Notch and β1-integrin interactions in neural stem cells

**Notch, EGFR and β1-integrin pathways are coordinated in Neural Stem Cells**

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**Summary**

Notch1 and β1-integrins are cell surface receptors involved in the recognition of the niche that surrounds stem cells through cell-cell and cell-extra cellular matrix interactions, respectively. Notch1 is also involved in the control of cell fate choices in the developing central nervous system [1]. Here we report that Notch and β1-integrins are co-expressed and that these proteins co-operate with the epidermal growth factor receptor in neural progenitors. We describe data that suggest that β1-integrins may affect Notch signalling through 1) physical interaction (sequestration) of the notch intra cellular domain fragment by the cytoplasmic tail of the β1-integrin 2) by affecting trafficking of the notch intra cellular domain via caveolin mediated mechanisms. Our findings suggest that caveolin1-containing lipid rafts play a role in the coordination and coupling of β1-integrin, Notch1 and tyrosine kynase receptor signalling pathways. We speculate that this will require the presence of the adequate β1-activating extra cellular matrix or growth factors in restricted regions of the central nervous system, and namely in neurogenic niches.

**Introduction**

Integrins and ECM (extra cellular matrix) molecules play crucial roles during embryogenesis [2] in mesoderm development, epithelial morphogenesis, neural tube closure, anchorage to the ECM basal lamina and central nervous system (CNS) development. In particular, β1-integrins (α6β1) are highly expressed in embryonic stem (ES) cells [3] and in neural stem cells (NSC) [4] and are required for cortical development [5]. Notch1 is a cell surface protein involved in the control of cell fate choices in the developing CNS [1]. This transmembrane receptor is involved in stem cell maintenance [6] and promotes glial and neural fates in a stepwise manner, first by inhibiting neuronal fate and promoting glial fate and secondly by promoting astrocyte differentiation [7]. Notch1 also plays a role in the control of neurite extension in mammalian cells and in axon growth in *Drosophila* [8-10]. Interestingly, in the immune system Notch1 serves two biologically contrasting functions: it is responsible for the apoptotic cell death of B lymphocytes [11] while it promotes the survival of T cells [12]. The diverse effects of Notch1 activation observed in multiple cell types and at different stages of development suggest the presence of context-dependent control.
mechanisms. Growth factors (GF) and ECM molecules (acting through integrins) belong to the complex environment that surrounds NSC during development [13] and that affect Notch signalling. For example, FGF1 and FGF2 inhibit neural differentiation by affecting (directly or indirectly) the Notch pathway [14] and EGFR activation leads to Notch signalling during pancreas tumorigenesis [15]. Integrins may also be involved in the Notch response during angiogenesis, when Notch4-expressing endothelial cells display β1-integrin in an active, high affinity conformation [16]. Furthermore, in Zebrafish the boundary cells between developing somites behave differently depending on the levels of Notch activation and it has been suggested that the extra cellular matrix (which differs at the rhombomere boundaries) plays a role in this process [17]. Nevertheless, the coordination between β1-integrin, Notch1 and GF pathways is poorly understood. 

Lipid rafts are special membrane regions that affect signalling by sorting proteins and lipids into specific membrane domains, where privileged interactions occur. Caveolae are specialized lipid rafts which contain cholesterol, sphingolipids and caveolins, (22-24Kda membrane proteins, required for the formation of the caveolae) and that serve as scaffolds for signalling molecules. In this manuscript we explore how some of the receptors for ECM and GF (that are present on the surface of the NSC) act together with the Notch1 pathway to control the NSC responses to changes in the micro environment. We discuss the possibility that lipid rafts may play important roles in directing the changes in signalling and the responses to environmental changes that occur during cortical development, and may act as integrators of parallel and simultaneous signals originated from integrins, growth factors and Notch receptors. We conclude that the GF and ECM composition of biological neural stem cell “niches” may affect NSC maintenance and differentiation by affecting Notch signalling, in a context- and time-dependent manner.

**Experimental procedures**

**Reagents and antibodies**

FGF-2 was obtained from PeProtech, EGF from Calbiochem and B27 supplement from Gibco life technologies. Antibodies for immunoprecipitation blocking experiments and western blots were obtained from Chemicon and pharmingen (anti-β1 integrins), Santa Cruz (EGFR), Upstate Biotechnology (EGFR), Cell Signalling Technology (phosphorylated MAPK, MAPK). Antibodies used for immunohistochemistry included polyclonal Notch1 and caveolin1 (Santa Cruz), monoclonal anti-Nestin (Pharmingen) and monoclonal anti-βIII tubulin (Sigma). All fluorescent secondary antibodies were obtained from Jackson Immunochemicals or Molecular Probes. The EGF receptor inhibitor AG1478, (Calbiochem) was used at 20 µM. Cocktails of protease and phosphatase inhibitors were from Calbiochem. Remaining products were from Sigma, if not otherwise specified.

**Primary neurosphere culture and preparation of ESC-derived NSC**

Primary cultures were prepared from newborn and embryonic day 14.5 (E14.5) C57BL/6 mice (postnatal day P0–P2), as previously described [18]. Briefly, spheres of neural precursors were grown in EGF or FGF-2 (20 ng/ml) from dissociated postnatal day 0-2 mouse forebrain in DMEM/Hams-F12 supplemented with B27[19,20].
The culture media was changed every 3 days.
To prepare ES cell-derived NSC we used a similar approach to the one developed by Bibel et al [21]. Briefly, we generated embryoid bodies (EB) that were exposed to retinoic acid (RA) for 4 days (as described by [22]). Taking into account that a switch in growth factor requirements (from FGF2 to EGF) occurs in vivo during mid-neurogenesis [23,24] we sequentially exposed the RA-primed EBs to FGF2 first and to a mix of FGF2 and EGF secondly, in order to simulate the changes in the GF micro-environment that occur during embryonic CNS development.

Secondary neurosphere formation assays
Intact primary neurospheres maintained 8 to 10 days in vitro (8-10 DIV) were mechanically dissociated and the same number of cells for each condition was plated at low density (5000 cells in 1.5ml, < 5 cells/µl) and grown for 10 days in 20 ng/ml of EGF and FGF2. The number of secondary neurospheres formed was counted and statistically significant differences between groups were calculated using Student t-tests.

Secondary neurosphere formation assays after Morpholino treatment or EGFR inhibition
Intact neurospheres (8-10 DIV) were exposed to β1 antisense morpholinos obtained from GeneTools, following the manufacturer protocols (www.gene-tools.com). Control groups were exposed to a mis-sense morpholino with a random sequence. The spheres were then mechanically dissociated and the same number of cells for each condition were plated at low density (3 to 4 cells/µl) and grown for 10 days in different growth factor concentrations (20 ng/ml or 2 ng/ml of either EGF or FGF2), as described above. The number of secondary neurospheres formed was then counted and statistically significant differences between groups were calculated using Student t-tests. Secondary neurosphere formation assays were also done in the presence of an EGFR inhibitor (AG1478, 20 µM) and these experiments were analysed as described above.

Immunohistochemistry
Neurospheres, ES cell-derived NSC, neonatal or embryonic brain tissue were fixed in 2-4% paraformaldehyde in phosphate buffered saline (PBS). Tissue samples were cryo-protected in 25% sucrose and sectioned (14 µm) prior to immunohistochemistry, except for cell monolayers. The samples were blocked in PBS (0.1% Triton X-100) containing normal blocking serum and incubated overnight with the appropriate antibodies at 4°C, followed by incubation with the secondary antibodies and counter-staining with DAPI or sytox green. Pictures were acquired using a Zeiss Axioplan 2 fluorescence microscope and Smartcapture 2 software.

GST-NICD construct preparation
Briefly, the NICD fragment was subcloned from the plasmid plabe-NICD (kind gift from G. Weinmaster) into the pGEX expression vector to prepare a GST-NICD fusion protein. The fusion protein was produced in E.coli, linked to agarose beads (Pharmacia) and purified using standard protocols. Pull-down assays were performed by incubating total lysates obtained from neurospheres with the GST-NICD or GST-alone beads, for two hours at room temperature. The beads were then collected, washed and boiled and the resulting supernatant was analysed by
SDS-PAGE and immunoblotting, as follows.

**Western blots and immunoprecipitations**

For western blotting, neurospheres or ESC-derived NSC were lysed (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl buffer, 1% Triton X-100) in the presence of protease and phosphatase inhibitors (5 µg/ml leupeptin, 2 µg/ml aprotinin, 2 mM PMSF, 1 µg/ml pepstatin, 2 mM Sodium Fluoride, 2 mM Sodium Vanadate, all from Sigma) or the equivalent Calbiochem cocktails. The supernatant was clarified by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentrations were determined with a Bio-rad protein assay with BSA as a standard and equal amounts of protein were loaded in each well. Proteins were separated by SDS-PAGE and electroblotted onto nitrocellulose membranes (Hybond-C, Amersham). Membranes were blocked in 10% non-fat dry milk in Tris-buffered saline (TBS) for 1 hour at room temperature. Blots were then incubated with the primary antibodies overnight at 4°C in milk-TBS containing 0.1% Tween-20 (TBS-T), followed by a 2 hour incubation with the appropriate secondary peroxidase-conjugated antibody (Amersham). Blots were developed using ECL reagents (Amersham), following the manufacturer’s instructions (Amersham). For immunoprecipitations, the samples were lysed as previously described. To remove non-specifically binding proteins 150-200 µg of proteins were pre-cleared by mixing with agarose beads (A/G plus, Santa Cruz) for 30 minutes at 4°C. The samples were then incubated with the adequate antibody in the presence of fresh agarose A/G beads, either at 4°C overnight or at room temperature for two hours on a rotating platform. The beads were then washed and boiled for 10 minutes in Laemli loading buffer. Equal amounts (as measured by protein assay) were loaded on 10% SDS-PAGE gels and processed for immunoblotting as previously described.

**Raft isolation**

Isolation of rafts was performed using sucrose gradients, as previously described [25]. Briefly, neurospheres were placed on ice and suspended for 30 min in 0.2 ml extraction buffer: 50 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, 1% Triton-X100, and a cocktail of protease and phosphatase inhibitors (Calbiochem). Cell lysates were then adjusted to 40% OptiPrep and overlaid with solutions 30 and 10% Optiprep in the extraction buffer. These gradients were centrifuged for 16 hr at 35,000 rpm at 4°C in a SW50.1 rotor (Beckman Instruments). Fractions of equal volume including the raft (floating fraction) and non-raft (bottom fraction) fractions were collected and analysed by SDS-PAGE (10%) followed by immunoblotting. Protein assays were performed on all fractions before immunoblotting, to ensure equal loading.

**RT PCR**

RNA was extracted using the RNAeasy kit (Qiagen) and 0.1 µg of RNA from each sample was used to generate cDNA using the transcriptor first strand cDNA synthesis kit (Roche). RT PCR was done using the published primers for Hes5 and GAPDH and following the conditions described in Zine et al. [26]

**Results**

β1-integrins and Notch1 co-localize in the Ventricular Zone, in Neurospheres and in ES cell-derived NSC
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Stem cells from the skin and prostatic epithelia can be identified and isolated by their high β1-integrins expression levels [27,28]. In the developing brain β1-integrin is expressed in the ventricular zone (VZ) [4] (Fig 1 C) by NSC that are exposed to a changing ECM and, possibly, to variable growth factor levels [13,29]. Likewise, Notch1 plays a role in NSC and is thought to direct radial glial cell (RGC) differentiation [30,31]. Not surprisingly Notch1 is expressed in the same VZ region [32] (Fig 1 B) and, interestingly, it is co-expressed with the β1-integrins (Fig 1 D). Co-expression of Notch1 and β1-integrins is also detectable in neurospheres (Fig 1 E-G), [4,29,33], and in NSC/radial glial cells cultures, derived from ES cells (Fig 1 H-J). The observation that both proteins are simultaneously expressed in neural progenitors and that their expression overlaps raises the hypothesis that they may co-operate or act in a coordinated fashion. To further test the hypothesis that β1-integrins and Notch pathways interact in neural progenitors/NSC we used primary neurospheres and ES cell-derived NSC, the later providing an NSC-enriched population positive for RGC markers (Fig 1 K-L)[21], (currently accepted to be NSC [34]). Notch1 is highly expressed in the ES cell-derived NSC cultures and co-localizes with the β1-integrin (Fig 1 H-J). The ES cell-derived NSC cultures are therefore suitable to study, in vitro, the cooperation between β1-integrin, growth factors and Notch pathways, all of which are known to be crucial for NSC and RGC maintenance and development [4,31,35].

β1-integrin and growth factor receptors are required for secondary neurosphere generation

To test the role of β1-integrin in NSC we treated primary neurospheres with morpholino antisense oligonucleotides against β1-integrin to decrease the β1 sub-unit protein levels in EGF- or FGF2-grown cells, prior to secondary neurosphere formation assays (see material and methods). The decrease in β1-integrin was confirmed by western blot (Fig 2 E). Spheres treated with anti-sense or mis-sense (control) morpholino oligonucleotides were dissociated and tested for their capacity to form new spheres (secondary neurosphere formation assay, see material and methods). These experiments show that a decrease in β1-integrin is associated with a moderate decrease in secondary neurosphere formation (Fig 2 A-D). Interestingly, in EGF-grown (A-B) and FGF2-grown (C-D) cells the decrease in secondary neurosphere formation is more significant at low EGF levels (2 ng/ml, Fig 2 B) than at higher EGF levels (20 ng/ml, Fig 2 A), suggesting that in the presence of high EGF levels the cells are less dependent on β1-integrin to maintain adequate levels of proliferation or survival. After morpholino treatment the decrease in secondary neurosphere formation in FGF2-grown cells (C-D) is already apparent at high levels of FGF2 (20 ng/ml Fig 2 C), when compared to spheres grown with low levels of FGF2 (2 ng/ml, Fig 2 D).

EGFR and β1-integrins interactions have been extensively demonstrated in three-dimensional breast culture systems [36] and it is conceivable that the high levels of EGFR found on the nestin positive FGF2-grown spheres (Fig 2 F, G, H) may be responsible for the more acute response to a decrease in β1-integrin observed in FGF2-grown cells. To test this hypothesis spheres grown in both EGF and FGF2 were used in a secondary neurosphere formation assay in the presence of FGF2 and an EGFR inhibitor, AG1478...
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(20 µM). The exposure to AG1478 resulted in a sharp decrease in the number of secondary neurospheres formed, indicating that even for FGF2-grown spheres the EGFR is the crucial pathway involved in proliferation (Fig 2 J), as indeed suggested by the high levels of EGFR found on the FGF2-grown cells (Fig 2 G and H). Consequently, in FGF2-grown spheres with decreased levels of β1-integrin (EGFR strongly positive/β1 depleted) lack of exposure to EGF will be severely felt (in spite of the high levels of EGFR expression), and can not be compensated by (lacking) integrin activation. In the EGF-grown spheres (EGFR positive/β1 depleted), even low levels of EGFR will be enough to respond to the EGF in the media. These results suggest that β1-integrin may be important for EGFR activation in neurospheres, and point towards a potential cooperation between the two pathways, as already described for epithelial cells and fibroblasts [37,38]. Furthermore it was recently shown that a decrease in β1-integrin causes a reduction in neurosphere size during secondary neurosphere formation assays, due to reduced progenitor proliferation and increased cell death[39]. The loss of β1-integrin in neurospheres also reduces the number of nestin positive cells in a growth factor-dependent manner and this phenotype can be rescued by exposing the cells to high growth factor levels[39]. This result is consistent with our morpholino experiments and both may be explained by the signalling confluence of EGFR and β1-integrins towards the MAPK pathway. In fact, in the absence of β1-integrins, the signalling through the MAPK pathway may become more dependent on the presence of EGF in the media [4]. Interestingly, we have also observed that the addition of EGF and FGF2 to starved neurospheres leads to an increase of the detectable levels of Notch intra cellular domain (NICD) expression by Western blot (Fig 2 I). Other authors have observed that growth factors, such as CNTF, increase NICD expression levels [40] or affect Notch activation [15,41]. Taking into account that β1-integrins modulate the response to GF in neural progenitors [39] we raise the hypothesis that the GF effects on the levels of NICD could be partially dependent or coordinated with the activation of β1-integrins. How the β1-integrins, GF receptors and Notch signalling pathways are coordinated in neural progenitors remains to be elucidated and we suggest that special membrane domains may be involved in the coordination of these pathways interactions, cross-talks and sequential and/or simultaneous effects.

Caveolin1, a lipid raft resident protein, is present on Neurospheres, on ES-derived NSC/RGC, and in the embryonic VZ

Lipid rafts are membrane domains that act as privileged signalling platforms [42], where interactions and cross-talk between different signalling pathways may take place. Recently, lipid rafts were found to be present on neuroepithelial progenitors, where they play a role in signal transduction [43]. We reasoned that the embryonic VZ, the ES-derived NSC and primary neurospheres could also contain lipid rafts. Using western blots and immuno-fluorescence we confirmed the existence of caveolin1 (a component of lipid rafts) on ES-derived NSC, in the embryonic VZ and on neurospheres (Fig 3 A-H).

Notch1 is present on caveolin1 enriched fractions isolated from Neurospheres and ES-derived NSC
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and GF can mobilize NICD out of the caveolin containing fractions
Lipid rafts may compartmentalize and direct signal transduction at the plasma membrane [44-46]. The context-dependent modulation of Notch (or other receptors) may require the simultaneous activation of (or the interaction with) other membrane receptors, like β1-integrins, promoted by specific signalling platforms like the lipid rafts, in a temporally and spatially controlled manner. Rafts can be isolated within detergent-insoluble glycosphingolipid-rich (DIG) microdomains by density gradient centrifugation at 4°C [47]. We examined the distribution of Notch1 in Optiprep density gradient fractions of Triton X-100 extracts prepared at 4°C. We reasoned that changes in the protein levels in rafts (membrane compartments of specific lipid composition that are privileged for receptor interactions and act as signalling platforms [42,48]) are more relevant than the overall changes in expression, and allow for subtle changes to be detected in functional fractions. Lipid rafts are insoluble in Triton X-100, float at the 10-30% interface of the density gradient and we used the presence of caveolin1 to confirm that the raft fraction had been correctly identified. Note that we also observed that Caveolin1 (a raft resident protein) is present on the cells located at the edge of neurospheres (Fig 3 C), in ES derived NSC (Fig 3 F) and in the embryonic VZ (Fig 3 B). To test the effect of growth factors on neural progenitors we starved (overnight) intact EGF- and FGF2-grown neurospheres and then added EGF or FGF2, respectively (20 ng/ml) for 24h. Using intact neurospheres ensured that only the cells located at the edge (enriched in β1-integrins, EGFR and Notch1 [29]) were exposed to the changing environment. The lysates from spheres treated in this manner were centrifuged on percoll gradients and the resulting fractions were analysed for the presence or absence of Notch1 protein in caveolin1-containing membrane compartments, with or without growth factor activation (Fig 4 A-B). When EGF is added to starved EGF-grown neurospheres (Fig 4 A, panel labelled EGF) an increase in NICD is detected in the non raft fraction (lane 1) at the expenses of the caveolin1 positive fractions (lanes 2 to 5 in Fig 4 B). Likewise FGF2 produces the same effect when added to starved FGF2-grown neurospheres (Fig 4 A, panel labelled FGF2).

Taking into account that β1-integrins modulate the response to GF in neural progenitors [39] we raised the hypothesis that the GF effects on the levels of NICD could be partially mediated by β1-integrins. If so, activation of β1-integrins should also lead to activation of the Notch pathway.

In neurospheres the activation of β1-integrins with Mn2+ mobilizes NICD out of the caveolin-containing fractions
To further characterize the mechanisms involved and to challenge the role of β1-integrins we stimulated β1-integrins in intact spheres and in ES-derived neural stem cells, using manganese (Mn2+). Mn2+ is a divalent cation known to activate β1-integrins [49] and to induce the re-distribution of LFA-1, α4β1 [50] or α6β1[47] into lipid rafts. While no significant change in Notch1 distribution occurred after Mn2+ treatment in EGF-grown spheres, Mn2+ leads to the redistribution of Notch from caveolin+ to caveolin-fractions in FGF2-grown neurospheres (Fig 4 A, panel labelled FGF2). The specificity of this effect on neurospheres was analysed by
exposing the suspension cultures to a β1-integrin blocking antibody (Ha2/5), prior to Mn2+ exposure (**Fig 4 C**). We observed that pre-incubation of the starved neurospheres with the blocking β1-integrin antibody decreases the Notch mobilization induced by the addition of Mn2+ (**Fig 4**) in both EGF- and FGF2-grown NS cultures, with a greater proportion of NICD remaining in the caveolar compartment. When combined with our β1-integrin loss of function (morpholino) data these results suggest that a decrease in β1-integrin may affect neurosphere formation assays by altering Notch1 processing/transfer to the nuclear fraction and thus preventing its downstream proliferative actions. Interestingly, integrin-mediated adhesion has been proposed to govern the presence of cholesterol enriched microdomains or lipid rafts on the plasma membrane, by controlling internalization via a caveolin-dependent pathway [51]. Together with our results these experiments raise the possibility that β1-integrins play a role in the growth factor-modulated transfer of NICD between membrane compartments and/or into the nucleus, possibly via lipid rafts.

### β1-integrin activation with Mn2+ leads to movement of NICD into the nucleus in ES-derived NSC

To test whether the activation of β1-integrins affects Notch internalization we exposed cells to Mn2+ (a divalent cation known to activate β1 integrins [49]), and evaluated the movement of NICD to the nucleus, by immunocytochemistry. Mn2+ is known to activate β1-integrins and to induce the re-distribution of β1-integrins into lipid rafts. Using an antibody specific for the Notch intracellular domain (NICD), (raised against an epitope exposed only after cleavage), we observed that β1-integrin activation with manganese led to a shift of NICD into the nucleus, detectable by immuno-histochemistry (**Fig 5**). This experiment was technically less demanding to do on monolayers of ES-derived NSC than on three dimensional neurospheres, where the cell-cell contacts and large amounts of ECM present account for activation of integrins, even when GF levels are low.

### β1-integrin activation with Mn2+ is followed by changes in HES5 mRNA expression

To study the effects of β1-integrin activation on the Notch1 downstream pathway we used Mn2+ to induce a change in the β1-integrin conformation and activation state, as previously described [49]. Other molecules known to activate integrins were also used, namely ECM molecules (laminin1, laminin2 and fibronectin) and EGF. It is noteworthy that fibronectin and laminin can cause EGFR phosphorylation, through β1-integrin activation [37,38,52], and EGF may also activate β1-integrin, through cross-talk between the EGFR and β1-integrin. Cells grown in EGF and FGF2 were starved overnight and then stimulated with Mn2+, GF or ECM for 3 hours. Changes in HES5 mRNA expression were evaluated by RT-PCR (**Fig 5**). This experiment revealed that Mn2+, EGF, EGF+FGF2 and ECM all lead to HES transcription, while FGF2 alone does not (**Fig 5**). The GF response pattern suggests that β1-integrin activation may affect Notch activation and HES transcription through the EGFR, which can be activated through β1-integrin stimulation by ECM ligands or EGF, but not by FGF2 alone. In fact, recent evidence shows that fibronectin and laminin can cause EGFR phosphorylation through β1-integrin
activation [52]. Furthermore, in breast tumour cells β1-integrin and EGFR pathways are known to be coupled and interdependent [36] and null β1-integrin NSC are more reliant on high growth factor levels (for survival and proliferation) than wild type NSC [39]. Finally, EGFR activation is important for neurosphere generation and by blocking this receptor with an inhibitor the number of secondary spheres produced after passaging decreases dramatically (as previously shown in Fig 2 J).

β1-integrins co-immunoprecipitate with NICD
To further understand how Notch1 and β1-integrins pathways could simultaneously affect stem cell behaviour we searched also for direct interactions between the two proteins. We used neurospheres lysates to look for physical interactions between Notch1 and β1-integrins, both of which are highly expressed by the cortical layer of the EGF-and FGF2-grown neurospheres [4,29]. Using lysates from 8-10 days *in vitro* neurospheres incubated with an antibody against β1-integrin and blotted with anti Notch1, we found that β1-integrin and Notch1 co-immunoprecipitate (Fig 6 A), indicating they are present in the same protein complex. The specificity of this interaction was confirmed by a reverse co-immunoprecipitation. For this purpose a GST-NICD protein (generated as described in materials and methods) was used to pull down the β1 and α6 subunits in EGF- and FGF2-grown neurospheres (Fig 6 B, C).

Discussion
In this paper we show that Notch1 and β1-integrins are co-expressed in ES-derived NSC, neurospheres and in the mouse embryonic VZ. We report that the Notch pathway cross-talks with the β1-integrin pathway, as indicated by the interaction detected between the NICD fragment and the β1-integrins. Furthermore, GF (EGF and FGF2) cause an increase of NICD levels and activation of β1-integrins with Mn2+ induces NICD internalization, via caveolin1-enriched rafts, with appearance of the NICD fragment in the nucleus. These results suggest that the GFs may play a role by enhancing the level of NICD, while the β1-integrins could modulate how much of it reaches the nucleus, by regulating the internalization and travel through the endocytic compartments. Therefore the β1-integrin may act dually, as an NICD buffering/sequestering system and as an internalization control, activated by extra cellular cues. The ECM/GF environment could play an important modulatory role on NSC behaviour through β1-integrins, which may act by controlling the nuclear availability of NICD in a context-dependent manner (possibly by playing a role in Notch internalization, through caveolin1-containing membrane domains), making the Notch pathway modulable by extrinsic factors such as GF and the ECM.
mechanisms of neural development. In particular, changes in protein signalling that occur during NSC generation and differentiation can be analysed in these cells, and the context-dependent effects of proteins such as Notch are more amenable to analysis using the ES-derived NSC cultures, than using the heterogeneous neurosphere and VZ explant cultures.

β1-integrins, ECM and GF participate in a complex network of interactions that affect NSC behaviour

The proteins that constitute the neurogenic niche participate in more than one regulation loops. For example during development FGF signalling plays a role in neural and mesodermal cell induction, mediated by Ets and GATA transcription factors [58]. FGF also promotes changes in the cell responsiveness to the environment by increasing the expression of β1-integrins, laminin and EGFR on neural progenitors [4,23,24,59,60]. Furthermore, FGF2 affects Notch signalling [61] and regulates neuronal differentiation by poorly defined interactions with Notch [14]. Taken together these observations suggest that FGF2 increases the levels of receptors and ligands and prepares the NSC to become responsive to subsequent waves of growth factors and to a changing ECM. Interestingly, two of the receptors up-regulated by FGF2 (EGFR and β1-integrins) are known to cross-talk in epithelial cells [36,52] and manipulation of either pathway can overcome deficits in β1-integrin or EGFR signalling in NSC [39]. Furthermore, in Drosophila a dynamic interplay exists between Notch and EGFR signalling and both antagonistic and synergistic/additive effects have been described [62,63]. In vertebrates, cross-talk between TGFβ and Notch occurs [64] and EGFR activation leads to Notch signalling during pancreas tumorigenesis [15]. These examples indicate that some functions of Notch may be context-dependent and could require complex interaction loops with other signalling pathways.

Direct interactions can also explain context-dependent signalling effects: our co-immunoprecipitation data (Fig 6 A-C) suggests that β1-integrins (which are very highly expressed in the VZ, together with laminin2, Fig 6 D-F) may sequester the NICD fragment. This raises the possibility that the β1-integrin associated Notch1 mobilization to the nucleus could be a context-dependent event, partially regulated by the extra cellular environment. In fact integrins can regulate Rac targeting by internalization of membrane domains such as lipid rafts [65] and in Drosophila selective endocytic pathways are required for the Delta/Serrate/LAG-2 (DSL) family to activate Notch [66,67]. Therefore, it is conceivable that the effect of integrin activation on Notch may require lipid raft internalization of Notch-containing membrane domains. Our finding that ES-derived NSC, the embryonic mouse VZ and neurosphere primary cultures are all very rich in caveolin1, (a component of lipid rafts), supports the hypothesis that these microdomains may play important roles in the coordination between signalling pathways in NSC. Lipid rafts may play a crucial role in controlling signalling in a spatial and temporal manner [68]. Therefore, the role of lipid rafts and their biological significance in NSC could be to bring together in the same domain two proteins (Notch and β1-integrin) at defined developmental time points, allowing for interactions to occur between multiple signalling pathways or, alternatively, implementing direct physical
interactions between the two proteins (Fig 6 G, H). The integrative role of lipid rafts may help to explain the context-dependency of receptor signalling. For example, while Notch1 and β1-integrins are both expressed in ES cells and ES-derived NSC, caveolin1 is more abundant in the later (data not shown), perhaps facilitating direct or indirect interactions between β1-integrin and Notch1 pathways preferentially in neural progenitors. Our results (Fig 6 A-C) suggest that the cytoplasmic tail of the β1-integrin interacts (directly or as part of a protein complex) with the NICD fragment and alters the biological availability of the NICD fragment, modulating the amount that can reach the nucleus. The interaction between the cytoplasmic domains of Notch1 and β1-integrins suggests a speculative model, whereby direct linkage of the two proteins could be affected by the ligation state of the integrin. We propose that β1-integrins “mop up” excess free NICD (Fig 6 G, H) under specific conditions, for example during cell division, in the presence of the adequate ECM like laminin alpha2-containing laminins (which abunds in the ependymal surface of the VZ and may “anchor” the progenitor/NSC cells) or through cross-talk with tyrosine kynase receptors (TKR) like the EGFR. This mechanism could be important to modulate the level of transcriptional regulation activity of NICD and to control effects on survival, proliferation and differentiation. In turn, the “release” of the NICD fragment allows it to reach the nucleus to promote the maintenance of an undifferentiated fate (Fig 6 G, H).

A model that relies on external factors to alter the equilibrium between bound and free intra cellular signalling molecules has also been suggested for the regulation of β-catenin signalling, where a balance between bound and free levels of β-catenin is partially controlled by multiple interactions between cadherins and receptor tyrosine kinases [69]. Likewise, proteins like Notch that depend on regulated intra-membrane proteolysis for signalling [70], require effective mechanisms to control downstream signalling. We suggest that spatial control can be achieved by a “buffering system” that keeps levels of NICD balanced, in the presence of the right ECM. The model we propose predicts that an adequate balance of free and bound NICD is maintained only in the cells that are in contact with the adequate matrix or in the presence of specific growth factors (Fig 6 G, H), further highlighting the important role played by niches during neural development. The model predicts that changes in β1-integrins levels could lead to an imbalance in NICD levels. Interestingly, NICD over-expression in a chondrogenic cell line inhibits differentiation and decreases proliferation [71] and, likewise, a decrease in β1-integrin in chondrocytes causes diminished proliferation and changes in the G1/S transition and cytokinesis [72]. Both these observations may be due to an imbalance (increase) in free NICD, provoked by the NICD over-expression or by the decrease in β1-integrins, respectively. If changes in β1-integrins expression causes an imbalance in NICD levels, the increase in NICD availability that we observe when GF are added to the neural progenitor cultures may explain the proliferation rescue of the β1-integrin null cells by GF [39]. Interestingly, the addition of GF to β1-integrin null cells also increases the number of nestin expressing cells and decreases differentiation, an effect that could also
be explained by activation of the Notch pathway.

An additional role for β1-integrin could be to provide survival signals (in a context-dependent manner that depends on the ECM composition), to counteract the potentially deleterious effect of excessive Notch activation. For example, it is known that in B lymphocytes Notch1 induces cell cycle arrest and apoptosis [11] while in T cells Notch1 has an anti-apoptotic function [12]. Inhibition of Notch by Numb in the Drosophila serotonin lineage causes cells to differentiate, while cells that retain Notch signaling initiate apoptosis [73]. Interestingly, the onset of mammary apoptosis in the mouse mammary gland coincides with a change of conformation between ligand-bound and unbound β1-integrin [74]. We speculate that the equilibrium between apoptosis and survival in neural progenitors could be due to a balance between the levels of β1-integrins and the levels of NICD, and therefore highly context-dependent. During cortical development, once the cells abandon the laminin-rich “VZ niche” [4] (Fig 6 G, H), they encounter a different matrix and/or growth factor environment, which may favor non-canonical Notch biological roles in differentiation or cell death. This proposed model could also account for the context-dependent effects of Notch1 described for different malignant cells [75].

In summary, Notch1 is a cell surface protein involved in the control of cell fate choices in the developing CNS [1]. Our data suggests that the Notch1 pathway is partially dependent on the integrin/ECM/GF environment. β1-integrins may act on Notch signaling through 1) physical interaction (sequestration) of the NICD fragment by the cytoplasmic tail of the β1-integrin 2) by affecting trafficking of the NICD. The ligation state of β1-integrins could therefore “fine tune” Notch activation/processing in a changing ECM and growth factor environment resulting in differential effects on cell fate according to the micro-environment present at a given moment in time. The different roles attributed to Notch during cortical development, such as the role in the sequential generation of radial glia and of neurons [30,31] or the later role of Notch in neurite extension [8], could be explained by a context-dependent modulation of the pathway, dependent on the ECM composition and GF availability [13]. We conclude that, through a β1-dependent Notch1 pathway modulation, the ECM and GF in the immediate vicinity of NSC may participate in neural stem cell fate determination in neurogenic niches. Furthermore, our findings suggest that caveolin1-containing lipid rafts play a role in the coordination and coupling of β1-integrin, Notch 1 and TKR signalling pathways. We speculate that this will require the presence of the adequate β1-activating ECM or GF in restricted regions of the CNS, and namely in neurogenic niches.

References

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Figure Legends
Figure 1
β1-integrins and Notch1 co-localize in the developing VZ and in neurospheres. Notch1 and β1-integrins are detected in the ventricular zone (VZ) during mouse gestation [4,32] and both play important roles in neural stem cell control. Ligands for the Notch1 and β1-integrins (delta1 and laminin, respectively) are available in the VZ and their expression levels change in time [4,76]. High levels of β1-integrins (red, C) and Notch1 (green, B) are present in the embryonic day 12.5 mouse VZ. Note that Notch1 (green, B) and β1-integrin (red, A) are co-expressed in the VZ (yellow, D). Note the lack of overlap in blood vessels (arrow, C and D). NSC can be cultured from embryonic or postnatal CNS tissue [19,77] in suspension cultures that give rise to spheroid structures (neurospheres) that contain NSC at the edge [29], where these cells express high levels of Notch1 [29] and lex/SSEA [33]. Notch1 is expressed in sectioned neurospheres (D) together with β1-integrin (E) and they partially overlap (yellow, F), predominantly at the edge. The edge of neurospheres contains a nestin-rich cell population, which expresses EGFR (see figure 2) and β1-integrins [4].

Neural progenitors derived from ES cells express radial glial markers, Notch1 and β1-integrin: embryonic stem (ES) cells can be driven towards a neural progenitor fate using diverse protocols [22,53,55] and also give rise to radial glial like (RGC)-cells [21] that express RGC markers (see Fig 1 J-K). Taking into account that RGCs are now generally accepted to be NSC [34,78] the ES cell-derived neural progenitors can be considered to be a NSC population, that can be readily grown and maintained in large numbers to analyse complex pathway interactions in NSC. The ES-derived neural progenitors express high levels of Notch1 receptor (G) and β1-integrins (H), like the mouse embryonic SVZ and the edge of primary Neurosphere cultures. Note that Notch1 and β1-integrins expression patterns overlap in the ES cell-derived NSC (I, yellow). The ES cell-derived NSC cultures are therefore suitable to study, in vitro, the cooperation between β1 and Notch1 pathways, both of which are known to be crucial for NSC and RGC maintenance and development [4,31,35].

Figure 2
β1-integrins and EGFR are required for secondary Neurosphere formation.
1) A decrease in β1-integrin affects secondary Neurosphere formation at low EGF and at high FGF2 concentrations: intact neurospheres were exposed to morpholino anti-sense to reduce β1-integrin expression and then dissociated and used in secondary neurosphere formation assays in the presence of 20 ng/ml EGF (A), 2 ng/ml EGF (B), 20 ng/ml FGF (C) and 2 ng/ml FGF (D). The experiments were carried out 5 times for each condition. A statistically significant decrease (P<0.001) in the number of secondary neurospheres formed after anti-sense treatment was observed.
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at 20 ng/ml of EGF, 2 ng/ml EGF and at 20 ng/ml FGF2. The strongest effect was seen for 20 ng/ml FGF2 (C), followed in order by EGF 2 ng/ml (B) and EGF 20 ng/ml (A), suggesting that the morpholino-treated FGF2-grown spheres are more dependent on β1-integrin than the EGF-grown ones. The decrease in β1-integrin was ascertained by Western blot (E). Samples grown in EGF or FGF2 and either exposed to the morpholino (m) or to the mis-sense (ms) were run in paralelle after protein quantification. Note that the EGFR is markedly up-regulated at the nestin-rich edge of FGF2-grown spheres, as detected by immunohistochemistry on sectioned neurospheres (F, nestin and G, EGFR) and confirmed with western blot (H). The nuclei in F and G are labelled with DAPI (blue). To test the role of the EGFR activation in neural progenitor proliferation intact mouse spheres (grown in a mix of EGF and FGF2 for 8 days) were used for secondary neurosphere formation assays in the presence of FGF2 and of an EGFR inhibitor (AG1478, 20 μM), as described in material and methods (J). Control cells were exposed to DMSO alone. Exposure to the EGFR inhibitor induces a statistically significant reduction (P<0.001, n=3) in the number of secondary spheres formed, in the presence of FGF2. Note that EGFR is highly expressed at the edge of neurospheres (G), in the region where nestin+ progenitors are abundant (F). Exposure of neurospheres to growth factors affects NICD levels: addition of EGF and FGF2 to 24-hours-growth factor starved neurospheres leads to an increase in detection of Notch intracellular domain (NICD) by western blot (I). The experiment shown is representative of various replicates (n > 3). Note that as a result of EGF or FGF2 addition (after growth factor starvation) MAPK is phosphorylated while total levels of MAPK remain similar, indicating a strong and specific response to the growth factors. Equal amounts of proteins were loaded in each lane.

Figure 3
Caveolin1, a resident lipid raft protein, is present in neural progenitor cells.
Lipid rafts act as privileged signalling platforms where cross-talk and interactions between signalling pathways may take place. Rafts contain resident proteins such as caveolins. Caveolin1 is expressed in the embryonic VZ (E12.5) (A, nuclear DAPI and B caveolin1 in green, and nuclear DAPI in blue), at the edge of neurospheres (C, caveolin1 in green) where β1-integrin predominates (D, red) and in the ES cell-derived NSC (E-H). E DAPI; F, caveolin1 (green); G, phalloidin-texas red; H, merge. LV: lateral ventricle.

Figure 4
Role of β1-integrins and lipid rafts in GF mediated NICD mobilization.
Exposure of neurospheres to growth factors or Mn2+ leads to NICD mobilization: Neurospheres grown in either EGF (top panel, A) or FGF2 (bottom panel, A) were starved overnight and exposed to culture media, culture media with growth factors, or culture media containing Mn2+ for 12hours. Panel A and and panel B show the same fractions of cell lysates, blotted with a Notch antibody (panel A) or a caveolin1 antibody (panel B), to identify fractions that contain caveolin1 and Notch1. This experiment shows that the NICD is mobilized from a caveolin+ fraction (lane2) to a caveolin1- fraction (lane1) when EGF is added to EGF-starved neurospheres and when FGF2 is added to FGF2-starved neurospheres. This suggests that the addition of growth factor stimulates Notch1 transfer between compartments. β1-integrins can be activated by Mn2+. When Mn2+ is added to FGF2-grown neurospheres NICD also moves between compartments but when Mn2+ is added to EGF-grown neurospheres
no NICD movement between compartments is detected. Note that the protein concentrations for each of the fractions (collected as described in materials and methods) were normalized prior to the western blot analysis, thus ensuring equal loading of the gels. The presence of caveolin1 was tested on all fractions but only fractions 6 to 1 are shown here. NICD: Notch1 intracellular domain. Cav1: caveolin1.

**Blocking β1-integrins with an antibody decreases the NICD mobilization induced by Mn2+:** Neurospheres grown in either EGF or FGF2 (C) were starved overnight and then exposed sequentially to a β1-integrin blocking antibody for 1 hour and to Mn\(^{2+}\) overnight. The controls were not incubated with the blocking antibody and were exposed to Mn\(^{2+}\) overnight. The neurospheres were then harvested, lysed and spun on Optiprep gradients to isolate different membrane compartments, as previously described. The same amounts of proteins per fraction were analysed by western blots with anti-Notch1 and anti-caveolin1 antibodies. This experiment shows that pre-incubation with a β1-integrin blocking antibody decreases the response of Notch1 to Mn\(^{2+}\), indicating that β1-integrins may play a role in the growth factor-dependent Notch1 activation. The growth factor- and integrin-dependent activation of Notch1 might explain the context-dependent effects of this protein throughout neurogenesis.

**Figure 5**

**β1-integrin activation in ES cell-derived NSC leads to movement of NICD into the nucleus and to changes in HES5 mRNA expression**

To evaluate if the NICD mobilization leads to transport into the nucleus ES cell-derived NSC were starved overnight and then exposed to Mn\(^{2+}\) for 30 minutes, to activate β1-integrins. The cells were then fixed and used for immunofluorescence. NICD was detected using a polyclonal antibody that is specific for the intracellular portion of Notch1, released after the second cleavage. Panel A-D shows the control cells expression of β1-integrin (A, I in red), Notch 1 (B, J in green), nuclear DAPI (C, blue in K) and a merged image (D, L) when the cells are **not exposed** to Mn\(^{2+}\). Note that Notch1 is detected mainly in the cytoplasm (B, D). Panel E-H shows the same stainings in cells that were starved overnight, and then **exposed** to Mn\(^{2+}\): β1-integrin (E, M in red), Notch1 (F, N in green) and nuclear DAPI (G, O in blue) and a merged image (H, P). Note that NICD is detected in both the cytoplasm and the nucleus in F, H (compare B and F), note also that in dividing cells Notch1 and β1-integrin overlap (yellow colour, arrow in L). This experiment was technically less demanding to do on monolayers of ES cell-derived NSC than on three-dimensional neurospheres, where the cell-cell contacts and large amounts of ECM present may account for activation of integrins, even when GF levels in the media are low. Furthermore these cultures represent faithfully the radial glial cell/NSC population [79]. Q) Addition of Mn\(^{2+}\), ECM and GF to ES cell-derived NSC increases the expression of HES5 mRNA, when compared to starved cells. Lanes 1-9: cells were starved overnight and then exposed to laminin1 (lane1, L1), laminin2 (lane 2, L2), fibronectin (lane3, FN), Mn\(^{2+}\) (lanes 6 and 7, Mn), a mix of EGF and laminin1 (lane 8, EL1) or a mix of EGF and laminin2 (lane 9, EL2). Lanes 4 and 5 show cells that were starved (st) overnight and not exposed to either GF or ECM molecules. HES5 mRNAs were detected by RT-PCR. GAPDH mRNA expression was used as a control.
Figure 6
β1-integrins co-immunoprecipitate with the intra cellular domain of Notch1 (NICD).
A) Western blot with anti-Notch1 of lysate prepared from 10 (days in vitro) neurospheres grown in FGF2 (lane 1) or EGF (lane 2). Co-immunoprecipitation of β1-integrin and Notch1 lysates obtained from EGF- or FGF2-grown neurospheres: lysates from EGF- or FGF2-grown neurospheres were used to immunoprecipitate β1-integrin with a polyclonal rabbit anti-β1 antibody. The proteins were resolved on SDS gels, transferred to membranes and blotted for the presence of Notch1 using a goat polyclonal antibody. This experiment indicates that Notch1 co-immunoprecipitates with β1-integrins. A band with the size of 110 (cleaved Notch 1) is detected in lanes 4 and 5 (FGF2-grown neurospheres) and in lanes 7 and 8 (EGF-grown neurospheres). Note that it is the same band as the one detected in the western blot (prepared from aliquots of the same lysates, lane 1 and 2). A larger band is also observable on the blot, possibly a multi-protein complex. Lanes 3 and 6 are pre-clear controls, where the lysate is incubated with beads-only, to detect non-specific attachment/interactions. (B, C) To check the specificity of the Notch-β1 interaction the reverse immunoprecipitation was done using a GST-NICD fusion protein (or GST protein alone, as a control) to pull down β1-integrins in lysates from EGF-grown (B) or FGF2-grown (C) neurospheres. The pool down with GST-NICD was followed by blotting with a polyclonal antibody against α6β1 and reveals the adequate α6 band (B, C, top arrowhead) and β1 band (B, C lower arrowhead). EGF: EGF-grown spheres. FGF2: FGF2-grown spheres. VZ: ventricular zone. IP: immunoprecipitation.

Role for a direct NICD-β1 interaction in the ventricular zone. β1-integrins are very abundant in dividing VZ cells. Arrows in D show cells that express high levels of integrins and arrows in E point at the dividing cells. Note the condensation of laminin (laminin α2-rich) in the VZ (F, arrows), indicating that the adequate ligand for β1-integrins is available in the VZ. Furthermore in the VZ, β1-integrins and Notch1 expression overlap (see Fig 1, D). We propose that in the presence of the adequate ECM or GF, β1-integrins in the VZ restrict the movement of the cleaved NICD, by tethering it to the membrane during symmetric (G) or asymmetric (H) divisions. In both cases (G, H) this insures that only cells anchored to the ECM proceed to retain NSC characteristics (sustained self-renewal, blockage of differentiation and survival). While in symmetric divisions (G) both cells are anchored and behave equally, in the second case (H) the anchorage to the ECM is not equal and the retention of β1-integrins in the most apical cell conditions the relative availability of NICD in the two daughter cells, ultimately affecting cell fate. Our co-immunoprecipitation data indicates that this tethering occurs and that β1-integrins interact with NICD (A-C). The overlap between β1-integrins and actin markers like phalloidin [13] in the VZ reinforces the hypothesis that β1-integrins help to anchor some crucial molecules and signalling complexes in a polarized manner in the VZ. Laminin in G and H is depicted in orange and predominates along the ependymal ventricular surface (as seen in panel F), while fibronectin is predominantly expressed outside the VZ, and towards the pial surface of the developing neural tube. NICD: Notch1 intra cellular domain; +++ NICD: high levels of NICD; +NICD lower levels of NICD; VZ: ventricular zone.
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