# Hedgehog pathway, microRNAs and neural stem cells

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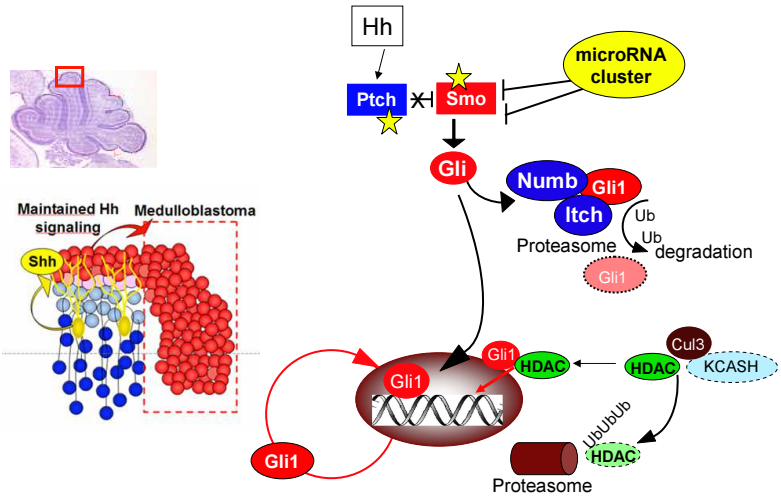
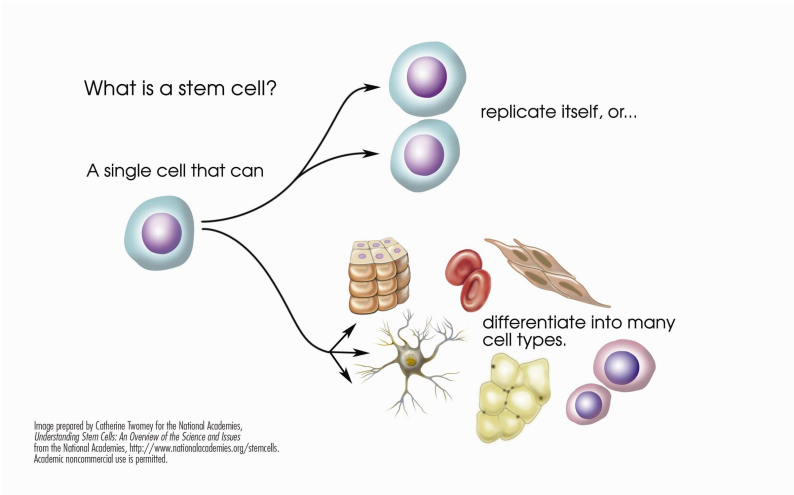
## Regulatory mechanisms in the Hh pathway




Di Marcotullio et al. *PNAS* 2004
Di Marcotullio et al., Nature Cell Biol 2006
Ferretti et al . Embo J 2008
Canettieri et al Nature Cell Biol 2011

# Stem cells



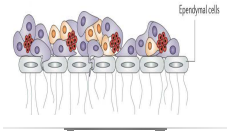

# Stem cells

Stem cells play a fundamental role in:

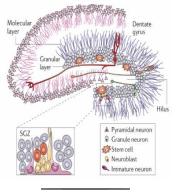
- Development
- Tissue regeneration
- Degenerative disorders

- Stem cells, directed to differentiate into specific cell types, offer the possibility of a renewable source of replacement cells and tissues to treat diseases including macular degeneration, spinal cord injury, stroke, burns, heart disease, diabetes, osteoarthritis, and rheumatoid arthritis.
- Furthermore, defects in stem cell biology may lead to developmental alterations and cancer.

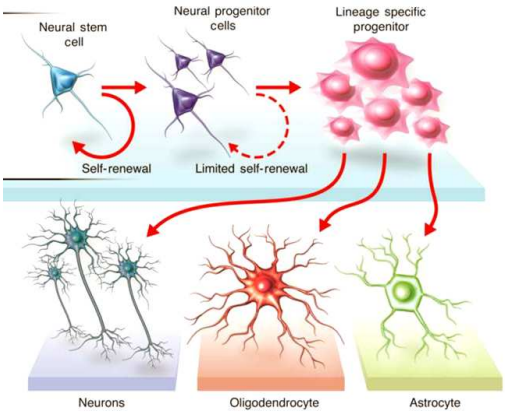
# Neural stem cells




**Subventricular zone (SVZ)**
Lateral ventricles




**Subgranular zone (SGZ)**
hyppocampus

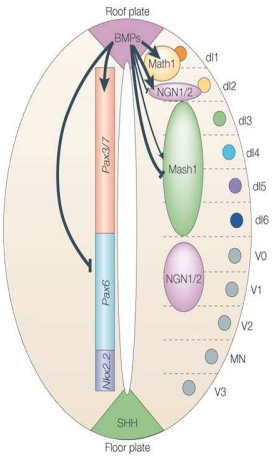



## Spatial and temporal control of cell fate are triggered by morphogens

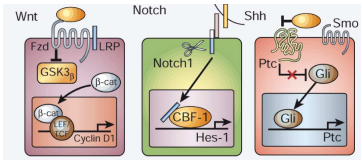
- ✓ **Morphogens**: molecules/signalling determining:
  - ✓ pattern of tissue development in morphogenesis
  - ✓ positions of the various specialized cell types within a tissue

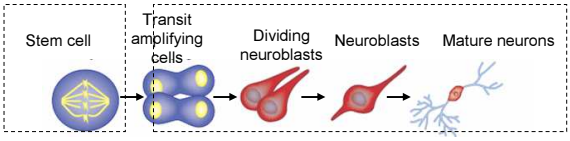
- ✓ M. diffuse through the tissues of an embryo during early development.
- ✓ **Concentration gradients** are set up driving the process of differentiation of **unspecialized (stem/progenitors)** cells into **different cell types, or the maintenance of undifferentiated/stem cells features in specific regions/niches** and determining the formation of all the tissues and organs of the body.

Developmental morphogens: BMPs, Wnts, FGFs and Hedgehog,



PATHWAYS most involved in Stemness signals:




Hh is one of the master developmental pathways which plays and essential role in the stem cell compartment.

To fully understand this role we are investigating the signaling pathway, its targets and regulatory mechanisms, in neural cerebellar stem cells.

## Hedgehog and neural stem cells

- Hh signaling controls embryonic and postnatal NSC of forebrain subventricular zone and of the hippocampus *(Ahn & Joyner, 2005; Lai et al, 2003; Machold et al, 2003; Palma et al, 2005; Palma & Ruiz i Altaba, 2004).*
- In cerebellum, Hh is critically required to keep transit-amplifying granule cell progenitors (GCPs) undifferentiated, to promote their proliferation *(Dahmane & Ruiz i Altaba, 1999; Wallace, 1999; Wechsler-Reya & Scott, 1999)*
- Hh-driven Gli transcription factors, act on target genes promoting cell proliferation and reducing cell differentiation.
- The identity of Hh/Gli target genes involved in the control of stemness in NSCs and cancer SCs is poorly understood

*Nat Neurosci*. 2005 June ; 8(6): 723–729.

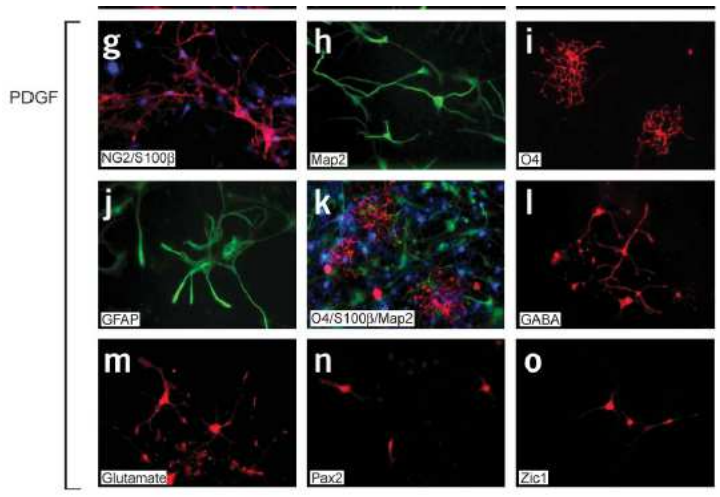
# Isolation of neural stem cells from the postnatal cerebellum

**Audra Lee**[1], **Jessica D Kessler**[1], **Tracy-Ann Read**[1], **Constanze Kaiser**[1], **Denis Corbeil**[2], **Wieland B Huttner**[2], **Jane E Johnson**[3], and **Robert J Wechsler-Reya**[1]

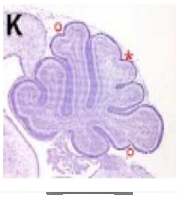
1*Department of Pharmacology & Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, USA*

## Abstract

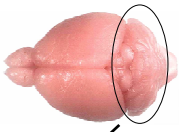
The cerebellum is critical for motor coordination and cognitive function and is the target of transformation in medulloblastoma, the most common malignant brain tumor in children. Although the development of granule cells, the most abundant neurons in the cerebellum, has been studied in detail, the origins of other cerebellar neurons and glia remain poorly understood. Here we show that the murine postnatal cerebellum contains multipotent neural stem cells (NSCs). These cells can be prospectively isolated based on their expression of the NSC marker prominin-1 (CD133) and their lack of markers of neuronal and glial lineages ($lin^-$). Purified $prominin^+lin^-$ cells form self-renewing neurospheres and can differentiate into astrocytes, oligodendrocytes and neurons *in vitro*. Moreover, they can generate each of these lineages after transplantation into the cerebellum. Identification of cerebellar stem cells has important implications for the understanding of cerebellar development and the origins of medulloblastoma.



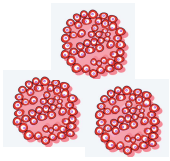
## NSC



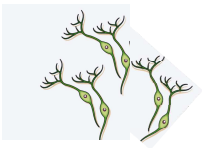

cerebellum



•NSCs isolated from cerebellum of 4 days old CD1+ mouse
•Grown in specific stem cell medium form neurosferes



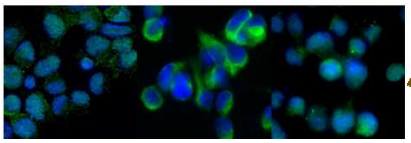
In vitro **differentiation** with PDGF.



## NSC (Postnatal cerebellar stem cells)

**Stemness markers and Hedgehog Molecules**

Sox2/DAPI Nestin/DAPI Gli1/DAPI

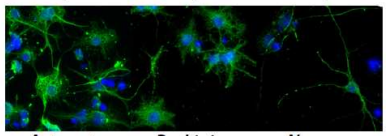


DIF

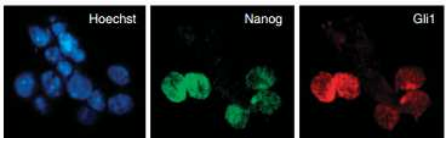
PDGF

**Differentiation markers**

s100/DAPI Pvalb/DAPI Tubb3/DAPI

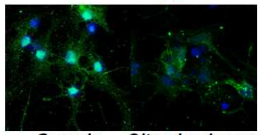


*Astrocyte* *Purkinje* *Neuron*




DIF

Zic1/DAPI Cspg4/DAPI



*Granule* *Oligodendrocyte*

## Transcriptome Profiles of :

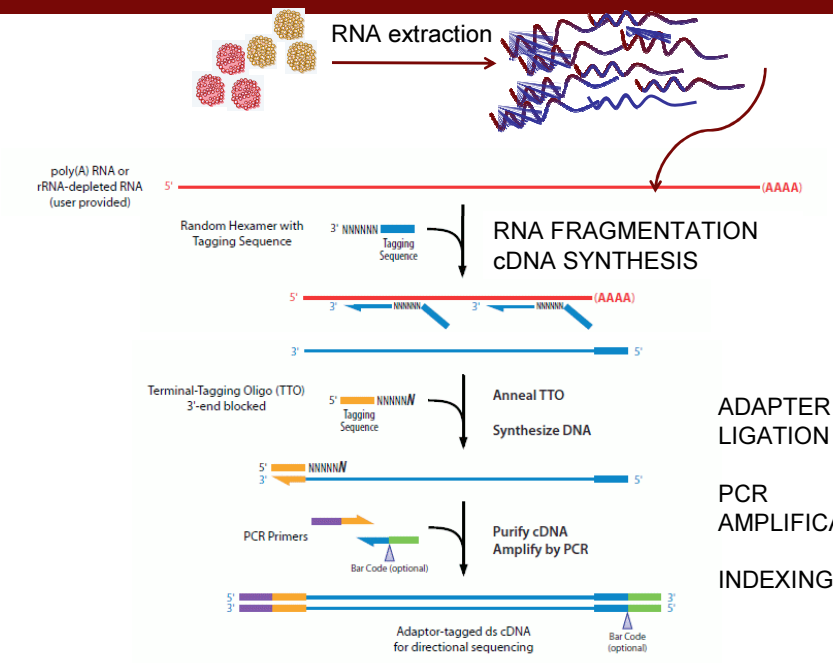
**1.NSC..**

To obtain a complete transcription pattern of cerebellar NSC

**2. … and differentiated NSC**

To identify transcripts differentially expressed during differentiation

## methods- RNASeq libraries prep




ADAPTER LIGATION

PCR AMPLIFICATION

INDEXING

# RNA-SEQ

RNA Next Generation Sequencing (RNA-Seq) technology

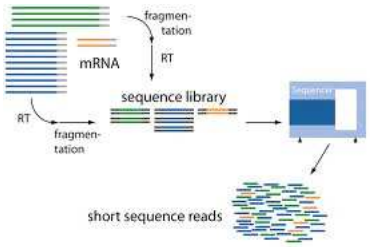
**A massive parallel deep-sequencing –**

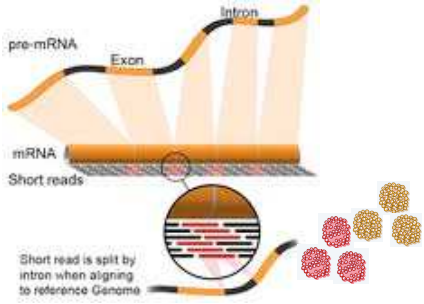
RNA-Seq is able to interrogate the genome-wide global transcriptome and identify

new transcripts,

alternative spliced isoforms

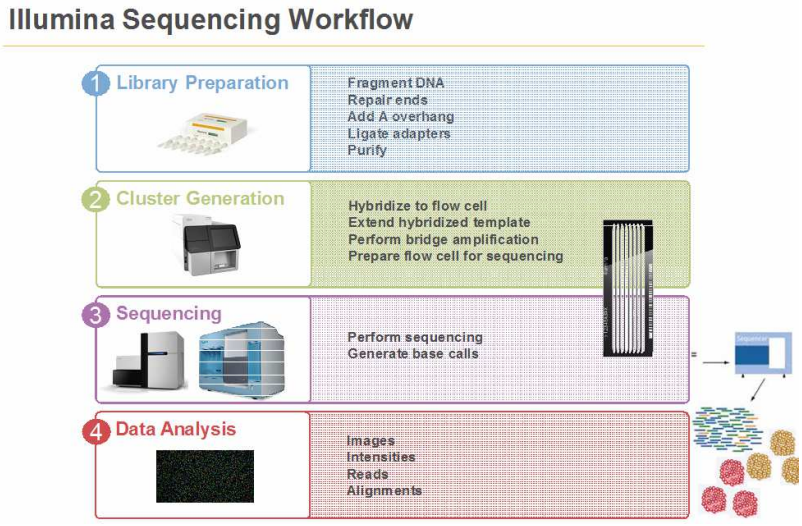
non-coding RNAs.



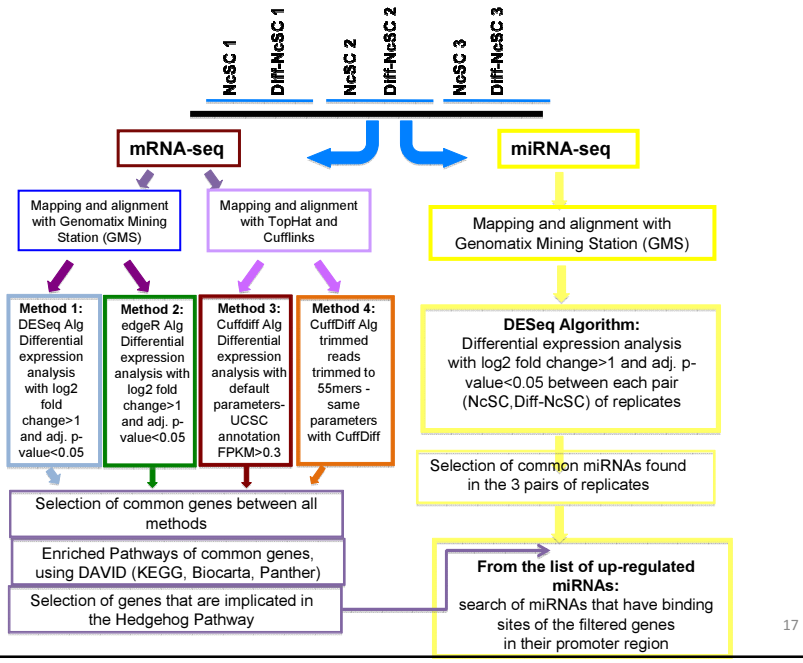




High throughput analysis of RNA expression profiles by using Illumina technology:

70 bp paired-end sequencing each run.

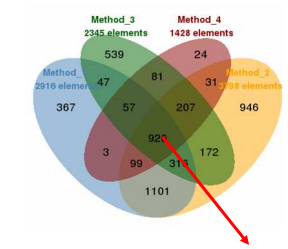


## Procedures

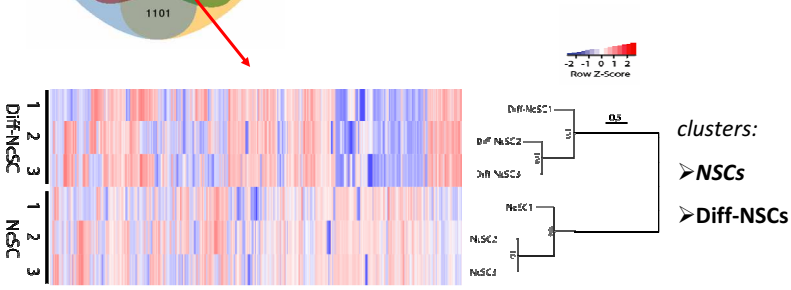





- Quality of sequencing and normalization validated: no biases
- In each sample there are about 170’000 expressed transcripts (80% annotated)
- Identified about 14000-15000 expressed genes (86% annotated)
- Data analyzed by 4 different approaches: transcripts emerging as differential in all 4 systems are selected (926 transcripts)



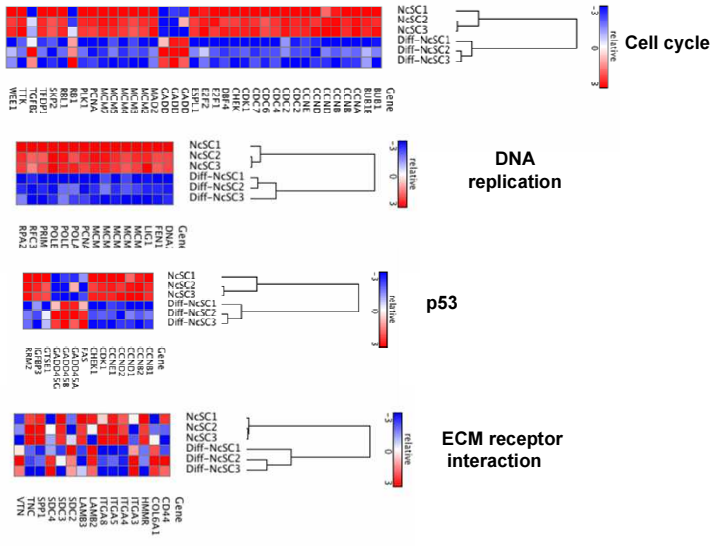

***Tophat and Cufflinks differentially expressed Trascripts.***




*clusters:*

- ***NSCs***
- **Diff-NSCs**

Common transcripts heatmap

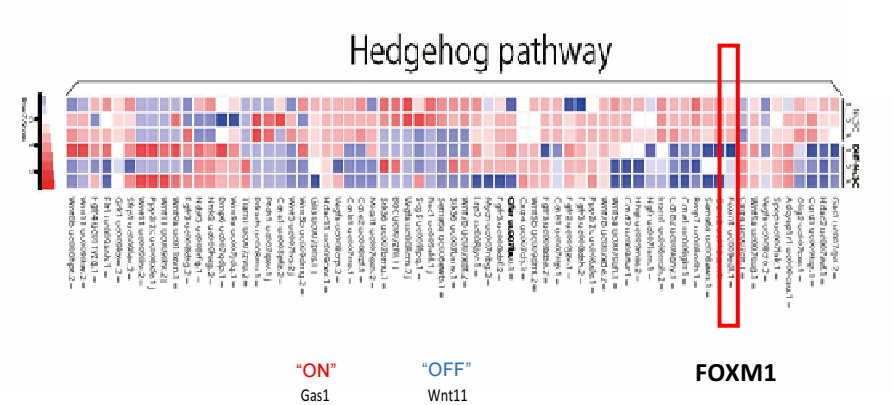



**Cell cycle**

**DNA replication**

**p53**

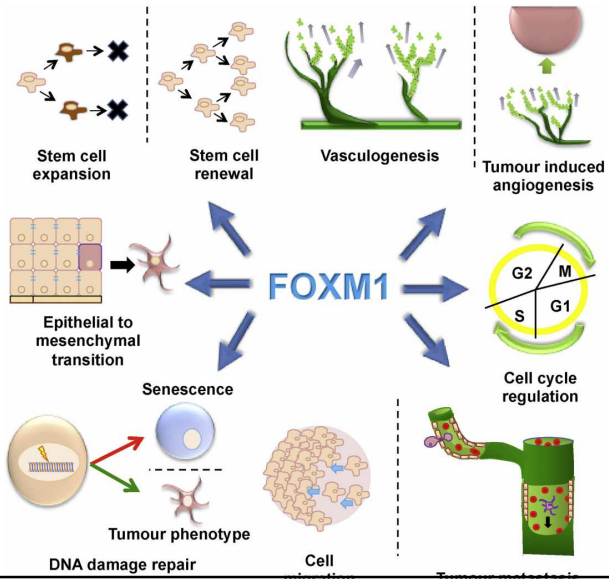
**ECM receptor interaction**




**FOXM1**

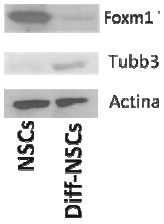
| "ON" | "OFF" |
|---|---|
| Gas1 | Wnt11 |
| Foxm1 | Vegfa |
| Hdac2 | Tiam1 |
| Ccnd1 | Cxcr4 |
| Olig2 | Ecc.. |
| Bmp7 | |
| fgfr3 | |
| Ecc.. | |

## Multiple roles of FOXM1

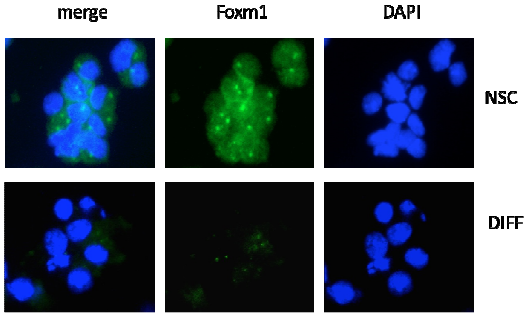


## *FOXm1 is expressed in NSC but not in differentiating neural cells*

**West. Blot**

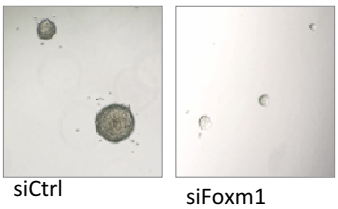


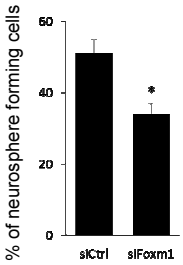

**Immunofluorescence**

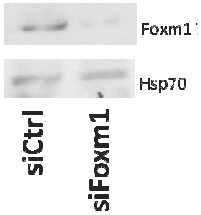



## Biological role of Foxm1 in NSCs

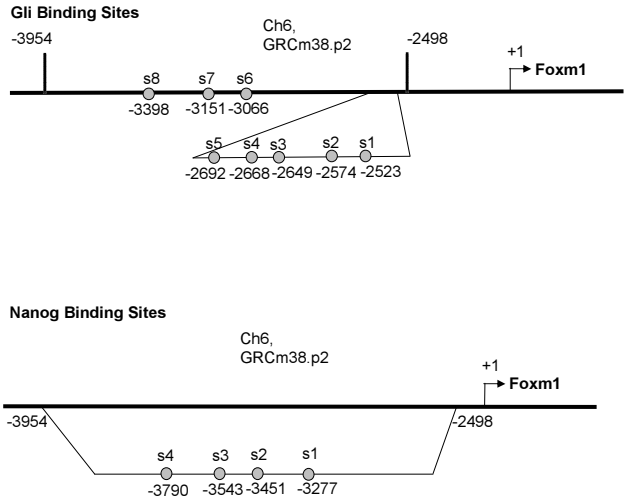
Neurosphere formation assay



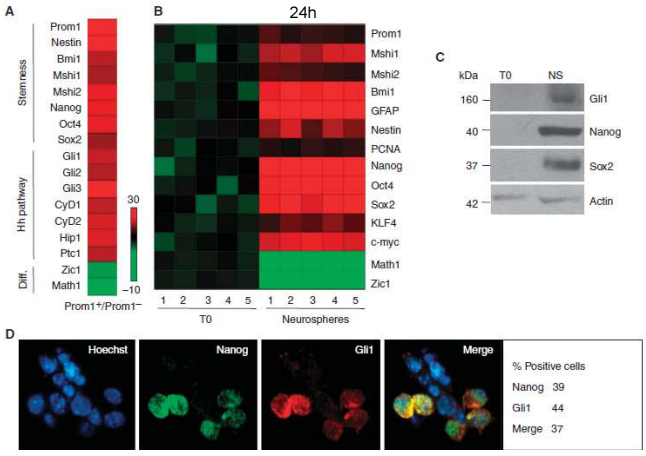
## Binding sites on FOXM1 regulatory regions





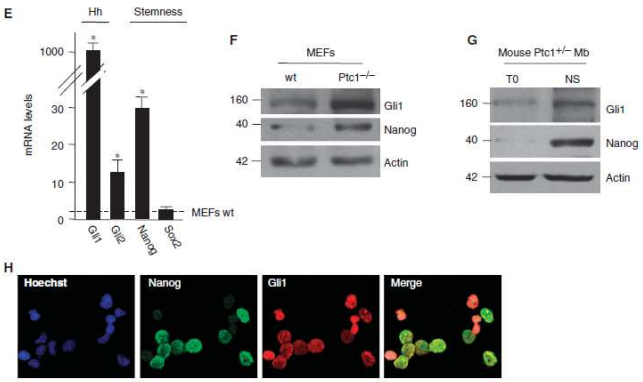

*Correlation between hedgehog and stem cell markers expression in cerebellar stem cells*

Primary cultures and neurospheres



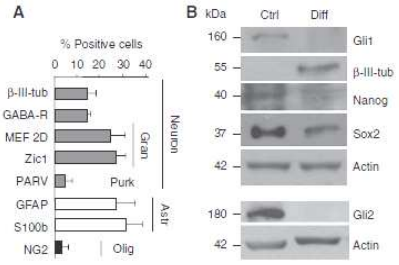
## *Correlation between Gli and Nanog* expression

Ptc-/- MEF

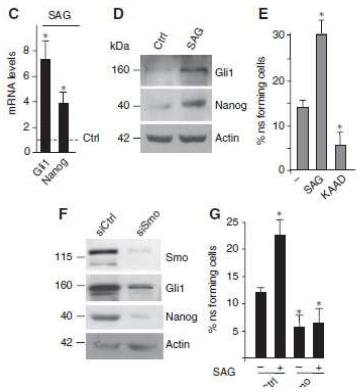



MB neurosferes

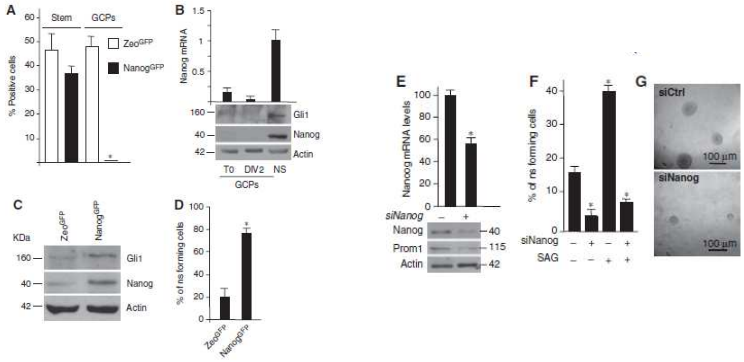
## *Role of Hedgehog-Gli1 and Nanog in cerebellar NSC*




NSC w/PDGF 7 days



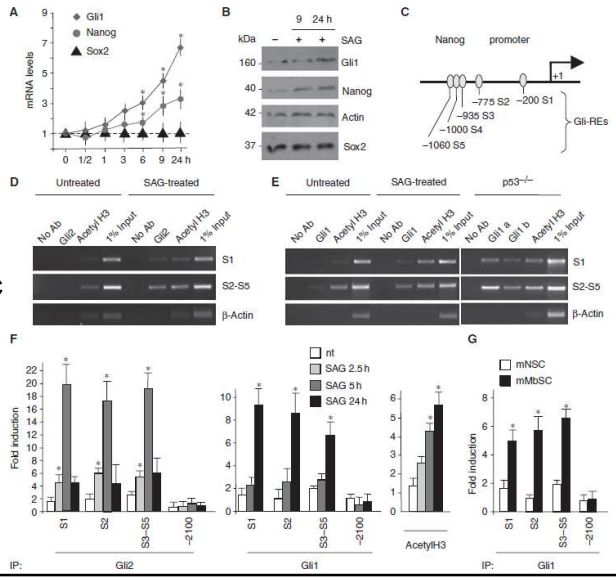
## *Nanog is required for NSC self-renewal induced by Hh*



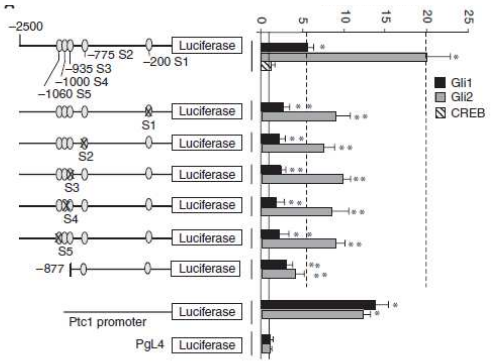

## Hedgehog/Gli activate Nanog transcription

Mouse NSC

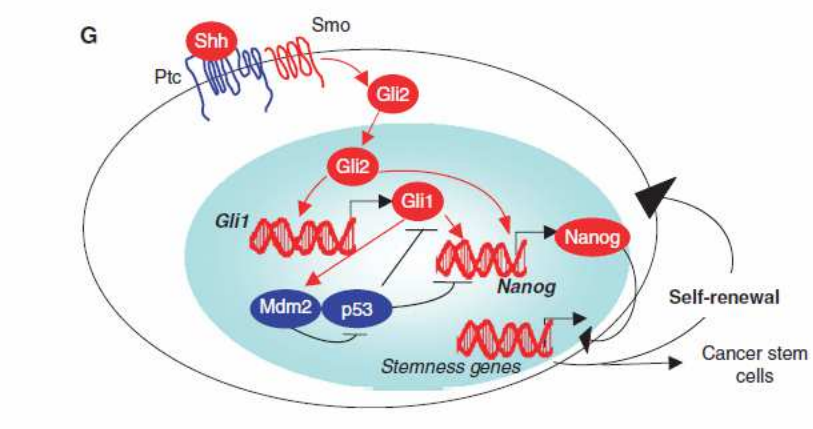
qPCR-CHIP

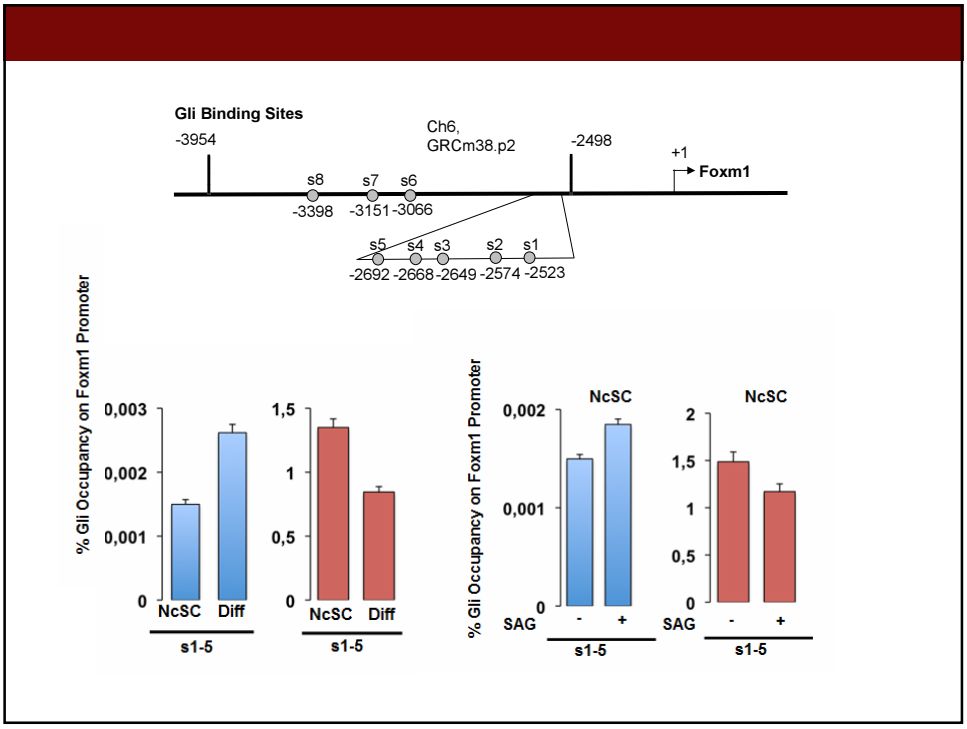


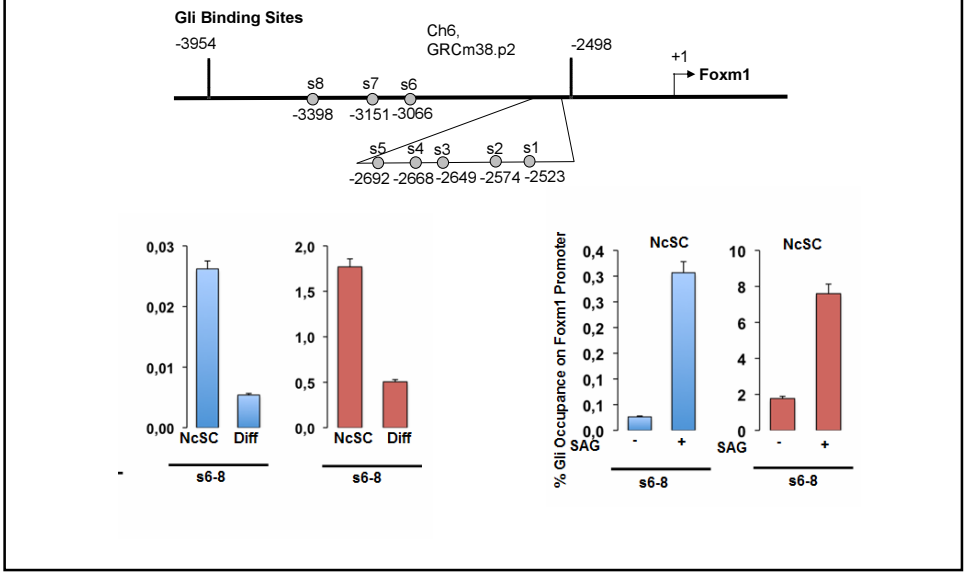
## Analysis of Gli1 responding elements in Nanog promoter

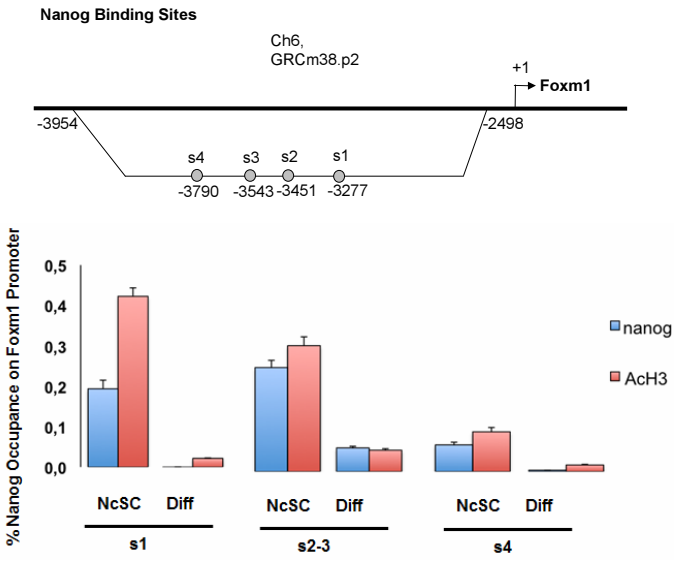


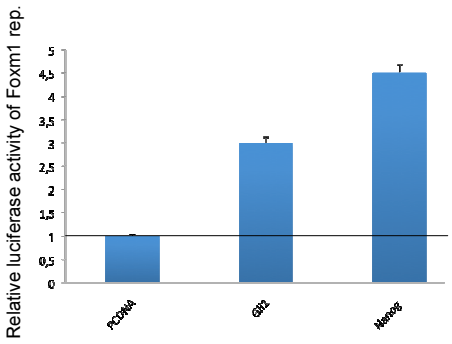

## Nanog is a direct target of Hh/Gli pathway




Gli Binding Sites
-3954
Ch6, GRCm38.p2
-2498
+1
Foxm1
s8 s7 s6
-3398 -3151 -3066
s5 s4 s3 s2 s1
-2692 -2668 -2649 -2574 -2523
% Gli Occupancy on Foxm1 Promoter
0,003
0,002
0,001
0
NcSC Diff
s1-5
1,5
1
0,5
0
NcSC Diff
s1-5
% Gli Occupancy on Foxm1 Promoter
NcSC
0,002
0,001
0
SAG - +
s1-5
NcSC
2
1,5
1
0,5
0
SAG - +
s1-5

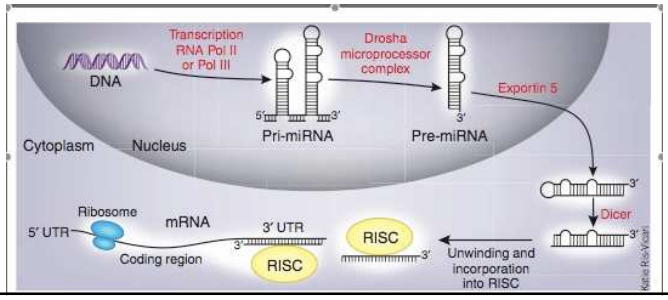

Gli Binding Sites
-3954
Ch6, GRCm38.p2
-2498
+1
Foxm1
s8 s7 s6
-3398 -3151 -3066
s5 s4 s3 s2 s1
-2692 -2668 -2649 -2574 -2523
0,03
0,02
0,01
0,00
NcSC Diff
s6-8
2,0
1,5
1,0
0,5
0,0
NcSC Diff
s6-8
% Gli Occupance on Foxm1 Promoter
NcSC
0,4
0,3
0,3
0,2
0,2
0,1
0,1
0,0
SAG - +
s6-8
NcSC
10
8
6
4
2
0
SAG - +
s6-8


Nanog Binding Sites
Ch6,
GRCm38.p2
+1
Foxm1
-3954
-2498
s4
s3
s2
s1
-3790
-3543
-3451
-3277
% Nanog Occupance on Foxm1 Promoter
0,5
0,4
0,3
0,2
0,1
0,0
nanog
AcH3
NcSC
Diff
NcSC
Diff
NcSC
Diff
s1
s2-3
s4


Relative luciferase activity of Foxm1 rep.
5
4,5
4
3,5
3
2,5
2
1,5
1
0,5
0
PCNA
Gli1
Nanog

# microRNAs

- **Short non-coding** RNA sequences encoded in either introns or exons DNA
- Have **post-transcriptional regulatory** functions (i.e., differentiation, metabolic homeostasis, apoptosis, and proliferation)
- Have a **complementary target mRNA** transcript to which they bind causing negative regulation
- Mode of action: either **degrade mRNA** transcript or **prevent translation**
- Hundreds of miRNAs in humans, each with many target sites

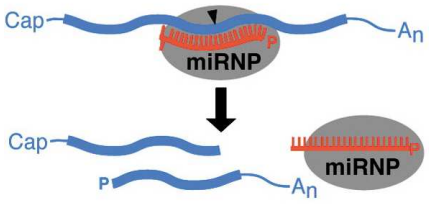



# Mode of action

**(A)** Perfect matching with target mRNA

↓

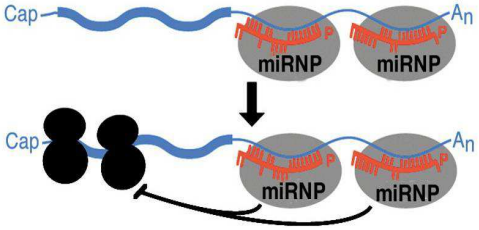
Endonucleolytic cleavage and transcript degradation



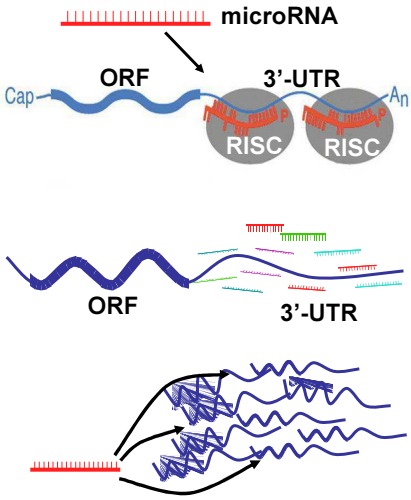

**(B)** Imperfect matching with target mRNA, limited to 3'UTR

↓

Translation repression

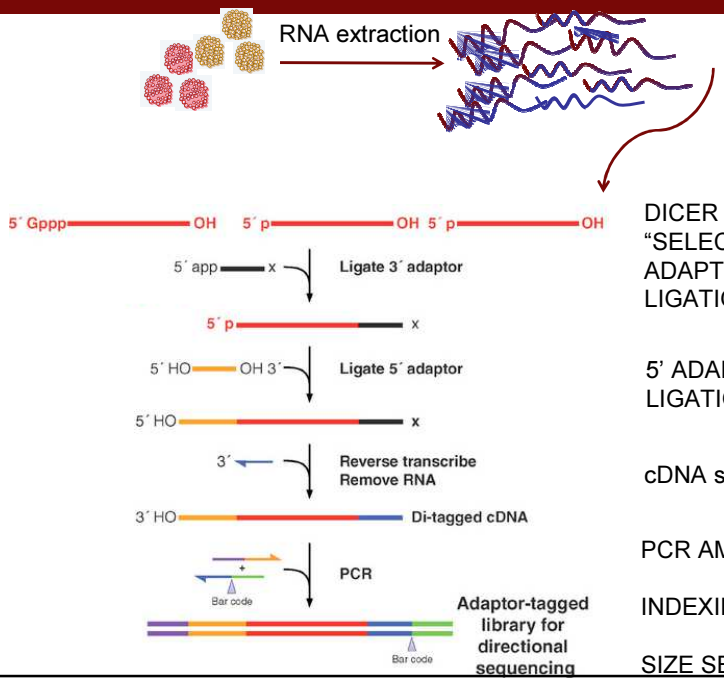


## MicroRNA: negative regulation of gene expression




- **MicroRNAs** imperfectly pair to in 3'-UTR of target mRNA
- Gene Expression inhibition degree depends on the numbers of microRNA binding sites.
- **A messenger RNA can have binding sites for several microRNAs**
- **Each microRNA often acts on several mRNA**

## methods- microRNASeq libraries pre




DICER CUT "SELECTION" – 3' ADAPTER LIGATION

5' ADAPTER LIGATION

cDNA synthesis

PCR AMPLIFICATION

INDEXING

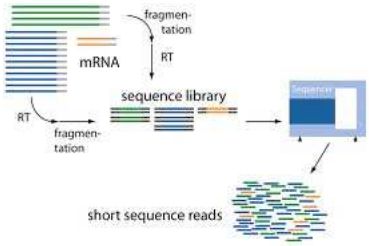
SIZE SELECTION

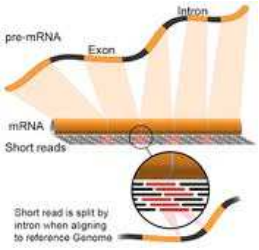
## hightroughput techniques - methods

### microRNA-SEQ→ miRnome

**A massive parallel deep-**sequencing

- ✓ miR expression analysis
- ✓ Deregulated miRNAs
- ✓ IsomiR 5'
- ✓ Putative new pre
  - ✓ **moRNA (miRNA-offset RNAs- genomic region flanking mature miRNA)**
  - ✓ **miRtrons**



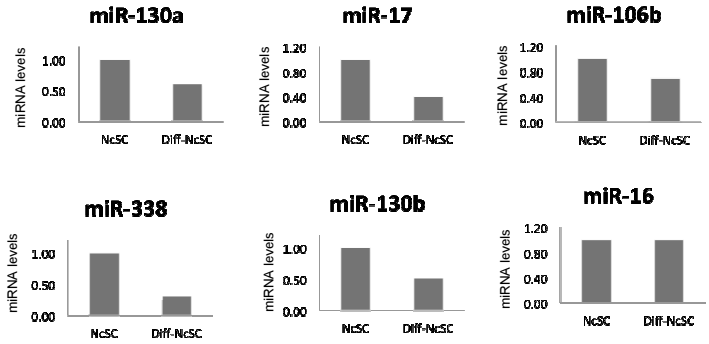




## microRNAs putatively regulated by Foxm1

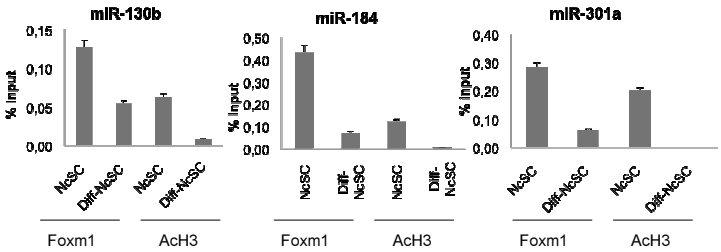
| miRNA | log fc | adj. P-value | Part of miRNA Cluster |
|---|---|---|---|
| miR-184-5p | 7,00 | 0,013652227 | - |
| miR-15B-3p | 4,09 | 0 | 15-16 |
| miR-130A-5p | 3,70 | 5,17E-15 | - |
| miR-92A-1-5p | 3,35 | 0 | 17-92 |
| miR-335-3p | 3,30 | 0 | - |
| miR-16-2-3p | 3,20 | 2,83E-54 | 15-16 |
| miR-25-5p | 2,96 | 8,20E-119 | 106b-25 |
| miR-130B-5p | 2,88 | 0 | - |
| miR-16-1-3p | 2,86 | 9,33E-188 | 15-16 |
| miR-301A-5p | 2,82 | 8,37E-121 | - |
| miR-15B-5p | 2,71 | 0 | 15-16 |
| miR-130B-3p | 2,57 | 0 | - |
| miR-93-3p | 2,49 | 2,03E-92 | 106b-25 |
| miR-92A-3p | 2,38 | 0 | 17-92 |
| miR-106B-5p | 2,17 | 0 | 106b-25 |

| miR-15-16 Cluster | miR-17-92 Cluster | miR-106b-25 Cluster |
|---|---|---|
| 15 | 17 | 106b |
| 16 | 18a | 93 |
| | 19a | 25 |
| | 20a | |
| | 19b-1 | |
| | 92a-1 | |

## validation in qpcr



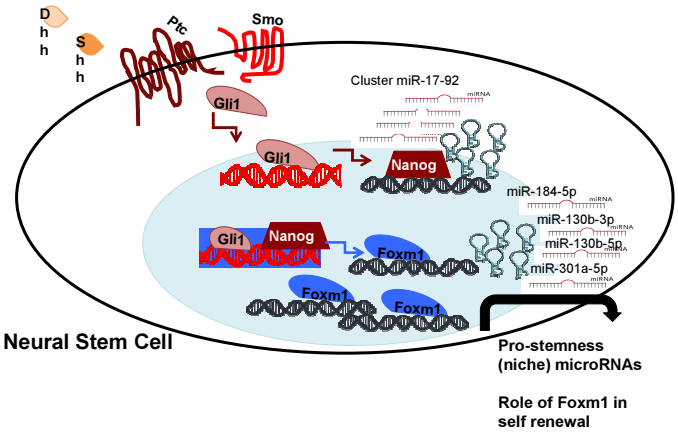

## 5C Chips (positive in main and negative in sup




Positive ChIPs of miRNAs

| miRNA | log fc | adj. P-value |
|---|---|---|
| miR-184-5p | 7,00 | 0,013652227 |
| miR-130B-5p | 2,88 | 0 |
| miR-301A-5p | 2,82 | 8,37E-121 |
| miR-130B-3p | 2,57 | 0 |

# Conclusions

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- Lucia Di Marcotullio
- Elisabetta Ferretti
  - Agnese Po
  - Evelina Miele
  - Mersini Besharat
  - Et many others…

## Thank You!

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1952-2014