Cell

Presenilin-Dependent ErbB4 Nuclear Signaling Regulates the Timing of Astrogenesis in the Developing Brain

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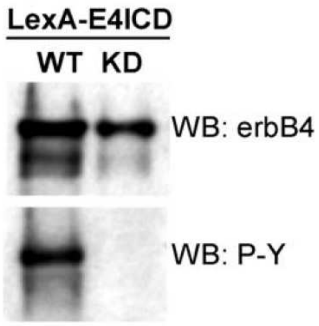
1

Identification of Proteins that Interact with Activated E4ICD

To identify ErbB4-binding proteins that are involved in transcriptional regulation they used a yeast two-hybrid system that facilitates the isolation of proteins that interact with activated RTKs

Screening of a cDNA expression library from rat embryonic day 14 (E14) spinal cord and dorsal root ganglia with a bait containing the entire E4ICD in an activated state led to isolation of several putative E4ICD-interacting proteins.



LexA-E4ICD fusion, when expressed in mammalian cells, dimerizes and becomes autophosphorylated.

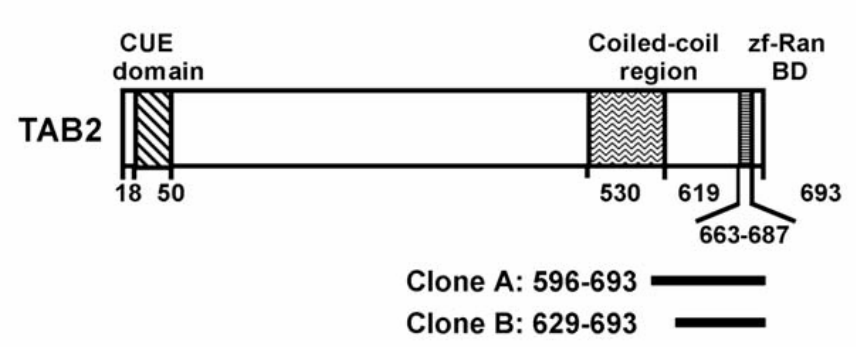
Yeast expressing wild-type (WT) and kinase-dead (KD) LexA-E4ICD fusion proteins were lysed and immunoblotted with ErbB4 or phosphotyrosine (P-Y) antibodies, showing that both proteins are expressed (top panel) but only the WT is tyrosine phosphorylated.

2

Two clones contained cDNAs encoding the C-terminal region of TAB2 (TAK1 binding protein 2), a protein first identified as an adaptor for TAK1 (transforming growth factor β-activated kinase 1)

Schematic diagram of TAB2

Regions of TAB2 included in two clones identified in the screen are indicated.
zf-Ran BD: zinc finger Ran-binding domain.



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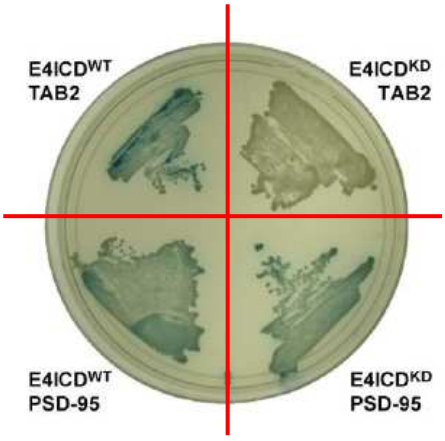
Wild-type E4ICD also interacted with full-length TAB2 in yeast, and this association was abolished when the tyrosine kinase activity of E4ICD was eliminated by a mutation in the ATP-binding site (E4ICDKD).



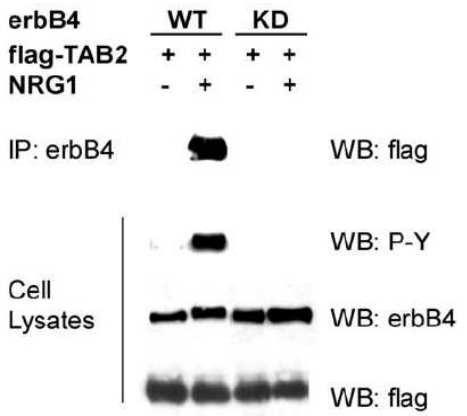
Tyrosine kinase activity of E4ICD is necessary for interaction with TAB2 in yeast. WT or KD LexA-E4ICD was coexpressed in yeast with full-length TAB2 or PDZ domains 1 and 2 of PSD-95 as fusion proteins with the activation domain.
TAB2 only interacts with phosphorylated E4ICD (blue color), whereas PSD-95 interacts with both baits.

→ binding of TAB2 to ErbB4 in yeast appears to occur only when the receptor is activated.

4

Does TAB2 binds to ErbB4 in mammalian cells?
Does this interaction depends on receptor activation by NRG1?

Cells were cotransfected with FLAG-TAB2 and full-length ErbB4 expression constructs and then subjected to immunoprecipitation with ErbB4 antibodies.



Cosa manca a questa IP?

→ TAB2 coprecipitated with ErbB4 only after NRG1 treatment

5

Presenilin-Dependent Cleavage of ErbB4 does not induce TAB2 phosphorylation

The dependence of ErbB4/TAB2 interaction on NRG1 suggested that ErbB4 activation could alter TAB2, particularly its state of phosphorylation or its cellular localization.

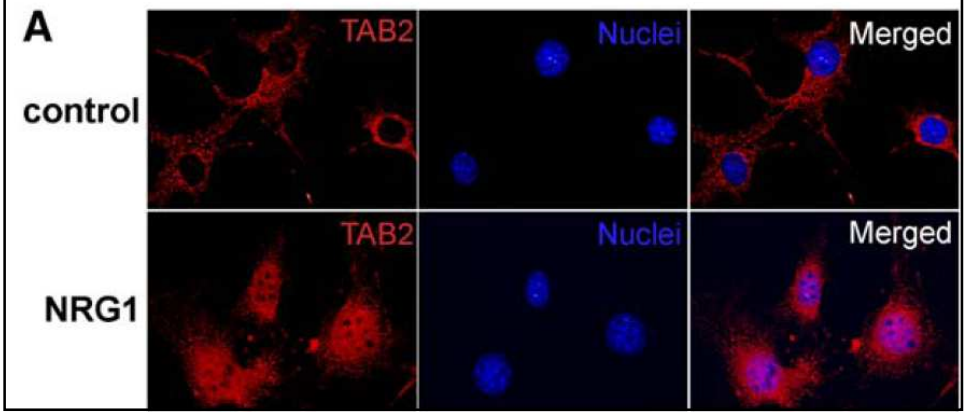
Phosphotyrosine western blot and ^{32}P incorporation assays in cells expressing ErbB4 did not demonstrate induction of TAB2 phosphorylation by NRG1 (data not shown).

→ It appears that ErbB4 activation does not induce TAB2 phosphorylation.

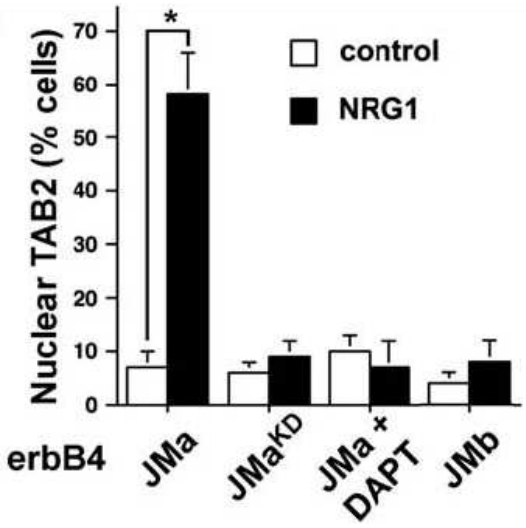
6

Presenilin-Dependent Cleavage of ErbB4 promotes TAB2 Nuclear Translocation

NRG1-ErbB4 signaling had dramatic effects on the cellular distribution of TAB2:
- in quiescent NIH 3T3 cells, TAB2 was excluded from the nuclei independently of whether the cells expressed ErbB4 or not.
- upon treatment with NRG1 for 2 hours, TAB2 translocated to the nucleus in cells expressing ErbB4-JMa.



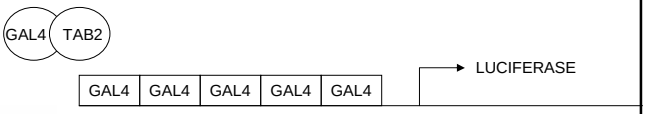
Does NRG1-induced TAB2 nuclear translocation depend on ErbB4 activation and cleavage?

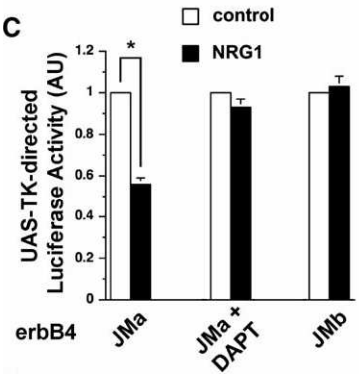


Whereas treatment with NRG1 for 2 hr induces a dramatic increase in TAB2 nuclear localization in cells expressing ErbB4 JMa, this translocation does not occur if cells are exposed to the presenilin inhibitor DAPT 30 min before NRG1 stimulation or if cells express the cleavage-resistant isoform ErbB4 JMb or ErbB4 JMa^{KD}

8

Could TAB2 be mediating potential effects of E4ICD on gene expression?





They tested this using a GAL4/TAB2 fusion protein and a 5x(GAL4)-TK-luciferase reporter (UAS-TK). The basal activity of this reporter in unstimulated cells is moderate, but it can be either increased or decreased by transcriptional activators or repressors.

Treating cells expressing ErbB4 JMa and GAL4/TAB2 with NRG1 significantly reduced 5x(GAL4)-TK-directed luciferase activity. NRG1 had no effects on 5x(GAL4)-TK-directed luciferase activity when cells expressed ErbB4 JMb or when the ErbB4 JMa-expressing cells were exposed to DAPT.

→ NRG1-mediated ErbB4 activation and cleavage induces nuclear translocation of TAB2 and raised the possibility that an E4ICD/TAB2 complex represses transcription.

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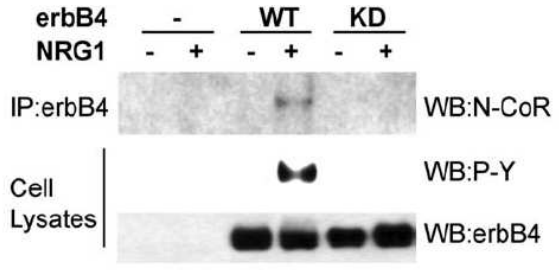
TAB2 can form a complex with the transcriptional corepressor N-CoR

Does N-CoR also interacts with ErbB4?

Cells were transfected with full-length ErbB4 or ErbB4KD, treated with NRG1, and immunoprecipitated with ErbB4 antibodies.
As with TAB2, only wild-type ErbB4 coimmunoprecipitated with endogenous N-CoR, and this occurred only when the cells were stimulated with NRG1.



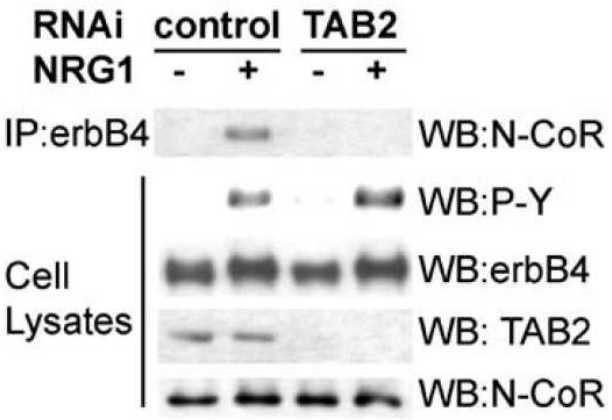
Cosa manca a questa IP?

No tyrosine phosphorylation of N-CoR was detected when ErbB4 was activated (data not shown).

10

→ NRG1 induces the formation of an E4ICD/TAB2/N-CoR complex

Which is the role of TAB2 in the interaction between E4ICD and N-CoR?
Could ErbB4 bind to N-CoR in the absence of TAB2?



Cosa manca a questa IP?

Lack of TAB2 expression (using RNAi) abolished the NRG1-dependent E4ICD/N-CoR association

→ suggesting that TAB2 forms the bridge between E4ICD and N-CoR

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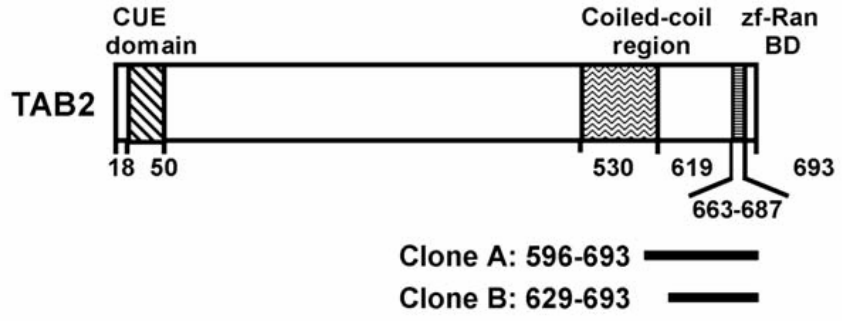
Which domain of TAB2 is involved in the interaction with E4ICD?

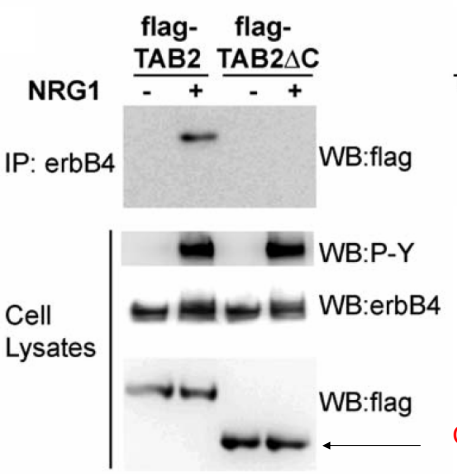


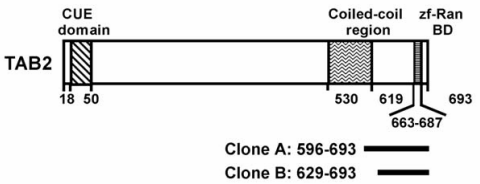
Which methods can you use to obtain deleted constructs (ΔN or ΔC)?

12

Which domain of TAB2 is involved in the interaction with E4ICD?

Truncated versions of TAB2 were used to define the specific domains within this protein responsible for its ability to link E4ICD to N-CoR.
As expected from the portion of TAB2 that binds to E4ICD in the yeast two-hybrid assay, a truncated TAB2 lacking its C-terminal end (TAB2ΔC) failed to coprecipitate with activated ErbB4.





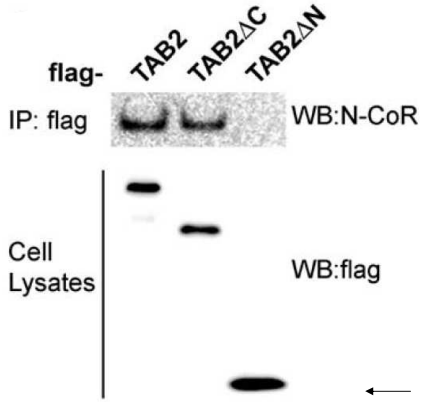
the C-terminal 64 amino acids of TAB2 are not only sufficient but also necessary for ErbB4/TAB2 interaction

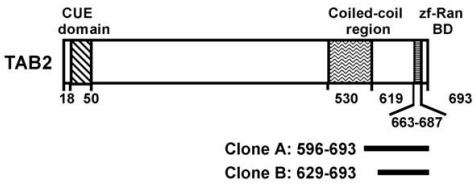
Cosa rappresentano queste bande **?**

13

Which domain of TAB2 is involved in the interaction with N-CoR?

TAB2 binds constitutively to N-CoR.
N-CoR physically interacts with TAB2ΔC but not with TAB2ΔN, the C-terminal E4ICD-interaction domain of TAB2.



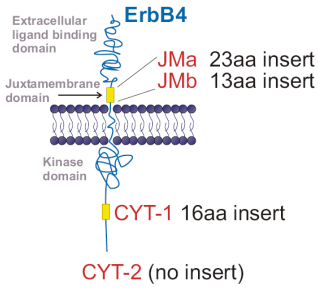


Cosa rappresentano queste bande?
Quali info mancano all'IP e al wb dei lisati cellulari?

→ TAB2 is a bifunctional molecule with nonoverlapping binding sites for ErbB4 and N-CoR and it provides the critical link for the formation of the ErbB4/TAB2/N-CoR complex.

14

Could E4ICD/TAB2/N-CoR complex be of biological significance for Primary Neuronal precursors in the central nervous system?

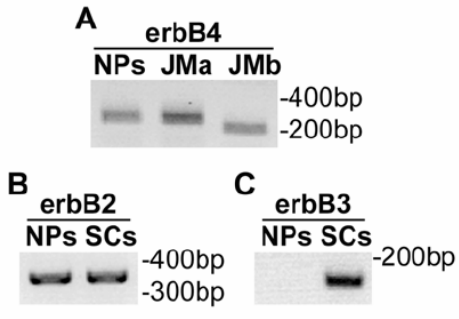


Come potete identificare quali isoforme di ErbB4 sono espresse dai precursori neuronali primari?

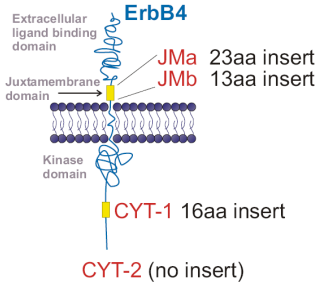
SC=Schwann cells (controllo positivo)

Quale tecnica è stata utilizzata per testare l'espressione degli ErbB ed identificare le isoforme di ErbB4?

Quali dati hanno ottenuto?

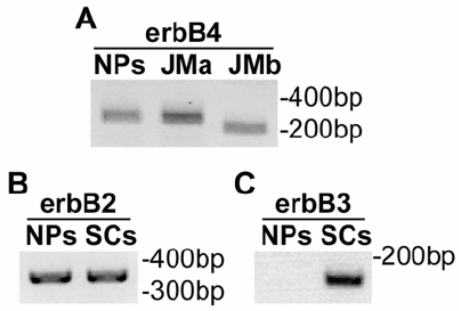


Could E4ICD/TAB2/N-CoR complex be of biological significance for Primary Neuronal precursors in the central nervous system?

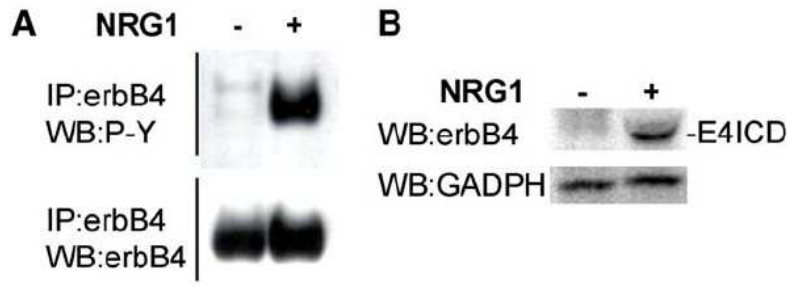


Primary Neuronal precursors (NPs) obtained from E14.5 rat cortices express only the ErbB4 JMa isoform (and ErbB2).

Since ErbB2 does not bind to NRG1, any response of NPs to NRG1 would require ErbB4, acting as either a homodimer or an ErbB4/2 heterodimer.



In Primary Neuronal precursors ErbB4 is readily activated by NRG1, leading to its cleavage and release of the 80 kDa E4ICD.

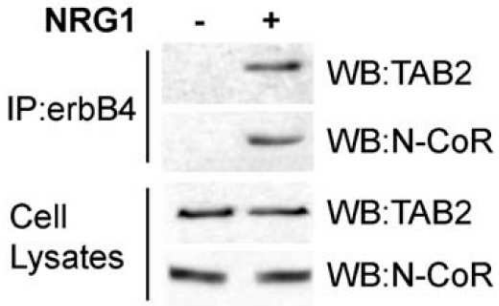


Perché corrono due gel diversi per studiare la banda alta e la banda bassa di ErbB4?
Non si potrebbero osservare in uno stesso gel?

17

Does ErbB4 endogenous to Primary Neuronal precursors interact with TAB2 and N-CoR?

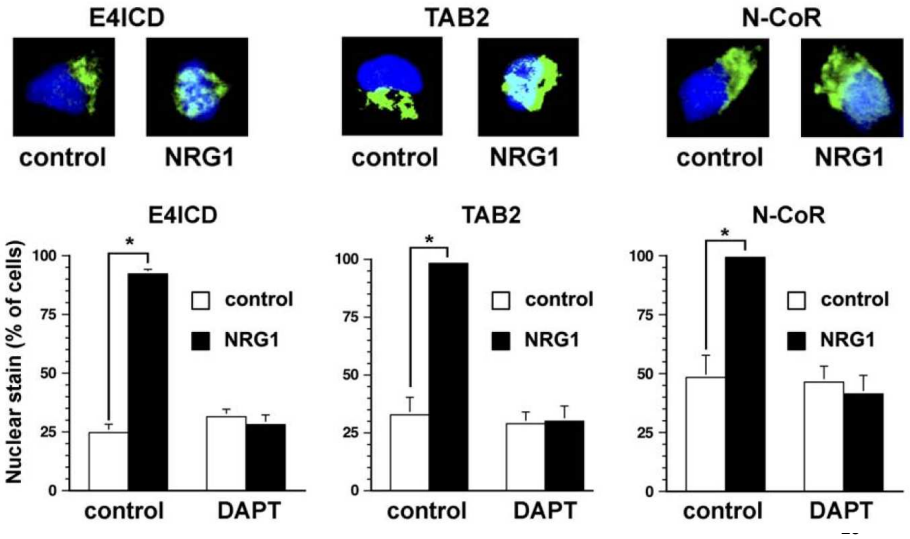
Immunoprecipitation assays show that NRG1 induces ErbB4 JMa association with both endogenous TAB2 and N-CoR.



(Cosa manca a questa IP?)

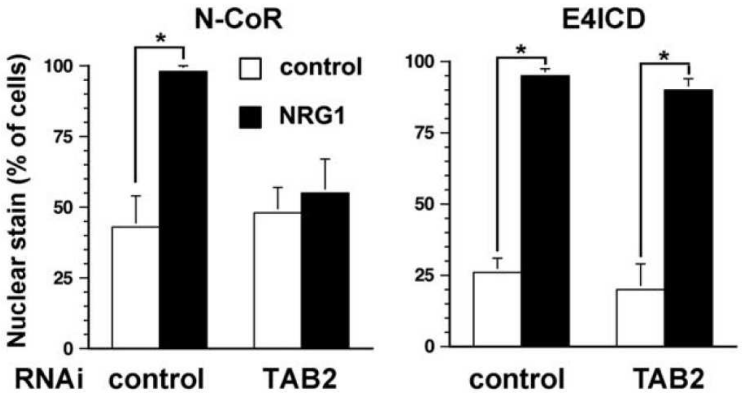
18

Immunostaining showed that NRG1 promotes nuclear translocation of E4ICD, TAB2, and NCoR in virtually all Primary Neuronal precursors and that this depends on presenilin activity



Quale differenza osservo fra E4ICD, TAB2 e N-CoR?

Lentivirus-mediated RNAi knockdown of TAB2 abolished the NRG1-induced nuclear translocation of N-CoR, whereas E4ICD nuclear translocation was not affected.



→ E4ICD might be responsible for nuclear shuttling of the E4ICD/TAB2/N-CoR complex in NPs.

20

Could NRG1 stimulation of ErbB4 JMa nuclear signaling regulate aspects of Primary Neuronal precursors biology?

Multipotent Primary Neuronal precursors can be isolated from embryonic brains and maintained in culture in a proliferative undifferentiated state or can be induced to adopt astrocytic or neuronal fates by extracellular signaling molecules.

ciliary neurotrophic factor (CNTF) -> astrocytic differentiation (GFAP+)

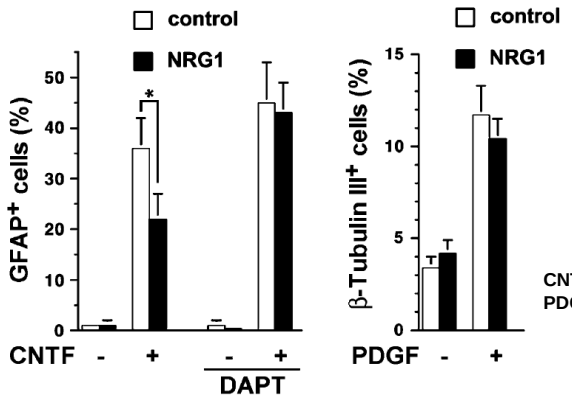
platelet-derived growth factor (PDGF) -> neuronal differentiation (β-tubulin III+)

NRG1 stimulation of Primary Neuronal precursors did not induce the acquisition of either neuronal or astrocytic fates, and did not modify survival or proliferation.

21

NRG1 antagonized the effects of CNTF on astrogenesis without altering the ability of PDGF to induce Primary Neuronal precursors to adopt a neuronal fate.

The presenilin inhibitor DAPT blocked the effect of NRG1 on CNTF-induced astrogenesis indicating that cleavage of the ErbB4 JMa receptor is required for this inhibition of astrogenesis.



Su 200 cellule trattate con CNTF quante differenziano in senso astrocitario?

Su 200 cellule trattate con PDGF quante differenziano in senso neuronale?

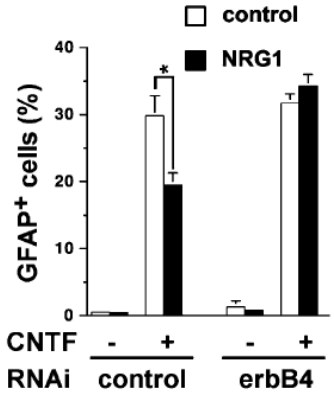
CNTF -> astrocytic differentiation (GFAP+)
PDGF -> neuronal differentiation (β-tubulin III+)

Activation and cleavage of ErbB4 JMa after NRG1 stimulation might contribute to maintenance of the Primary Neuronal precursors pool in a neurogenic state by preventing their differentiation into astrocytes.

22

Is ErbB4 the NRG1 receptor implicated in this differentiation effect?

ErbB4 expression in Primary Neuronal precursors was eliminated using lentivirus mediated RNAi knockdown. Infection with control lentivirus did not alter the number of GFAP-positive astrocytes found in untreated, NRG1-treated, and/or CNTF-treated cultures.
Knockdown of ErbB4 completely abolished the ability of NRG1 to antagonize the CNTF-induced astrogenesis, indicating that this receptor is essential for the NRG1-mediated effect.



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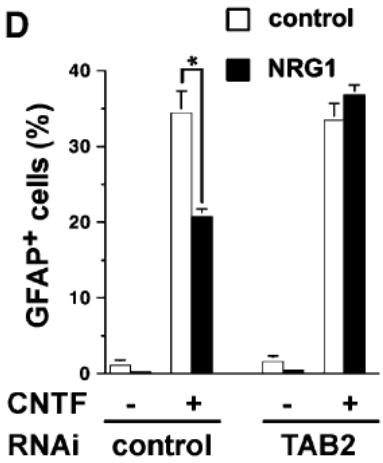
Similar experiments carried out with TAB2 RNAi produced identical results showing that TAB2 is required for the inhibition of the NRG1-dependent inhibition of astrocyte differentiation.



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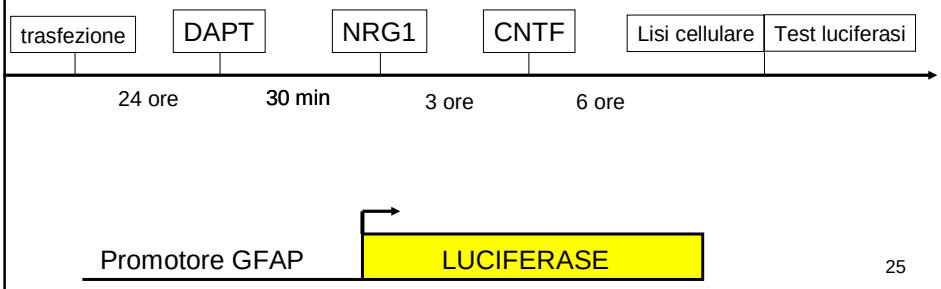
Does E4ICD Nuclear Signaling Inhibit Astrogenesis through Transcriptional Repression of Astrocytic Genes?

1. Primary Neuronal precursors were cotransfected with GFAP-luciferase
The next day, cells were treated with NRG1, CNTF, and/or DAPT:
2. DAPT was added 30 min prior to NRG1
3. NRG1 was added 3 hr prior to CNTF
4. Six hours after CNTF addition, Primary Neuronal precursors were lysed and luciferase activity was measured



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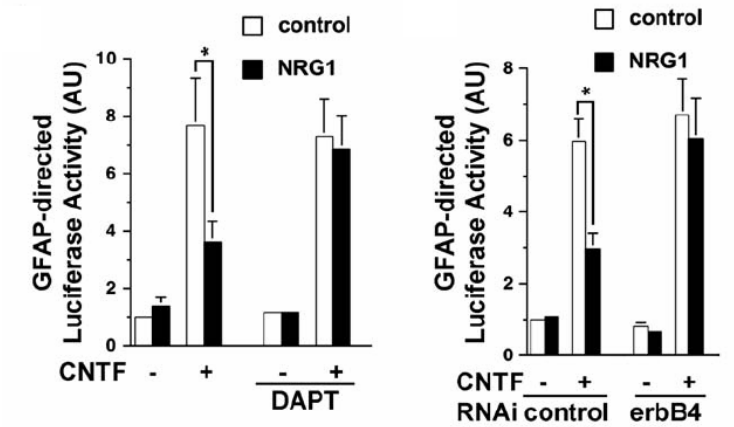
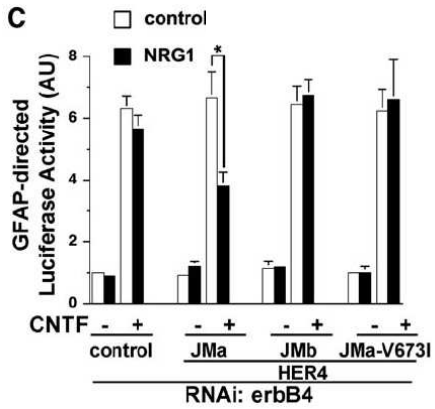


NRG1 had no effect on GFAP promoter basal activity in a luciferase reporter assay in Primary Neuronal precursors, but it significantly reduced the effects of CNTF on this promoter activity.
This antagonistic effect of NRG1 was blocked by the presenilin inhibitor DAPT, expression of dominant-negative forms of presenilin, and knockdown of ErbB4.

Importantly, NRG1 had similar effects on S100β, another astrocyte protein.[26]

Can ErbB4 variants with diverse sensitivities to proteases rescue the effects of NRG1 after RNAi knockdown of endogenous ErbB4?



Cos'è il "rescue"?

Senza rescue, a cosa potrebbe essere imputabile il fenotipo che osservo?

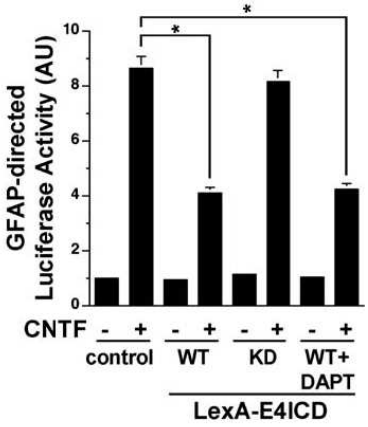
Per quale motivo ErbB4 JMa e JMb trasfettato esogenamente è resistente all'azione dei siRNA? Perché il suo RNA non viene degradato?

ErbB4 endogenous to NPs was knocked down by RNAi. After 3 days, the human ErbB4 juxtamembrane isoforms (HER4 JMa or JMb) or the presenilin-resistant HER4 JMa V673I, which are resistant to the RNAi, were transfected along with the reporters.

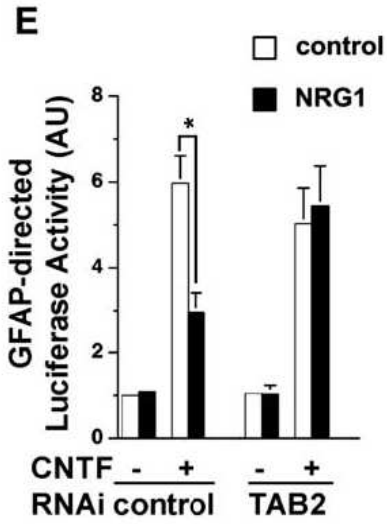
Only the cleavable isoform HER4 JMa rescues the ErbB4 knockdown phenotype.[27]

Is E4ICD activity sufficient to promote the GFAP transcriptional repression?

LexA-E4ICD fusion, when expressed in mammalian cells, dimerizes, becomes autophosphorylated, and interacts with TAB2.
Expression of LexA-E4ICD in NPs had no effect on the activity of the GFAP promoter, but it antagonized the CNTF-mediated GFAP activation. This effect of LexA-E4ICD was abolished when E4ICD was rendered kinase dead but was not affected by the presenilin blocker.



Similar to ErbB4, knockdown of TAB2 eliminated the antagonistic effect of NRG1 on GFAP expression.



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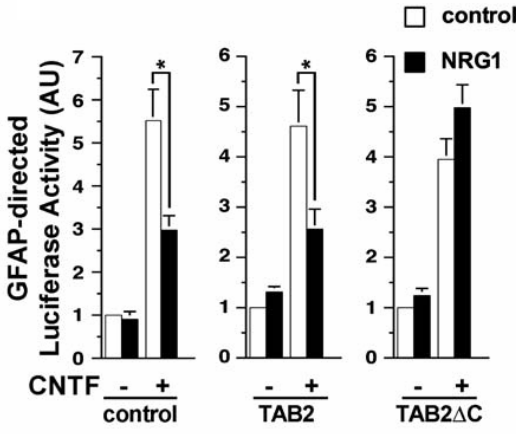
Overexpression of TAB2ΔC, the truncated TAB2 that binds to N-CoR but not to ErbB4, also blocked the antagonistic effect of NRG1 on CNTF-mediated transcriptional activation of GFAP, whereas full-length TAB2 did not.



→ TAB2ΔC acts as a dominant-negative molecule by preventing formation of the complex.

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These results

❊ indicate that repression of the GFAP promoter by ErbB4 nuclear signaling requires the presence of TAB2, which brings together E4ICD and N-CoR.

❊ suggested that E4ICD could be part of the transcriptional repressor complex that mediates the NRG1 inhibition of GFAP and S100β expression.

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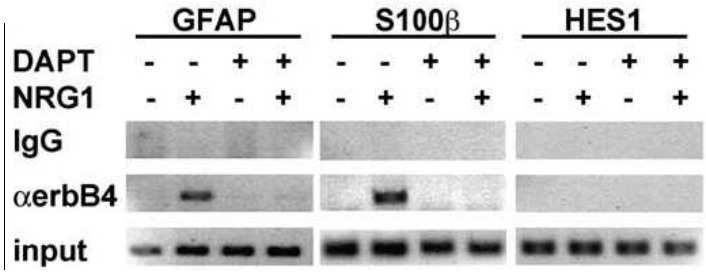
To test this possibility, they used a chromatin immunoprecipitation (ChIP) assay. Immunoprecipitation with ErbB4 antibodies showed that E4ICD associates with the GFAP and S100β promoters in NPs, but only after treatment with NRG1. Moreover, these associations were blocked by presenilin inhibition.



The association of E4ICD with the glial promoters was specific since normal rabbit immunoglobulin G (IgG) failed to immunoprecipitate these promoters and ErbB4 antibodies did not precipitate a control promoter, HES1.

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Is ErbB4 Necessary for the Correct Timing of Astrogenesis *In Vivo*?

The *in vitro* studies suggested that presenilin-dependent ErbB4/TAB2/N-CoR nuclear signaling may regulate astrogenesis *in vivo*.

To test this possibility, they analyzed the expression of the astrocytic markers GFAP and S100β in the cortex of ErbB4 knockout embryos.

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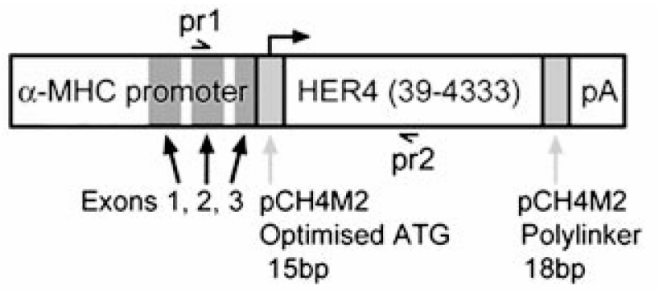
to study knockout embryos they used a line of mutant mice that is rescued from early embryonic lethality by reintroduction of ErbB4 in the heart.



ErbB4 $^{-/-}$ HER4^{heart} ($^{-/-\ ht+}$)

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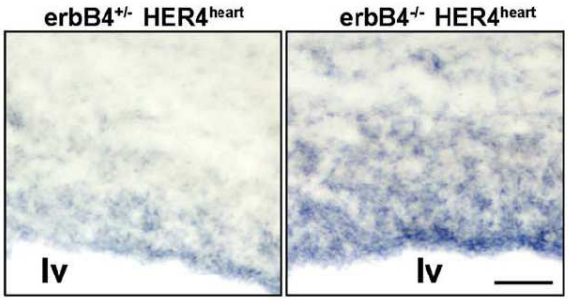
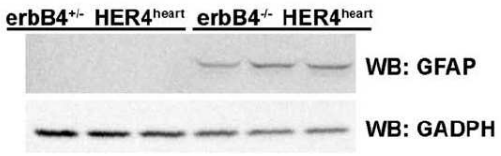
At E17.5, a time at which astrogenesis is just beginning, the levels of GFAP mRNA and protein were dramatically elevated in the cortical neurogenic layers of the ErbB4 $^{-/-}$ HER4^{heart} mice compared to their control littermates (ErbB4$^{+/-}$ HER4^{heart}).



in situ hybridization

lv:lateral ventricle



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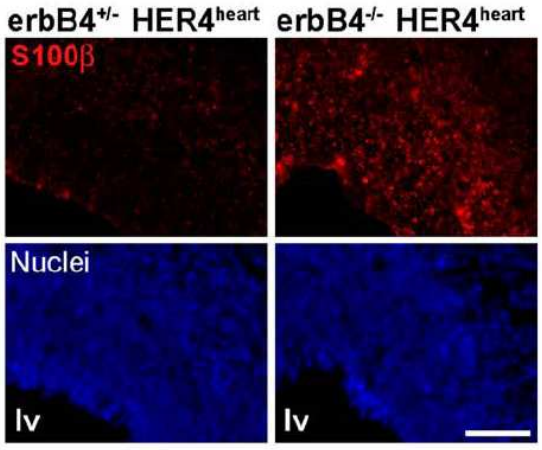
Furthermore, expression of S100β was also increased in the cortex of ErbB4-/- HER4^{heart} mice.



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❊ The results described above clearly indicated that ErbB4 signaling plays a critical role in controlling the onset of astrogenesis *in vivo* but did not provide insights into the importance of ErbB4 cleavage in this process.

❊ To investigate this, they tested whether the alterations in GFAP expression in the ErbB4-/- mice could be rescued by re-expression of the different ErbB4 isoforms using *in utero* electroporation.

❊ cDNAs encoding either HER4 JMa or HER4 JMb were transfected into the cortices of E13.5 ErbB4$^{-/-}$ HER4^{heart} mice together with a GFP expression plasmid.

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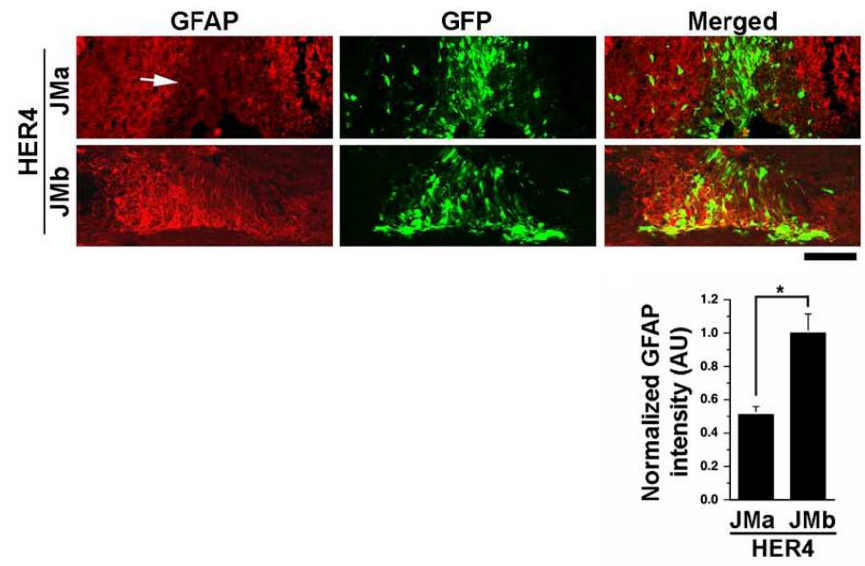
When embryos were dissected immediately after transfection and slices of the forebrains incubated for 4 days, expression of cleavage-sensitive HER4 JMa, but not cleavage-resistant HER4 JMb, significantly reduced the GFAP expression levels.



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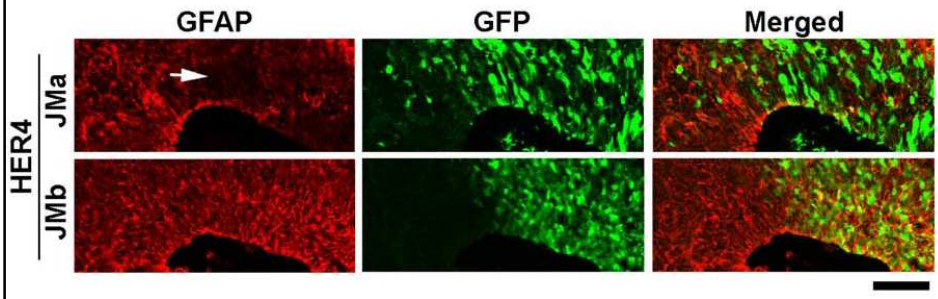
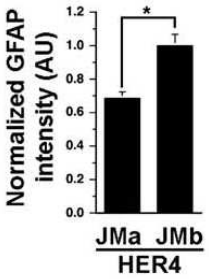
Identical results were obtained when the embryos were allowed to develop in utero until E17.5 and then analyzed histologically.



These results indicate that canonical RTK signaling by ErbB4 is not sufficient for normal control of GFAP expression in the developing cortex and that ErbB4 cleavage is necessary to prevent precocious astrogenesis.



❊ to determine whether ErbB4 nuclear signaling is sufficient to repress astrogenesis *in vivo*, they tested whether expression of E4ICD would reduce GFAP expression in wild-type mice.

❊ plasmids encoding either active or kinase-dead LexA-E4ICD were transfected into the brains of E13.5 wild-type embryos together with a GFP expression plasmid.

❊ after electroporation, the embryos were allowed to continue developing in utero until E18.5.

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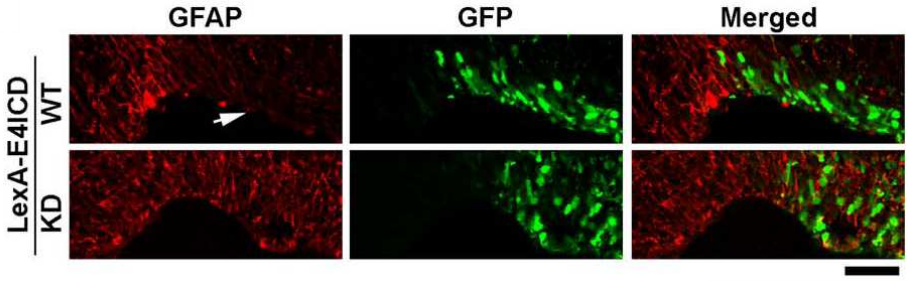
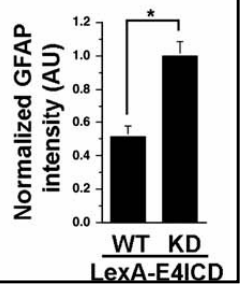
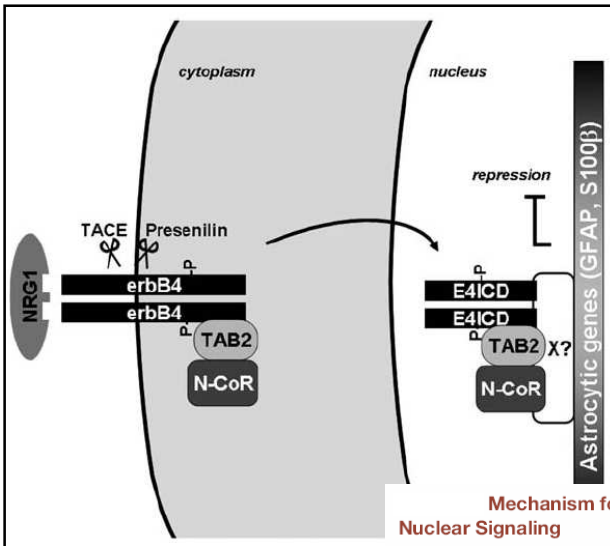
Similar to what observed *in vitro,* expression of wild-type LexA-E4ICD significantly reduced the levels of GFAP compared to untransfected areas, whereas kinase-dead LexA-E4ICD did not.



→ These results show that E4ICD is necessary and sufficient to regulate astrogenesis in the intact developing cortex.

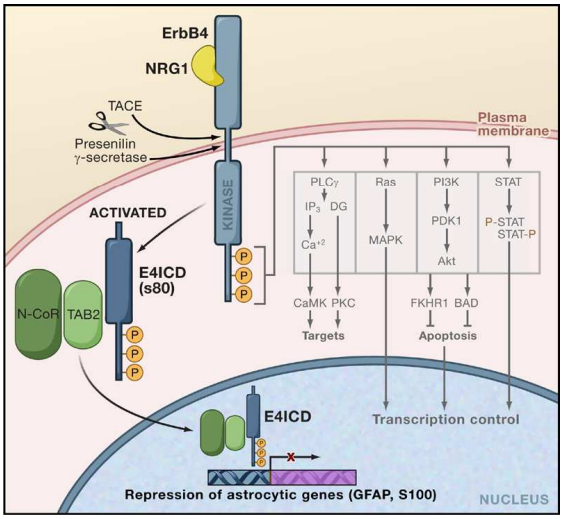




Mechanism for the Regulation of NP Fate by ErbB4 Nuclear Signaling

ErbB4 activation by NRG1 promotes receptor phosphorylation and its association with TAB2 and N-CoR. Ligand-induced ErbB4 cleavage by TACE and presenilin allows for the nuclear translocation of the E4ICD/TAB2/N-CoR complex. This complex binds to specific promoters (*GFAP* and *S100*β), leading to their transcriptional repression and preventing their activation by factors that induce astrocyte differentiation, such as CNTF.

In this paper authors show that NRG1-induced presenilin-dependent ErbB4 nuclear signaling regulates the timing of astrogenesis in the developing brain. Upon activation and presenilin-dependent cleavage of ErbB4, E4ICD forms a complex with the signaling protein TAB2 and the corepressor N-CoR.



This complex translocates to the nucleus of undifferentiated neural precursors and inhibits their differentiation into astrocytes by repressing the transcription of glial genes.

Consistent with this observation, cortical astrogenesis occurs precociously in ErbB4 knockout embryos, a phenotype that is rescued by re-expression of human ErbB4 JMa but not by the uncleavable ErbB4 JMb.

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❊ This is a novel mechanism by which an RTK signals directly to the nucleus to regulate transcription and influence cell fate choices of NPs.
During embryonic development, cortical NPs first generate neurons and then produce, or themselves become, astrocytes.

❊ The signaling mechanisms regulating the timing of these fate choices are not well defined:
- it is unclear whether astrogenesis occurs later than neurogenesis because factors that induce astrocyte formation are produced after those inducing neurogenesis or
- because factors that inhibit astrogenesis are present at early stages of brain formation.

❊ These results support the latter possibility, indicating that, during the early stages of brain development, NPs are exposed simultaneously to extracellular signals that induce neuronal and astrocyte production but that neurogenesis is favored by presenilin-dependent ErbB4 nuclear signaling that antagonizes the actions of astrogenesis-promoting signals.

❊ At later stages, reduction in ErbB4 signaling, most likely due to reduction in the levels of ErbB4 expression by the NPs, would favor the generation of astrocytes.

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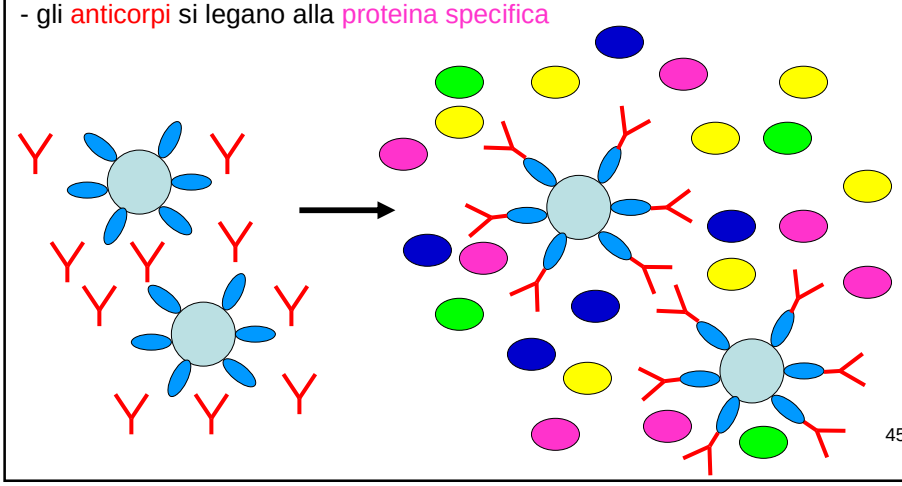
IMMUNOPRECIPITAZIONE

• cellule in coltura o tessuto
• estratto proteine totali
• mescolo estratto proteico + anticorpo + sferette di sepharose-protein A nella stessa provetta
 - la proteina A (legata alle sferette di sepharose) si lega agli anticorpi,
 - gli anticorpi si legano alla proteina specifica



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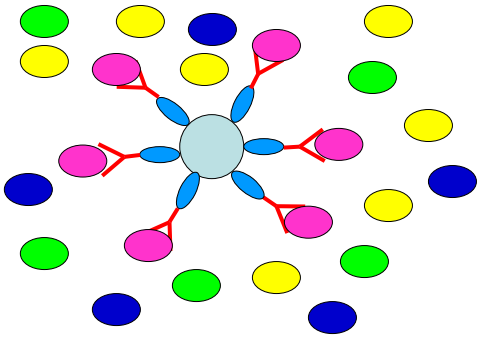
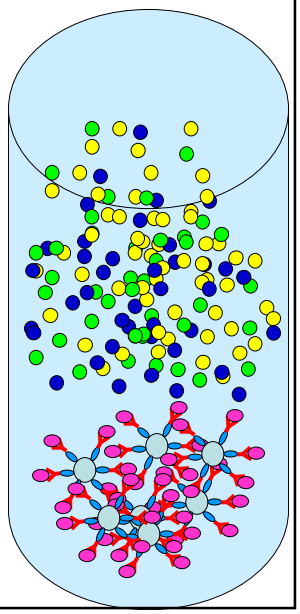
• metto in agitazione (rotazione) a 4℃ 2h o O/N aff inché gli anticorpi vadano in contatto con tutte le proteine presenti nell'estratto





• centrifugo a bassa velocità (3000 rpm, 1 min, 4℃)

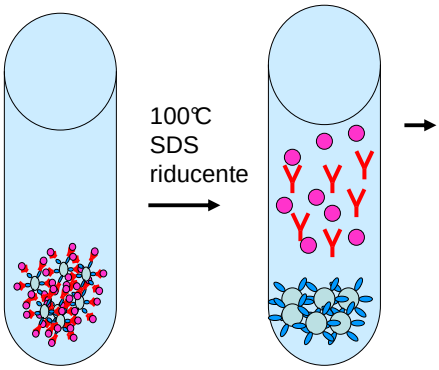
sul fondo della provetta si depositano:

sferette di sepharose-proteina A-anticorpo-proteina

in soluzione restano:

proteine non legate dall'anticorpo

• scarto il **surnatante**
• aggiungo SDS (che denatura le proteine), beta-mercapto-etanolo (che riduce i ponti disolfuro) e porto 5' a 100℃
→ le sferette di sepharose-proteina A si staccano dall'anticorpo che si stacca dalla proteina
• le sferette di sepharose-proteina A si depositano sul fondo
• in soluzione restano l'anticorpo e la proteina (più eventuali proteine legate alla proteina specifica)



• la soluzione contenente l'anticorpo e la proteina viene caricata su gel di polyacrylamide (SDS-PAGE)

• il gel viene fatto correre e poi viene trasferito su membrana di nitrocellulosa

• western blotting

(se prima di bollire le proteine è stato fatto un saggio chinasico utilizzando ATP radioattivo, sul gel verrà esposta una lastra radiografica)

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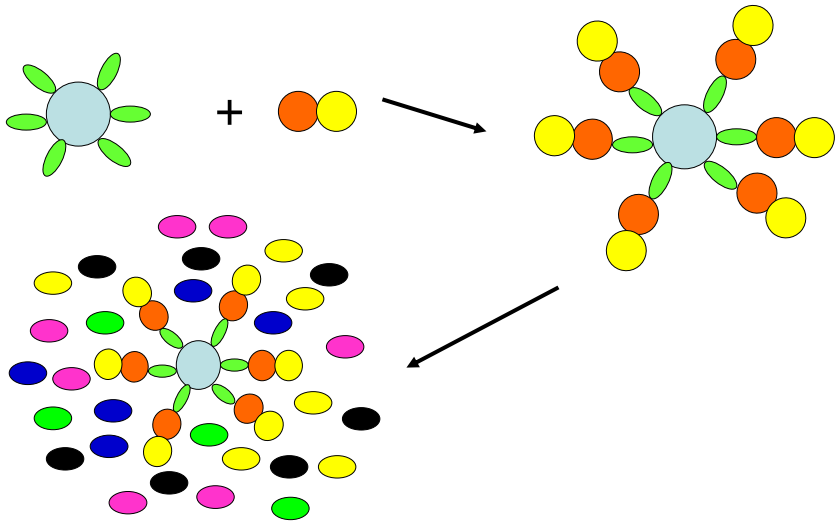
PULL-DOWN

sferette di sepharose-glutatione

GLUTATIONE TRANSFERASI **PROTEINA ESCA**



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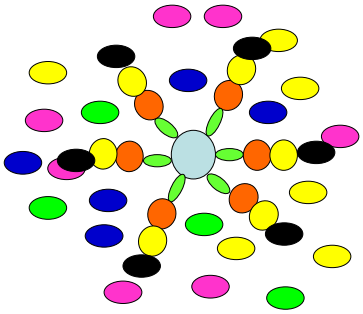
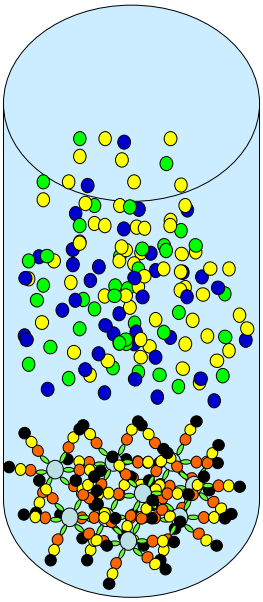
• metto in agitazione (rotazione) a 4℃ 2h o O/N aff inché la proteina “esca”, (fusa con la glutatione transferasi) vada in contatto con tutte le proteine presenti nell’estratto





• centrifugo a bassa velocità (3000 rpm, 1 min, 4℃)

- sul fondo della provetta si depositano:sferette di **sepharose-glutatione--glutationetransferasi-proteina esca**

- in soluzione restano:
proteine non legate alla proteina “esca”

• procedo come per l’immunoprecipitazione