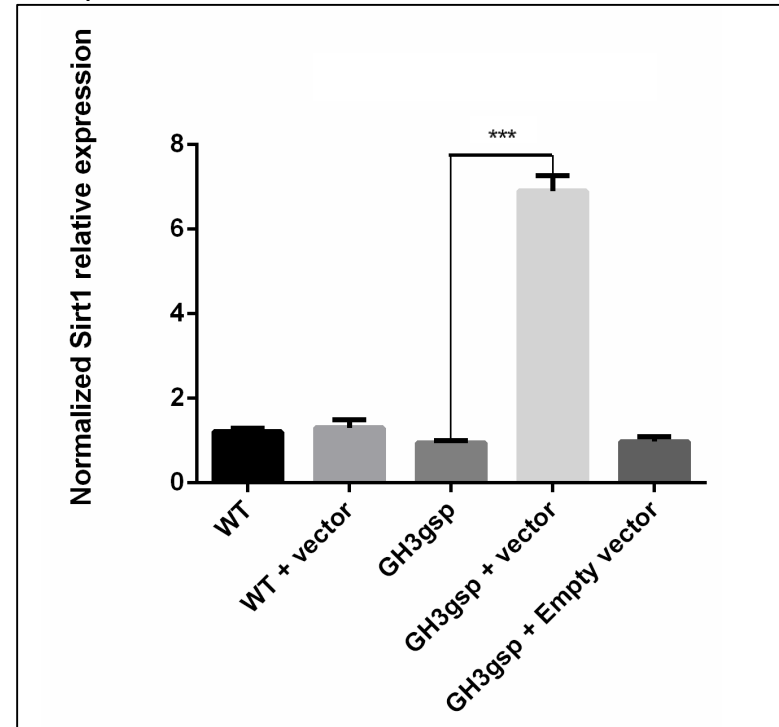
In vitro experiments

Sirt1 is overexpressed in adenoma cells

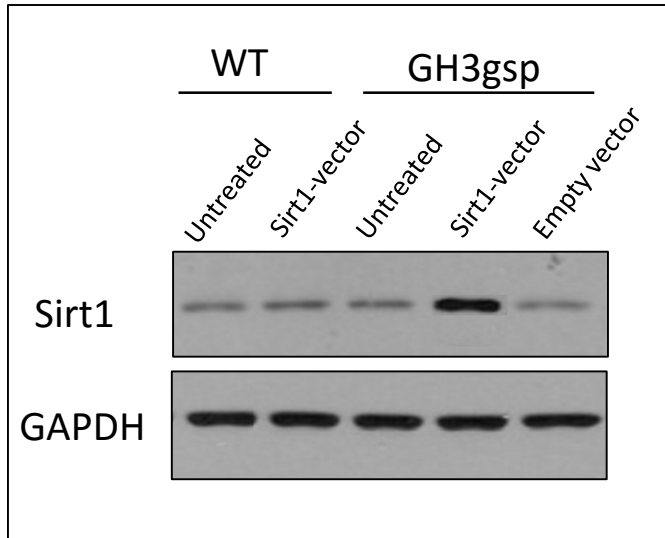
fluorescence



RT-qPCR



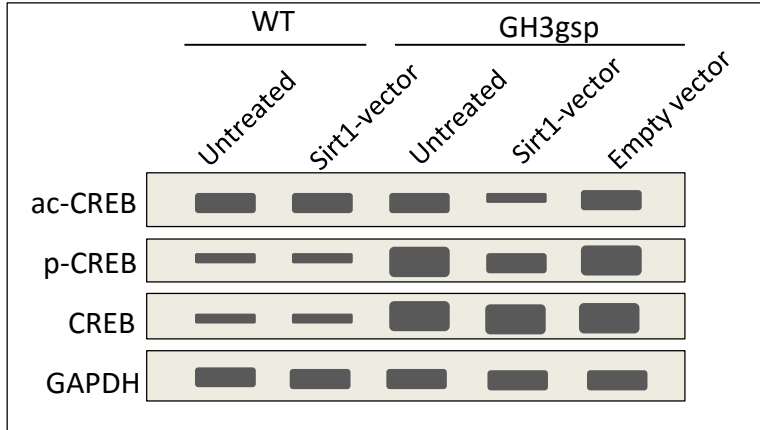
WB



In vitro experiments

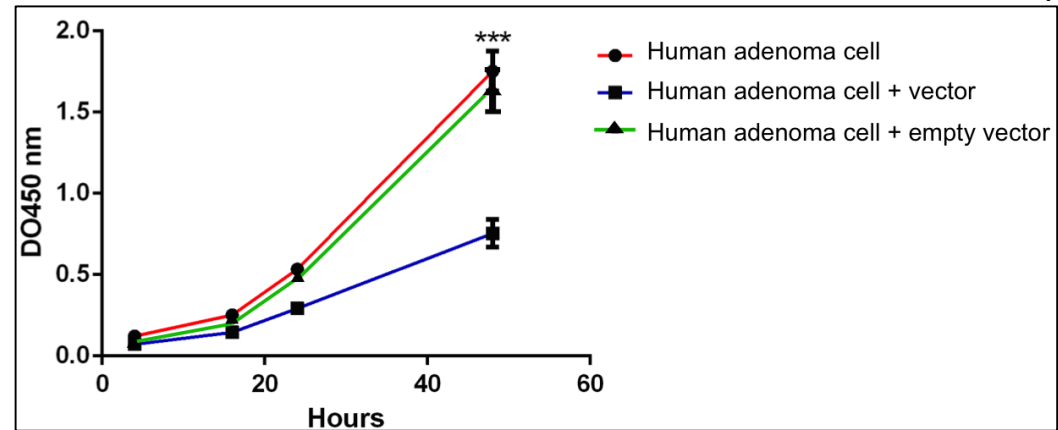
CREB pathway is affected

WB



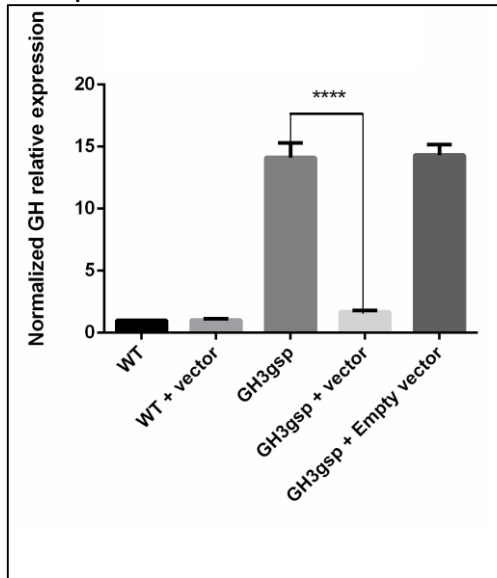
Proliferation is reduced

Proliferation assay

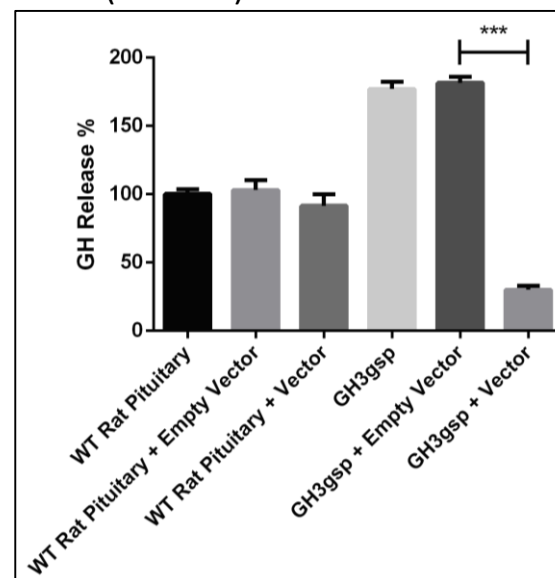


GH production is reduced

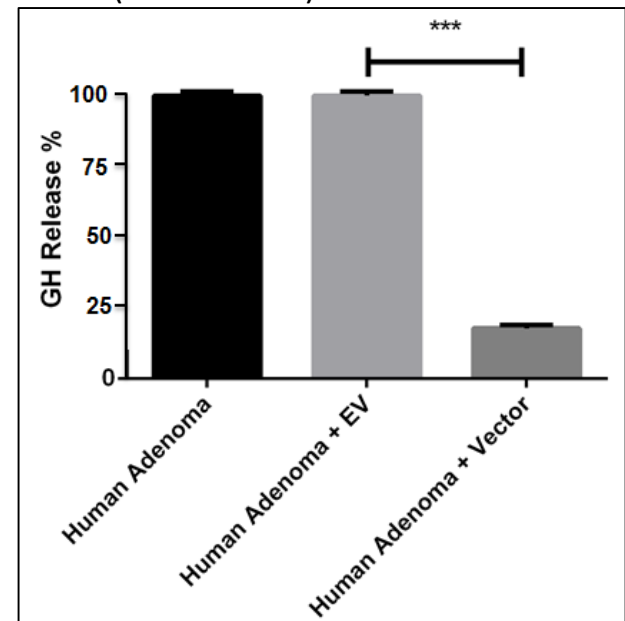
RT-qPCR



ELISA (rat cells)



ELISA (human cells)

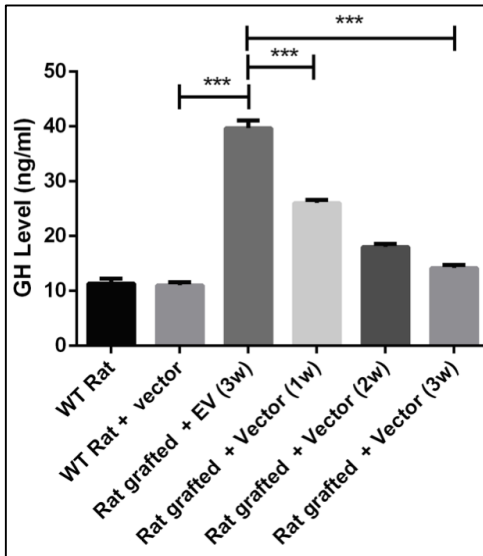


In vivo experiments

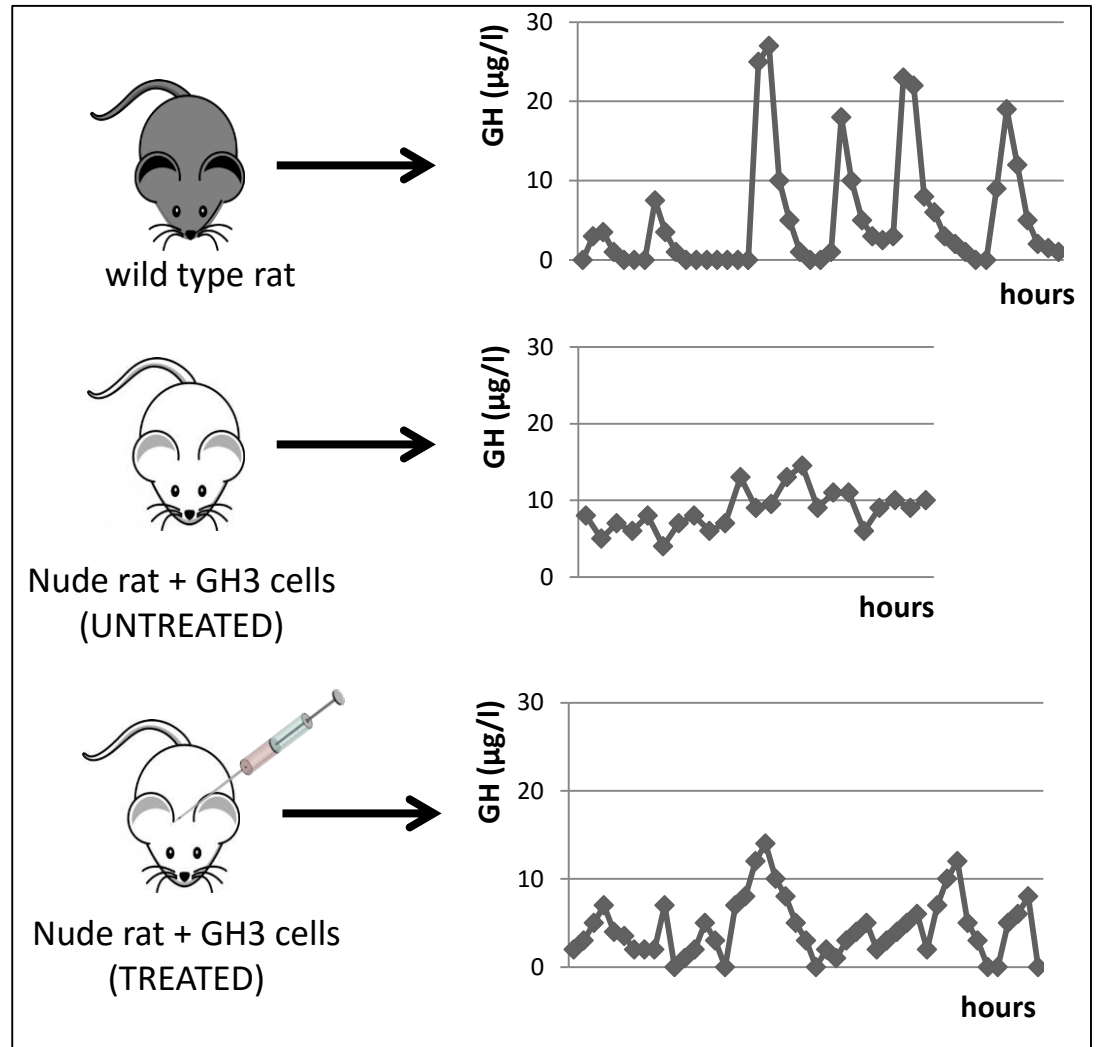
Stereotactic injection



ELISA



Pulsatil GH secretion in rats serum for 24 hours (ELISA)



Total number of rats: 45

Concentration of viruses: 5×10^9 evg/injection

Pitfalls and solutions

Since nude rats injected with adenoviral vector would not show inflammatory reaction, we don't have the guarantee of what could be the reaction *in vivo*.

So an improvement in the *in vivo* model will be done.

We haven't used pituitary human wild type cells because don't know for certain how long they could live in culture.

So further experiments could eventually involve this kind of cells.



Future perspectives

Ameliorate the Adenoviral vector in order to be safer in human.

Ameliorate detargeting strategy identifying new miRNAs.



Co\$t\$t

Rats (Charles River) (4 weeks old)	1.000 \$
GH3gsp cell line	417,50 \$
Proliferation test kit MTT	133 \$
SYBR® Green Quantitative RT-qPCR Kit	622 \$
GH ELISA kit	420 \$
Anti-CREB Rabbit	321 \$
Anti-pCREB Rabbit	279 \$
Anti-acCREB Rabbit	300 \$
Anti-GAPDH Rabbit	330 \$
Cloning kit	275 \$
B-Gal (lacZ) Assay kit	477 \$
Stabulation	30.000 \$/year



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MyBioSource.com
Antibody - Protein - ELISA Kit



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 NEW ENGLAND
BioLabs_{GmbH}

Thermo
SCIENTIFIC

pFiber plasmid: kindle gift of Yosuke Kawakami (JA Onomichi, General Hospital)

**Thank you
for your
attention!**

