# Transcriptome profiles of cancer stem-like cells in patient derived Diffuse Intrinsic Pontine Glioma (DIPG)

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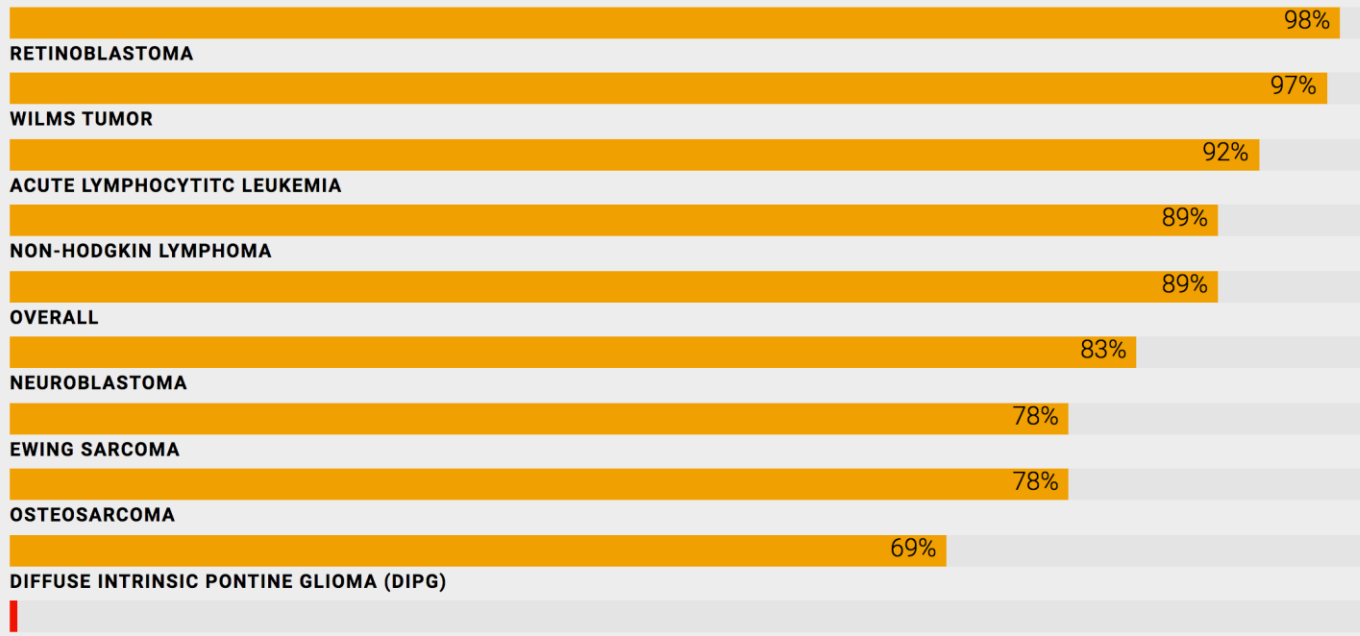
# Diffuse Intrinsic Pontine Glioma: most lethal brain tumor in children

## WHAT IS DIPG?

Diffuse Intrinsic Pontine Gliomas (DIPGs) are the most lethal brain tumors afflicting children. The tumor grows inside the brain stem and is not resectable. Most patients succumb to their disease within a year of diagnosis and long term survival is less than 1%. Pediatric patients are currently treated with radiation therapy and often chemotherapy; however, tumors exhibit rapid resistance to these therapies and commonly start to grow again within months of treatment completion. Understanding the biology of DIPG is critical in order to identify new effective treatments for this disease.

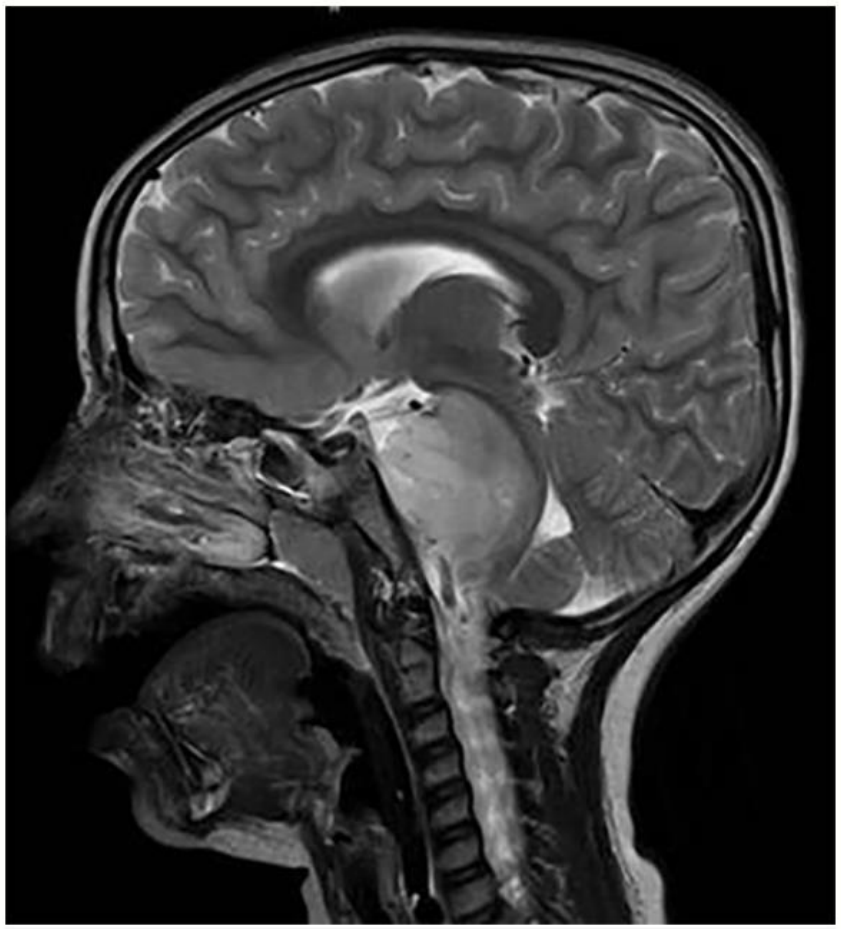
While considerable progress has been made in the identification of molecular subtypes of many cancers, the transfer of these findings into improved therapies for children with DIPG has not yet been realized. Genetic studies of DIPG have recently shed light on their mutations, but much remains to be learned about how this tumor starts growing and maintains itself.

**5-year survival rates of childhood cancer. DIPG has a 5-year survival rate of less than 1 percent.**




Source: American Cancer Society.

# Current therapeutic options for pediatric patients with brain stem tumors



Brain scan of a child with a DIPG tumor, located in the brain stem.
Credit: Rishi Lulla, M.D., Northwestern University Feinberg School of Medicine and Lurie Children's Hospital of Chicago

## Treatment Option Overview

### KEY POINTS

- There are different types of treatment for children with brain stem glioma.
- Children with brain stem glioma should have their treatment planned by a team of health care providers who are experts in treating childhood brain tumors.
- Childhood brain stem gliomas may cause signs or symptoms that begin before the cancer is diagnosed and continue for months or years.
- Treatment for childhood brain stem glioma may cause side effects.
- Six types of standard treatment are used:
  - Surgery
  - Radiation therapy
  - Chemotherapy
  - Cerebrospinal fluid diversion
  - Observation
  - Targeted therapy
- New types of treatment are being tested in clinical trials.
- Patients may want to think about taking part in a clinical trial.
- Patients can enter clinical trials before, during, or after starting their cancer treatment.
- Follow-up tests may be needed.

NIH NATIONAL CANCER INSTITUTE

# New Therapeutic Strategies for DIPG

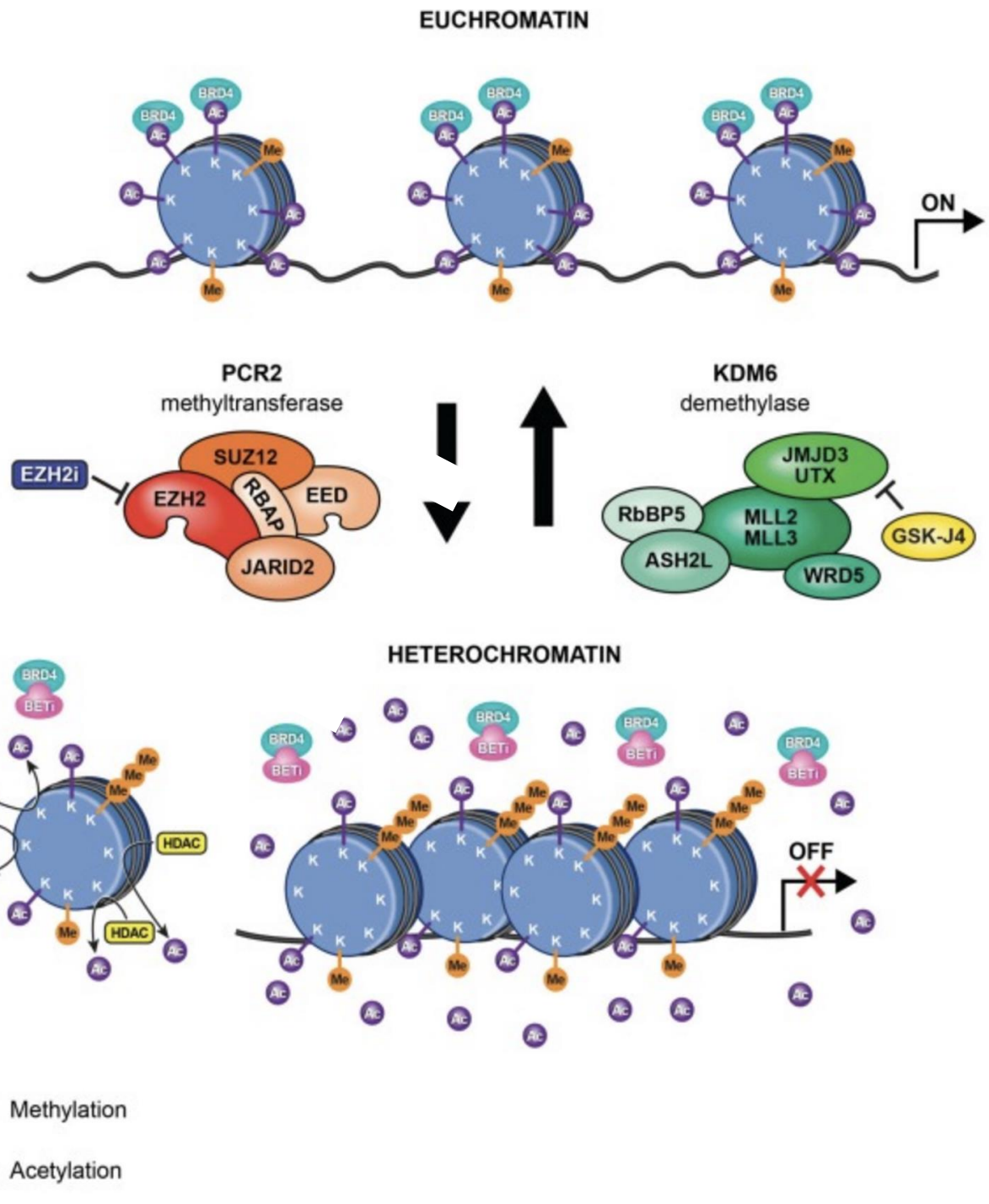
Epigenetic therapies:
Inhibit the aberrant transcriptional program:
Histone deacetylase inhibitors (HDAC)
Bromodomain (BET inhibitors) JQ1
EZH2
PRC2 inhibitor

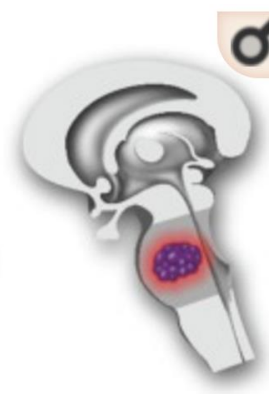
Targeting co-occuring mutations:
PI3K/mTOR
MAPK

Inhibit "stemness" self renewal properties:

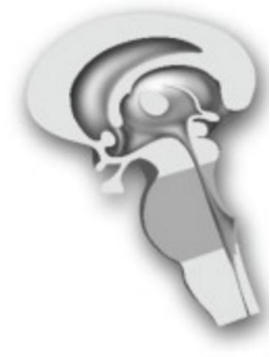
Differentiation?
Metabolism?
DNA damage and repair?

# Epigenetic targeted Therapies



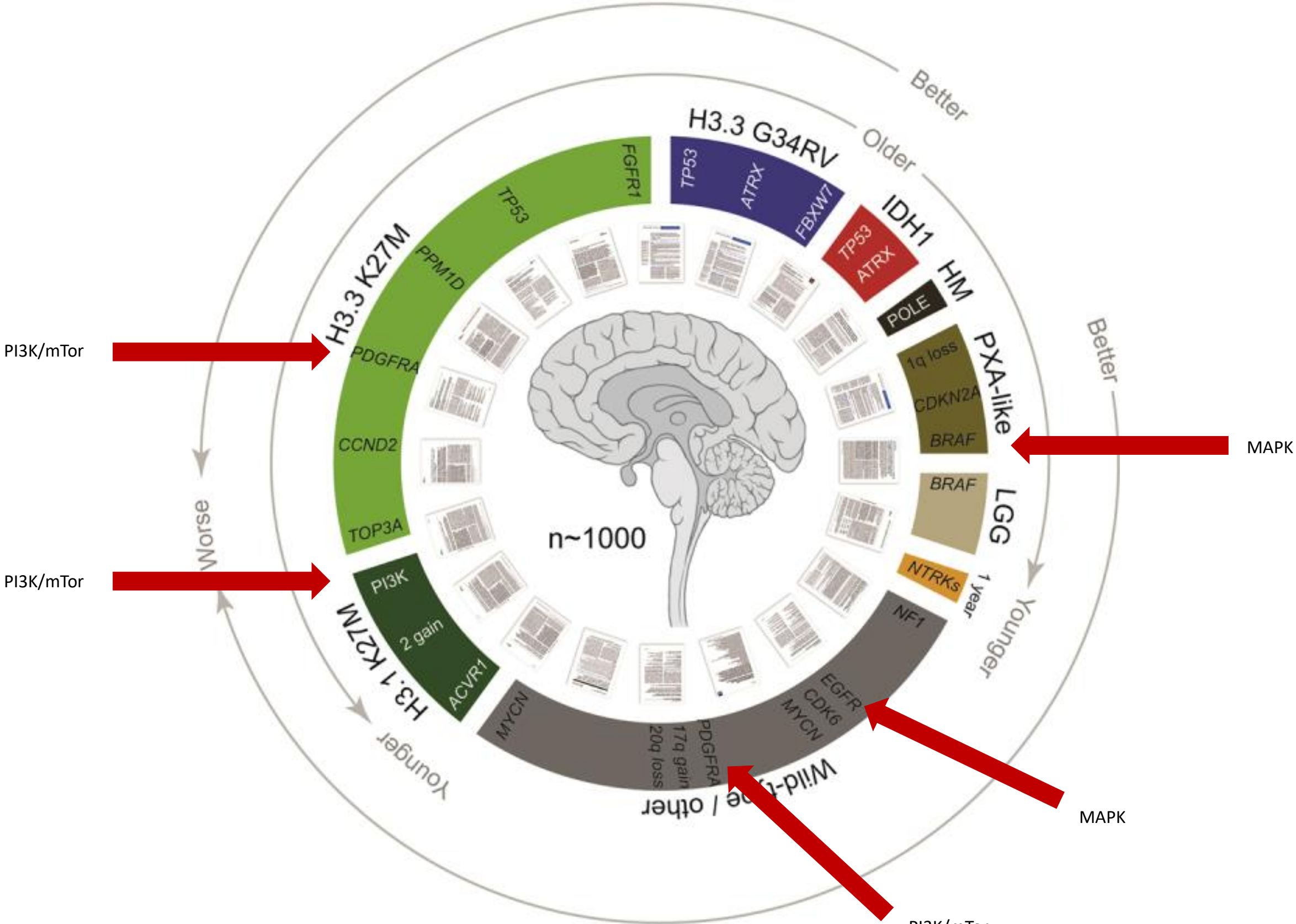



DIPG



Hashizume et al, Neurol Med Chir (Tokyo). 2017 Jul; 57(7): 331–342

# Targeting Co-segregating mutations




Mackay et al.

# DIPGs: glioma-derived cells with progenitor cell-like characteristics

DIPGs are very similar to normal neural progenitor cells (NPCs) or oligodendrocyte precursor cells (OPCs).

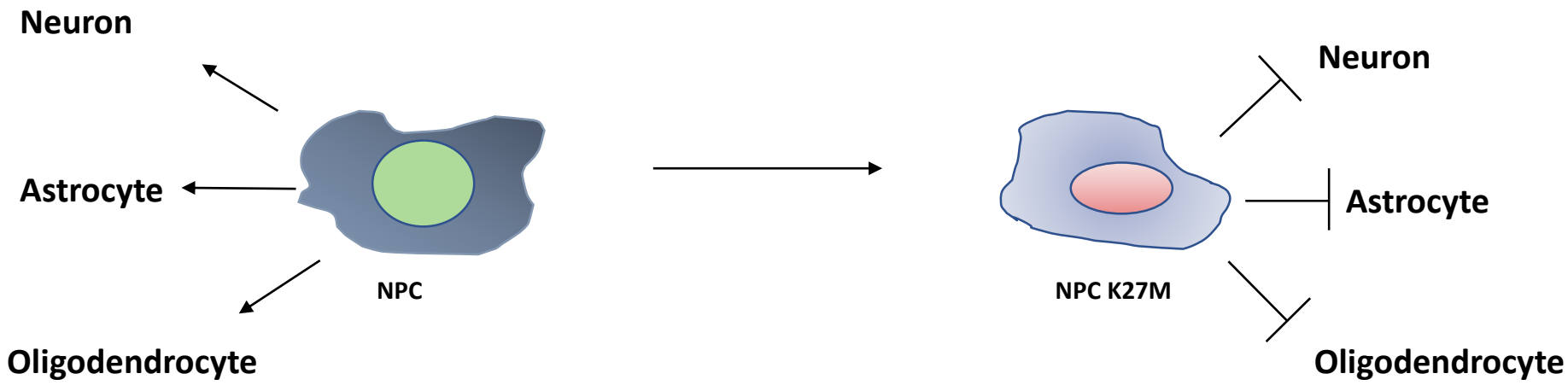
**DIPG are positive for neural stem cell linages markers: Nestin**

**DIPG are also positive for oligodendrocyte progenitor cell marker: Oligo2 and PDGFRa**

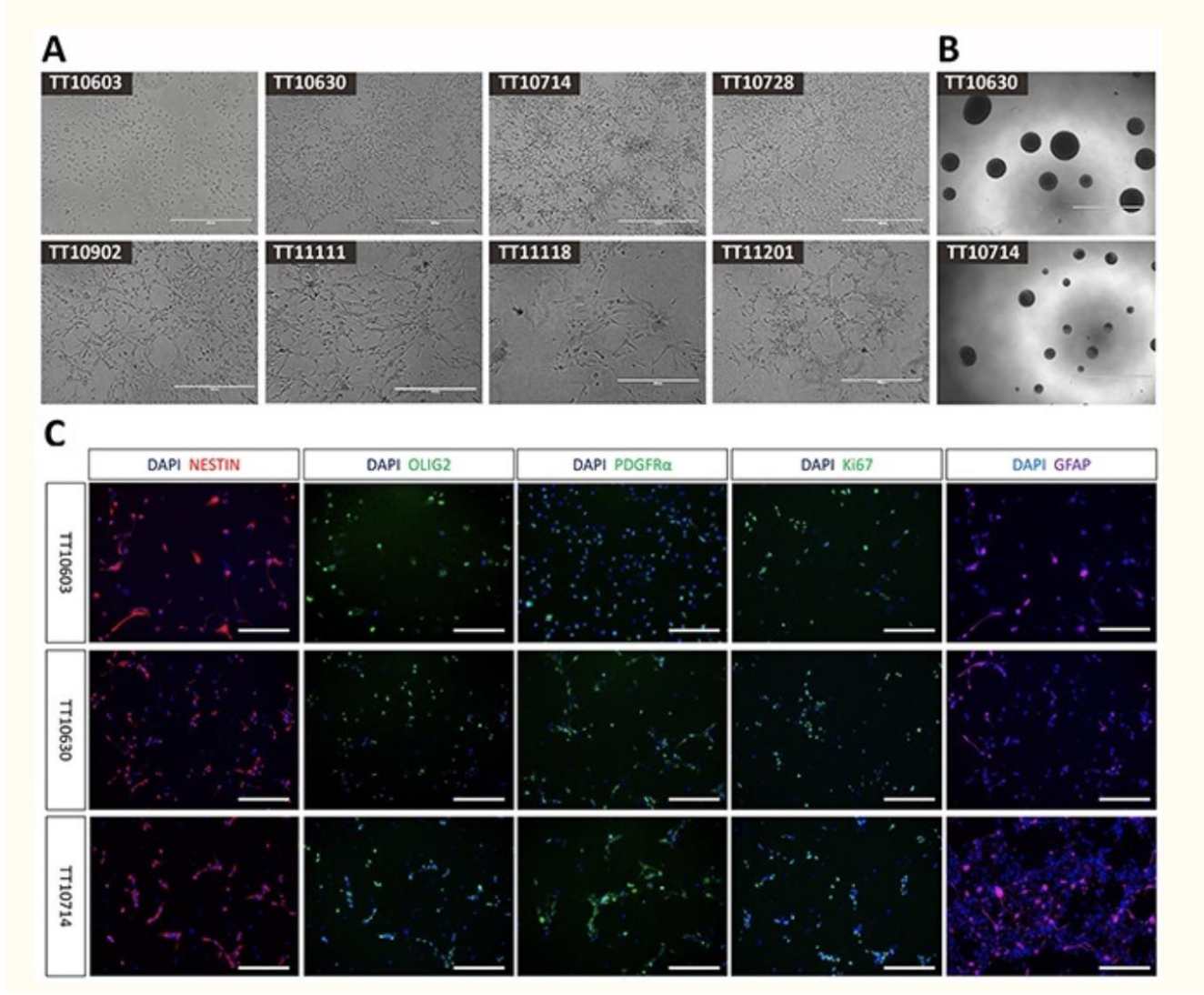
**DIPG are also positive for an astrocyte marker: GFAP**

**Normal Neurogenesis**

**H3K27M mutation results in 'Self renewal, multipotency, no differentiation'**



# Patient-derived DIPG cells preserve stem-like characteristics





**Undifferentiated lines are more reflective of patient material**

Xu et al. Oncotarget. 2017 Sep 29; 8(44): 76644–76655

Filbin et al *Science* 20 Apr 2018

DIPG.org

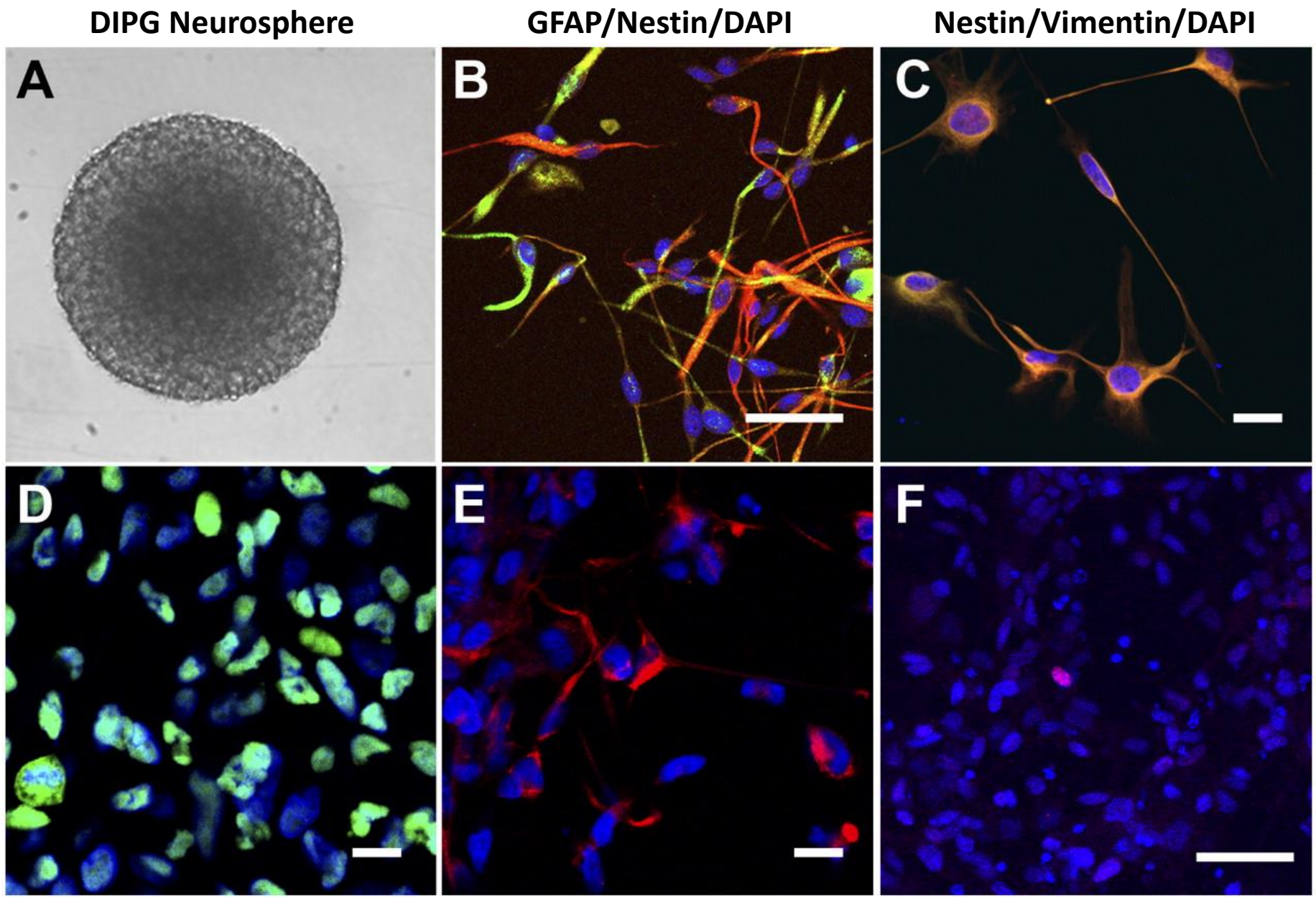
The Diffuse Intrinsic Pontine Glioma Resource Network

# Cancer Stem Cells

**Cancer stem cells (CSCs)** represent a subpopulation of cells that can generate all cell types found within a tumor and are thought to be responsible for tumor growth and spread. Like normal stem cells, cancer stem cells possess the capacity for self-renewal and multi-potency. (Multi-potent cells can make all the cell types in a tissue. In this case the tissue is the tumor.) The first cancer stem cells were described in acute myeloid leukemia, and have now been shown in many solid tumors, including many brain tumors such as glioblastoma and ependymoma. CSCs isolated from primary brain tumors possess many of the characteristics of normal neural stem cells, and can recapitulate the tumor *in vitro* and *in vivo*, whereas other cell types from the tumor cannot. CSCs are thus a small proportion of a tumor, but are solely responsible for tumor propagation.

The relationship of normal neural stem cells to cancer stem cells is somewhat controversial, but there is an emerging consensus that **many brain tumors arise from stem or precursor cell populations** in both children and adults. Excellent examples of this point include “radial glia” cells (a type of stem cell) giving rise to ependymoma and subventricular zone neural stem cells giving rise to central neurocytomas. With respect to more lineage-restricted precursors, Shh-responsive granule cell precursor cells of the cerebellum give rise to medulloblastoma in many cases, and recent animal model data indicate that oligodendrocyte precursors give rise to periventricular low grade gliomas in a mouse model of platelet-derived growth factor (PDGF) overexpression. Brain tumor stem cells exhibit many of the same marker proteins and utilize many of the same signaling pathways as normal neural stem cells. Understanding normal neural stem or precursor cells in the brainstem may thus shed light on brainstem tumor pathogenesis.

# Immunocytochemistry for CD133 reveals a $CD133^+$ fraction of about one-third of cells




Monje et al, PNAS March 15, 2011 108 (11) 4453-

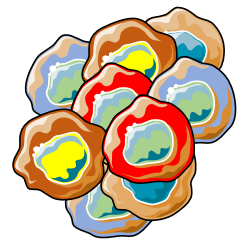
# Rational for targeting Tumor heterogeneity-Stemness

**Conventional Therapy**

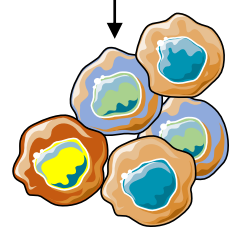
'Bulk' tumor shrinkage

Tumor regrowth due to CSC plasticity

**Targeted Cancer Stem Cell therapy**

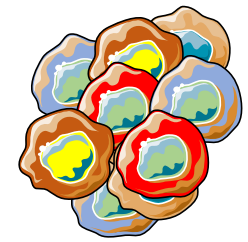


CSC eradication

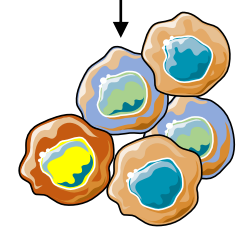


Inability to regenerate

**Combination therapies?**



CSC eradication



Bulk tumor eradication

Inability to regenerate

**Remission/Cure of the Patient**

# Ongoing projects in the Galban lab

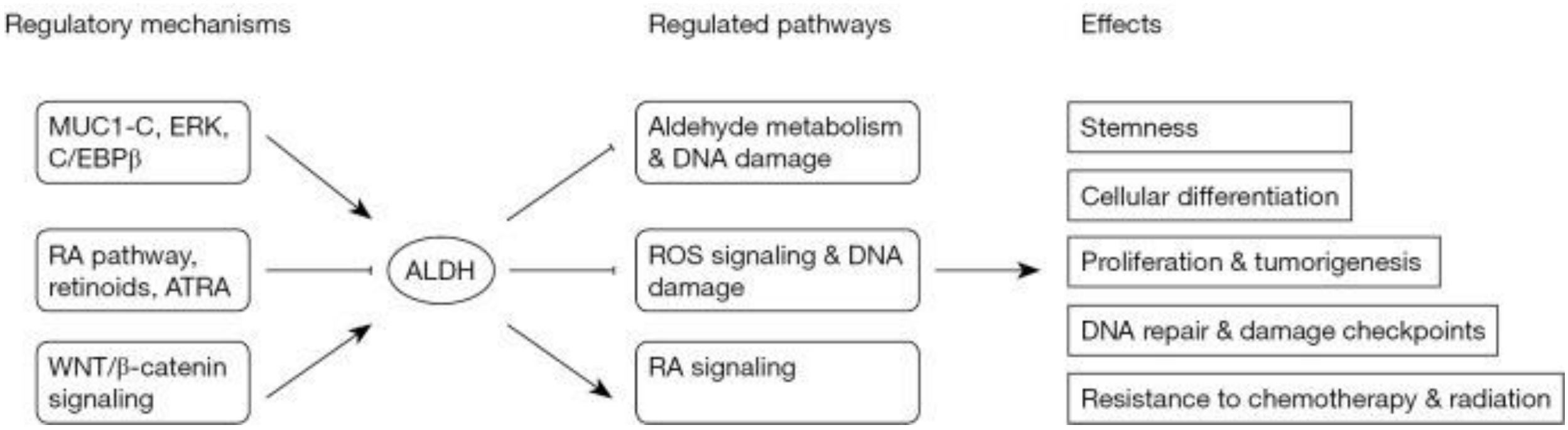
**Targeting Stemness:**

- Identification of CSC in DIPG
- Characterization of CSC in DIPG
- In vivo characterization of CSC in DIPGs
- Metabolism of CSC in DIPGs
- Targeting of CSC in DIPGs

**Identification of resistance mechanism:**

- Co-targeting metabolism and or epigenetic changes
- Developing epigenetic imaging strategies for DIPG

# ALDHs regulate multiple pathways to contribute carcinogenesis and stem cell signaling




Clark et al, Ann Transl Med. 2016 Dec; 4(24): 518.

# Aldehyde dehydrogenase (ALDH) superfamily

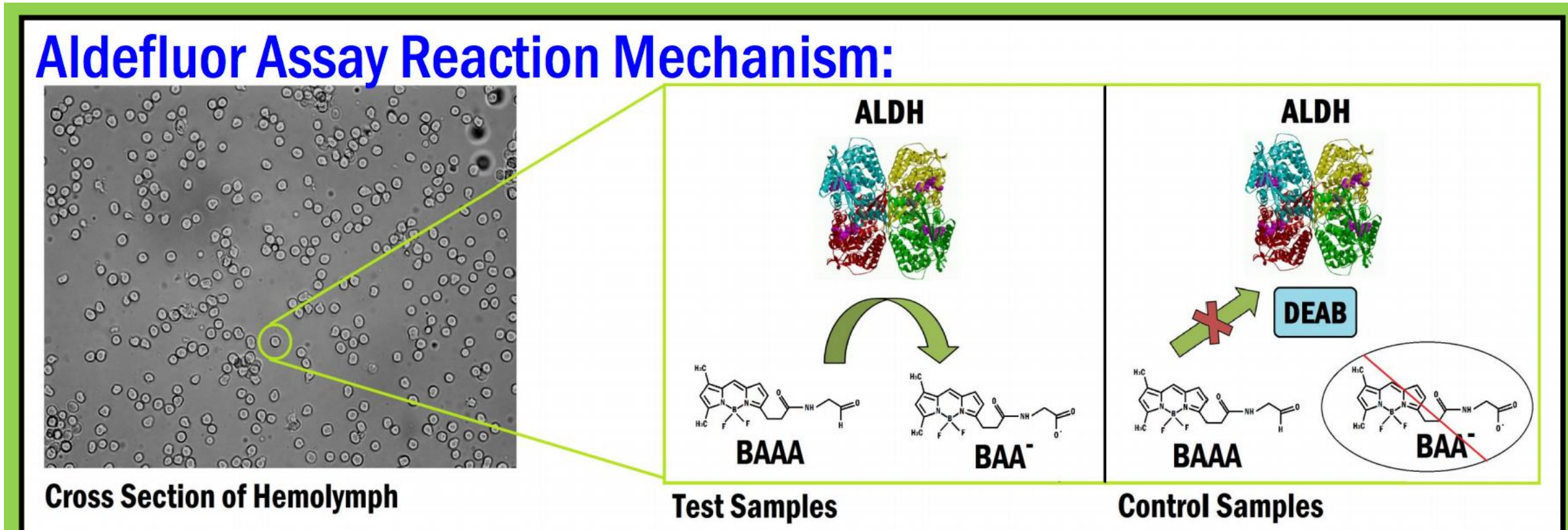
**Table 1: Human ALDH isoenzymes**

| Isoenzymes | prefered substrates | Subcellular distribution | Organ and tissue distribution | Chromosomal locaization |
|---|---|---|---|---|
| ALDH1A1 | Retinal | Cytosol | Liver, kidney, red blood cells, skeletal muscle, lung, breast, lens, stomach, brain, pancreas, testis, prostate, ovary | 9q21,13 |
| ALDH1A2 | Retinal | Cytosol | Testis, liver, kidney | 15q21.3 |
| ALDH1A3 | Retinal | Cytosol | Kidney, skeletal muscle, lung, breast, stomach, salivary glands | 15q21.3 |
| ALDH1B1 | Acetaldehyde, lipid peroxidation-derived aldehdes | Mitochondria | Liver, kidney, heart, skeletal muscle, brain, prostate, lung, teastis, placenta | 9p11,1 |
| ALDH1L1 | 10-Formyltetrahydrofolate | Cytosol | Liver, skeltal muscle, kidney | 3q21.3 |
| ALDH1L2 | Unknown | Cytosol | | 12q23.3 |
| ALDH2 | Acetaldehyde, nitroglycerin | Mitochondria | Liver, kidney, heart, skeletal muscle, lens, brain, pancreas, prostate, spleen | 12q24.2 |
| ALDH3A1 | Medium-chain aliphatic and aromatic aldehydes | Cytosol, nucleus | Stomach, cornea, breast, lung, lens, esophagus, salivary glands, skin | 17p11.2 |
| ALDH3A2 | Long-chain aliphatic aldehydes | Microsomes, peroxisomes | Liver, kidney, heart, skeletal muscle, lung, brain, pancreas, placenta, most tissues | 17p11.2 |
| ALDH3B1 | Lipid peroxidation-derived aldehydes | Mitochondria | Kidney, lung, pancreas, placenta | 11q13 |
| ALDH3B2 | Unknown | Mitochondria | Parotid gland | 11q13 |
| ALDH4A1 | Proline metabolism | Mitochondria | Liver, kidney, heart, skeletal muscle, brain, pancreas, placenta, lung, spleen | 1p36 |
| ALDH5A1 | Succinic semialdehyde | Mitochondria | Liver, kidney, heart, skeletal muscle, brain | 6p22 |
| ALDH6A1 | Methylmalonate semialdehyde | Mitochondria | Liver, kidney, heart, skeletal muscle | 14q24.3 |
| ALDH7A1 | Betane aldehyde, lipid peroxidation-derived aldehydes | Mitochondria, nucleus, cytosol | Fetal liver, kidney, heart, lung, brain, ovary, eye, cochlea, spleen adult spinal cord | 5q31 |
| ALDH8A1 | Retinal | Cytosol | Liver, kidney, brain, breast, testis | 6q23.2 |
| ALDH9A1 | γ-Aminobutyraldehyde, aminoaldehydes | Cytosol | Liver, kidney, heart, skeletal muscle, brain, pancreas, adrenal gland, spinal cord | 1q23.1 |
| ALDH16A1 | Unknown | Unkown | Neuronal cells | 19q13.33 |
| ALDH18A1 | Glutamatic γ-semialdehyde | Mitochondria | Kidney, heart, skeletal muscle, pancreas, testis, prostate, spleen,ovary, thymus | 10q24.3 |

**-> Aldehyde dehydrogenase 1A1 most important in stem cells and cancer**

Tomita, cancer, stem cell Received: July 11, 2015 Accepted: January 07, 2016

# ALDEFLUOR assay: viable, non-ab based stain, specific for ALDH1A1 isoform



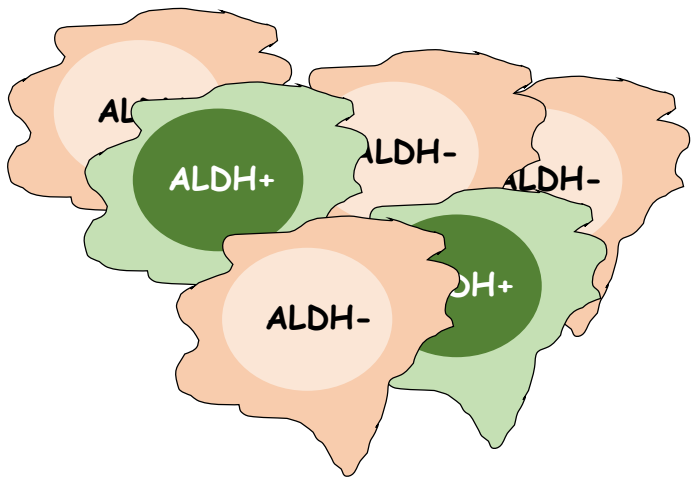

**Reaction Mechanism:** ALDH converts the Aldefluor reagent (BAAA) to its fluorescent form ($BAA^-$). In the control-treated samples, DEAB prevents BAAA from binding to ALDH. Thus, no $BAA^-$ is generated, and the amount of fluorescence is reduced.

BAAA(BODIPY-aminoacetaldehyde)
BAA-(BODIPY-aminoacetate)
Diethylaminobenzaldehyde (DEAB)

# Patient derived cell lines

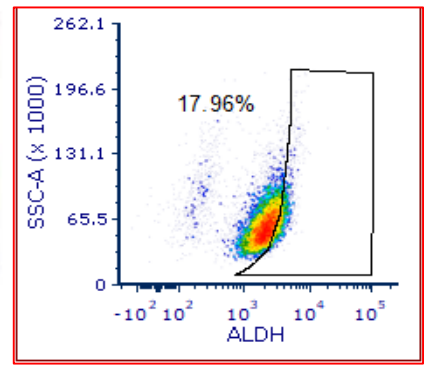
| Cell line | Mutations | Tissue Obtained | Prior Therapy | Source/Ref. |
|---|---|---|---|---|
| SU-DIPG IV | H3.1K27M, MDM4 amplified, ACVR1 G328V | Early postmortem autopsy | Radiotherapy, cetuximab, irinotecan | Grasso et al. *Nature Medicine (2015)* |
| SU-DIPG XIII | H3.3K27M | Early postmortem autopsy | Radiotherapy | Grasso et al. *Nature Medicine (2015)* |
| SF7761 | H3.3K27M | Surgical biopsy | None | Millipore |
| SF8628 | H3.3K27M | Surgical biopsy | None | Millipore |

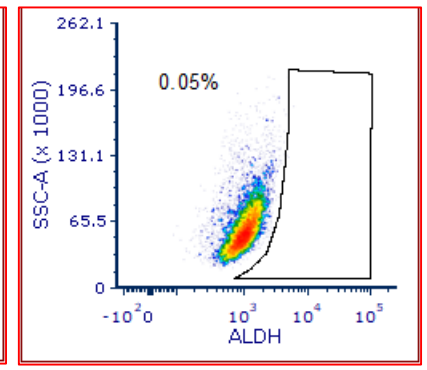
# Staining, FACS and microscopy of ALDH+ SU-DIPG-13 cells



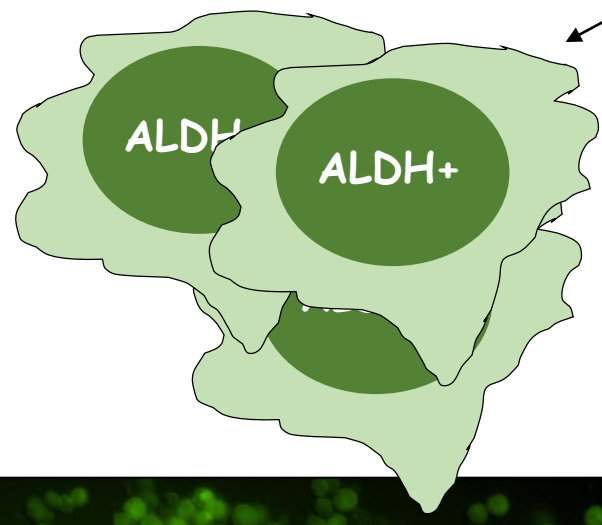

**ALDEFLUOR assay**

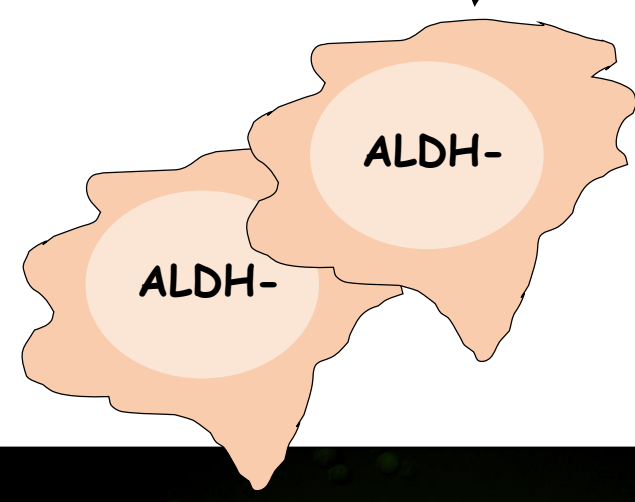
**FACS**

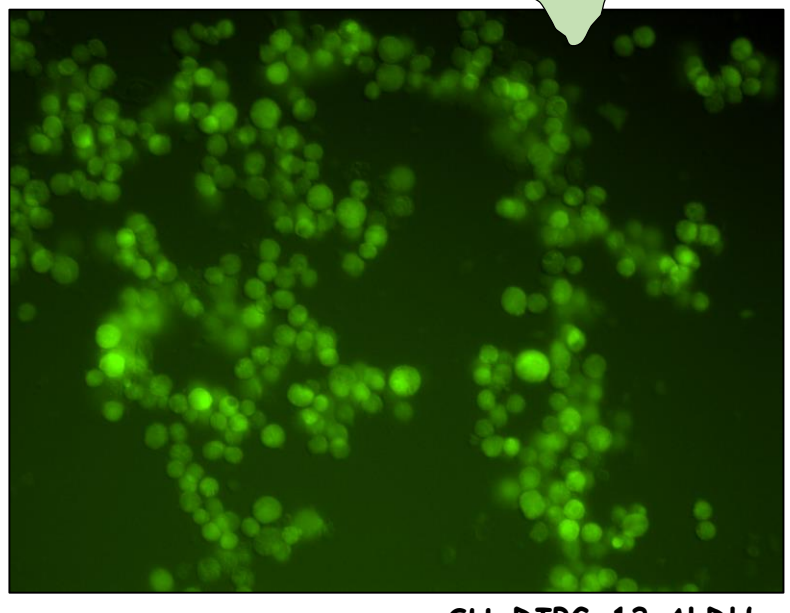


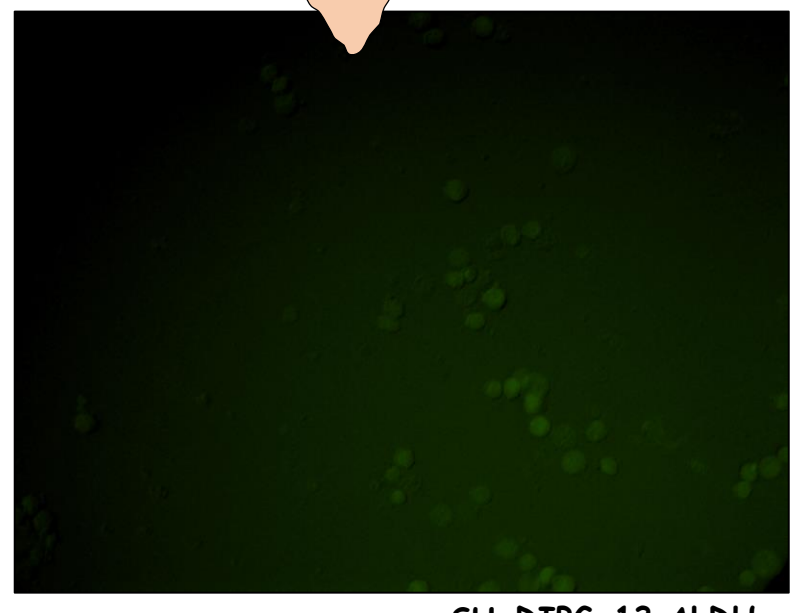

DEAB negative control



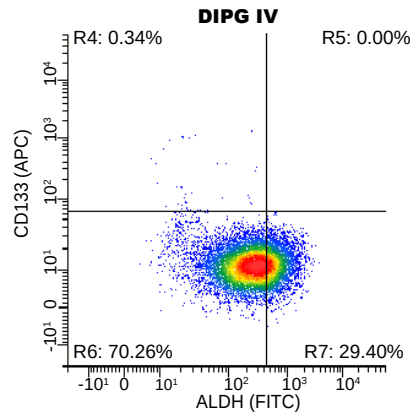



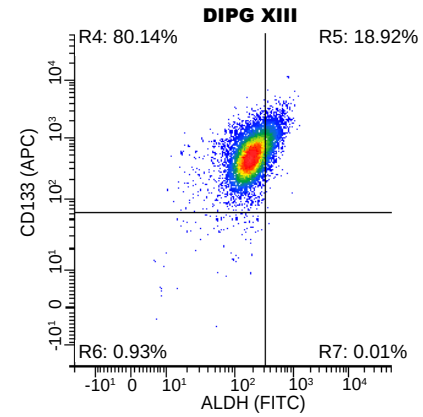
SU-DIPG-13 ALDH+

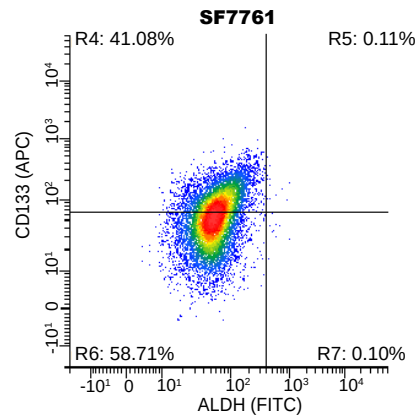


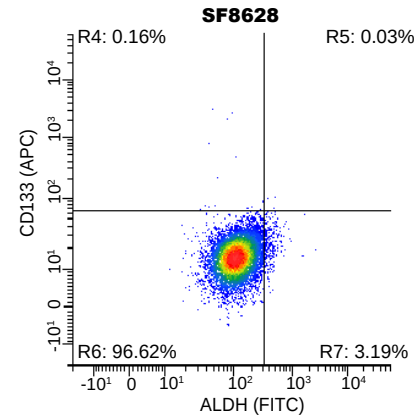
SU-DIPG-13 ALDH-

# Phenotypic diversity in stem cell marker expression of Aldehyde dehydrogenases and CD133 among DIPGs



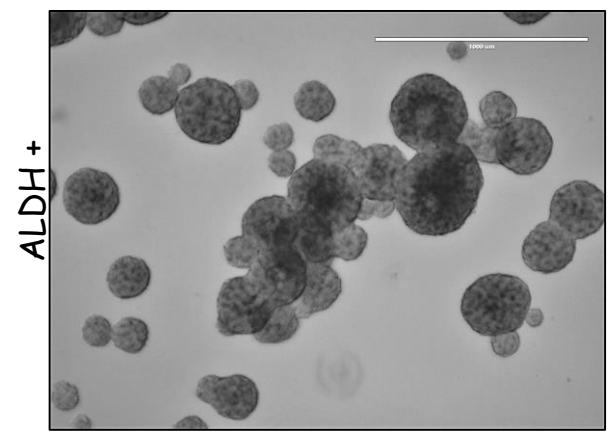



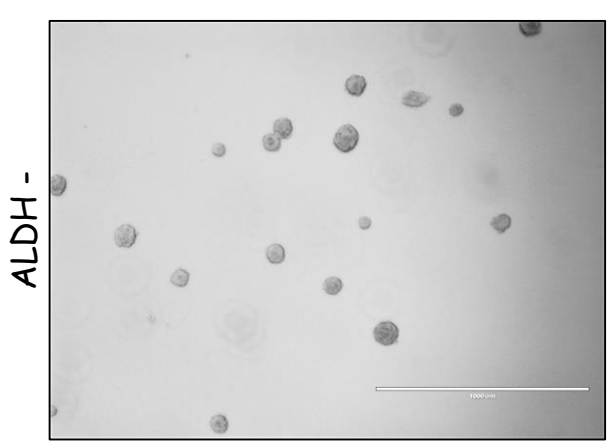




| Cell line | ALDH + (FITC) | CD133 + (APC) | ALDH+/CD133+ |
|---|---|---|---|
| DIPG IV | 31.41 ± 3.17 | 0.16 ± 0.14 | 0.08 ± 0.083 |
| DIPG XIII | 24.54 ± 7.80 | 98.42 ± 0.25 | 18.21 ± 2.28 |
| SF8628 | 8.27 ± 4.29 | 0.35 ± 0.35 | 0.027 ± 0.027 |
| SF7761 | 0.01 ± 0.01 | 46.3 ± 22.75 | 0.14 ± 0.090 |

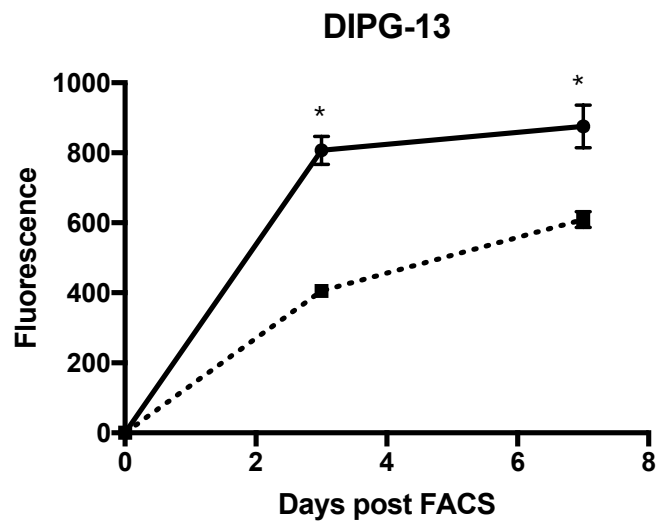
-> ALDH$^+$ Cell Populations Are Highly Variable among Individuals

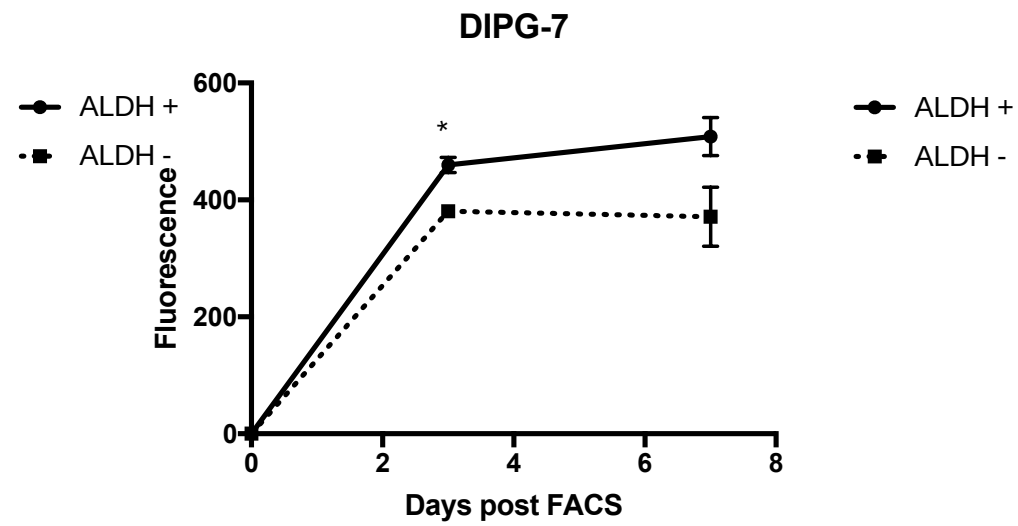
# Increased proliferation and sphere formation in ALDH+ cells

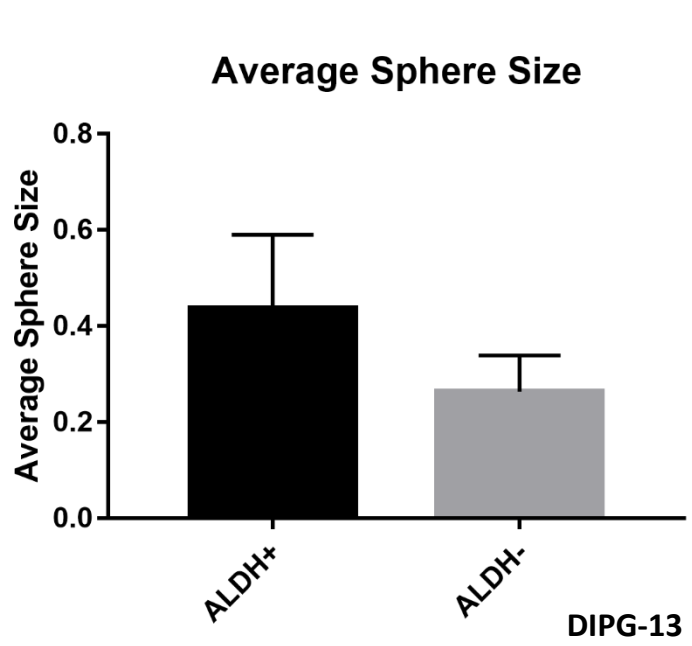


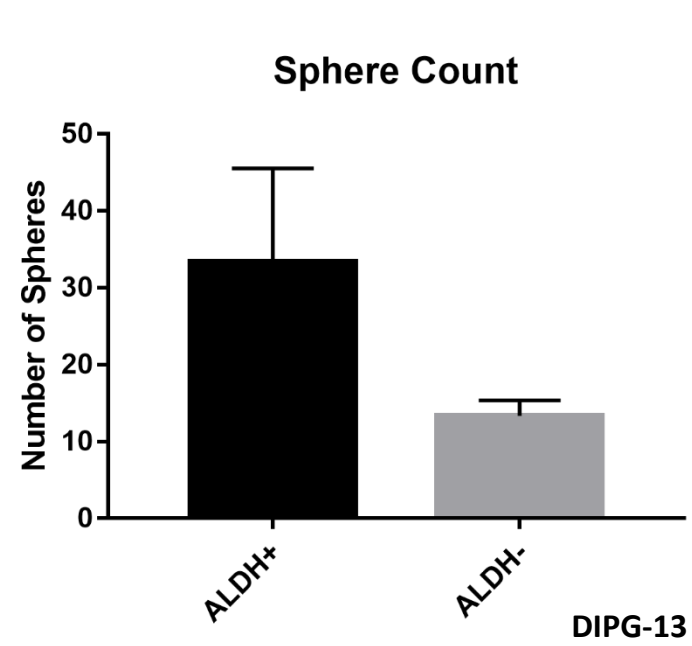




**DIPG-13** 7 days post FACS

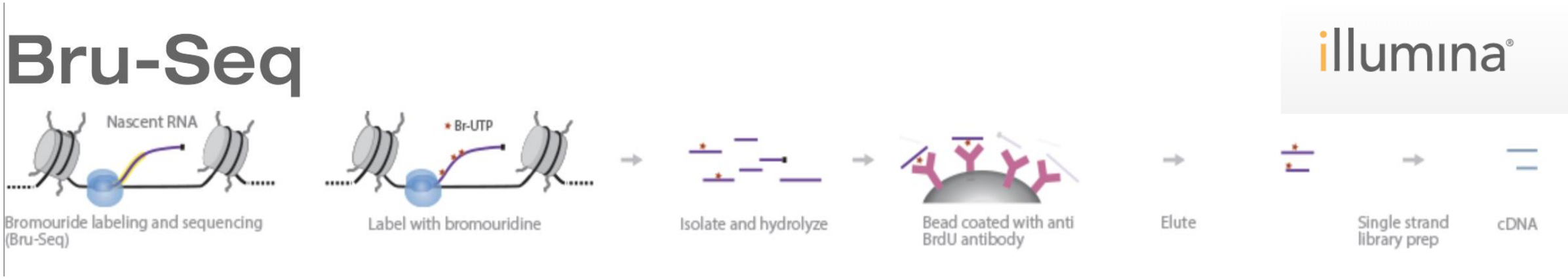


# Transcriptome analysis to determine 'stemness' of ALDH+ DIPG cells

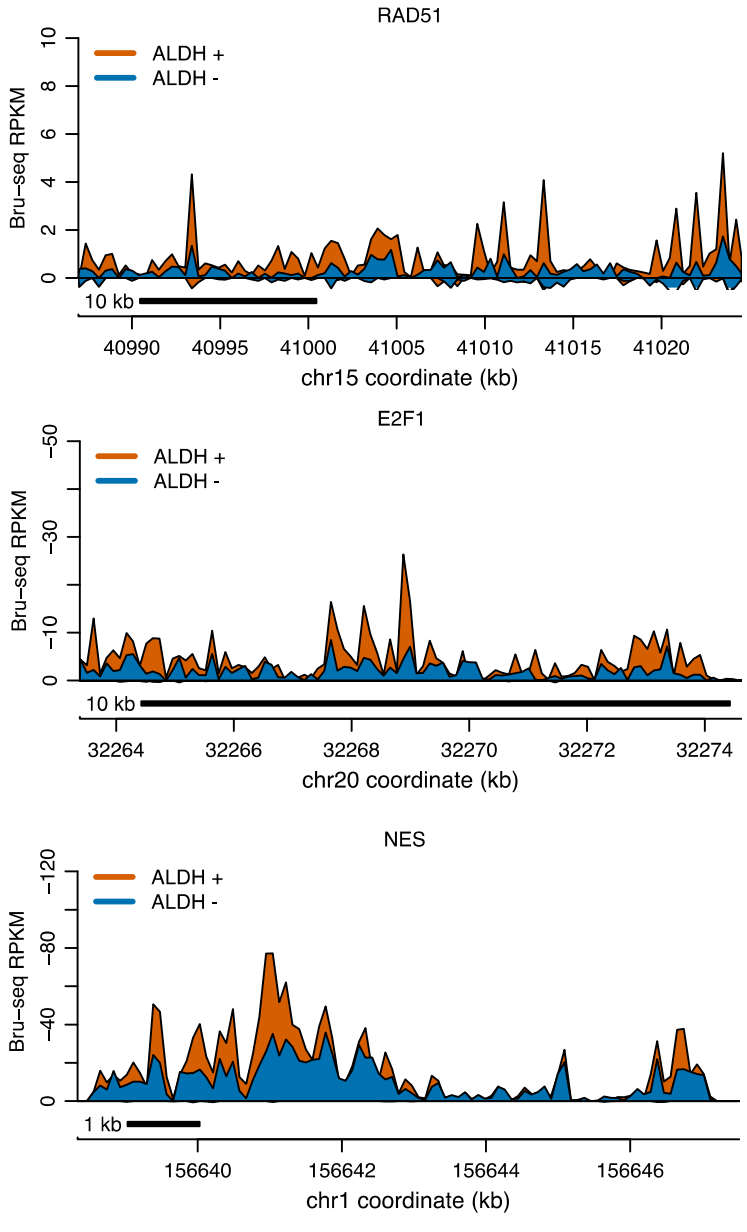


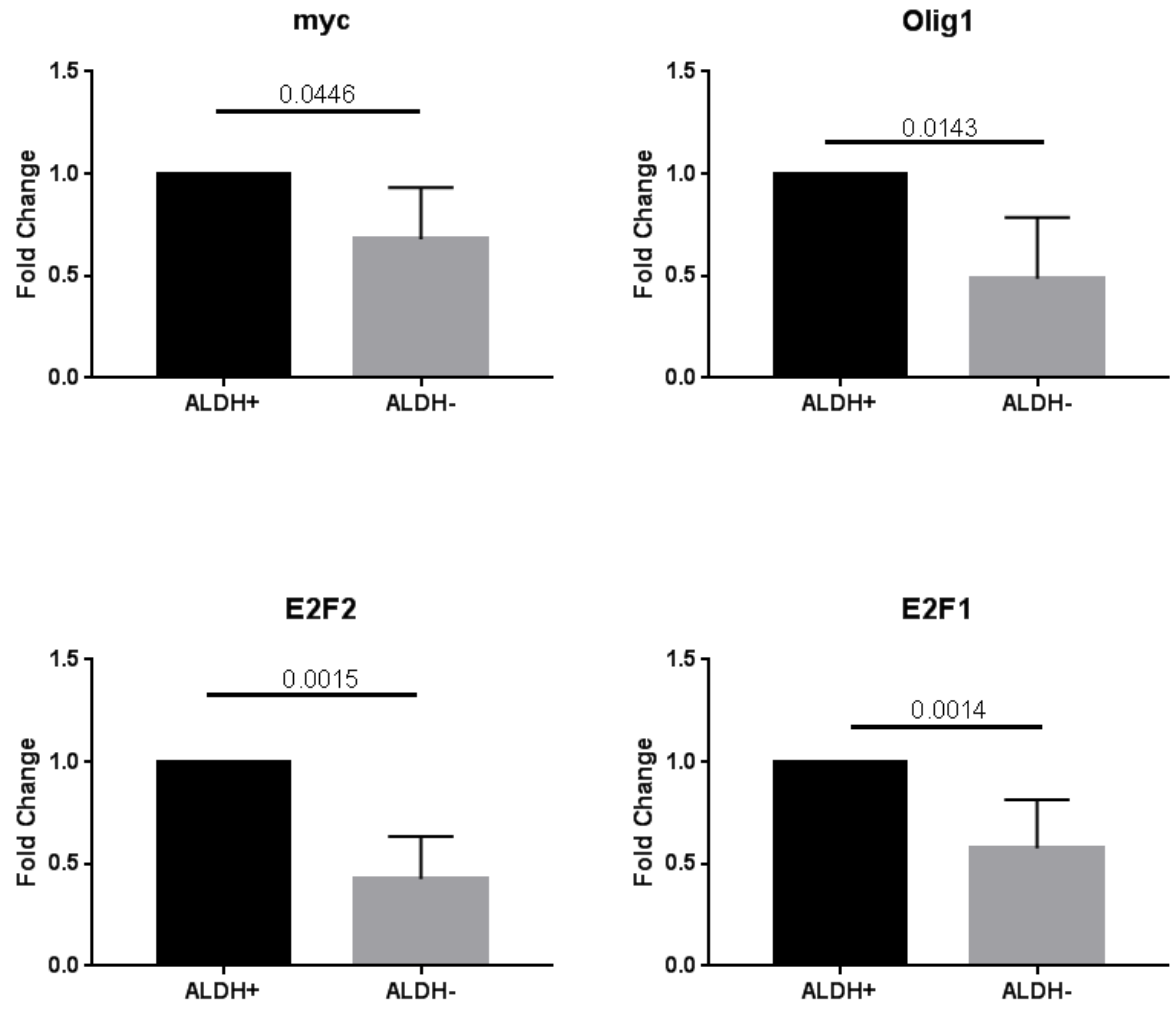

Bromouridine labeling of RNA
ALDEFLUOR staining
**FACS sorting in ALDH- and ALDH+ cells**


Collection of total RNA
Isolation of Bru-RNA
Generation of cDNA libraries
Sequencing and data analysis

**Collaboration with Mats Ljungman**

# ALDH positive DIPG cells exhibit a more "stem-like" transcriptome profile

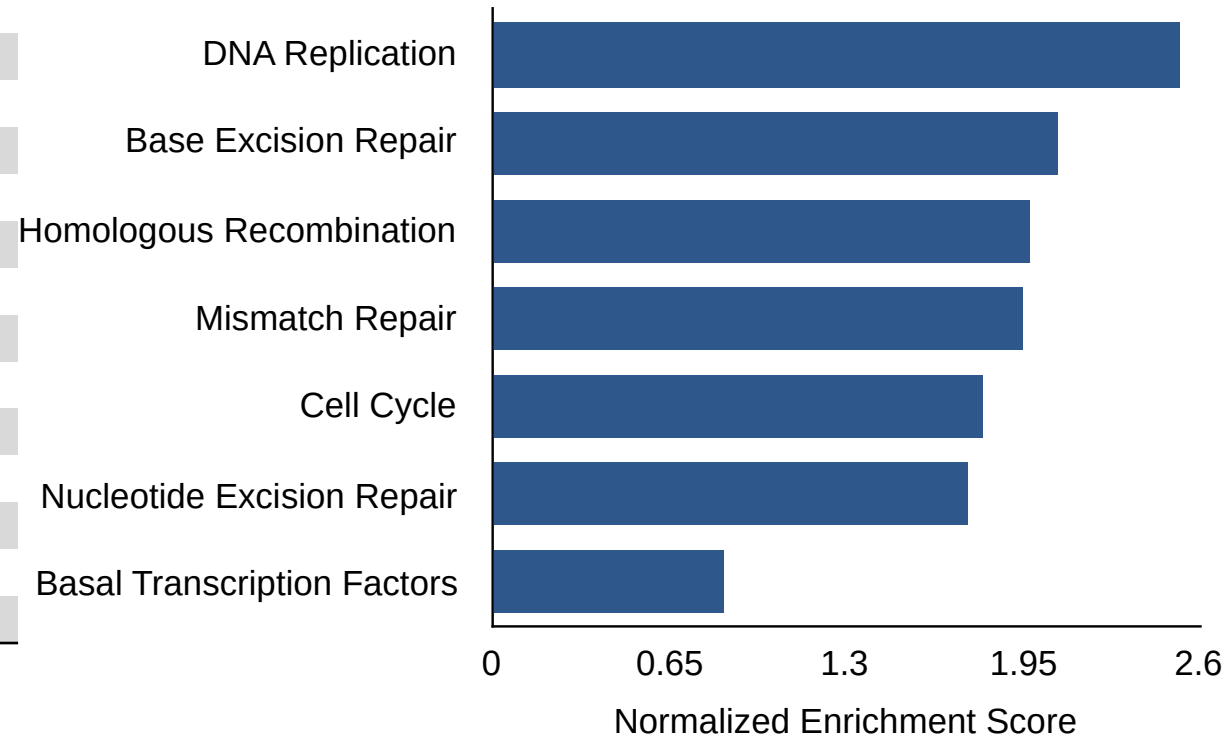


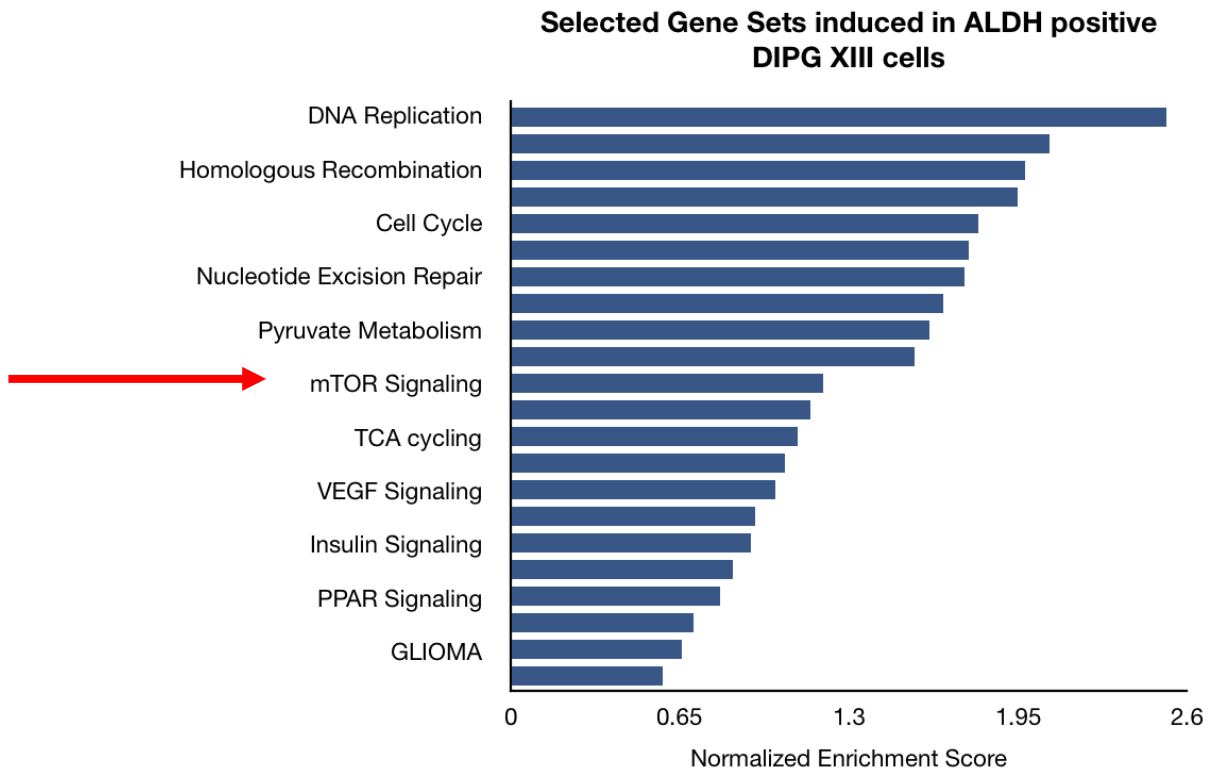
# Correlation between therapeutic resistance and protection of genome integrity

| Upregulated Gene | log2 Fold Change | Function |
|---|---|---|
| RECQL4 | 1.808 | Homologous Recombination |
| PKMYT1 | 1.78 | inactivates G2 to M transition |
| CEBPA | 1.5 | increases arrest of G1-S |
| GINS2 | 1.492 | interacts with chk2, initiation of replication forks |
| RAD51 | 1.326 | Homologous Recombination |
| TK1 | 1.325 | upregulated in S- phase |
| MCM2 | 1.213 | DNA replication initiation |
| E2F1 | 1.02 | stem cell , Cell cycle |
| E2F2 | 1.002 | stem cell, Cell cycle |
| RFC2 | 0.943 | DNA Damage Response |
| PCNA | 0.915 | DNA Damage Response |
| BRCA1 | 0.681 | Homologous Recombination |
| APEX1 | 0.625 | DNA Damage Response |
| OLIG1 | 0.62 | Stem cell marker |
| PARP1 | 0.589 | Base Excision Repair response |
| NES | 0.573 | Stem Cell marker |
| CHEK2 | 0.529 | DNA repair, cell cycle arrest |
| MYC | 0.189 | stem cell, proliferation |
| ATM | 0.008 | DNA Damage Response |

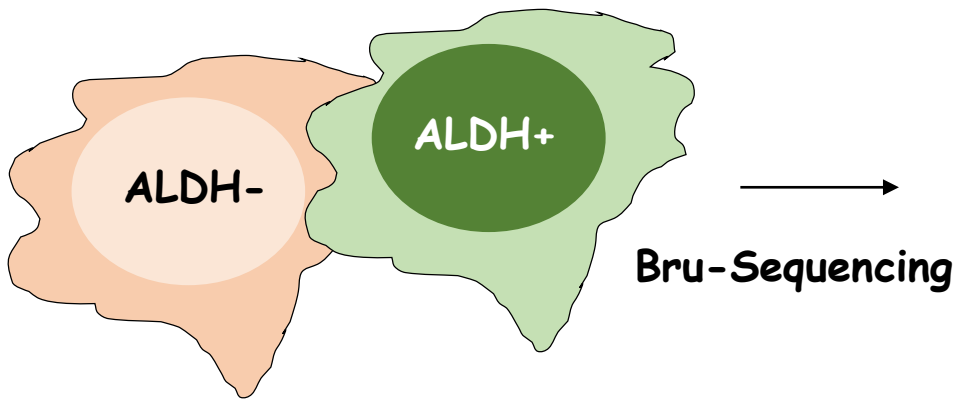
**Selected Gene Sets induced in ALDH+ DIPG XIII cells**



# Rationale for Targeting mTOR pathway



# Increased metabolism in ALDH+ cells




Bru-Sequencing

| Gene | Log2Fold change |
|---|---|
| LDHB | 0.661 |
| HK1 | 0.39 |
| ENO1 | 0.309 |
| PHGDH | 0.222 |
| PDHA1 | 0.142 |

LDHB = Lactate dehydrogenase B

HK1 = Hexokinase 1

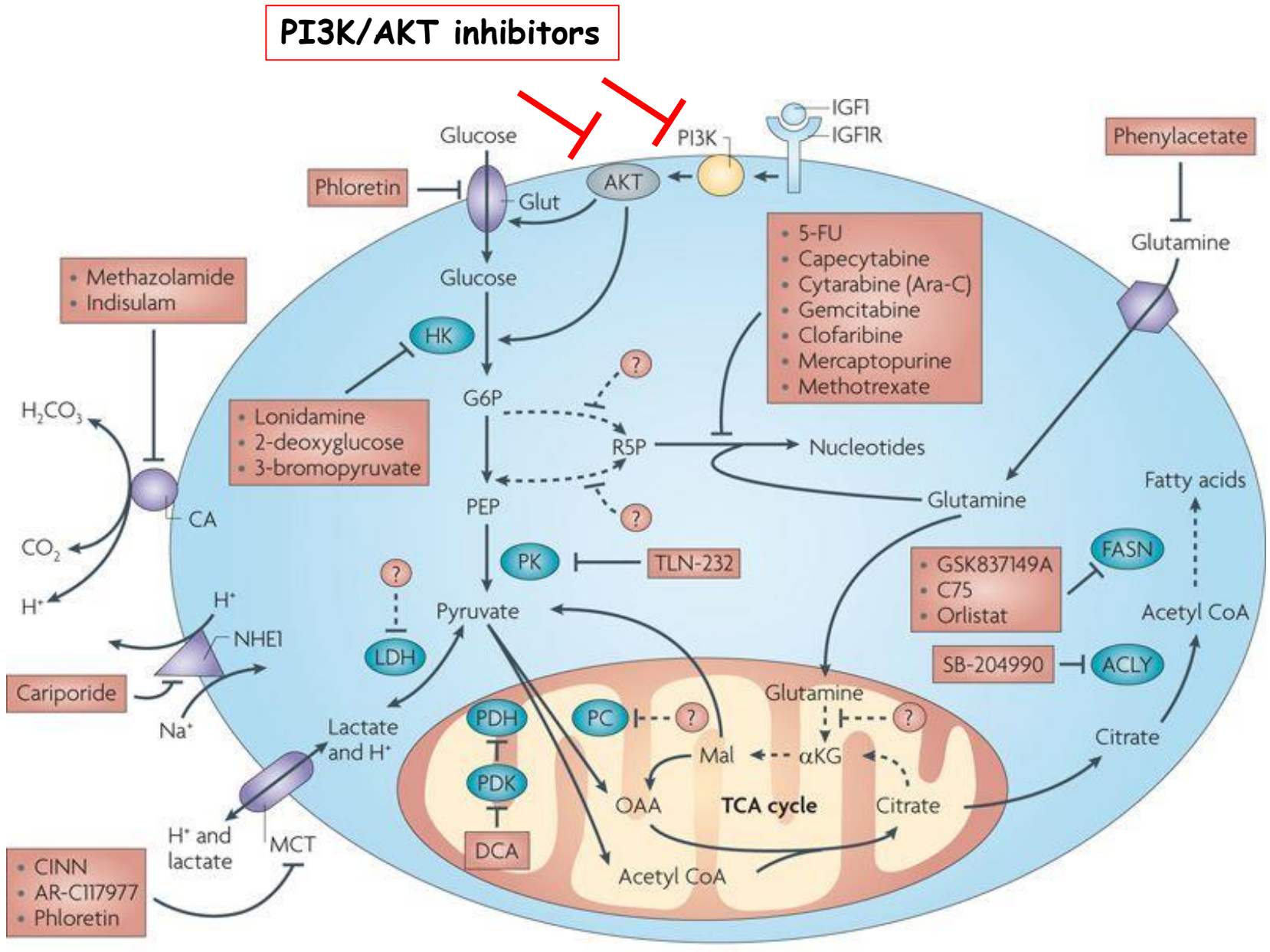
ENO1 = Enolase one

PHGDH = Phoshoglycerate dehydrogenase

PDHA1 = Pyruvate dehydrogenase

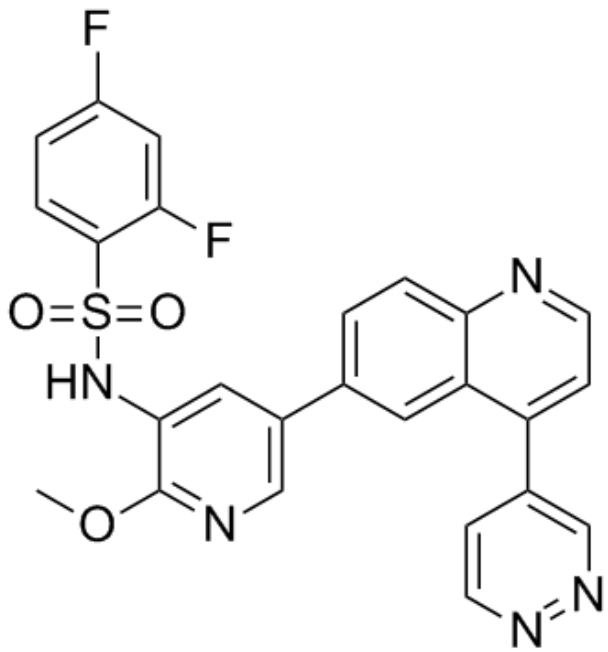
**Increased metabolism:** pentose, glycolysis, glyconeogenesis, pyruvate metabolism and oxidative phosphorylation

# Metabolic pathways and enzymes against which compounds are already in the clinic.




Nature Reviews | Cancer

# Strategies for developing multifunctional kinase inhibitors

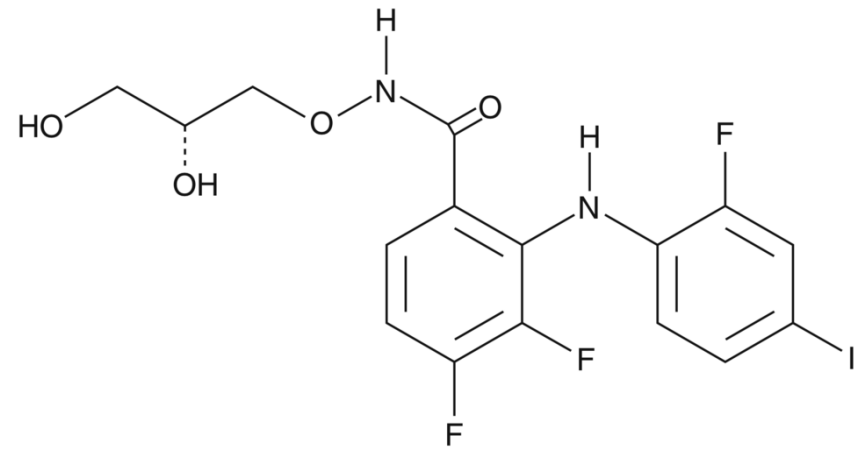


$C_{25}H_{17}F_2N_5O_3S$

**GSK2126458**

GlaxoSmithKline

**ATP-competitive PI3K/mTOR inhibitor**



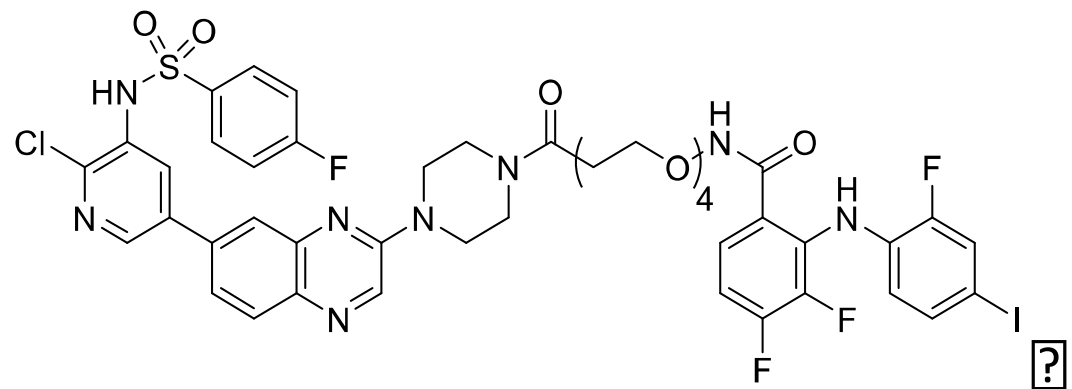
$C_{16}H_{14}F_3IN_2O_4$

**PD 0325901**

**Pfizer**

**ATP non-competitive MEK inhibitor**

?



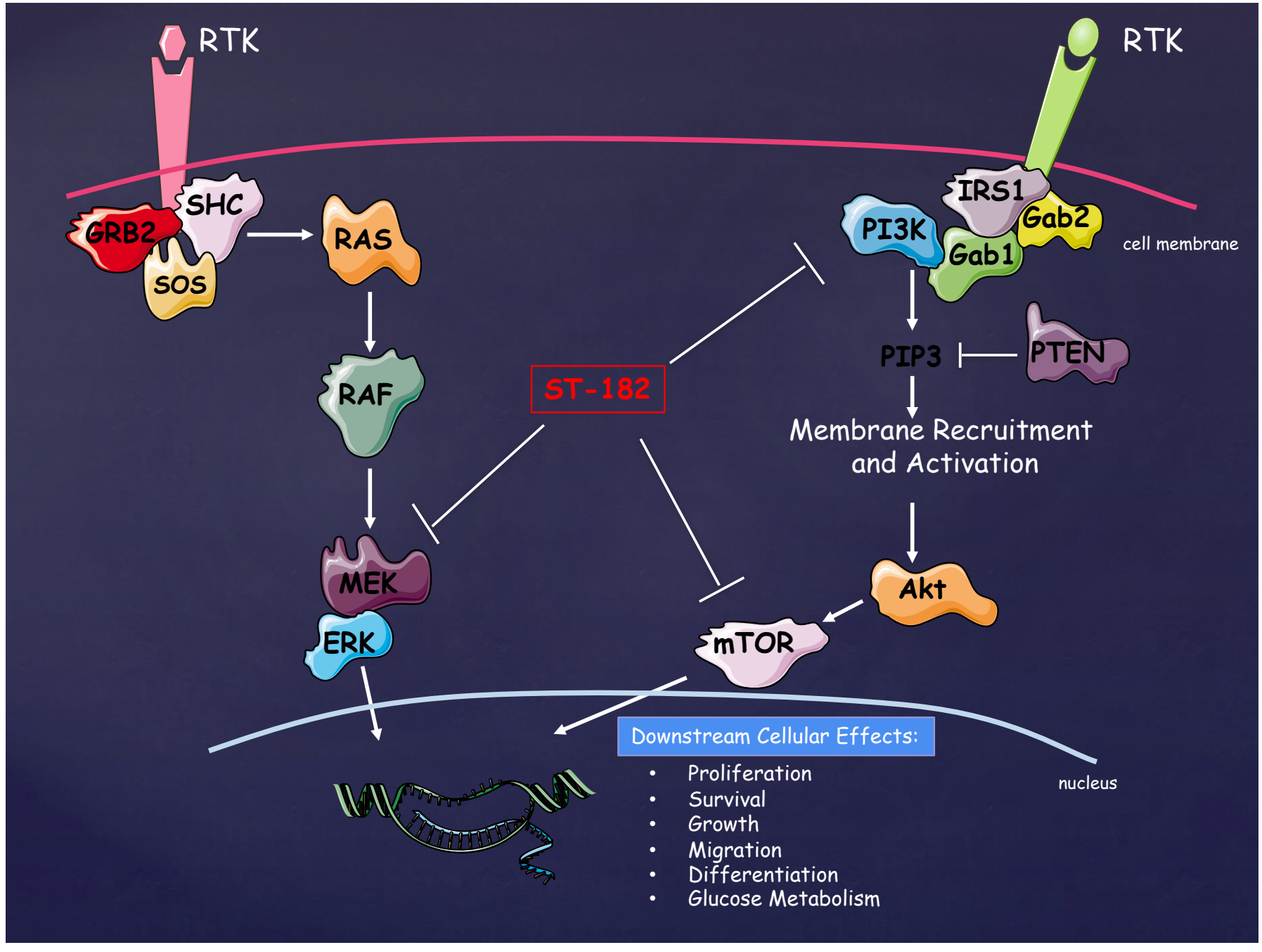
?

**ST-182**

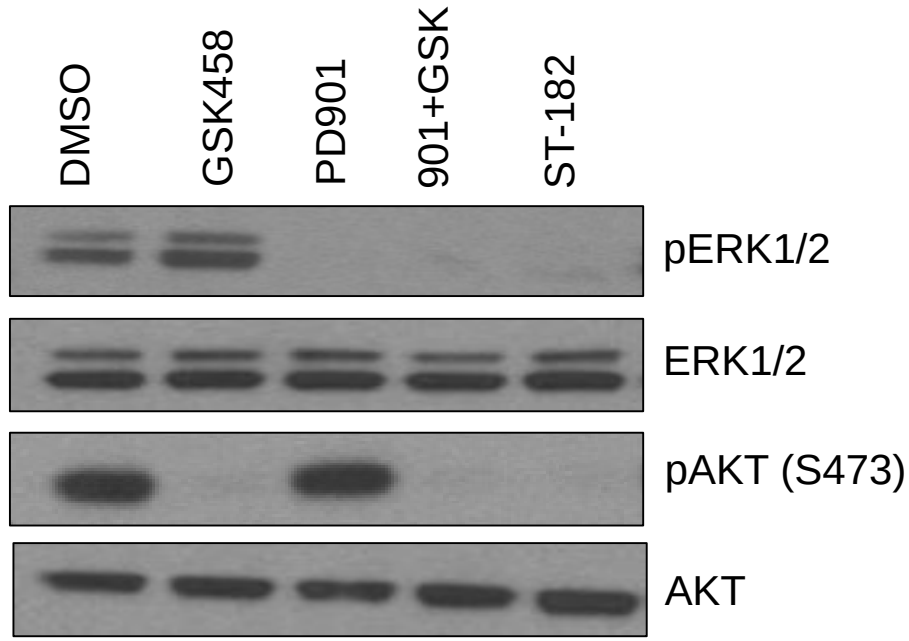
**multifunctional kinase inhibitor**

Ross/van Dort/Galban

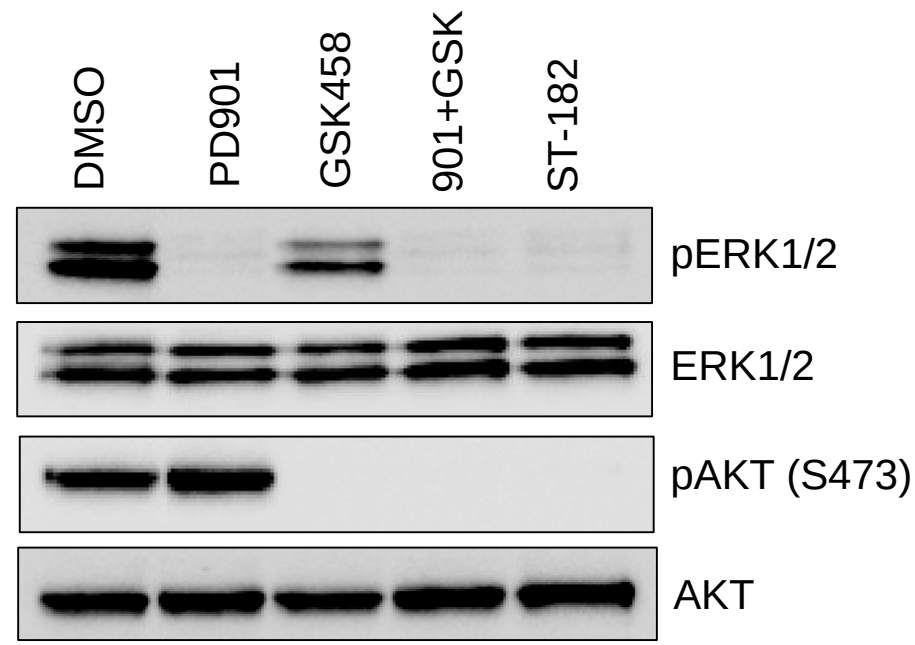
# Rational for simultaneously targeting the MAPK and PI3K pathway



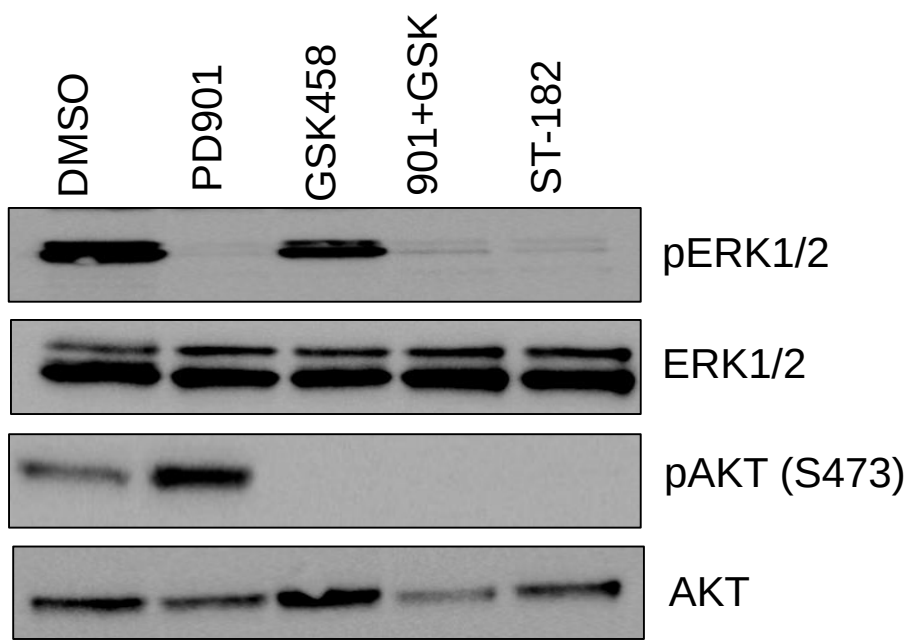
# ST-182 is a Multifunctional Kinase Inhibitor of MEK and PI3K/mTOR in Patient-Derived DIPG Cell Lines



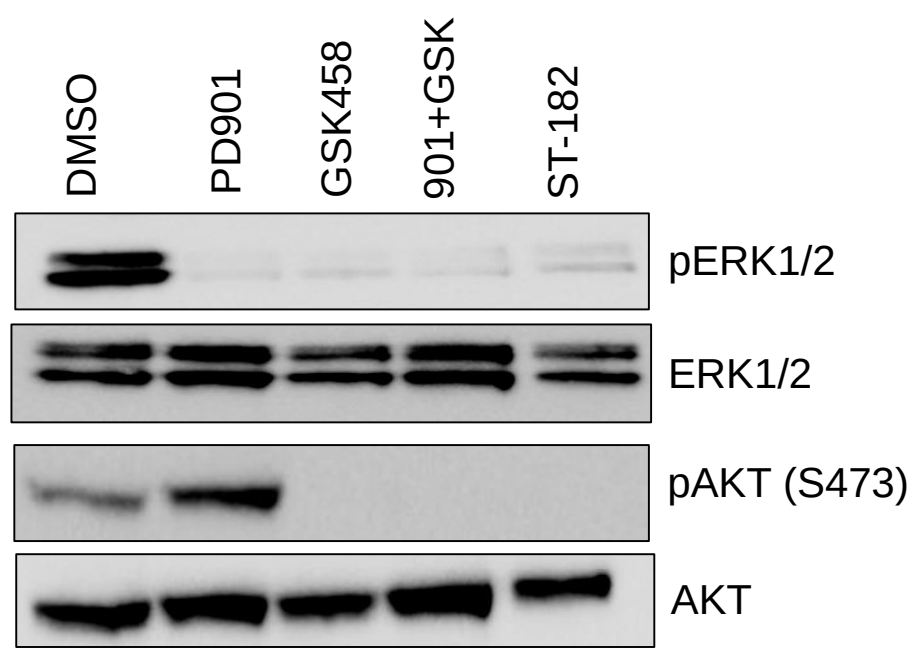

**SU-DIPG XIII**




**SU-DIPG IV**

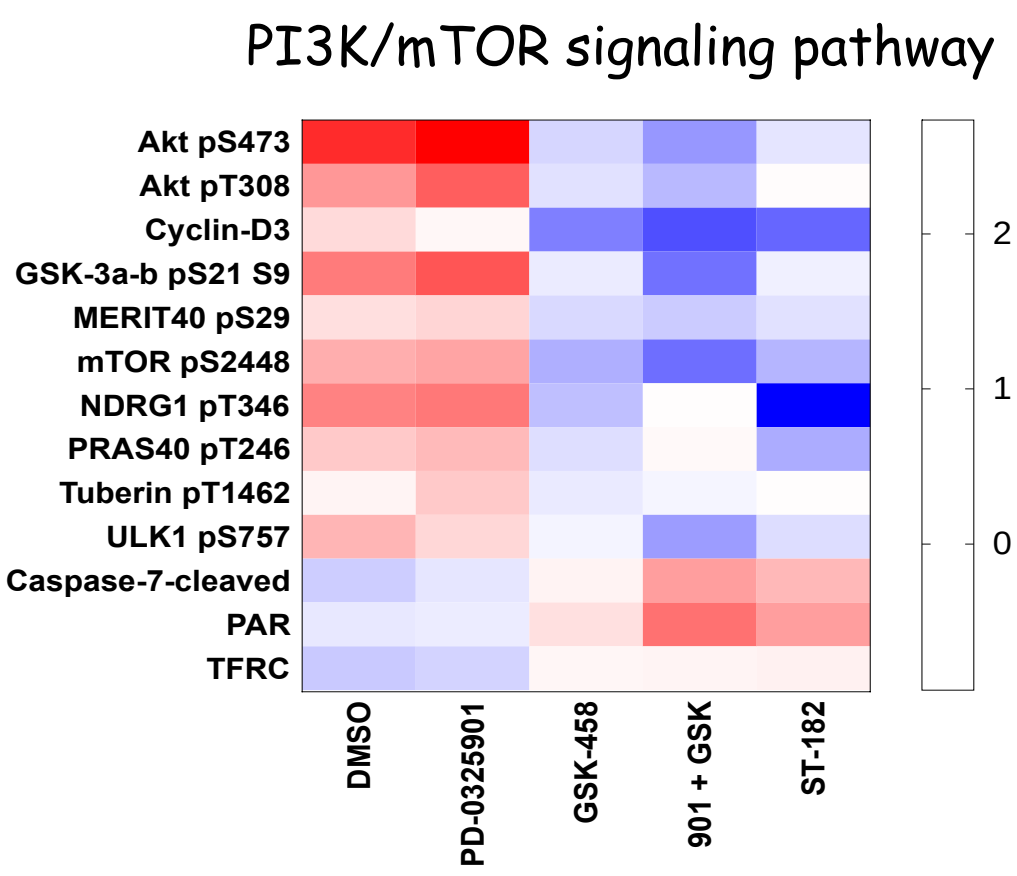


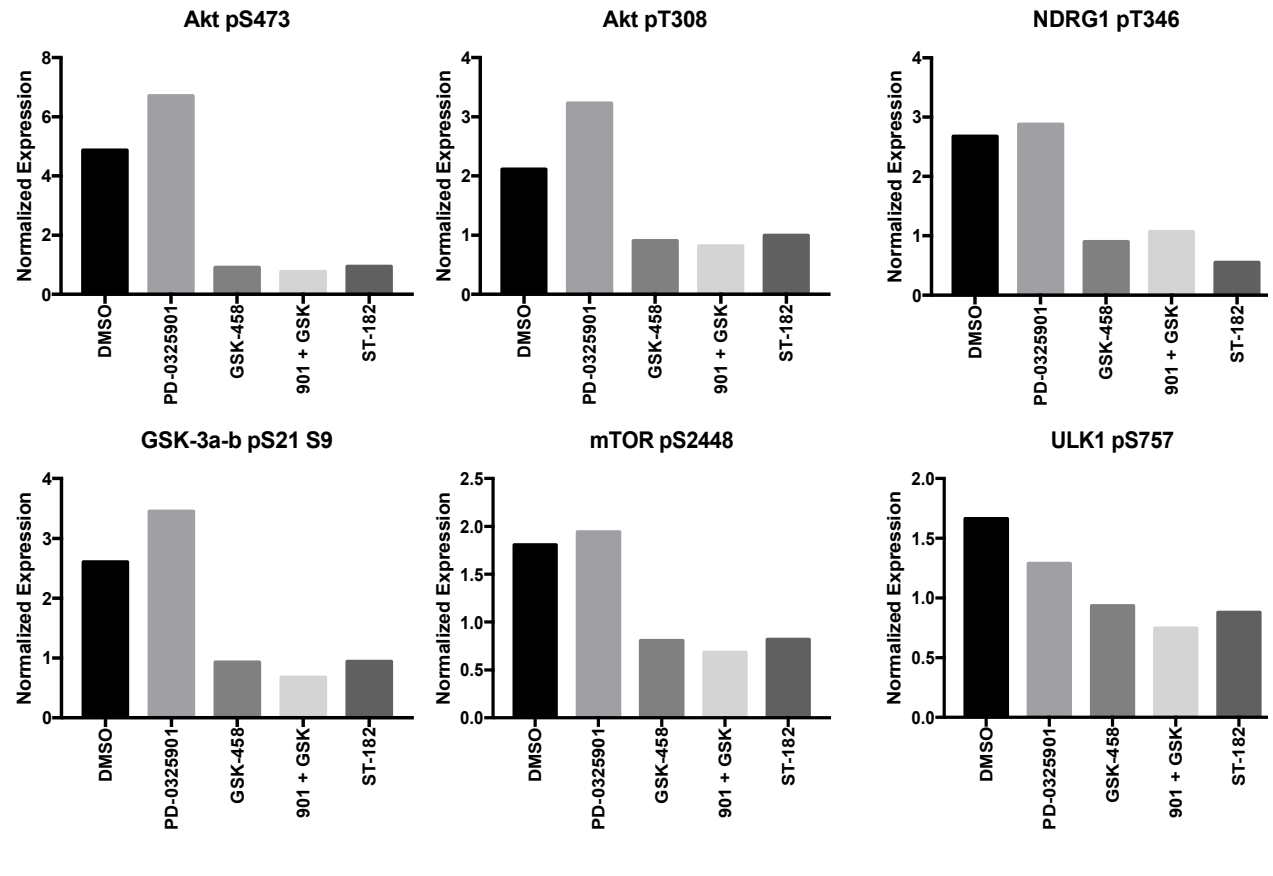

**SF7761**



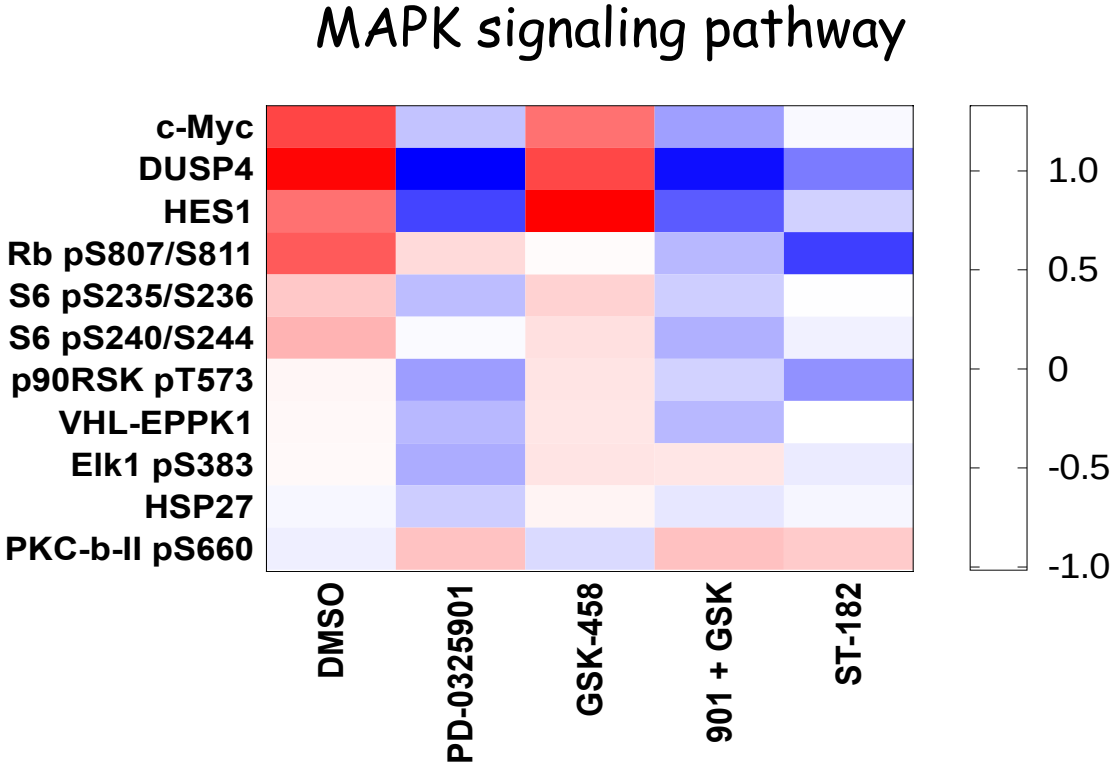

**SF8628**

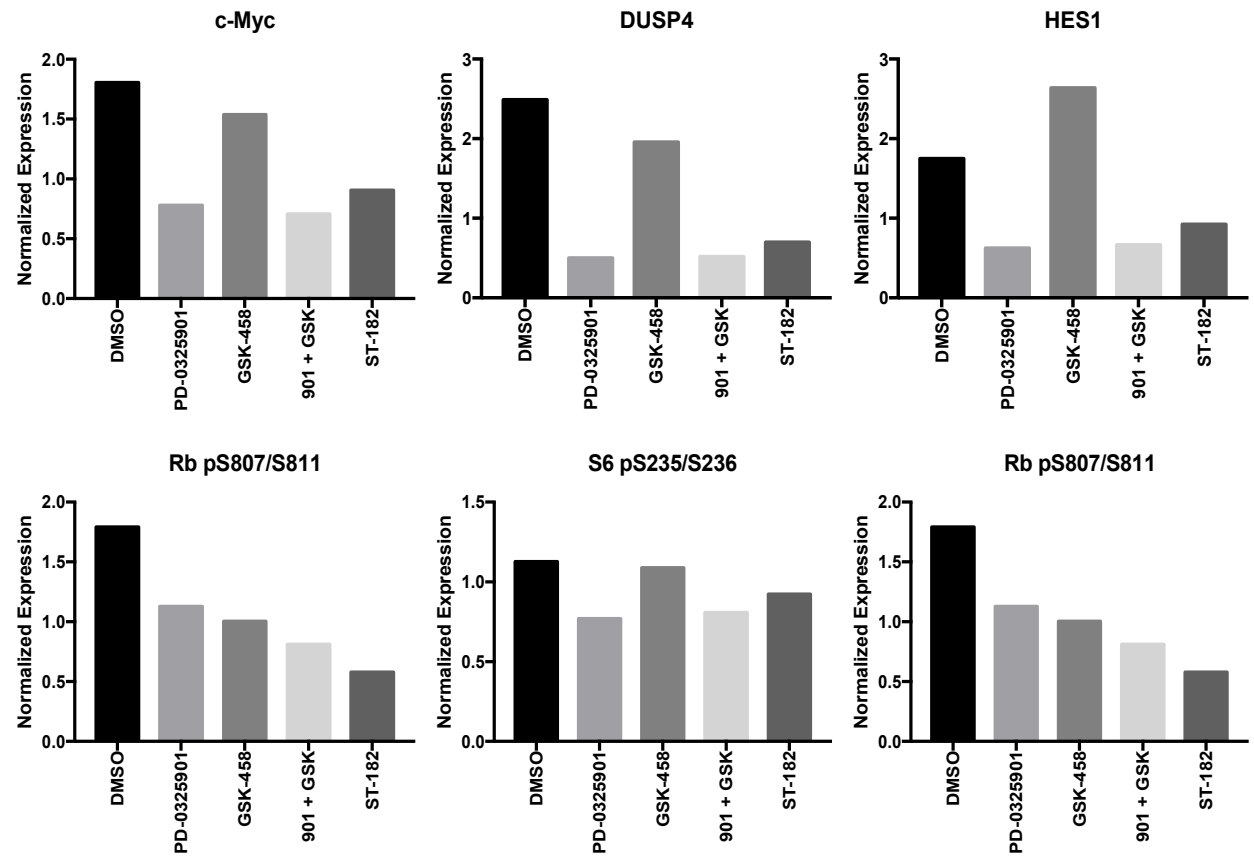
# PI3K inhibition by ST-182 affects downstream signaling



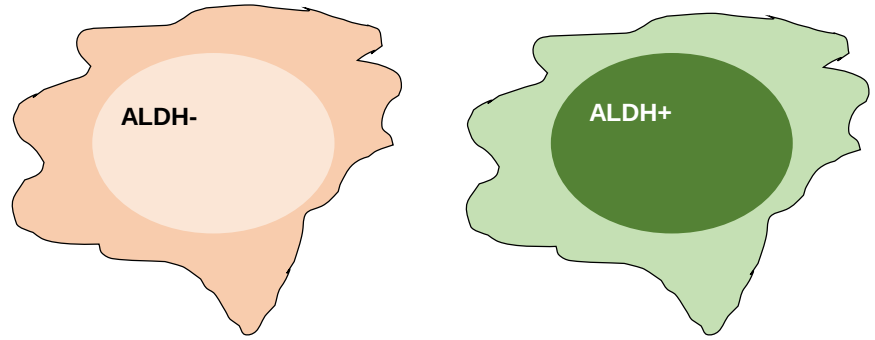
# MEK inhibition by ST-182 affects downstream signaling



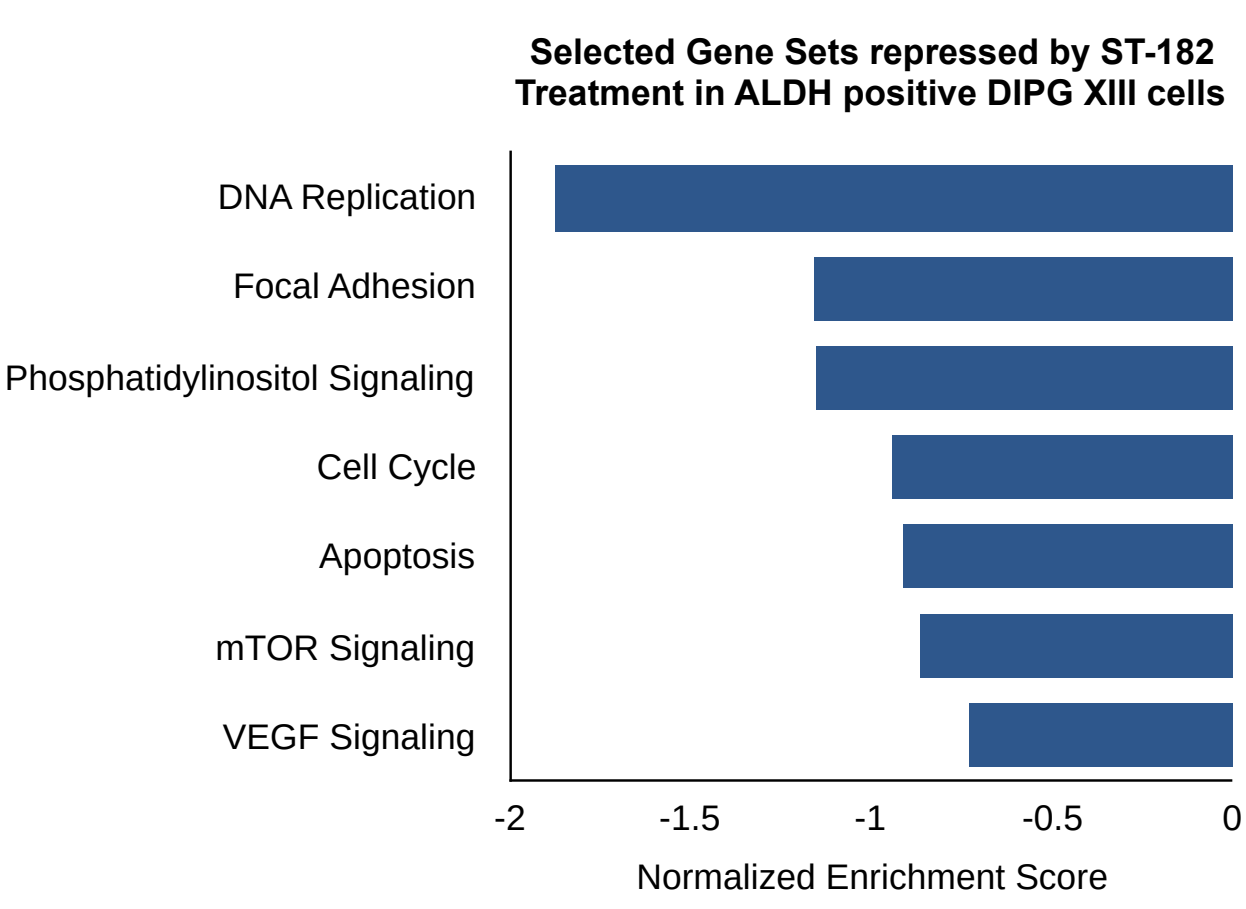
# Transcriptome analysis of ALDH+/- DIPG cells treated with ST-182

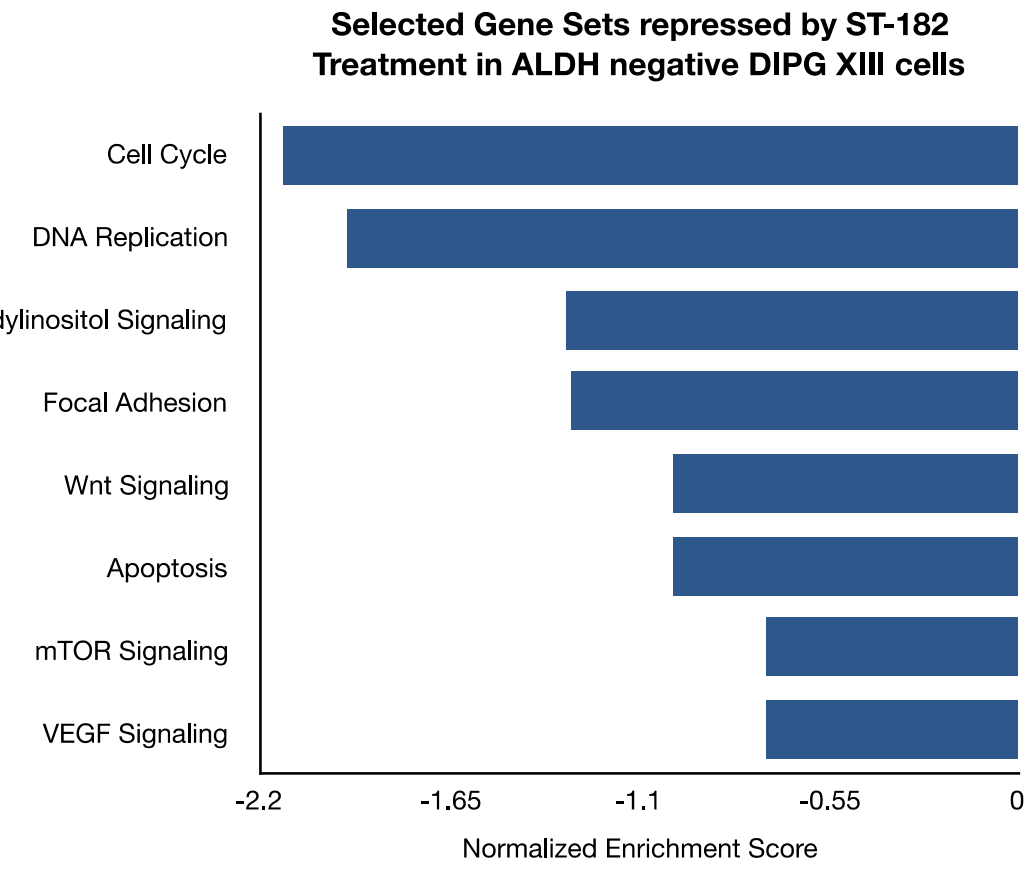
1. Treatment of cells with ST-182
2. Bromouridine labeling of RNA
3. ALDEFLUOR staining
4. FACS sorting in ALDH- and ALDH+ cells




5. Collection of total RNA
6. Isolation of Bru-RNA
7. Generation of cDNA libraries
8. Sequencing and data analysis

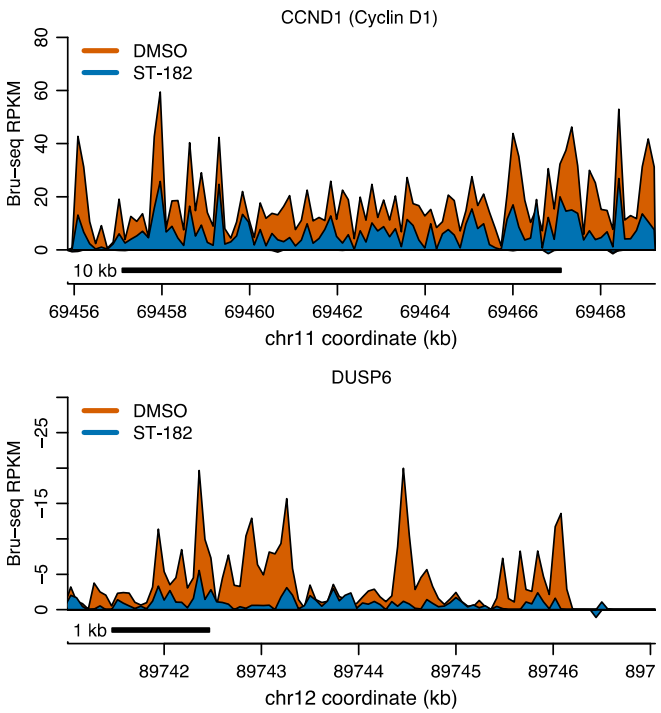
# Efficacy of ST-182 in inhibiting downstream signaling is similar in ALDH+ or ALDH- cells

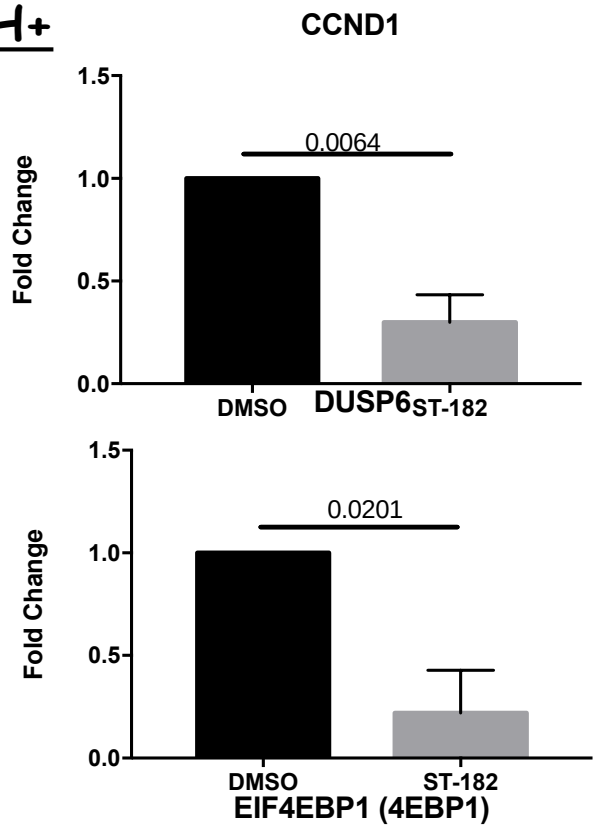


# Both downstream pathways are affected in ALDH+/- by ST-182 treatment

## ALDH+

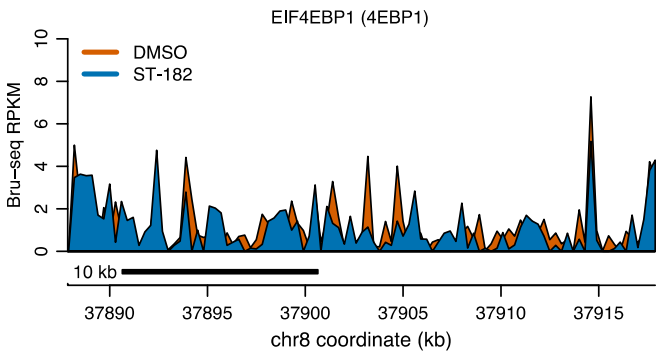


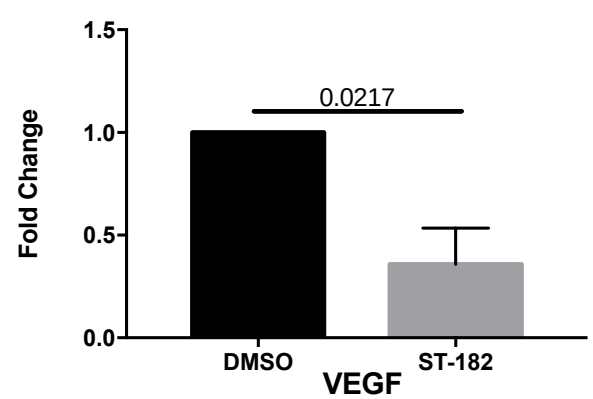



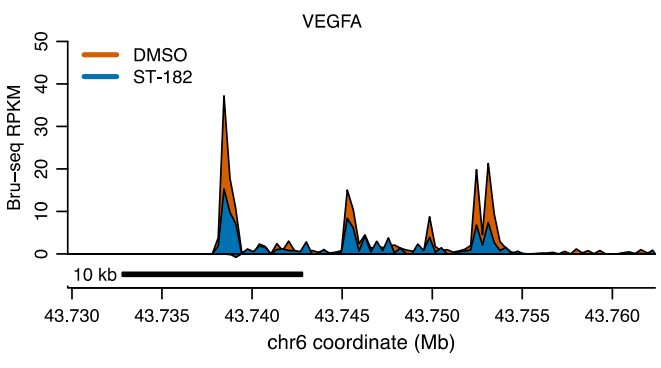


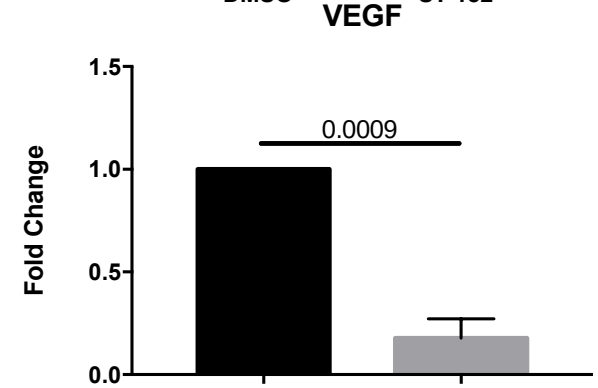




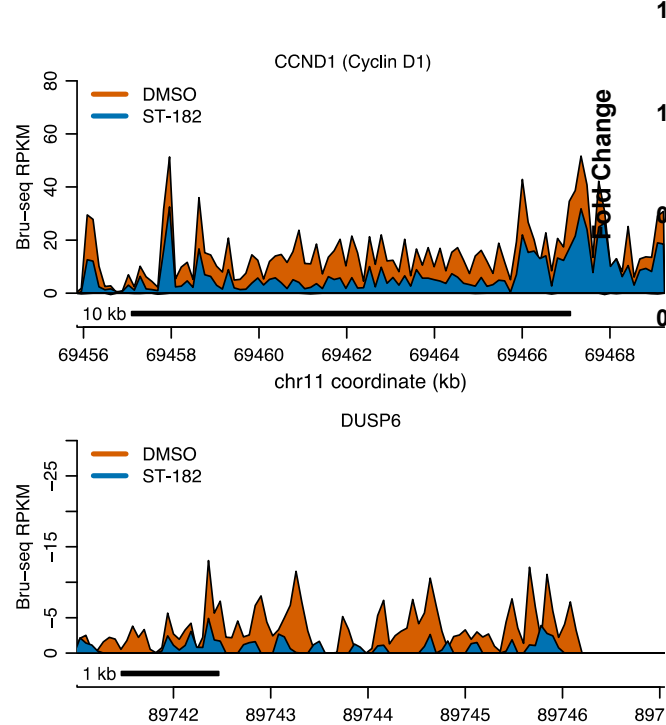



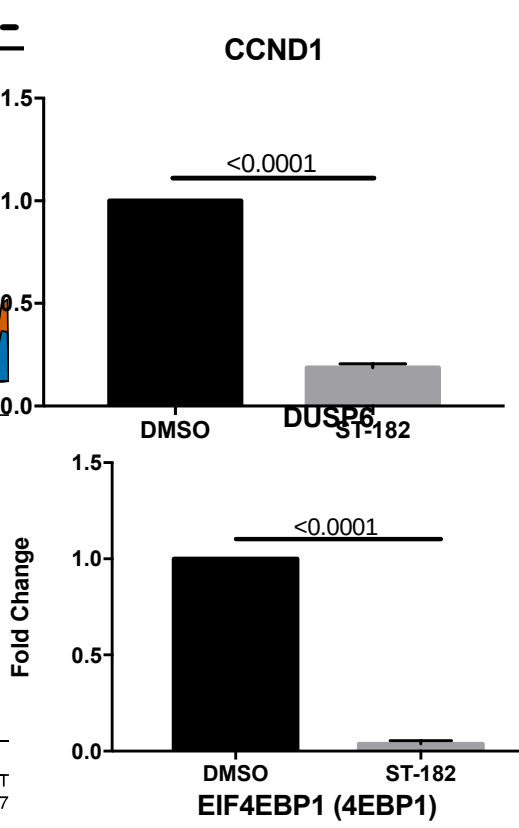




## ALDH-

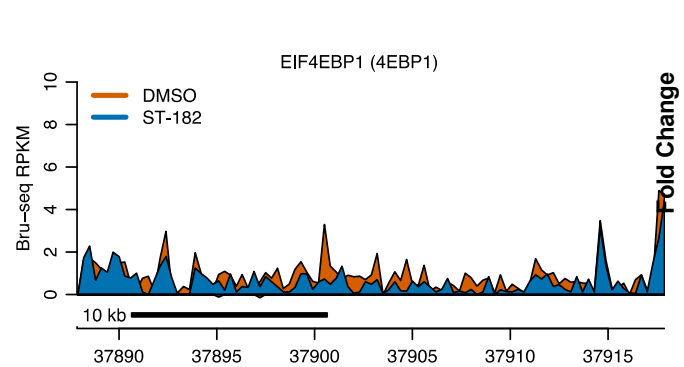


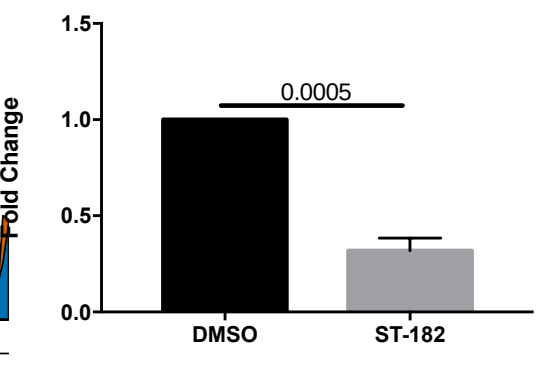



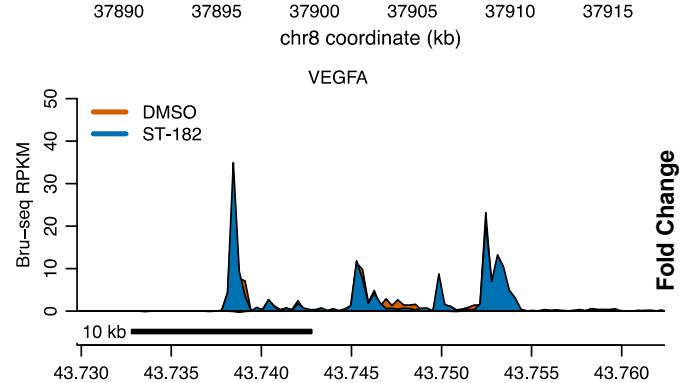


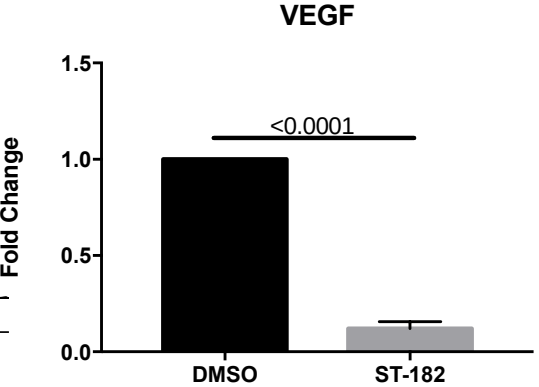




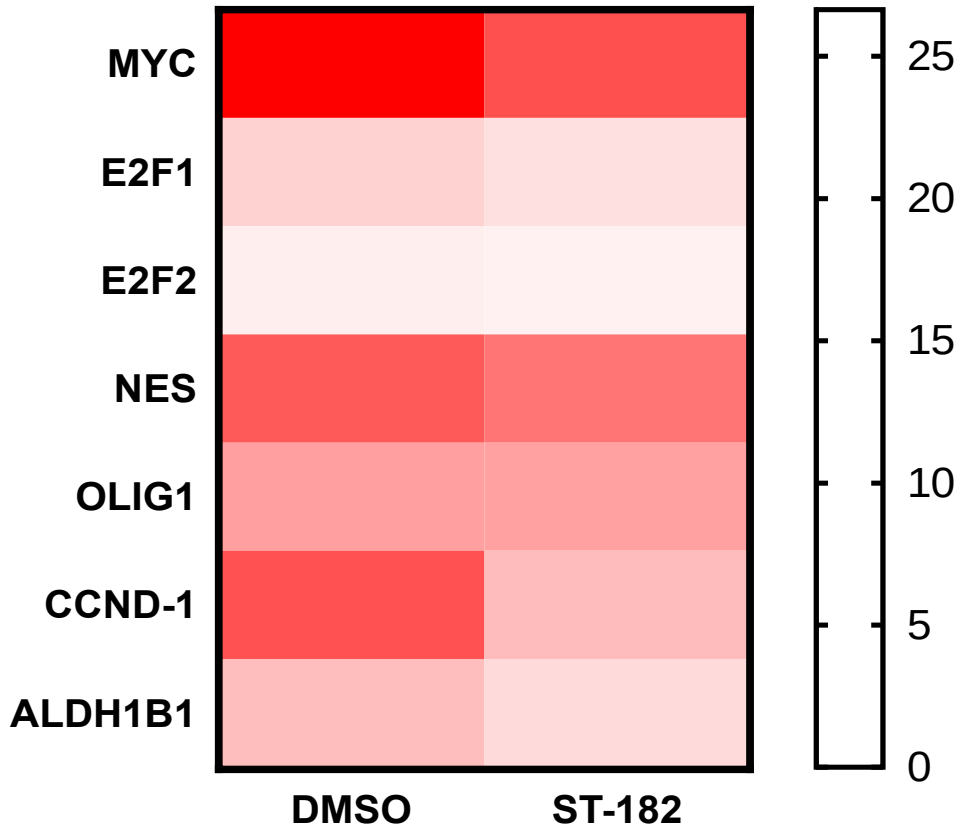



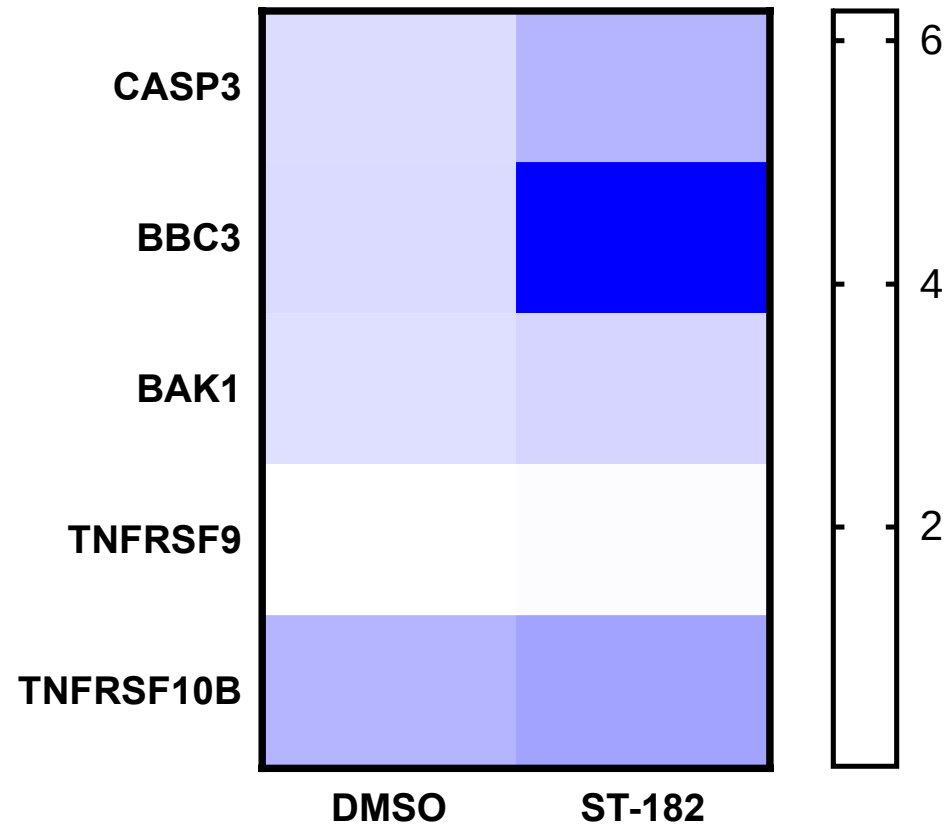



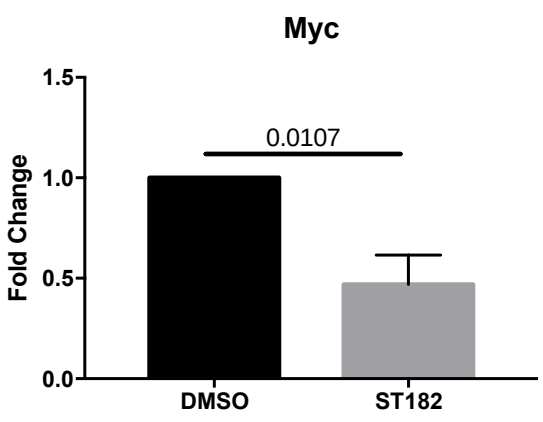
But how about the tumor initiating cells?

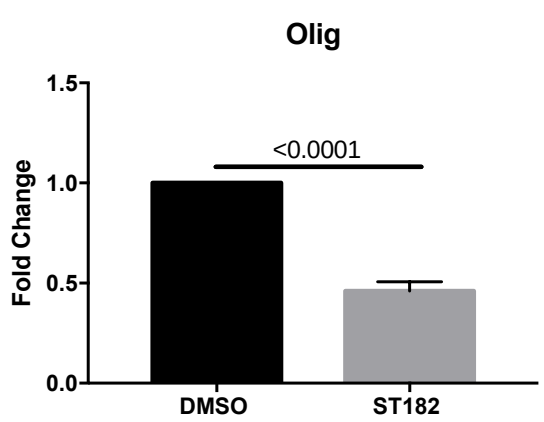
Does it affect Stemness?

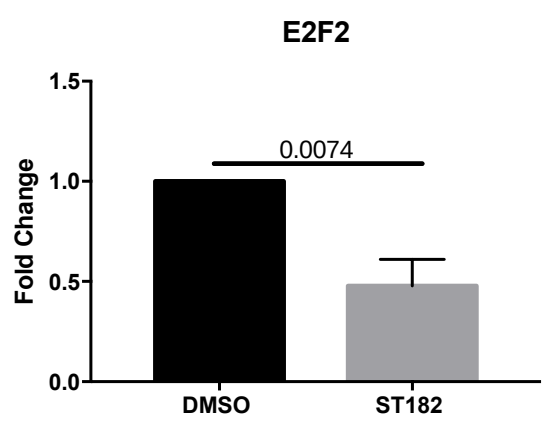
# Decreased stem cell reprogramming and induction of cell death in ALDH+ cells by ST-182

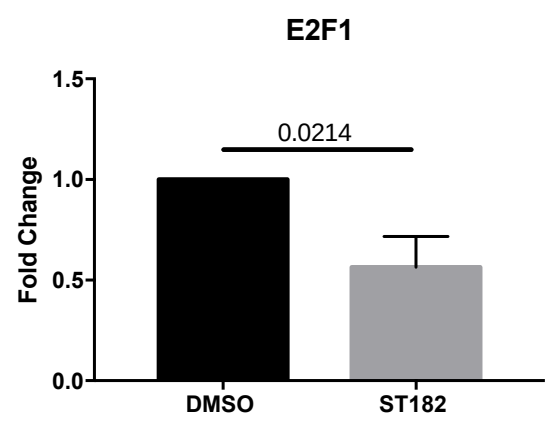


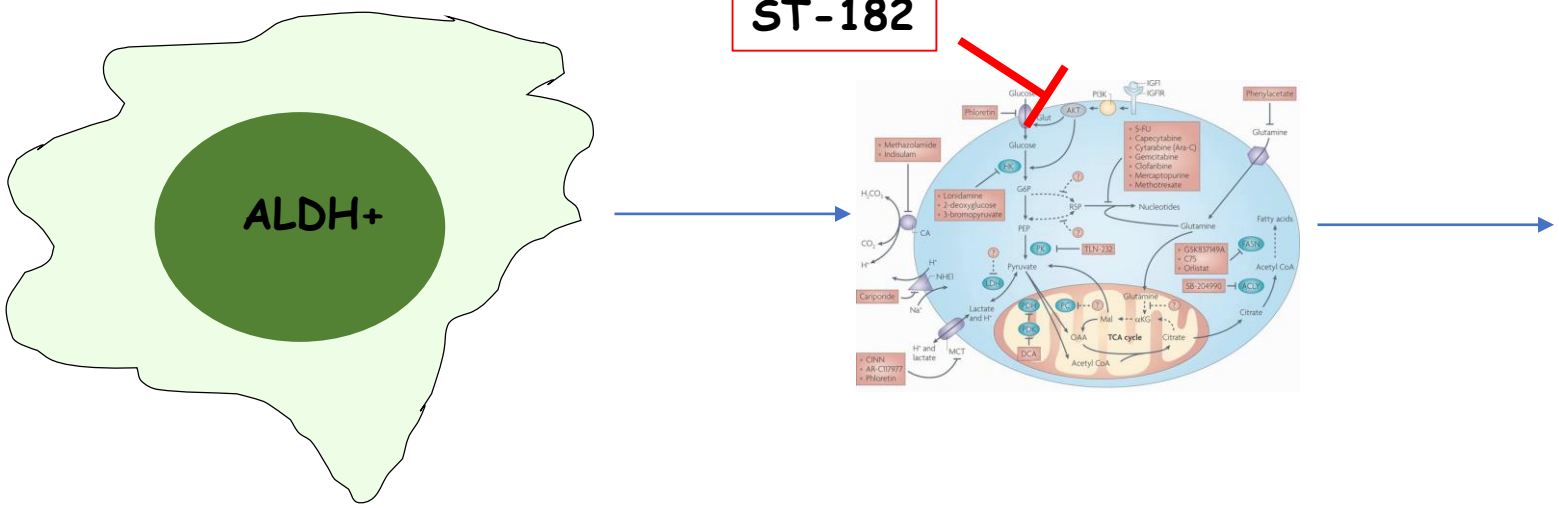



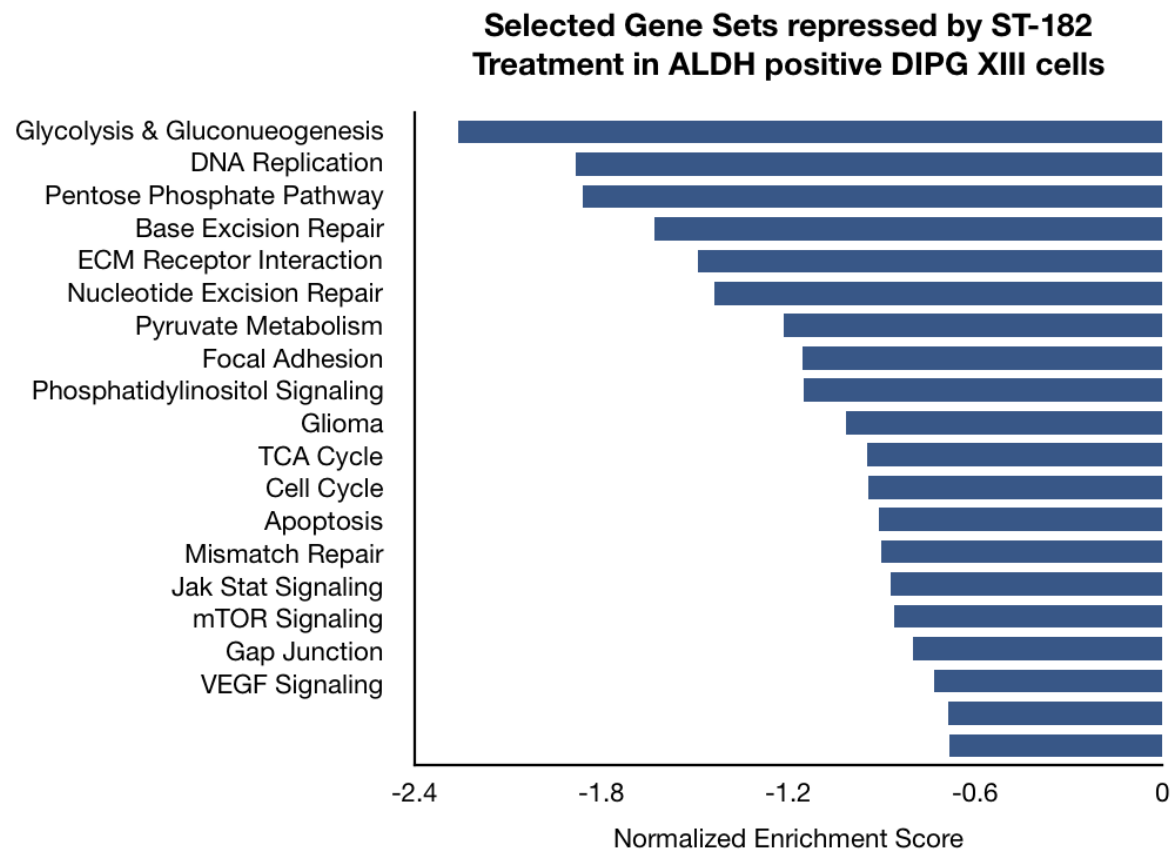



# Molecularly targeted therapy with ST-182 resulted in downregulation of metabolic genes and pathways

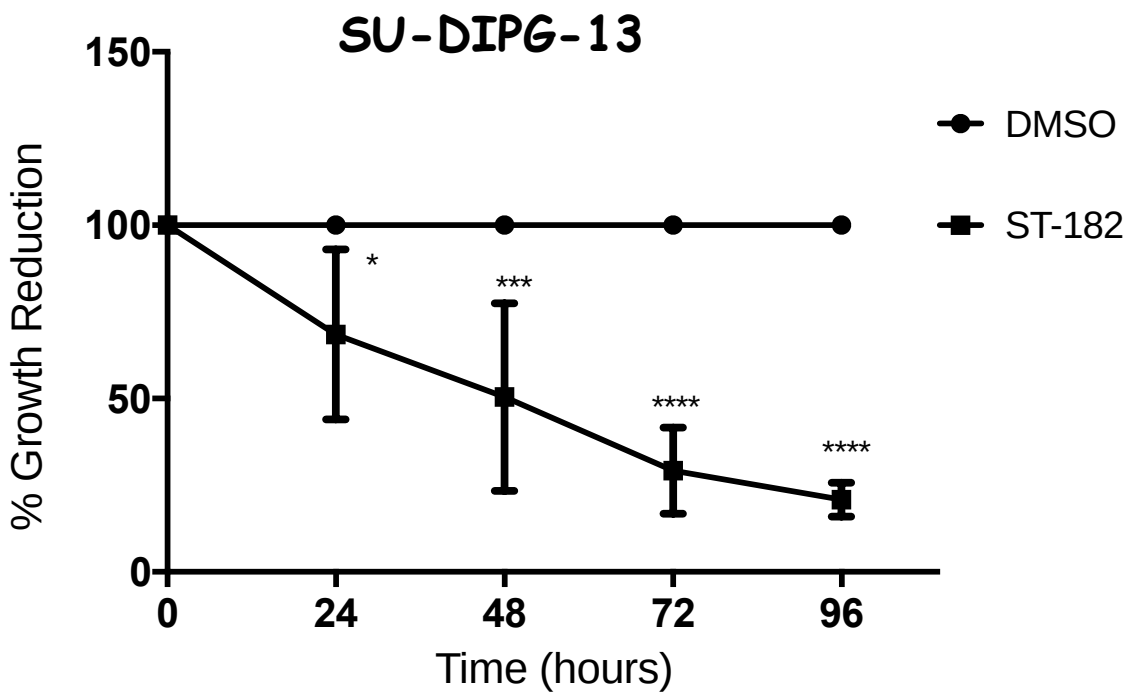


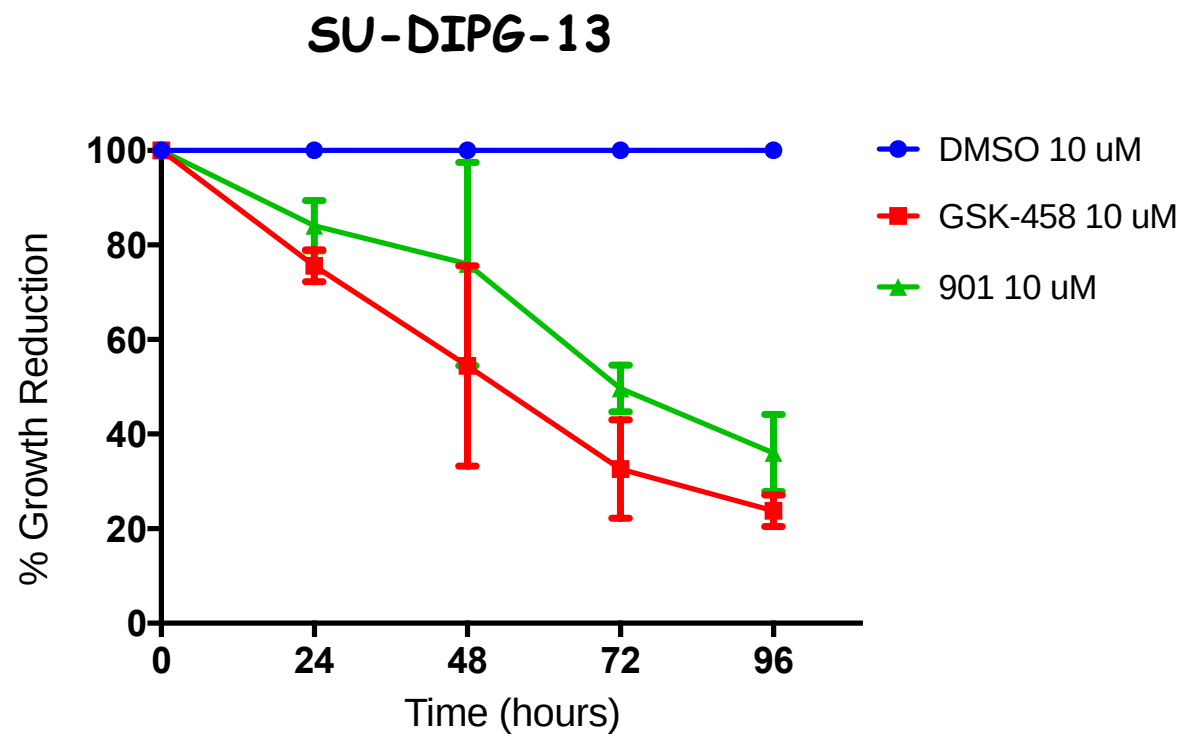

| Gene | Log2Fold change |
|---|---|
| LDHD | 1.132 |
| MAT2A | 0.114 |
| PHGDH | 0.024 |
| PSAT1 | -0.190584 |
| PDHA1 | -0.237849 |
| GAPDH | -0.260528 |
| HK1 | -0.682309 |
| LDHB | -0.91606 |
| ENO1 | -1.170154 |

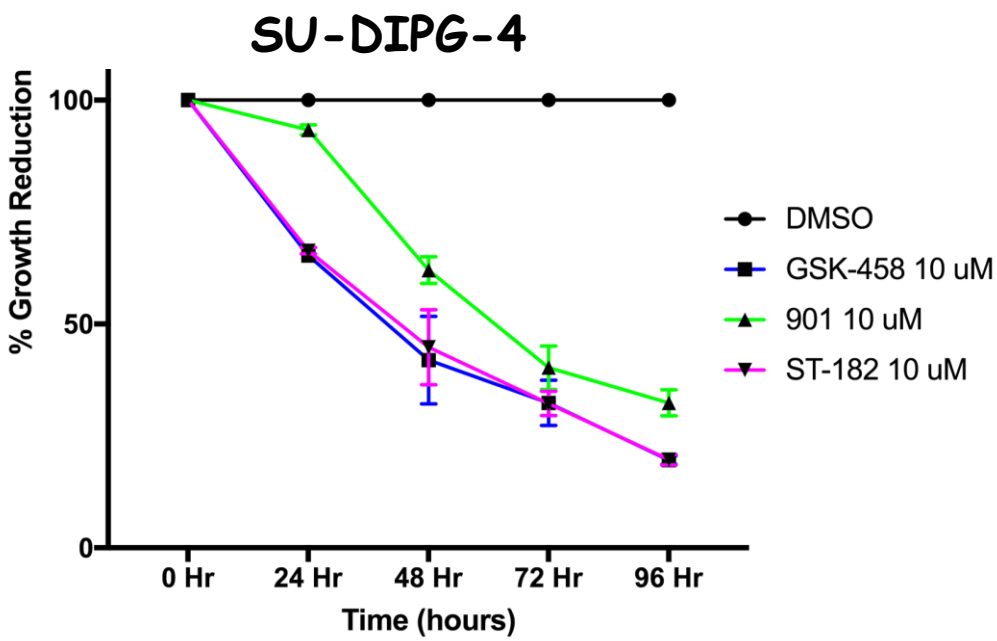
| | NES |
|---|---|
| Glycolysis & Gluconueogenesis | -2.263269 |
| DNA Replication | -1.8837976 |
| Pentose Phosphate Pathway | -1.8601029 |
| Base Excision Repair | -1.6294979 |
| ECM Receptor Interaction | -1.4928834 |
| Nucleotide Excision Repair | -1.4402269 |
| Pyruvate Metabolism | -1.2166411 |
| Focal Adhesion | -1.1562096 |
| Phosphatidylinositol Signaling | -1.1532564 |
| Glioma | -1.0161988 |
| TCA Cycle | -0.94789463 |
| Cell Cycle | -0.9433225 |
| Apoptosis | -0.9119114 |
| Mismatch Repair | -0.90364456 |
| Jak Stat Signaling | -0.8714247 |
| mTOR Signaling | -0.8633705 |
| Gap Junction | -0.8015549 |
| VEGF Signaling | -0.7326856 |
| Ubiquitin-Mediated Proteolysis | -0.68998206 |
| Endocytosis | -0.68429935 |

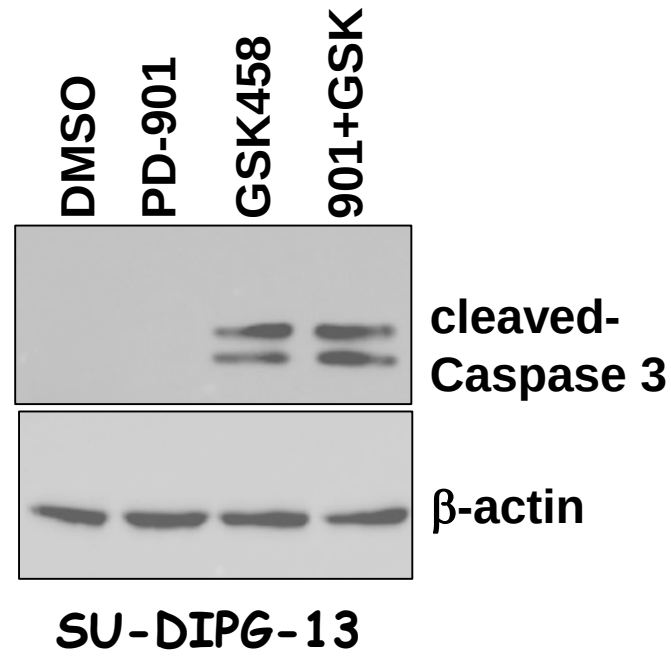


# MAPK/PI3K/mTOR inhibition induces Caspase-3 dependent cell death



# ALDH+ DIPG cells reside higher in the cellular hierarchy *in vivo*

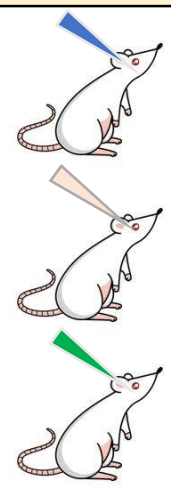
**FACS and intracranial implantation**

**Brain tumor formation assessed by Bioluminescence imaging**

**Not sorted** →

**ALDH-** →

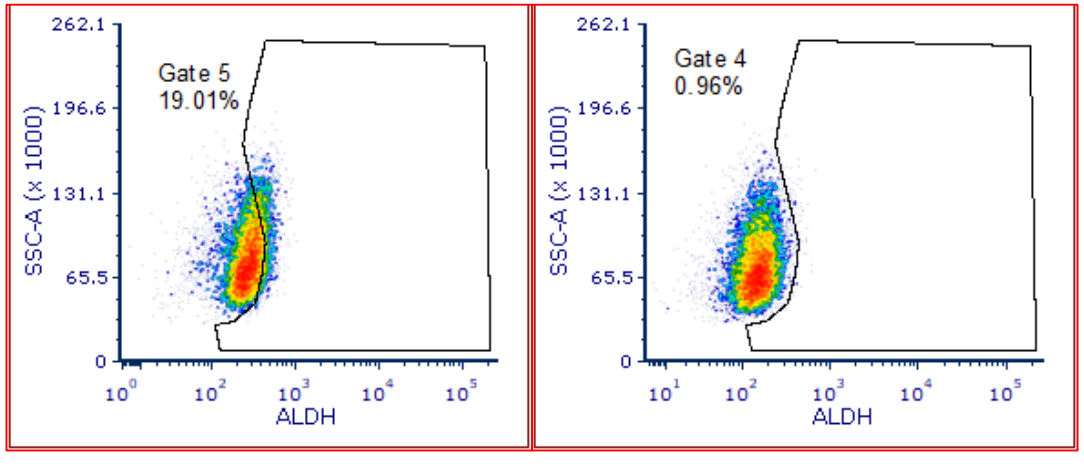
**ALDH+** →



**100 days later** →

- **Bioluminescence**
- **Histological analysis**
- **Flow cytometric analysis**

## DIPG-7-luc cells FACS and intracranial implantation into NSGs




DEAB

# Do ALDH+ have stem cell potential in vivo?

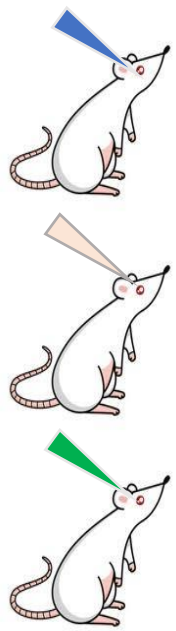
**FACS and intracranial implantation**

**Brain tumor formation assessed by Bioluminescence imaging**

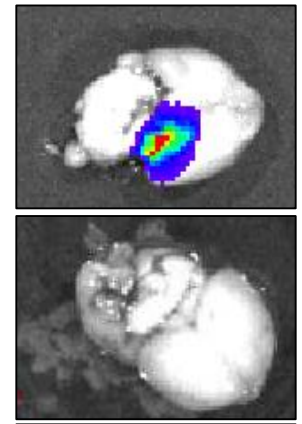
**Not sorted**

**ALDH-**

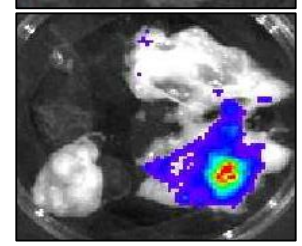
**ALDH+**



**100 days later**



unsorted

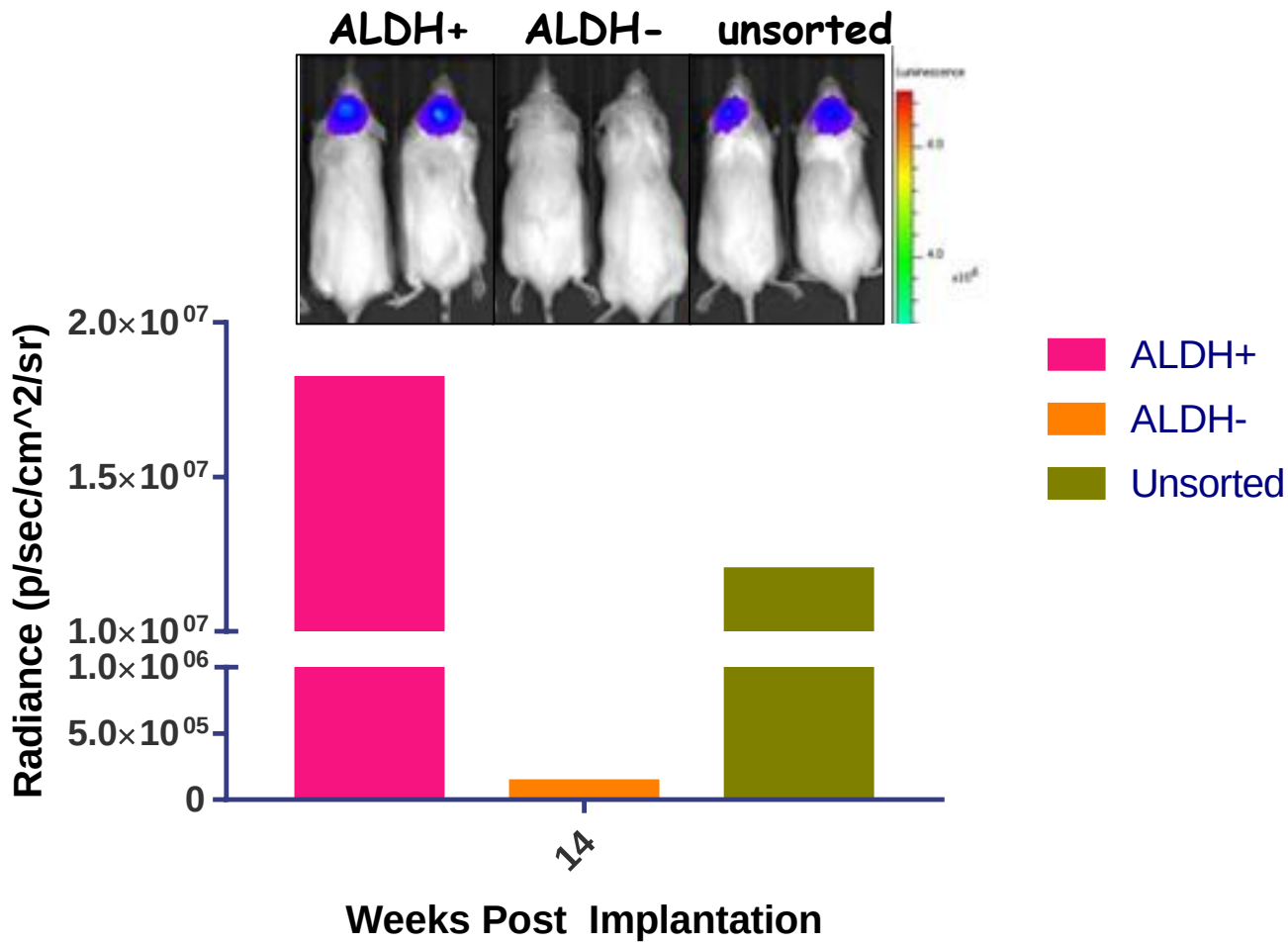


ALDH-



ALDH+

# ALDH+ DIPGs proliferate faster in vivo than ALDH- DIPG 7 cells

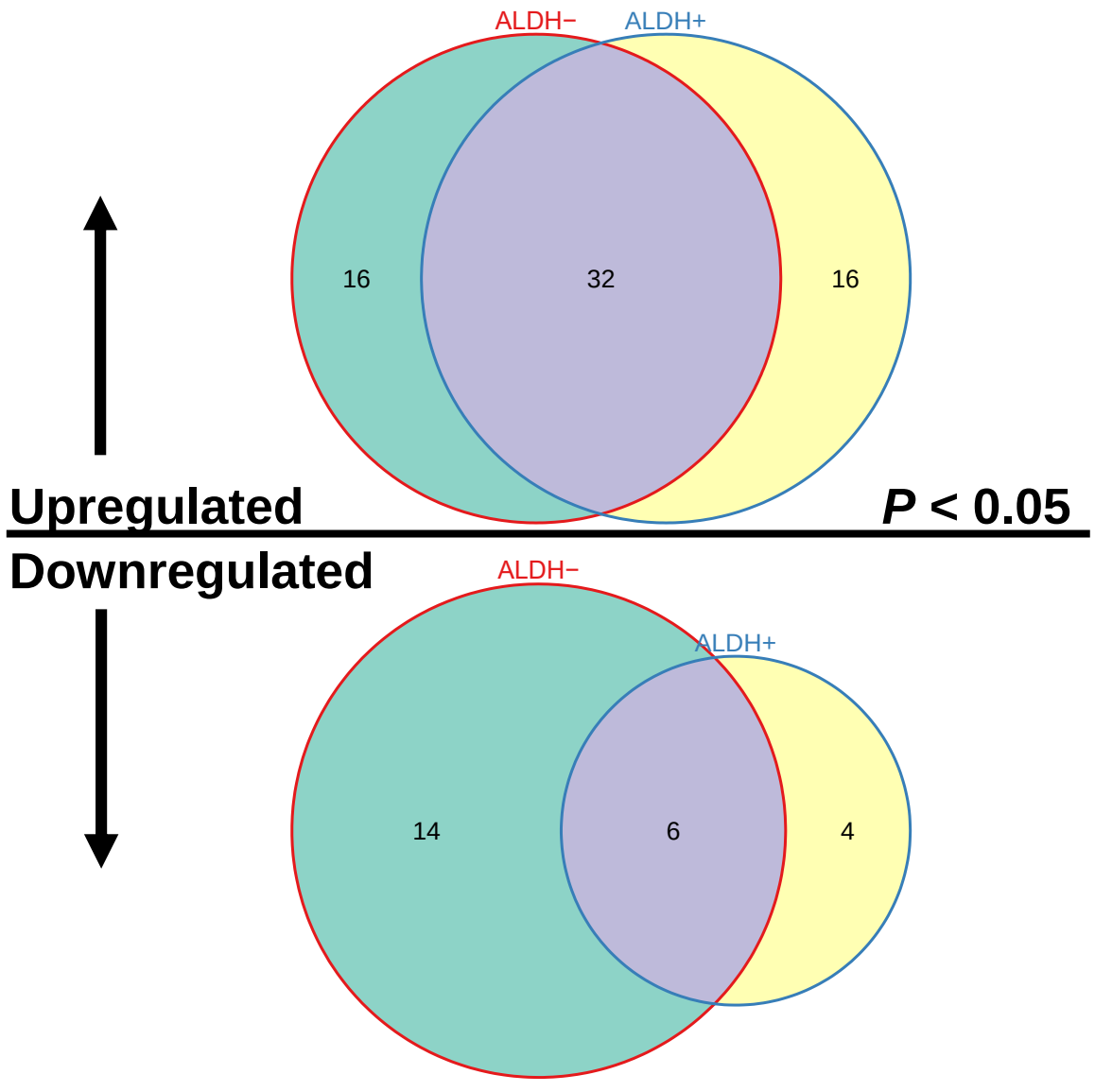


# Anticipating drug resistance to molecularly target therapies

- > 250 clinical trial for DIPG
- Confounding initial efficacy of PI3K inhibitions : LY294002
- Tumor relapse  common

**How can we anticipate resistance and identify drug targets before relapse occurs?**

# ALDH+/- cells represent distinct populations in DIPG with common as well as differential signaling responses to PI3K/mTOR/MAPK inhibition



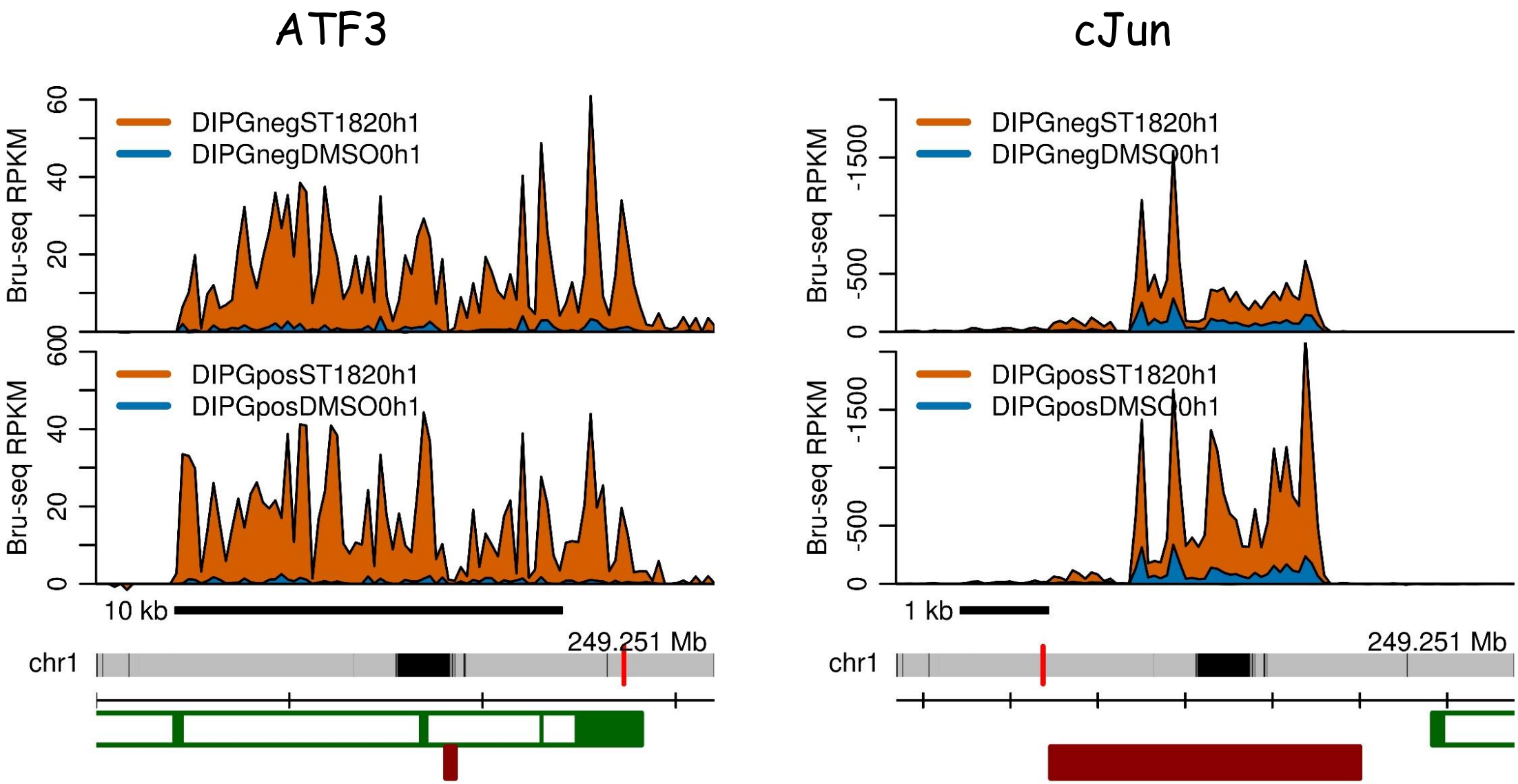

## Top 10 genes upregulated

| | | ALDH- | | ALDH+ | |
|---|---|---|---|---|---|
| chr.x | name | log2FC | FC | log2FC | FC |
| chr19 | KLF2 | 4.2302 | 18.767 | 4.7906 | 27.677 |
| chr1 | ATF3 | 3.9965 | 15.961 | 4.6943 | 25.89 |
| chr2 | RHOB | 3.5369 | 11.607 | 4.3941 | 21.026 |
| chr9 | KLF4 | 3.0486 | 8.2743 | 3.3909 | 10.49 |
| chr2 | ARID5A | 2.1944 | 4.577 | 2.8761 | 7.3416 |
| chr19 | BBC3 | 2.2686 | 4.8185 | 2.787 | 6.9021 |
| chr2 | KLF7 | 2.4917 | 5.6246 | 2.7412 | 6.6865 |
| chr1 | JUN | 2.1358 | 4.3948 | 2.6747 | 6.3849 |
| chrX | TSC22D3 | 2.542 | 5.8241 | 2.6679 | 6.3549 |
| chr10 | KLF6 | 1.8836 | 3.6899 | 2.5693 | 5.9353 |

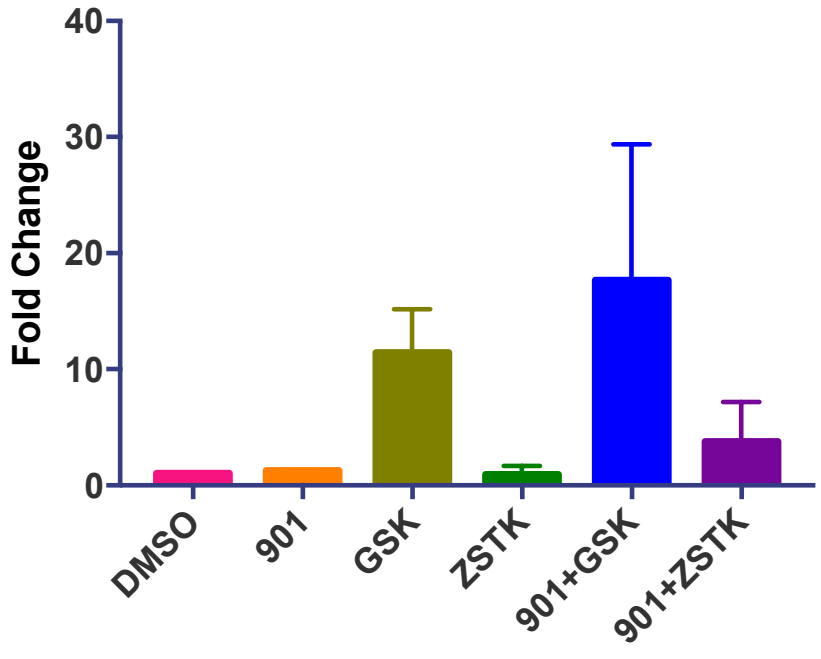
## Downregulated

| | | ALDH- | | ALDH+ | |
|---|---|---|---|---|---|
| chr.x | name | log2FC | FC | log2FC | FC |
| chr5 | SPRY4 | -2.667 | 0.157 | -3.044 | 0.121 |
| chr3 | ETV5 | -2.061 | 0.24 | -2.268 | 0.208 |
| chr2 | AC064875.1 | -1.679 | 0.312 | -2.133 | 0.228 |
| chr8 | LZTS1 | -1.501 | 0.353 | -1.856 | 0.276 |
| chr18 | ST8SIA5 | -1.654 | 0.318 | -1.711 | 0.305 |
| chr2 | SNED1 | -1.474 | 0.36 | -1.553 | 0.341 |

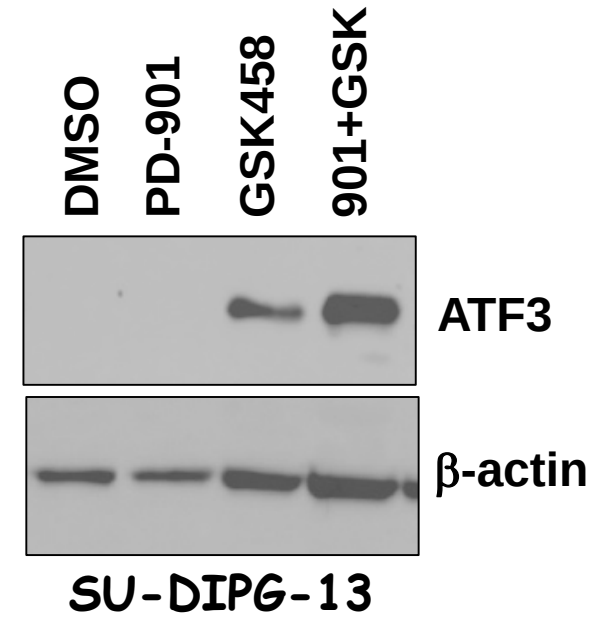
# Upregulation of Transcription factor ATF3 and ATF3 TF complex (cJun, JunD) in ST-182 treated cells



# Activating Transcription Factor 3' (ATF3) regulation by molecularly targeted therapy




SU-DIPG-13




SU-DIPG-13

# Comparison of GSK-458 with GDC-0084: both PI3K/mTOR inhibitors, but GDC-0084 is in clinical trials for DIPG

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Trial record **1 of 1** for: GDC | DIPG

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**Study of GDC-0084 in Pediatric Patients With Newly Diagnosed Diffuse Intrinsic Pontine Glioma or Diffuse Midline Gliomas**

⚠ The safety and scientific validity of this study is the responsibility of the study sponsor and investigators. Listing a study does not mean it has been evaluated by the U.S. Federal Government. Know the risks and potential benefits of clinical studies and talk to your health care provider before participating. Read our disclaimer for details.

ClinicalTrials.gov Identifier: NCT03696355

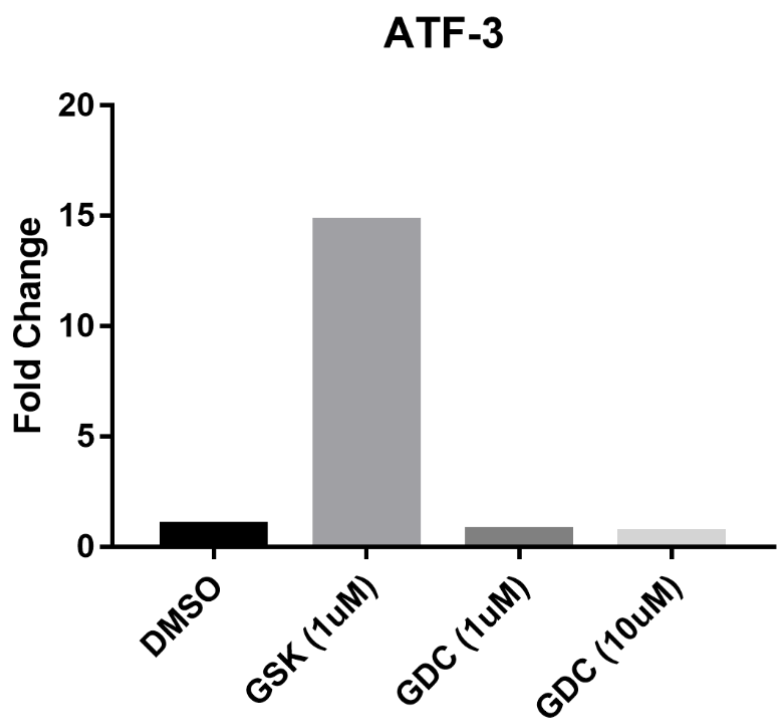
Recruitment Status : Recruiting
First Posted : October 4, 2018
Last Update Posted : April 22, 2019

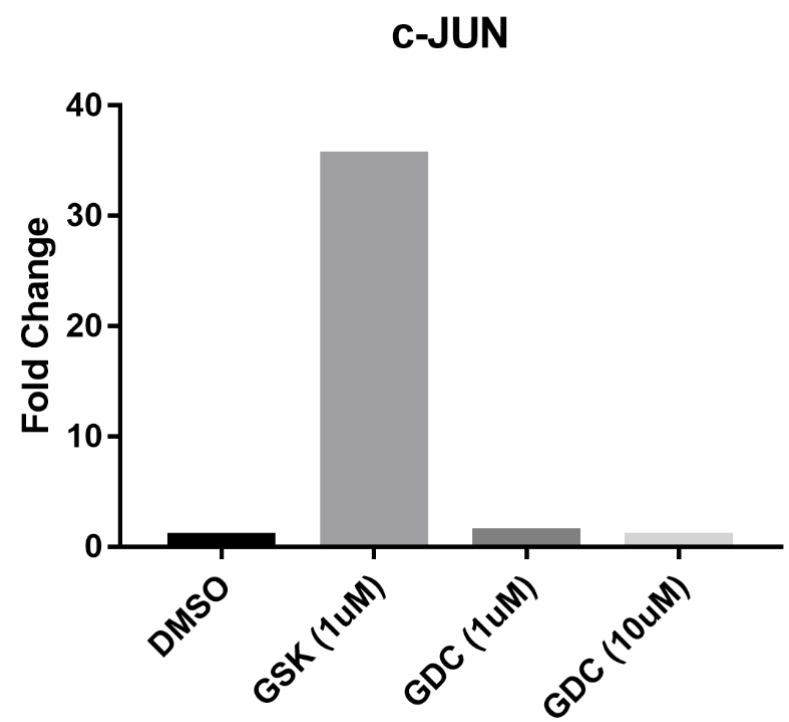
See Contacts and Locations

**Sponsor:**
St. Jude Children's Research Hospital

**Collaborator:**
Kazia Therapeutics Limited

**Information provided by (Responsible Party):**
St. Jude Children's Research Hospital



# You know that you are on the right track when somebody else does the same work ☹

## Targeting PI3K using the blood brain barrier penetrable inhibitor GDC-0084 for the treatment of diffuse intrinsic pontine glioma (DIPG)


Ryan J. Duchatel[1,2], Dilana Staudt[1,2], Evangeline Jackson[1,2], David Skerrett-Byrne[3], Muhammad Jamaluddin[1,2], Adjanie Patabendige[1,4], Jason Cain[5], Maria Tsoli[6], Michelle Monje[8], Frank Alvaro[2,9], David Ziegler[6,7], Matthew D. Dun[1,2]

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[6]Childrens Cancer Institute, University of NSW, Randwick, AUSTRALIA
[7]Kids Cancer Centre, Sydney Children's Hospital, Randwick, NSW, Australia
[8]Departments of Neurology, Neurosurgery, Pediatrics, and Pathology, Stanford University School of Medicine, Stanford, CA, USA.
[9]John Hunter Children's Hospital, Lambton, NSW, AUSTRALIA


### PI3K activation is an oncogenic driver in DIPG

- Diffuse Intrinsic Pontine Glioma (DIPG) is an incurable childhood brain cancer. More than 80% of patients harbour a point mutation in Histone-H3 which sees a substitution of lysine 27 for a methionine (K27M) in either *H3F3A*/H3.3 or *HIST1H3B*/H3.1 variants.
- H3K27M mutations result in global hypomethylation promoting oncogenic transcription.
- The RTK-AKT/PI3K/mTOR pathways (**Fig. 1**) are altered in more than 2/3rds of DIPG patients, therefore, represent an attractive therapeutic target. However, PI3K inhibitors have been notoriously ineffective due to their inability to cross the blood-brain-barrier (BBB).
- Targeting downstream components of the PI3K-AKT-mTOR signaling axis is a promising paradigm for PI3K mutant patients.
- This project aims to overcome the limitations of targeting downstream effectors of the PI3K pathway by utilising a novel PIK3CA specific, BBB permeable inhibitor, GDC-0084, currently in clinical trials for Glioblastoma (GBM) and DIPG

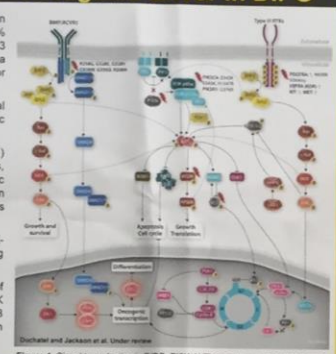


Figure 1. Signal transduction in DIPG. PI3K/AKT promote growth and survival.

### GDC-0084 is a BBB permeable inhibitor of PI3K

- GDC-0084 is an oral blood brain barrier (BBB) permeable inhibitor of the PI3K pathway.
- The BBB permeability of GDC-0084 was confirmed in glioblastoma multiforme (GBM) xenograft models using U87-GBM cells by MALDI imaging (**Fig. 2A-B**) following oral administration of 15mg/kg [1].
- *In vivo* efficacy of GDC-0084 in U87 glioblastoma xenograft model determined via micro-CT images of brains from control and 2 weeks of GDC-0084 treated mice (**Fig. 2C**). Significantly reduced U87 brain tumour volume was seen in mice treated with GDC-0084 for 2 weeks compared to control (**Fig. 2D**) [1].
- GDC-0084 is currently in **Phase II clinical** trials for GBM (NCT03522298), and recently commenced a **Phase I clinical** trial in DIPG (NCT03696355)

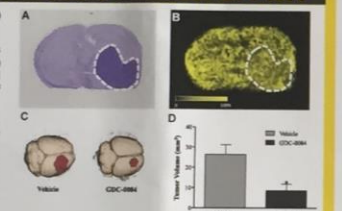



Figure 2. Brain distribution of GDC-0084 in xenograft mouse models of GBM. Significant reduction in tumour volume seen following 2 weeks of oral administration 15mg/kg. Images adapted from: Salphati et al. *Drug Metab Dispos* 2016;44:1881-1889.

### DIPG vs GBM *in vitro* sensitivity to GDC-0084

- We compared the relative sensitivities of GBM and DIPG cell lines to GDC-0084 (**Fig. 3**). DIPG cell lines (n=6) were more sensitive to PI3K inhibition by GDC-0084 compared to GBM cell lines (n=4. p=<0.001) highlighting the therapeutic potential of GDC-0084 for the treatment of DIPG.
- GDC-0084 significantly reduced the growth of all DIPG cell lines regardless of whether they harboured PI3K mutations, and was significantly more cytotoxic than the mTORC1 inhibitor Rapamycin ($p = <0.05$).
- GDC-0084 induced significant levels of Annexin V positivity in a PIK3CA mutant (SU-DIPG-33) but not wild type (SU-DIPG-4) (**Fig. 4**) after 24hr treatment with 5μM GDC-0084.

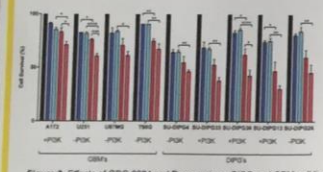



Figure 3. Effects of GDC-0084 and Rapamycin on DIPG and GBM cell lines

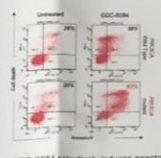



Figure 4. GDC-0084 selectively induces apoptosis in a PI3K mutant DIPG cell line

**Hypothesis:** Analysis of signal transduction pathway alterations following PI3K inhibition using GDC-0084 via phosphoproteomic profiling, will uncover DIPG survival dependencies and reveal novel drug targets for combinatorial therapies.

### Quantitative phosphoproteomic profiling

Quantitative phosphoproteomic profiling was performed in biological triplicate using SU-DIPG-36 (H3.1 K27M+, *ACVR1 G328E*, *PIK3R1 M326I*) treated with 1μM GDC-0084 for 6hrs and vehicle control (DMSO).

Cells were subjected to our standard phosphopeptide preparation techniques [2,3] and purified peptides from each of the 6 samples were labeled using tandem mass tags (TMT-10plex. Thermo Fisher Scientific) and mixed 1:1.

Non-modified, phosphorylated and glycosylated peptides were isolated after labelling and fractionated by Hydrophilic interaction chromatography (HILIC) [3]

Peptides were then analysed via liquid chromatography-tandem mass spectroscopy (LC-MS/MS) using Dionex nanoflow HPLC coupled to a Q-Exactive Plus hybrid quadrupole-Orbitrap MS system (Thermo Fisher Scientific).

Proteome Discoverer 2.1 (Thermo Fisher Scientific)

### Results: Quantitative phosphoproteomic profiling

- Using our standard quantitative phosphoproteomic workflow we reveal the **first DIPG phosphoproteomic profiling** results *in vitro*. Below we report proteomic events using a false discovery rate (FDR) of <1%.
- Our approach dramatically expands the current catalog of quantified DIPG specific proteins (proteome). Of these **6010**, we sequenced **2623** phosphoproteins, **1172** glycosylated proteins, **222** acetylated proteins and **177** methylated proteins.
- This revealed **4171** unique non-modified proteins, **1395** unique phosphoproteins, **238** unique glycosylated proteins, **129** unique acetylated proteins, **77** unique methylated proteins.
- Following GDC-0084 treatment, identified **99** phosphoproteins decreased by ≤-2 fold ($p = <0.05$) compared to the vehicle treated control.
- Following GDC-0084 treatment, identified **69** phosphoproteins increased by ≥ 2 fold ($p = <0.05$) compared to the vehicle treated control.

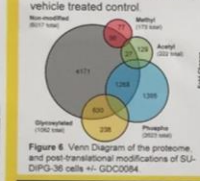



Figure 6. Venn Diagram of the proteome, and post-translational modifications of SU-DIPG-36 cells +/- GDC0084.

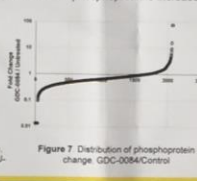



Figure 7. Distribution of phosphoprotein change. GDC-0084/Control

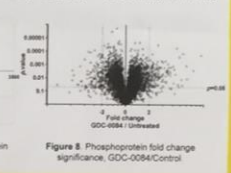



Figure 8. Phosphoprotein fold change significance, GDC-0084/Control

### GDC-0084 targets the PI3K

- Phosphoproteomic profiling of SU-DIPG-36 cells treated with GDC-0084 revealed decreased phosphorylation of key signaling proteins associated with the PI3K-AKT-mTOR axes (**Fig. 9A**), apoptosis (**Fig. 9B**), Mitogen Activated Protein Kinase (MAPK) family (**Fig. 9C**), Cyclin Dependent Kinase (CDK) family (**Fig. 9D**) and BCL family (**Fig. 9E**):
  - **PI3K-AKT-mTOR:** Decreased phosphorylation of AKT3, RAPTOR, RPS6K1 and BAD.
  - **TP53:** Increased phosphorylation of p53 and programmed cell death protein 5 (PDCD5).
  - **MAPK:** Decreased phosphorylation of MAPK1 and MAPK14.
  - **CDK:** Decreased phosphorylation of CDK9 and CDK5.
  - **BCL:** Decreased phosphorylation of BCL2 and BCL7.
- Combining PI3K-AKT-mTOR with BCL, MAPK or CDK inhibitors may induce synergistic lethality in DIPG.

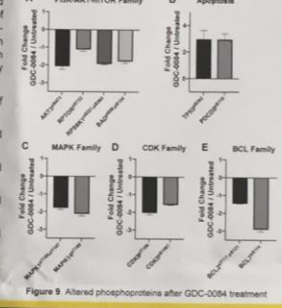



Figure 9. Altered phosphoproteins after GDC-0084 treatment

### GDC-0084 modulates the epigenetic landscape

- Interestingly, our quantitative proteomic profiling experiments simultaneously identified peptides methylated and acetylated, as well as phosphorylated and glycosylated.
- Within the allelic heterogeneity of K27M mutations (3-17%), we were able to identify significant alterations to the H3 epigenetic landscape after PI3K inhibition using GDC-0084.
- Decreased phosphorylation of HDAC2 and SUZ12 were seen.
- Dramatic increases in methylation of H3.3 at K27, and H3.1 at K27.
- Corresponding decreases in H3.1 K27 acetylation were also seen.
- Highlights a potential role for PI3K in regulating the PRC2 complex.

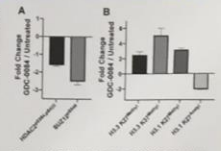
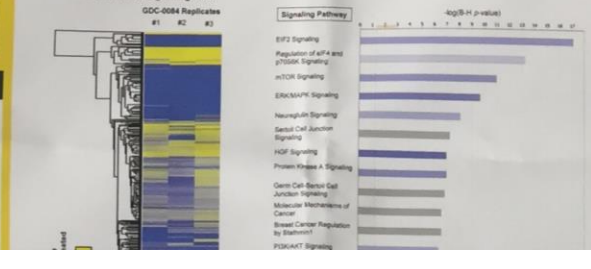



Figure 10. PI3K inhibition using GDC-0084 alters methylation and acetylation of H3.1 and H3.3

### Pathway analysis following GDC-0084 treatment

- IPA pathway analysis revealed a number of signaling pathways negatively regulated after treatment with GDC-0084 as well as some that might act as survival compensatory mechanisms. These pathways included regulators of the PI3K-AKT-mTOR signaling axis.




### Validation of phosphoproteomic changes

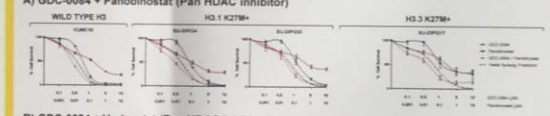

Figure 13. Western blot validation of proteomic changes following GDC-0084 and Rapamycin treatment

- Orthogonal validation of phosphoproteomic profiling results were achieved using SU-DIPG36 cells treated with 1μM GDC-0084 (GDC), or 1μM rapamycin (Rapa) for 3 or 6 hours and measured by western blotting.
- Again, GDC-0084 dramatic reduced PI3K signalling through the inhibition phosphorylated AKT (p-AKT), p-mTOR and p-RPS6K1.
- However, GDC-0084 did not reduce p-PTEN or p-p42MAPK.
- In comparison, treatment with rapamycin increased levels of p-AKT, and reduced levels of p-RPS6K1 but not p-mTOR.
- These data highlight the potent potential for PI3/AKT signalling pathway inhibition elicited by GDC-0084 in PIK3CA mutant DIPG cells.
- Work continues in our laboratory to determine whether PI3K wild-type DIPG cells show signaling reduced oncogenic signalling using GDC-0084.

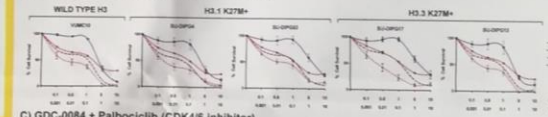
### Phosphoproteomic profiling predicts synergistic treatment combinations

- Using GDC-0084 phosphoproteomic profiling we set about designing combination strategies with the most clinically relevant inhibitors to help fast-track new clinical trials for patients with DIPG.
- GDC-0084 was tested in combination with Panobinostat (A), Vorinostat (B), Palbociclib (C), Ribociclib (D), Adavosertib (E), Erlotinib (F) and Venetoclax (G), across a wide variety of DIPG cell lines *in vitro*.
- Synergism was determined using the Fractional product method of Webb [4]

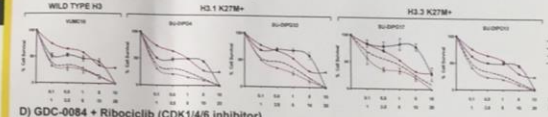
**A) GDC-0084 + Panobinostat (Pan HDAC inhibitor)**



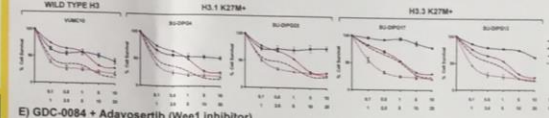

**B) GDC-0084 + Vorinostat (Pan HDAC inhibitor)**



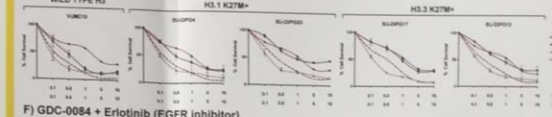

**C) GDC-0084 + Palbociclib (CDK4/6 inhibitor)**



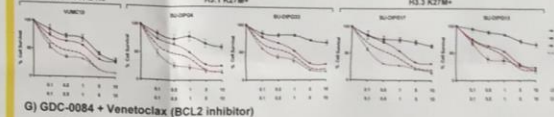

**D) GDC-0084 + Ribociclib (CDK1/4/6 inhibitor)**



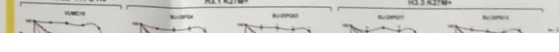

**E) GDC-0084 + Adavosertib (Wee1 inhibitor)**




**F) GDC-0084 + Erlotinib (EGFR inhibitor)**

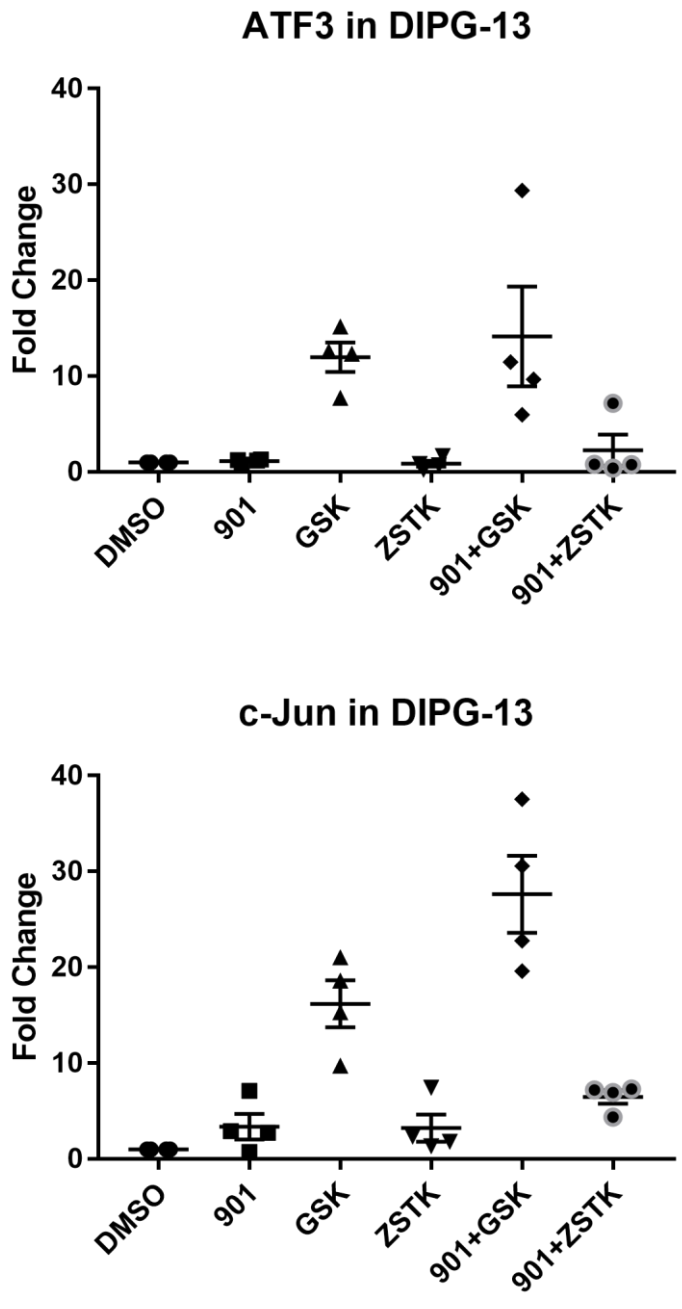



**G) GDC-0084 + Venetoclax (BCL2 inhibitor)**



# Comparison between H3 K27M mutant pediatric glioma (DIPG-13) and H3 WT adult glioma (D54)

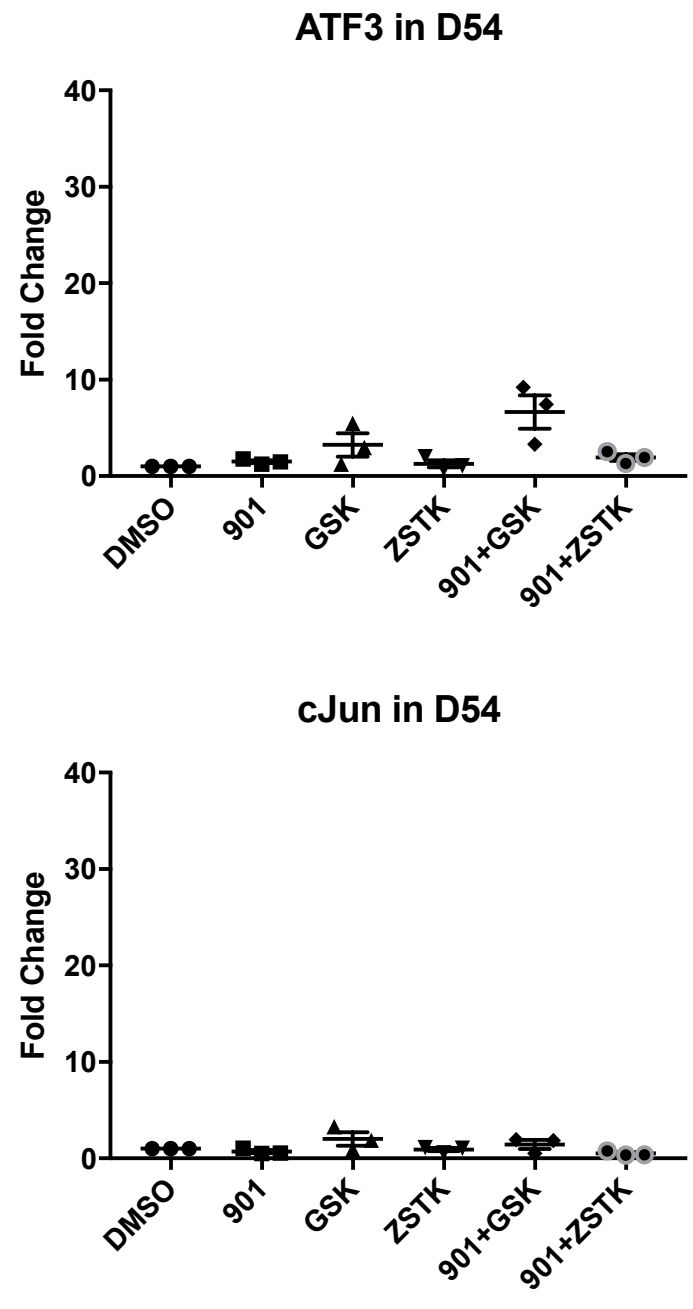
## H3K27M



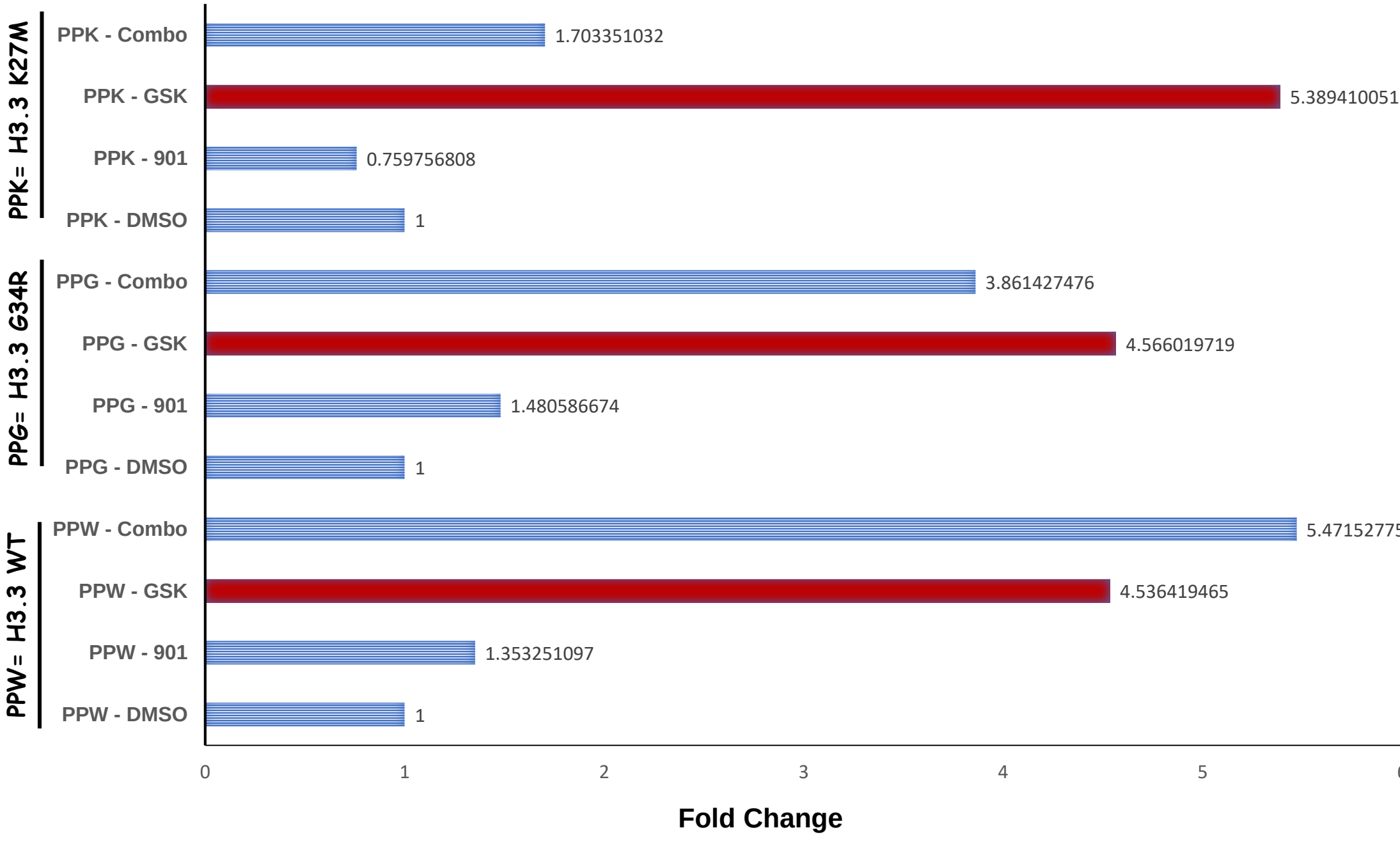




## H3 WT



# ATF 3 expression in murine isogenic HGG cells with mutations in PDGFRA and P53




**Collaboration with Vivek Yadav and Carl Koschmann**

# Resistance to targeted therapy mediated by upregulation of KLF4?

Format: Abstract Send to

Stem Cell Reports. 2017 Jun 6;8(6):1617-1629. doi: 10.1016/j.stemcr.2017.04.025. Epub 2017 May 25.

**Inhibition of KLF4 by Statins Reverses Adriamycin-Induced Metastasis and Cancer Stemness in Osteosarcoma Cells.**

Li Y[1], Xian M[1], Yang B[1], Ying M[2], He Q[3].

Author information

**Abstract**

Adriamycin-based combination chemotherapy is the standard first-line treatment for osteosarcoma, but tumor recurrence and metastasis occurs in most cases. Recent evidence suggests that microenvironmental stress such as chemotherapy can lead to the enrichment of cancer stem cells (CSCs), which result in cancer metastasis, recurrence, and drug resistance. However, the exact mechanisms underlying this phenomenon and how to target CSCs are still open questions. Herein, we report that Adriamycin treatment induces a stem-like phenotype and promotes metastatic potential in osteosarcoma cells through upregulating KLF4. KLF4 knockdown blocks Adriamycin-induced stemness phenotype and metastasis capacity. We further screen that statins remarkably reverse Adriamycin-induced CSC properties and metastasis by downregulating KLF4. Most strikingly, simvastatin severely impaired Adriamycin-enhanced tumorigenesis of KHOS/NP cells in vivo. These data suggest that Adriamycin-based chemotherapeutics may simulate CSCs through activation of KLF4 signaling and that selective inhibition of KLF4 with statins should be considered in the development of osteosarcoma therapeutics.



**KEYWORDS:** Adriamycin; KLF4; cancer stem cells; metastasis; osteosarcoma; statins

PMID: 28552603 PMCID: PMC5470096 DOI: 10.1016/j.stemcr.2017.04.025

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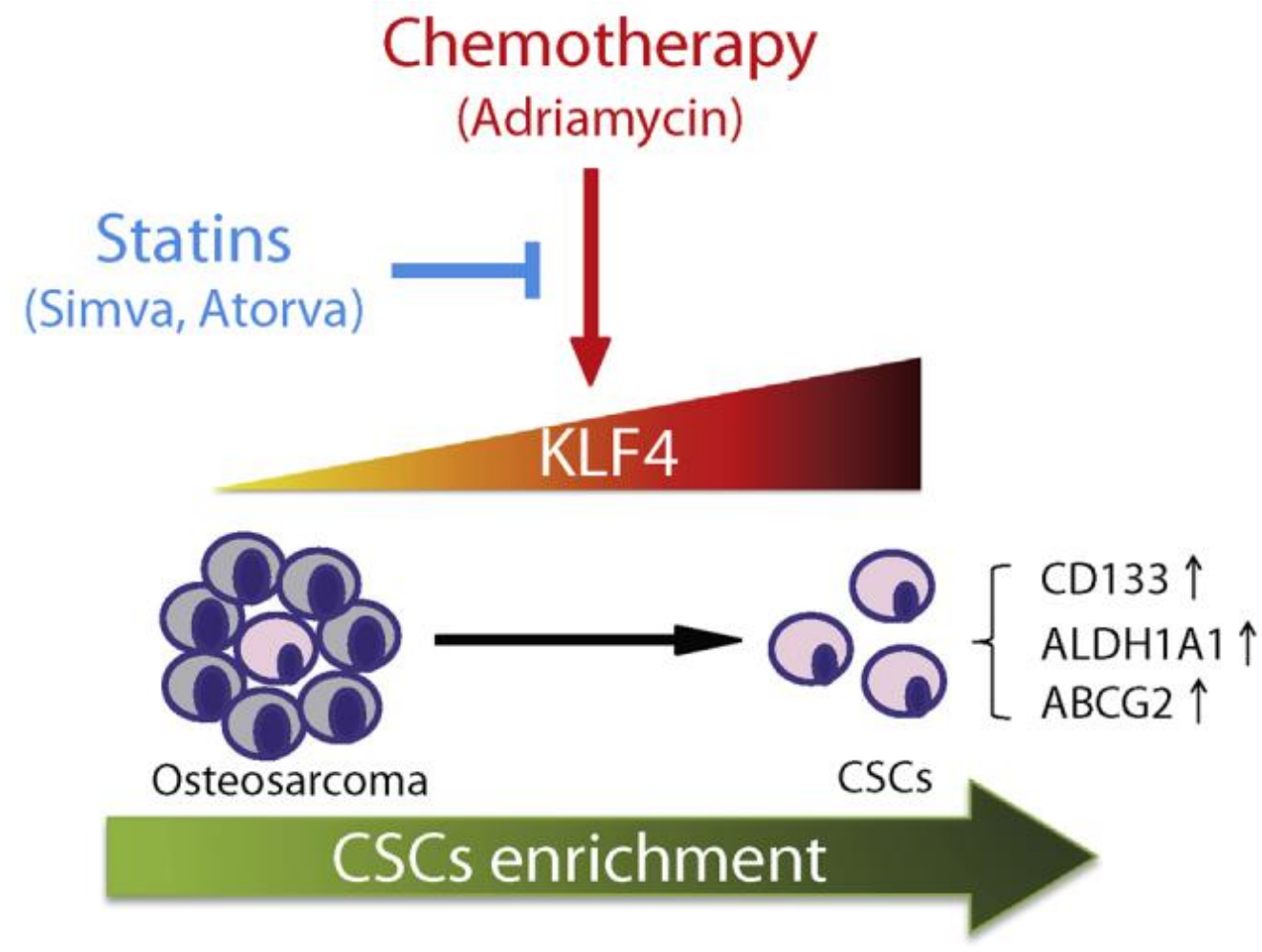


**-> new therapeutic strategy: combination of molecular targeted PI3K/mTOR inhibition with Statins???**

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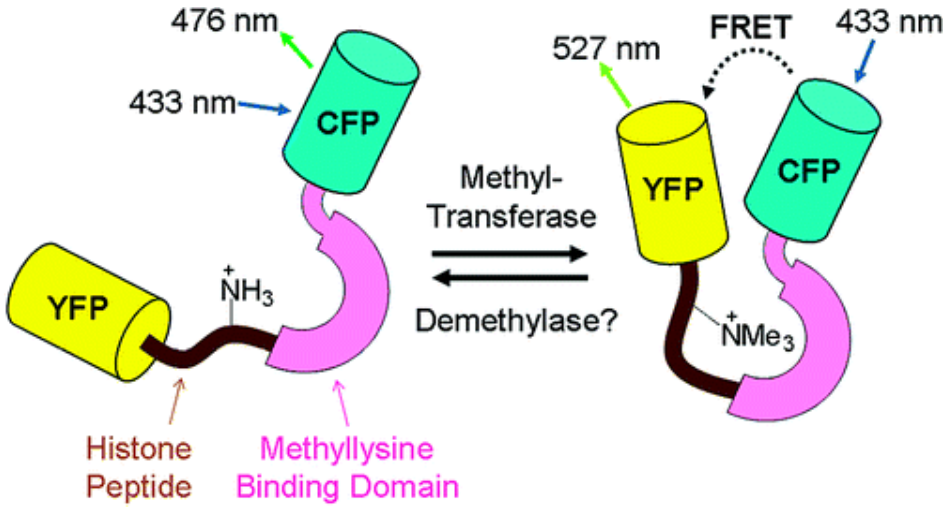
**Efficacy and Safety of Atorvastatin in Combination With Radiotherapy and Temozolomide in Glioblastoma (ART)**

The safety and scientific validity of this study is the responsibility of the study sponsor and investigators. Listing a study does not mean it has been evaluated by the U.S. Federal Government. Read our disclaimer for details.

ClinicalTrials.gov Identifier: NCT02029573

Recruitment Status : Completed
First Posted : January 8, 2014
Last Update Posted : August 18, 2017

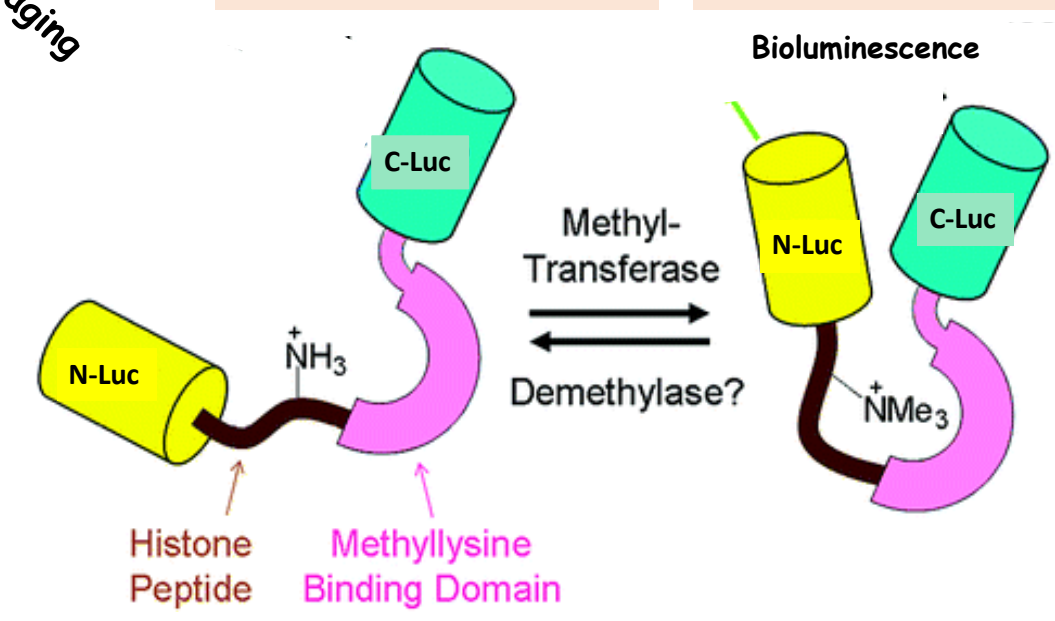
# Adaptations to the Histone K27 methylation reporter for *in vivo* bioluminescence imaging to test new therapeutic paradigms




Adaptations for in vivo imaging

**Mono-methylation transcription ON**

**Tri-methylation transcription OFF**




Lin CW et al, J Am Chem Soc. 2004 May 19;126(19):5982-3.

# Summary

- Identification and characterization of ALDH+ and or CD133+ CSC subpopulation in DIPG

- Stem cell properties in vitro/in vivo of DIPG CSCs

- Molecular targeted therapy:
  1. PI3K/mTOR efficacy in DIPG tumor cell kill of bulk tumor
  2. Effects of PI3K/mTOR/MAPK inhibition on "Stemness"

- Identification of resistance mechanisms: ATF3, KLF4

- Tools to develop therapeutic efficacy of single agent therapies as well as combination therapies: methylation reporter

# Future directions-1

**Targeting DIPG cancer stem cells by MAPK/PI3K/mTOR inhibition:**

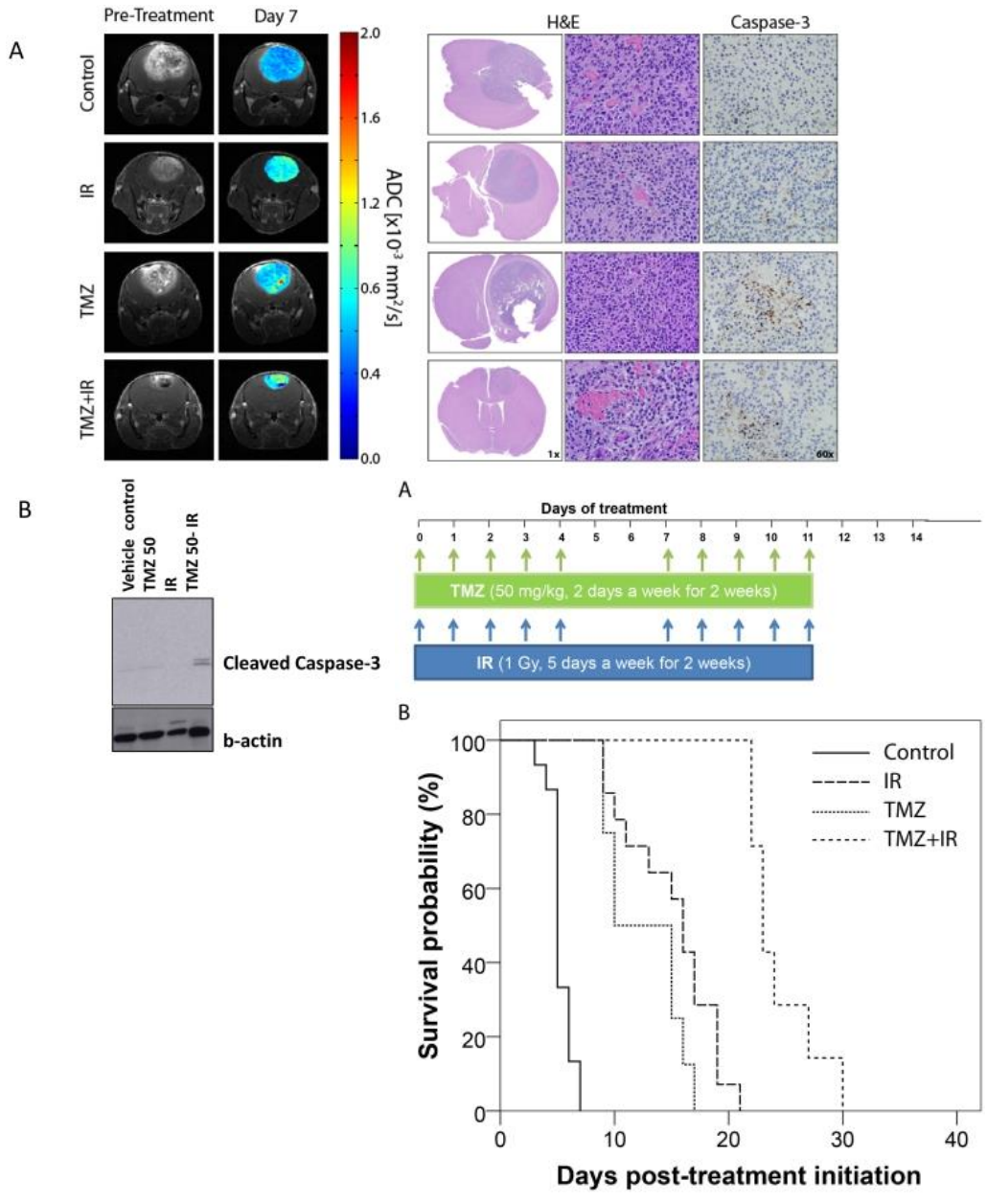
- cell death, cell cycle, proliferation, sphere formation, metabolism and stem cell gene expression as well as expression of pro-apoptotic genes in ALDH+ and CD133+ DIPGs

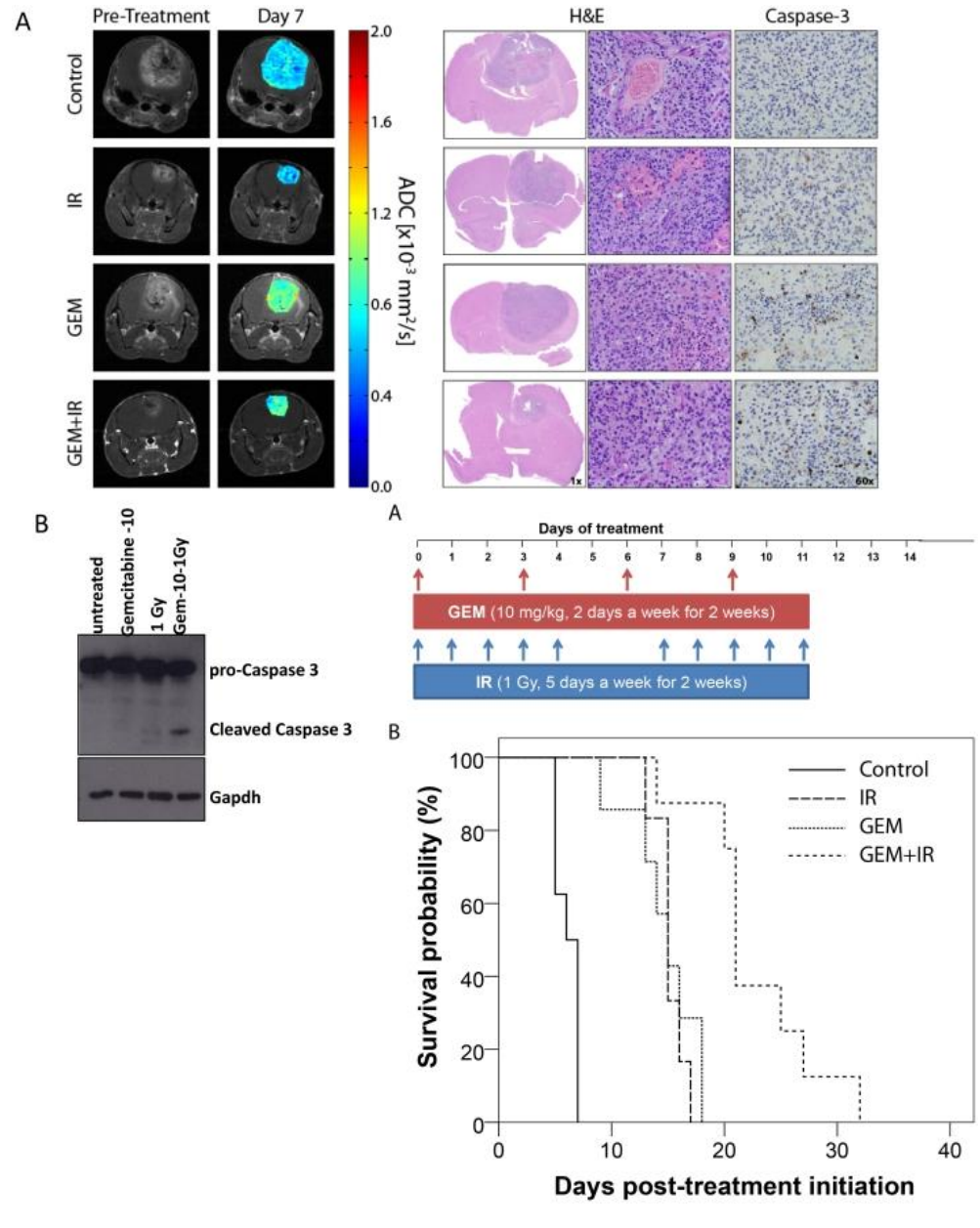
- Determine cancer stem cell characteristics *in vivo* ALDH+ DIPGs

- Efficacy of targeted MAPK/PI3K/mTOR inhibition in orthotopic DIPG mouse models:
  a) using a bioluminescent caspase 3 imaging reporter which allows for *in vivo* quantification of apoptosis

  b) clinically translatable diffusion-weighted MRI (DW-MRI) as a surrogate for cell death and early indicator of therapeutic efficacy

# DW-MRI as a biomarker to compare therapeutic outcomes in radiotherapy regimens incorporating temozolomide or gemcitabine in glioblastoma.



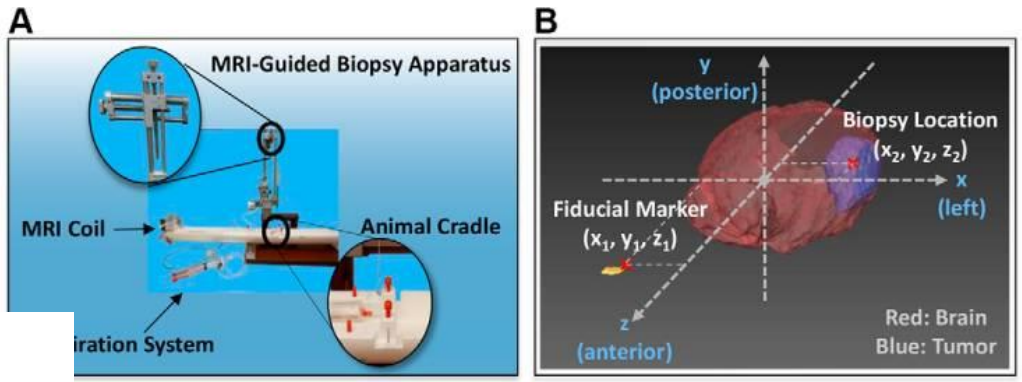

Galbán et al, PLoS One. 2012;7(4):e35857.

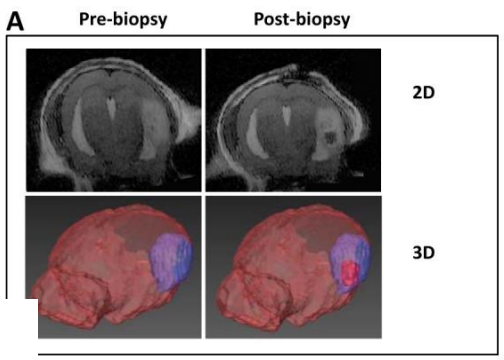
# Future directions-2

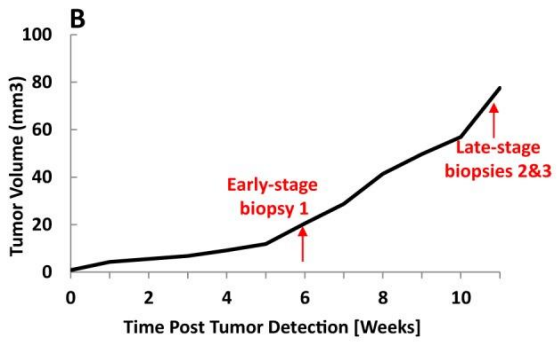
**Evaluate radio-sensitizing properties of MAPK/PI3K/mTOR inhibitors:**

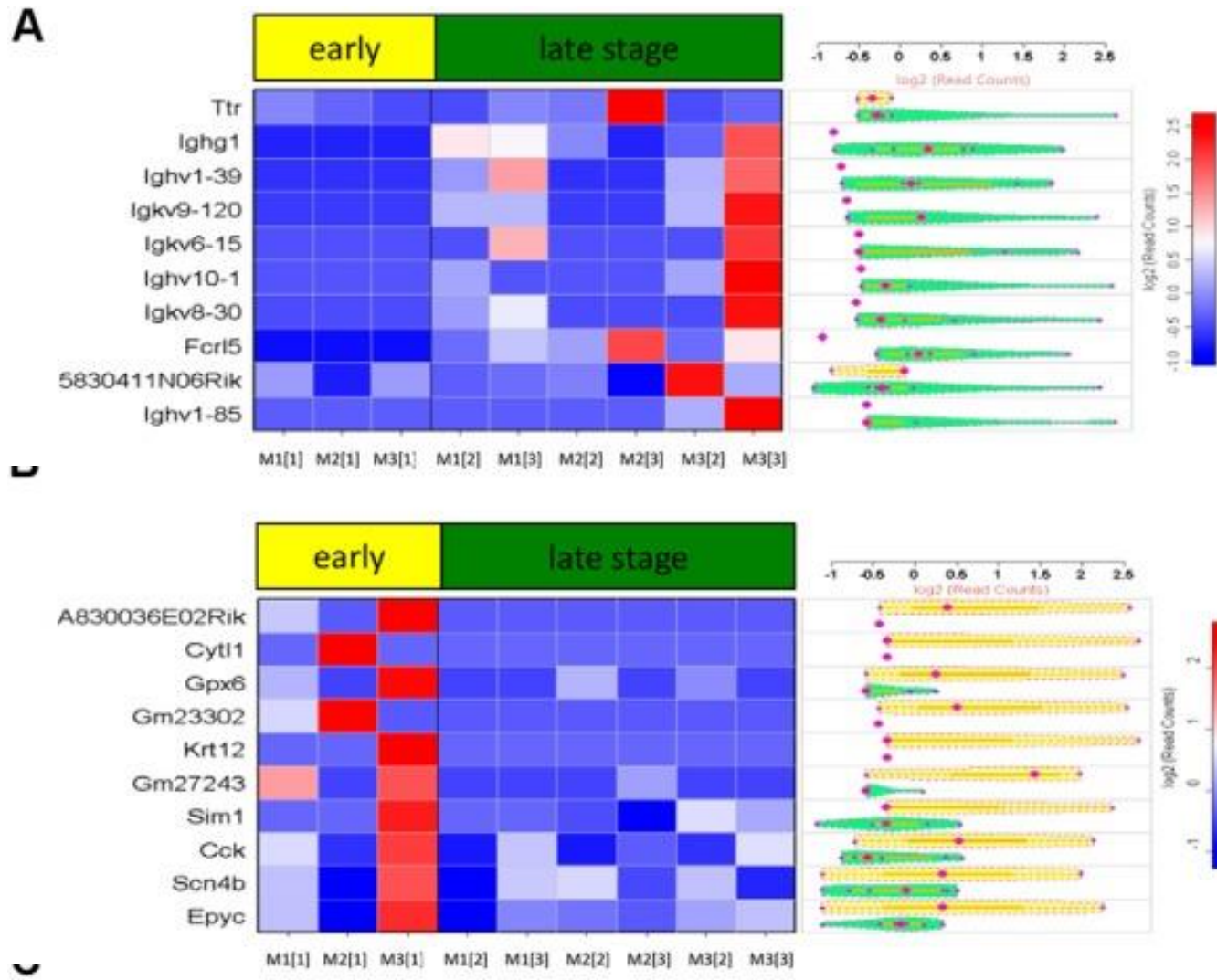
- efficacy of radiotherapy plus MAPK/PI3K/mTOR inhibition with single agents, combination or ST-182 in DIPGs
- stereotaxic MRI-guided sampling of DIPG tumors pre and post treatment coupled with transcriptome analysis to identify signaling pathways of aggressive and drug resistant DIPG subpopulations.

# MRI guided biopsies: identifying heterogeneity



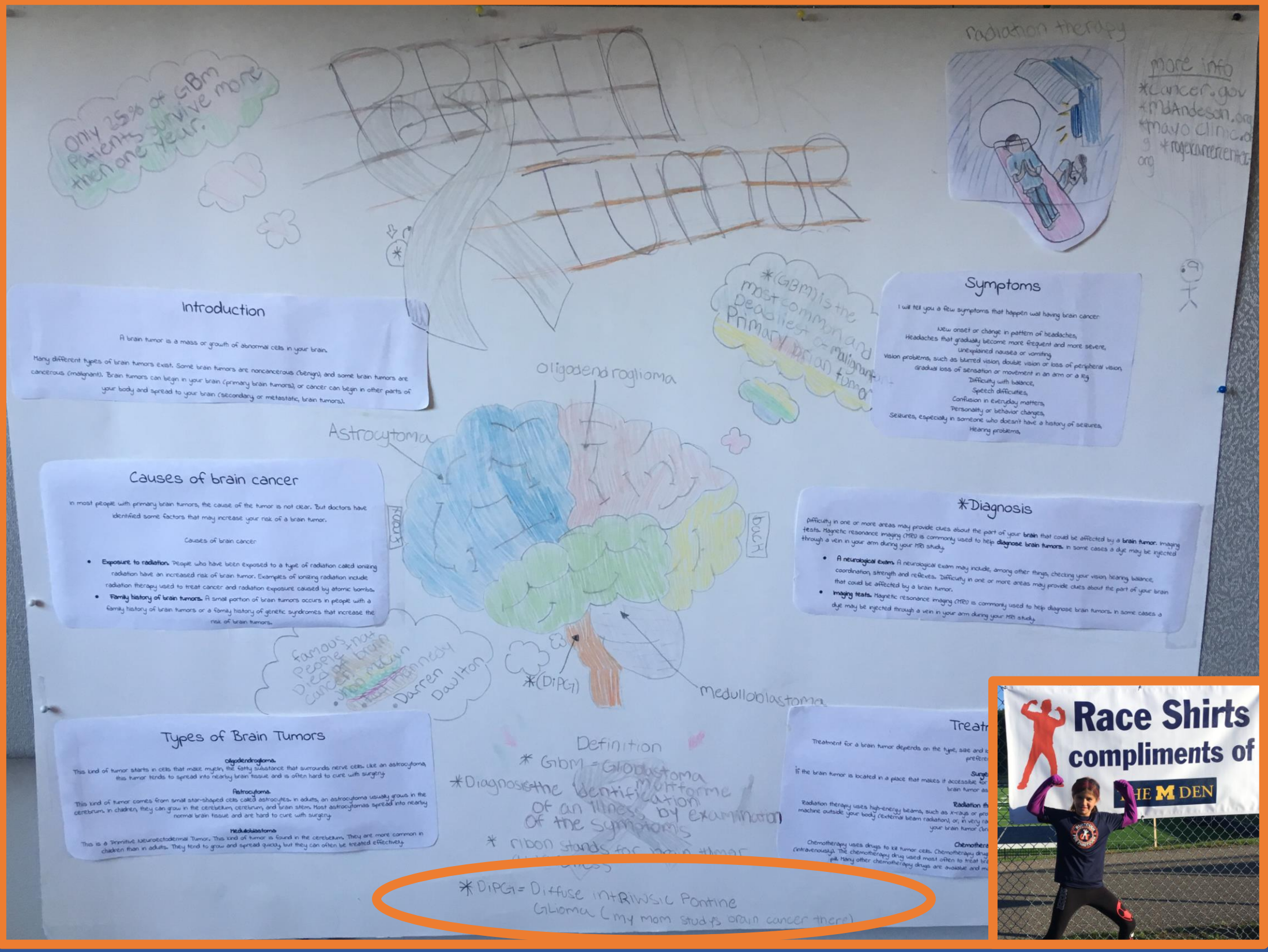




Galbán et al Tomography. 2017 Mar; 3(1): 9–15,

# Collaborative projects

- The use of histotripsy to treat " immune cold DIPG' with Dr. Zhen Xu
- Testing Oncoceutics drugs for targeting CSC in DIPG with Oncoceutics
- Combination with Ferroptosis inhibitors with Nneka/Costas's lab
- Glyco-gene expression in DIPG for novel target identification with Dr. Arun Everest-Dass
- Radio- and chemo-therapeutic induced tumor neoantigen Discovery with Dr. Rumble/Cayman chemicals

# Raising awareness




June Galban

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Uday Bhanu Maachani

**Bru-Sequencing**
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Karan Bedi
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**Metabolomics studies**
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Nneka Mbah

**Histotripsy studies**
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Tyler Gerhardson
Sang Won Choi

**Drug discovery**
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Marcian van Dort
Gary Luker

**Reporter studies**
Kathy Luker

**Target identification**
Arun Everest-Dass
Julie Rumble

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