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## Surfaceome Profiling Reveals Regulators of Neural Stem Cell Function

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**Key Words.** Neural stem cells • Membrane glycoproteins • Embryonic development •  
Developmental gene expression regulation

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Received January 23, 2013; accepted for publication July 24, 2013; first published online in *STEM CELLS EXPRESS* September 10, 2013.

© AlphaMed Press  
1066-5099/2013/\$30.00/0

[http://dx.doi.org/  
10.1002/stem.1550](http://dx.doi.org/10.1002/stem.1550)

### ABSTRACT

The composition of cell-surface proteins changes during lineage specification, altering cellular responses to their milieu. The changes that characterize maturation of early neural stem cells (NSCs) remain poorly understood. Here we use mass spectrometry-based cell surface capture technology to profile the cell surface of early NSCs and demonstrate functional requirements for several enriched molecules. Primitive NSCs arise from embryonic stem cells upon removal of Transforming growth factor- $\beta$  signaling, while definitive NSCs arise from primitive NSCs upon Lif removal and FGF addition. In vivo aggregation assays revealed that N-cadherin upregulation is sufficient for the initial exclusion of definitive NSCs from pluripotent ectoderm, while c-kit signaling limits progeny of primitive NSCs. Furthermore, we implicate EphA4 in primitive NSC survival signaling and Erbb2 as being required for NSC proliferation. This work elucidates several key mediators of NSC function whose relevance is confirmed on forebrain-derived populations and identifies a host of other candidates that may regulate NSCs. *STEM CELLS* 2014;32:258–268

### INTRODUCTION

During mammalian development, lineages diverge from pluripotent epithelium and acquire cell-autonomous properties including distinct adhesive profiles that facilitate morphogenetic movements and cell-sorting as well as distinct arrays of receptors which restrict the range and output of morphogen responsiveness. Cell surface protein composition at the earliest stages of NSC development is poorly understood, and improved characterization would help elucidate the regulators of critical NSC properties. A comprehensive surfaceome profile of NSCs would provide a starting point to in vivo identification, purification, and manipulation of NSCs. At present, the means of selectively manipulating the function of rare NSCs are limited, in part because so few markers are known.

Neural tissue is induced and segregated from ectoderm by release of bone morphogenetic protein (BMP) antagonists such as Noggin and Chordin from anterior visceral endoderm at E7.5 of murine development [1]; BMP antagonism effectively relieves repression of neural fate, as factors downstream of BMPs including SMADs and inhibitors of differentiation (ID) inhibit the neural program [2, 3]. These events can be modeled using pluripo-

tent embryonic stem (ES) cells that self-renew when cultured with BMP4 and leukemia inhibitory factor (Lif) [4] but undergo neural induction when placed at low density in serum free media (SFM) with Lif but without exogenous BMPs [3]. Greater than 90% of ES cells which survive in primitive neural stem cell (pNSC) culture conditions translate sufficiently abundant Sox1, a specific neural marker, after 20 hours that it is detectable by immunohistochemistry [5], consistent with Lif signaling through Stat3 mediating a block of non-neural differentiation via Nanog [2, 4, 6, 7].

Lif-dependent NSCs arise coincident with neural specification at E7.5 then decrease in abundance at E8.5, coincident with an increase in the prevalence of fibroblast growth factor (FGF)-dependent NSCs that persist through adulthood [8]. When ES cells are transferred from self-renewal conditions to serum-free media with Lif, a subset referred to as primitive NSCs (pNSCs) proliferate extensively as clones [3]. pNSCs produce definitive NSC (dNSCs) that are not Lif-dependent but require exogenous FGF comparable to embryo-derived NSCs [3]. FGF is required for proliferation of neural populations [5, 9], and downstream signaling via ERK—at least transiently—contributes to neural maturation [10]. We reasoned

that an unbiased profile of the cell-surface constituents of each cell in the early NSC lineage and functional interrogation would help elucidate the molecules that dictate their respective identities.

Here we profile the cell surface of ES cells, pNSCs, and dNSCs in this clonally derived sequential neural lineage using cell surface capture (CSC) technology to identify glycosylated plasma membrane proteins with mass spectrometry (MS) [11]. We also demonstrate the utility of this data in making predictions about protein function in NSCs. Specifically, we demonstrate that Neural-cadherin (N-Cad) is sufficient for exclusion of dNSC from the pluripotent inner cell mass (ICM), that c-kit signaling limits NSC-derived progeny, perhaps by promoting pNSC quiescence or limiting specification of alternative fates, that signaling through EphA4 is required for viability of pNSC populations, and that ErbB2 signaling is required for the proliferation of NSCs. Moreover, we show the relevance of these findings to NSCs derived from adult mice.

## MATERIALS AND METHODS

### Cell Culture

R1 ES cells (a gift from A. Nagy) were used for experiments unless otherwise specified. For maintenance, ES cells were grown on mitotically inactive embryonic fibroblast layers in Dulbecco's modified Eagle's medium (DMEM) plus 15% fetal calf serum (FCS) (Hyclone, Logan, UT, <http://www.hyclone.com>) culture medium containing LIF (1,000 U/mL; Millipore, Billerica, MA, <http://www.millipore.com>). pNSCs and dNSCs were generated as described in [3]. For CSC profiling, R1 ES cells were passaged three times to expand from a starting population of approximately  $1 \times 10^6$  cells to approximately  $5 \times 10^7$  ES cells before seeding pNSC cultures. Each population of cells analyzed by CSC ranged from  $1 \times 10^8$  to  $6 \times 10^8$  cells. YC5/eYFP ES cells (a gift from A. Nagy) were used to generate pNSC and dNSCs for aggregation experiments [38].

For the colony formation assays assessing the effect of small molecule inhibitors and siRNA, ES cells were plated in DMEM + 15% FCS (Hyclone) and LIF (1,000 U/mL; Millipore) on gelatin-coated plates [39]. pNSCs and dNSCs were plated in serum-free media supplemented with LIF (1,000 U/mL; Millipore) or FGF2 (10 ng/mL; Sigma) + Heparin (2  $\mu$ g/mL; Sigma) + B27 (1:50; Invitrogen), respectively [3, 5]. Colonies  $\geq 50$   $\mu$ m in diameter were counted in the quantification. Exclusively for the comparison of ES cells cultured in serum-free media (Supporting Information Fig. S1), ES cells were grown on gelatin in '2i' media 3  $\mu$ M chir99021 (Reagents Direct) and 1  $\mu$ M PD0325901 (Reagents Direct) [32].

### Inhibitor Assays

Inhibitors were added to the cultures during plating. EphA4 blocking peptide (Alta Biosciences), ErbB2 inhibitor II (Calbiochem, San Diego, CA, <http://www.emdbiosciences.com>), Gleevec (Toronto Research Chemicals Inc.), N-cad function blocking antibody (Sigma), or Cyclopamine (Toronto Research Chemicals Inc.) that were dissolved in PBS, DMSO, PBS, and EtOH, respectively. Control inhibitors were applied at the high end of the established efficacy range: cyclopamine efficacy increases up to 10  $\mu$ M in some assays [27, 40], so it was applied at 5  $\mu$ M; efficacy of the N-cad antibody was titrated previously [30], and half

the maximal dose (1  $\mu$ g/mL) applied. Inhibitors of candidate molecules were applied at concentrations ensuring efficacy and maintaining specificity to the extent possible. 50  $\mu$ M exceeds the concentration providing maximal inhibition of ephrin binding by the EphA4 function blocking peptide in one assay ( $\sim 10$   $\mu$ M) but was used to ensure efficacy as it retains specificity for EphA4 over other Eph-family receptors [41]. The reported IC<sub>50</sub> of Gleevec varies from 0.2  $\mu$ M [42, 43] to 9.5  $\mu$ M [44] dependent on the cell type and assay. Gleevec is known to inhibit multiple kinases at concentrations where it inhibits c-kit function [43], but escalating efficacy with respect to c-kit inhibition has been observed above 2  $\mu$ M [26], so we chose 4  $\mu$ M to ensure efficacy. Depending on the cell type and assay, the reported IC<sub>50</sub> of the ErbB2 inhibitor II (Calbiochem) ranges from 6  $\mu$ M to 30.9  $\mu$ M (Calbiochem). At concentrations below 100  $\mu$ M, inhibition is specific for ErbB2 and not other Erb family members (Calbiochem). Viability was assessed using trypan blue exclusion after 24 hours. Proliferation deficits were inferred in situations where inhibitors did not cause significant effects on viability but did prevent sphere formation. Viability and proliferation of ES cells was assessed on both gelatin (Millipore) and mitotically inactivated feeders.

### siRNA Assays

All siRNAs were obtained from Dharmacon and used according to the manufacturer's instructions. Each treatment group received siRNA for 2 days before the beginning of the colony forming assay. For example, application of siRNA to pNSCs consisted of treating ES cells for 2 days with a particular siRNA and then applying the same siRNA to pNSC for the duration of their 7-day culture period. All siRNAs were applied at a final concentration of 50 nM, along with DharmaFECT1 (1:500). The following were used: cKit ON-TARGETplus siRNA J-042174-05-0010, ErbB2 ON-TARGETplus siRNA J-064147-05-0010, EphA4 ON-TARGETplus siRNA J-055030-05-0010, Scramble ON-TARGETplus Non-targeting siRNA #1.

### Cell Surface Capturing

CSC was performed as described previously [11]. In brief, colonies were dissociated by manual trituration after incubation in 1 mM EDTA and 10% fetal bovine serum (Hyclone) for 15 minutes at 37°C. Extracellularly exposed aldehydes were then oxidized with 1.6 mM sodium meta-periodate (NaIO<sub>4</sub>) (Piercenet) and reacted for 1 hour with 5 mM biocytin hydrazide (Biotium.com). The cells were then washed and lysed by dounce homogenization in a hypotonic lysis buffer, and the nuclei were pelleted by centrifugation. The supernatant containing the membranes and the cytoplasm was mixed with membrane prep buffer (280 mM sucrose, 50 mM MES pH 6, 450 mM NaCl, 10 mM MgCl<sub>2</sub>) and subjected to ultra centrifugation. The microsomal pellet was collected after 1 hour centrifugation at 35,000 rpm and solubilized by addition of 0.1% RapiGest (Waters) and sonification. After overnight trypsin digestion, the biontinated peptides were coupled to streptavidin beads (Piercenet), thoroughly washed, and released by PNGaseF (NEB), which cleaves N-glycosylated peptides at their backbone. Peptides were then clean-up over C18-tips (The NestGroup) and subjected to LC-MS/MS.

### MS

Each peptide sample was analyzed on an Eksigent Nano LC system (Eksigent Technologies) connected to a hybrid linear ion trap LTQ Orbitrap (Thermo Scientific) mass spectrometer, which

was equipped with a nanoelectrospray ion source (Thermo Scientific). Peptide separation was carried out on a RP-HPLC column (75  $\mu\text{m} \times 10\text{ cm}$ ) packed in-house with C18 resin (Magic C18 AQ 3  $\mu\text{m}$ , Michrom BioResources) using a linear gradient from 90% solvent A (water, 0.2% formic acid, 1% acetonitrile) and 10% solvent B (water, 0.2% formic acid, 80% acetonitrile) to 65% solvent A and 35% solvent B more than 60 minutes at a flow rate of 0.2  $\mu\text{L}/\text{minute}$ . The data acquisition mode was set to acquire one high-resolution MS scan in the ion cyclotron resonance cell followed by three collision induced dissociation MS/MS scans in the linear ion trap. For a high-resolution MS scan,  $10^6$  ions were accumulated over a maximum time of 500 ms, and the full width at half maximum resolution was set to 60,000 (at  $m/z$  300). Only MS signals exceeding 500 ion counts triggered a MS/MS attempt, and  $10^4$  ions were acquired for a MS/MS scan over a maximum time of 250 ms. The normalized collision energy was set to 35% and one microscan was acquired for each spectrum. Singly charged ions were excluded from triggering MS/MS scans.

All acquired MS/MS spectra were searched against the International Protein Index database (Version 3.26) using the Sequest algorithm. The Sequest database search criteria included: 0.2 Da mass tolerance for the precursor ion, 0.5 Da mass tolerance for the fragment ions, variable modifications of 0.984016 Da for asparagines (representing formerly N-glycosylated asparagines after deamidation through the PNGaseF treatment) and 15.994915 Da for methionines (covering rapidly oxidizing methionines), carbamidomethylation as static modification for cysteines, at least one tryptic terminus, two missed cleavage sites. Statistical analysis of the data, including peptide and protein identification was performed using the Trans-Proteomic Pipeline v4.3 (TPP) including PeptideProphet and ProteinProphet [45–47]. Peptides and proteins were detected and quantified at a confidence score of  $>1.3$  using the TPP in combination with Progenesis software. The ProteinProphet probability score was set such that the FDR was less than 1% determined by ProteinProphet.

All MS data from this study can be downloaded in the open source mzXML format from (<http://www.peptideatlas.org/repository/>) and was integrated into the publicly accessible PeptideAtlas database at (<http://www.peptideatlas.org/>).

### Label-Free Quantification

Protein quantification was performed using Progenesis (Non-linear Dynamics). After manually improving the alignment, quantified peaks were filtered for identification by sequence search and overall protein abundances were calculated thereof. Significance of detected differences was assessed with ANOVAs.

### Animal Husbandry

All of the mice used in this study were CD1 mice obtained from the Jackson Laboratories (Bar Harbor, ME, <http://www.jax.org>) and housed in accordance with the Institutional and Governmental Animal Care Committee guidelines of the University of Toronto.

### Morula Aggregations

Eight-cell ES, pNSC, or dNSC colonies were aggregated with diploid CD1 morula for 24 hours as described in [48], and embryos were then transplanted into pseudo-pregnant CD1 females.

### N-Cadherin Aggregation Assay

pNSCs were passaged into SFM +FGF2 +B27 as described previously, with the addition of 0.8  $\mu\text{g}/\text{mL}$  N-Cad function-blocking antibody (Sigma) during plating. 0.8  $\mu\text{g}/\text{mL}$  was also added to KCI Simplex Optimization Medium (KSOM) during the overnight aggregation.

### Imaging

Sections of chimeras were imaged using a  $\times 40/0.60$  Olympus IX81 inverted microscope with the Olympus Microsuite Version 3.2 Analysis imaging system software (Soft Imaging System). Overnight integration into the ICM was visualized with the same setup and confirmed in a subset of cases with confocal imaging. Sections of embryoid bodies were taken with ZeissAxio Observer.D1 inverted fluorescent microscope equipped with an AxioCamMrm digital camera run by AxioVision v4.8 software.

### Confocal

Images of morula aggregations involving the N-cad function-blocking antibody were taken with an Olympus IX81 inverted microscope with the Olympus FV10-ASW v2.01. Images of dsRed chimeric embryos were taken every 5  $\mu\text{m}$  with a Leica TCS2 confocal microscope.

### Immunocytochemistry

Sections were permeabilized with 0.3% Triton X-100 detergent (Sigma), sequentially blocked with 1% bovine serum albumin (Sigma), followed by 10% normal goat serum (NGS) (Sigma) in Stockholm's PBS + 0.3% Triton X, pH 7.3 for 30 minutes at room temperature before application of primary antibodies overnight in Stockholm's PBS, 1.0% NGS, and 0.3% Triton X-100. Primary anti-smooth muscle actin was applied at 1:250 (Sigma). Sections were washed and re-blocked as above before applying the secondary antibodies at 37°C for 2 hours in Stockholm's PBS + 1.0% NGS. Goat anti-mouse 568 nm Alexa Fluor antibodies (1:333; Invitrogen) were used as a secondary. Nuclei were counterstained with 10  $\mu\text{g}/\text{mL}$  Hoechst 33258 (Sigma) before samples were mounted and coverslips applied using Gel Mount (Biomedica).

### QPCR

QPCR was performed using Taqman Assays (Applied Biosystems, Foster City, CA, <http://www.appliedbiosystems.com>; AB) according to the manufacturer's instructions on a 7900 HT Fast-Time PCR System (AB) and analyzed with SDS v2.3 software. The following Taqman Assays were used: EphA4 (Mm00433056\_m1; AB), Rplp0 (Mm99999223\_gH; AB), and cKit (Mm00445212\_m1; AB). We tried to measure Erbb2 abundance using two Taqman Assays (Mm00658541\_m1 and Mm01306783\_m1; AB) but did not obtain reliable results due to low transcript abundance.

## RESULTS

### Cell Surface Profiling Reveals Distinct NSC Signatures

Discrepancies exist between the abundance of protein and corresponding mRNAs [12]. These data suggest that meaningful regulation of protein profiles occurs at the level of

translation and supports the merit of directly assessing proteins for both identification and quantification.

During development, lineage maturation and terminal differentiation are governed by a variety of factors including adhesion molecules that dictate cell sorting and receptors that dictate morphogen responsiveness. Proteins at the cell surface are of particular interest in understanding these processes since they mediate cell-cell communication, signal transduction, migration, and adhesion. To elucidate plasma membrane proteins involved in maturation of the neural lineage, we compared the cell surface protein profile of ES, pNSCs, and dNSCs using CSC [11, 13]. CSC offers a major advantage over antibody profiling approaches to cell-surface protein characterization in that CSC enables protein identification and relative quantification of the broad spectrum of plasma membrane glycoproteins in parallel. As such, the technique offers insight into the complement of environmental stimuli to which cells may be responsive independent of the antibodies available for detection. Moreover, CSC distinguishes itself from comparable MS-based techniques in that labeling is performed on viable cells. In brief, the glycans of plasma membrane proteins were labeled, purified with streptavidin, then enzymatically released, and identified by reverse-phase liquid chromatography coupled to tandem MS (LC MS/MS).

Using CSC and LC MS/MS, 813, 1,134, and 880 peptides were detected in the three respective cellular subsets (ES, pNSCs, and dNSCs). After collapsing peptides that mapped to the same protein, a total of 378, 563, and 456 proteins were identified using Occam's razor approach of ProteinProphet with a false-discovery rate (FDR) of  $<0.01$  (Fig. 1A). In each cell type, more than 200 membrane glycoproteins were identified, many of these are shared between the three cell types (Fig. 1B).

In order to quantify the differential expression of the cell surface proteins, we analyzed them by label-free quantification [14] with Progenesis software. We focused on those proteins that were identified via at least one peptide containing the N-glycosylation motif: asparagine, any amino acid, serine or threonine (NXS/T) and a deamidation on this asparagine. Such motifs are a distinguishing feature of cell surface proteins compared to cytoplasmic proteins in that most cell surface proteins are N-glycosylated [15, 16]. Since we specifically target, enrich, and modify such glycopeptides during the CSC workflow, their specific and repetitive identification is strong evidence of the cell surface location of the respective protein at the time of labeling.

Overall, 228 proteins were quantified which differed significantly ( $p < .05$ ) in relative abundance between at least two of the three cell types. Use of precursors from within the same sequential lineage as background during comparisons with NSCs should enrich for differences that define the biology of each distinct NSC in the lineage. Proteins that are significantly ( $p < .05$ ) different and a minimum of five times more abundant based on the MS signal in one cell type in the lineage than in either of the other two are depicted in Figure 1C (150 proteins). Proteins are connected by an edge to the cell type in which they are five times higher [17]. More detailed summaries of the top 20 proteins by fold enrichment in NSCs can be found in Supporting Information Tables S1–S3, and a complete list of peptides with their corresponding normalized abundance in each cell type can be found in Supporting Information Dataset S1.

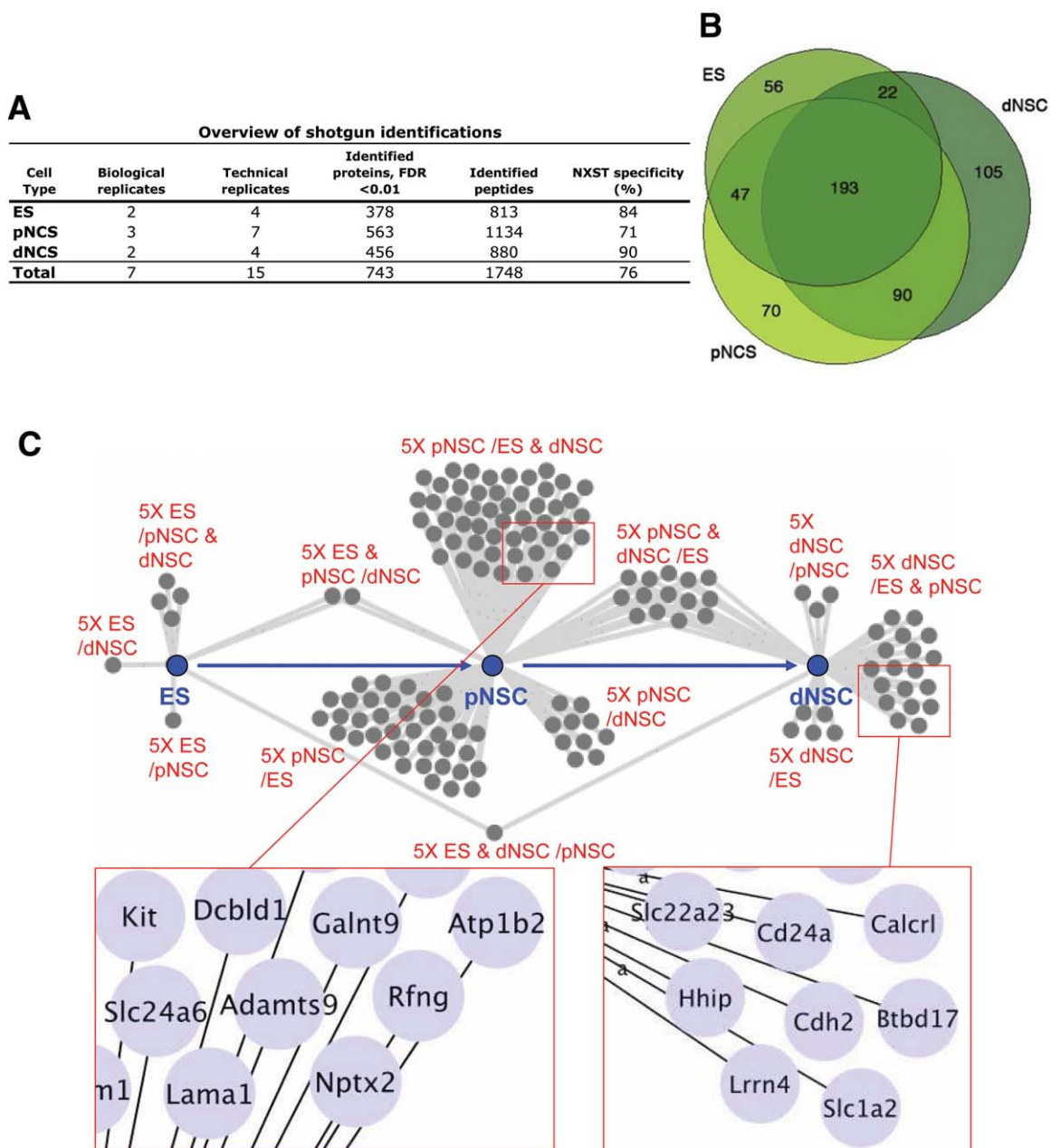
Cell surface protein profiling yielded enrichment of proteins whose relevance in the NSC lineage is established. Plectin, a cell surface marker commonly used for neural precursor enrichment [18] exhibited 50 times the MS signal intensity in pNSC than ES cells and 95 times the signal intensity in dNSC than ES cells. While the ratio of signal intensity between cell types is not an exact readout of the fold difference in copy number for a particular protein between cell types, it is an accurate predictor of upregulation or downregulation and can be used to estimate the magnitude of these differences. Several components of the hedgehog signaling pathway (Hh), known to be important in NSC function, were upregulated in NSCs; these included hedgehog-interacting protein (HhIP), which is 813 times higher in dNSC than ES cells ( $p < .05$ ), as well as Smoothened (Smo) which is 18 times higher in dNSCs than ES cells, and Patched 1 (Ptch1) which is 12 times higher in dNSCs than ES cells. Similarly, EphA4, which is an established mediator of viability in the adult subependymal [19, 20] was 31 times higher in pNSC than ES cells ( $p < .05$ ). As expected, lineage markers of NSC progeny are also upregulated in the surfaceome profile. For example, CD24, a marker of NSC-derived neuroblasts that is expressed specifically in the adult subependymal zone [21, 22], was 25 times higher ( $p < .05$ ) in dNSCs compared to ES populations based on the MS signals. While the numerical accuracy of differences in protein abundance between cell types would be improved by additional repetitions and absolute quantitative selective reaction monitoring assays using internal standards [23], they are useful estimates and suggest that the profiling data are likely to contain other molecules relevant to the biology of NSCs.

### Increased N-Cadherin Is Sufficient for Exclusion of dNSCs from Ectoderm

The greatest relative difference in abundance detected between dNSCs and their precursor pNSCs through CSC profiling was in N-cad, a calcium-dependent adhesion molecule where we detected an MS signal 5,850 times higher in dNSCs (Fig. 3A; Supporting Information Table S3). Adhesive differences between cell types are essential during development to organize tissues and signaling niches. To assess whether the differences in adhesion molecules detected by MS are functionally relevant and constitute candidate mediators of NSC sorting from unspecified ectoderm, we performed morula aggregation assays. The intent was to test whether increased N-cad was sufficient to exclude NSCs from the pluripotent ICM. Knowing that ES cells will colonize the ICM when aggregated with morula, we started by asking whether the two neural populations in the lineage were equally proficient in doing so. pNSC colonies incorporated within the ICM 88% ( $n = 1,302/1,486$ ) of the time, comparable to the 89% rate of ES cells ( $n = 146/164$ ) (Fig. 2B). Conversely, dNSC colonies rarely colonize the ICM ( $n = 33/814$ ) (Fig. 2B).

To assess whether the difference in adhesive compatibility between pNSC and dNSC was due to the loss of Lif signaling or addition of FGF + B27, we assessed the adhesive properties of pNSCs passaged into Lif, FGF, and B27. Under these culture conditions, NSCs colonized the ICM in 51% ( $n = 391/769$ ) of cases indicating that loss of Lif-based signaling is important but not solely responsible for the altered adhesive profile of dNSCs (Fig. 2B).

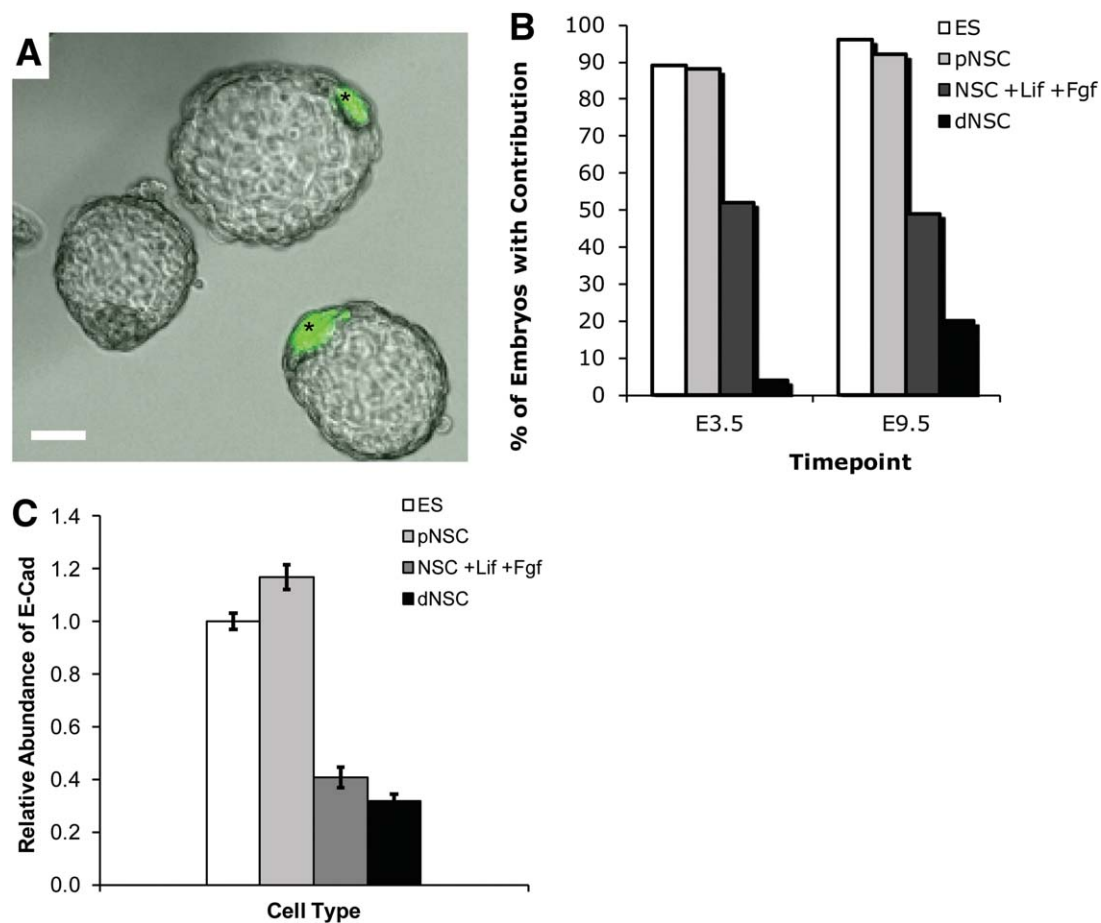
E-cadherin (E-cad) is primarily responsible for adhesion of preimplantation embryos [24], suggesting that Lif-dependent



**Figure 1.** Overview of cell surface capture-based protein identifications. **(A):** Summary of the experimental replicates as well as aggregate peptide and protein identifications in each cell type. **(B):** Schematic depicting the number of unique membrane proteins, predicted by Transmembrane Hidden Markov Model, identified in each cell type by ProteinProphet and the overlap between them. **(C):** The schematic depicts proteins (gray balls) which differ significantly in abundance between at least two cell types in the lineage ( $p < .05$ ) and are five times more abundant in the cell type (blue balls) to which they are connected with an edge than those to which they are not connected. Abbreviations: dNSC, definitive neural stem cell; ES, embryonic stem cell; FDR, false discovery rate; pNSC, primitive neural stem cell.

colonization of the ICM by dNSCs might be due to maintenance of E-cad by Lif. Because the NXS/T motif used to select membrane proteins is absent in a small subset of surface bound proteins, including E-cad, we estimated its relative abundance by quantitative polymerase chain reaction (PCR) (Fig. 2C). E-cad transcript abundance declines in ES-derived dNSCs as it does during lineage maturation of NSCs *in vivo* [25]. However, addition of Lif to dNSC cultures did not maintain the E-cad levels of ES and pNSCs ( $p < .05$ ), indicating E-cad is not responsible for the Lif-dependent rescue of ICM colonization by dNSCs.

To test the prediction that adhesion molecules showing elevated expression in dNSCs relative to ES and pNSCs mediated the exclusion of dNSCs from ectoderm, we blocked N-cad, which showed the greatest difference in abundance between dNSCs and their precursor population, pNSCs. We previously observed upregulation of N-cad in embryonic and adult NSC populations relative to ES cells suggesting that this feature of the *in vitro* model recapitulates development [25]. Culturing dNSCs in an N-cad function-blocking antibody partially “rescued” their ability to colonize the ICM (Fig. 3B). Confirmation that rescued cells made bonafide contributions to



**Figure 2.** NSCs lose the ability to colonize the inner cell mass (ICM) as they mature through the lineage. **(A):** Contribution of YFP+ ES cells (green) to the ICM (\*) is apparent in two of three blastocysts in this representative image. Scale bar = 50  $\mu$ m. **(B):** Quantification of the frequency with which donor cells colonize the embryo at the developmental time points indicated. Contribution at E3.5 was scored positive if integration within the ICM occurred after overnight integration and at E9.5 if embryos were partially fluorescent. Note that embryos were sorted at E3.5 such that only embryos with donor contribution to the ICM were reimplanted into surrogate mothers. The latter time point reflects this. pNSCs persisted to E9.5 in 48 of 52 cases compared to 9 of 19 blastocysts colonized by NSC + Lif + Fgf, and 1 of 5 in the case of dNSCs. **(C):** The relative abundance of E-cad was measured using quantitative polymerase chain reaction ( $\pm$  SEM). The data are normalized to abundance in ES cells. Abbreviations: dNSC, definitive neural stem cell; ES cell, embryonic stem cell; lif, leukemia inhibitory factor; pNSC, primitive neural stem cell.

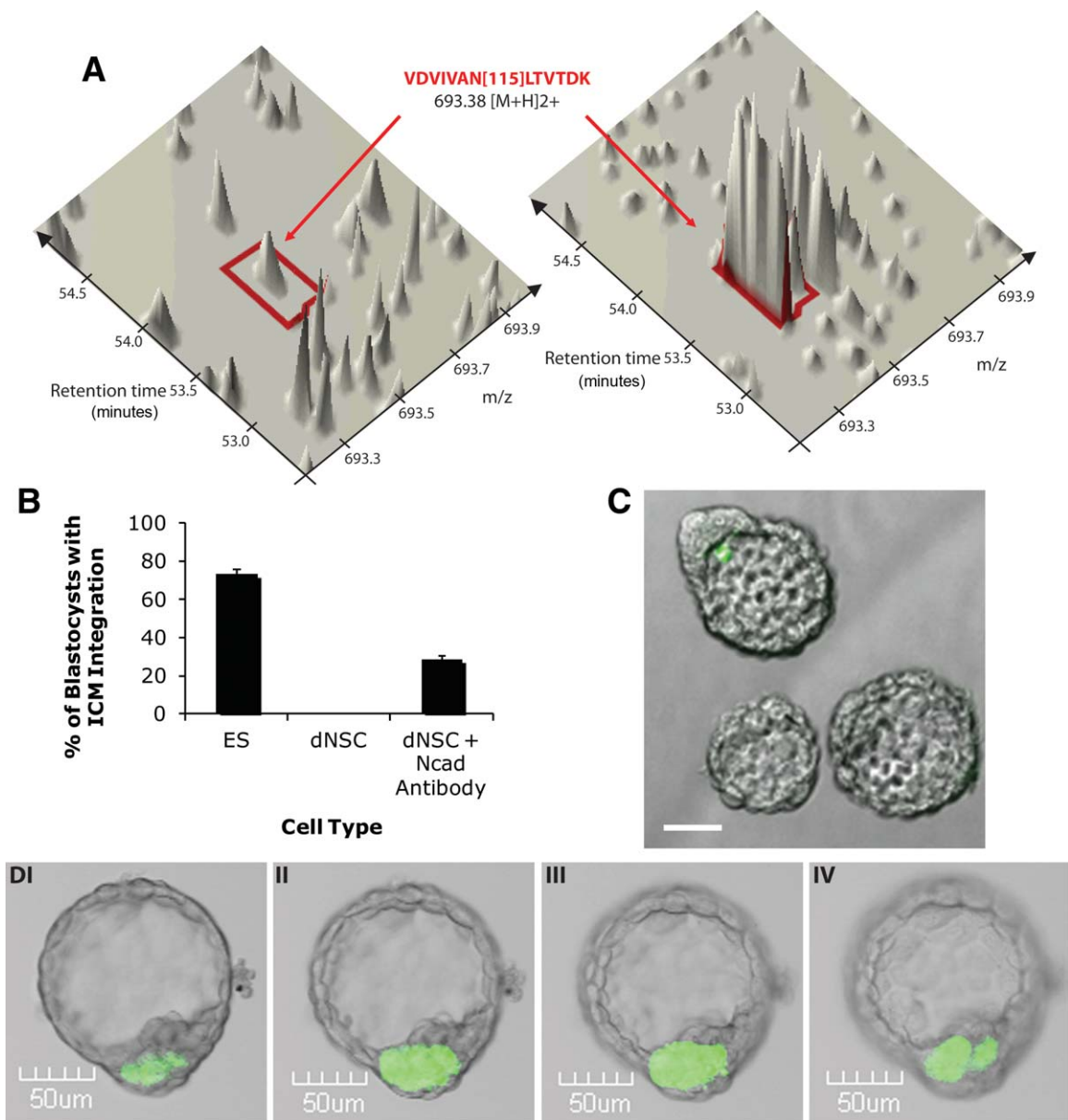
the ICM was achieved by confocal microscopy in all eight samples that were optically sectioned. A sample confocal stack of rescued dNSC aggregation is shown in Figure 3D. While the minimal culture conditions used in these cell and embryo-culture experiments are not physiological, they are ideal for teasing apart biology that may be buffered by redundancies in vivo. Despite being heterochronic, we note that these experiments collectively suggest that the N-cad upregulation contributes to exclusion of dNSCs from the pluripotent ectoderm.

#### Cell Surface Protein Profiling Identifies Molecules Required Specifically for NSC Function

We reasoned that molecules enriched in NSCs relative to the ES cells from which they were derived might mediate functions specifically in NSCs. For further analysis, we chose candidates that are five times enriched in either pNSCs or dNSCs relative to ES cells ( $p < .05$ ) and had an available selective inhibitor. To assay whether enrichment within the neural populations predicted functionally relevant molecules, we compared the

colony-forming ability of ES cells to each of the pNSC and dNSC populations in the presence of inhibitors targeting candidates of interest. Inhibition that selectively affected NSCs, but not ES cells, would suggest the candidate mediated proliferation, survival, quiescence, or cell fate specification of NSCs. These possibilities can be distinguished by additional experimentation. Colony formation in each inhibitor was normalized to the appropriate vehicle control at a concentration of inhibitor previously established to be selective for the target (see Materials and Methods section for detail) [26–30].

Of the proteins enriched on the cell surface of NSCs compared to ES cells, we chose inhibitors directed against N-cad, Hh, EphA4, ErbB2, and c-kit. Aside from the N-cad function-blocking antibody, each of these had significant effects on at least one of the ES-derived NSC populations without affecting ES cells ( $F_{3,15} = 8.27$ ,  $p < .05$ ). Inhibition of Hh, a pathway whose relevance to NSCs in vivo is established [31], blocked formation of both pNSCs and dNSCs without affecting ES cells ( $p < .05$ ) (Fig. 4A). This indicates that aspects of the in vivo lineage are being recapitulated effectively. Similarly, EphA4



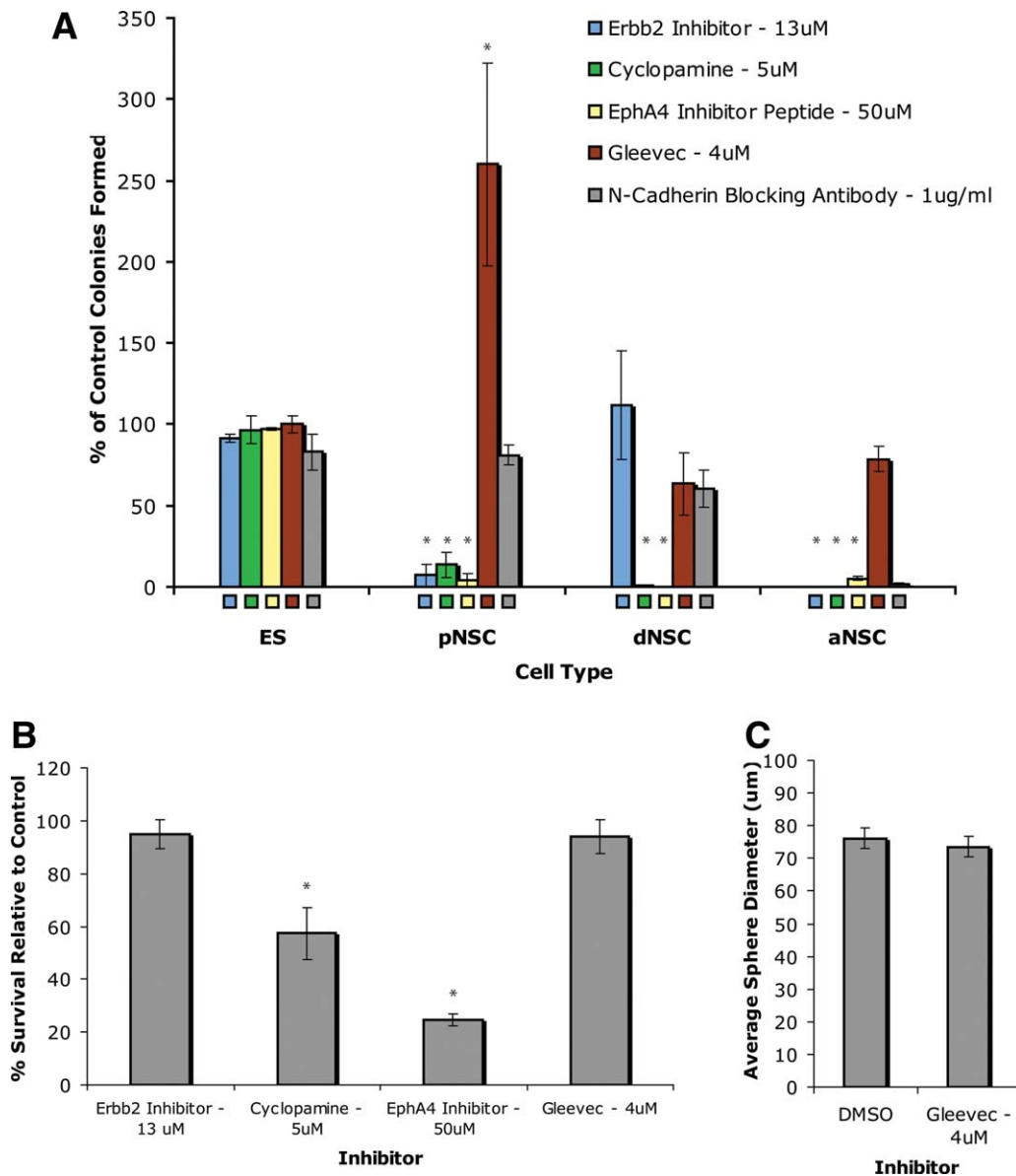
**Figure 3.** Upregulation of N-cad prevents dNSCs from aggregating with morula. **(A):** Three-dimensional peaks used to quantify the abundance of N-cad. The relative mass spectrometry (MS) integrated peak area signal of the N-cad peptide in the ES cell sample (left) is compared to the equivalent peak area signal in the dNSC sample (right). The peak area within the red box was selected, fragmented, and resulting spectra was identified as N-cad peptides by SEQUEST-based database searching. **(B):** Culturing dNSCs in N-cad function-blocking antibody partially rescues the ability of dNSCs to colonize the ICM. Integration frequency within the ICM was quantified and is depicted  $\pm$  SD (aggregate counts are: ES  $n = 45$ , dNSC  $n = 25$ , dNSC + N-cad antibody  $n = 83$ ). **(C):** Sample image of dNSC aggregation (scale bar = 50  $\mu$ m). **(DI-IV):** Representative optical sections (spaced by 10  $\mu$ m in a single confocal Z-stack) confirming that blocking N-cad in dNSCs rescues ICM colonization. Abbreviations: dNSC, definitive neural stem cell; ES cell, embryonic stem cell; ICM, inner cell mass.

has been implicated in viability of subependymal NSCs, and inhibition blocked dNSC colony formation without affecting ES colony growth ( $p < .05$ ) (Fig. 4A). Conversely, the same N-cad function-blocking antibody that disrupted adhesion of NSCs (Fig. 3D) did not impact functions assayable in this experiment: N-cad inhibition had no effect on colony formation of either pNSCs or dNSCs ( $p > .05$ ). An earlier role for EphA4 had not been explored previously, and we found that pNSCs exhibited a comparable response to dNSCs in requiring EphA4 for colony formation ( $p < .05$ ) (Fig. 4A). Erbb2 has not been implicated in NSC biology previously and blocked formation of

pNSC colonies ( $p < .05$ ) (Fig. 4A). This assay also revealed a novel function of c-kit signaling in NSC biology: its inhibition selectively increased colony formation of pNSCs ( $p < .05$ ) (Fig. 4A). The novel effects of the inhibitors on colony formation by pNSCs and dNSCs were specific to NSCs and unrelated serum-based ES culture media as repetition of the experiment culturing ES colonies in serum-free conditions [32] and inhibiting Erbb2, EphA4, or c-Kit yielded the same NSC-specific effects (Supporting Information Fig. S1).

We then asked whether the inhibitors were blocking signaling involved in viability or proliferation. Hh mediates

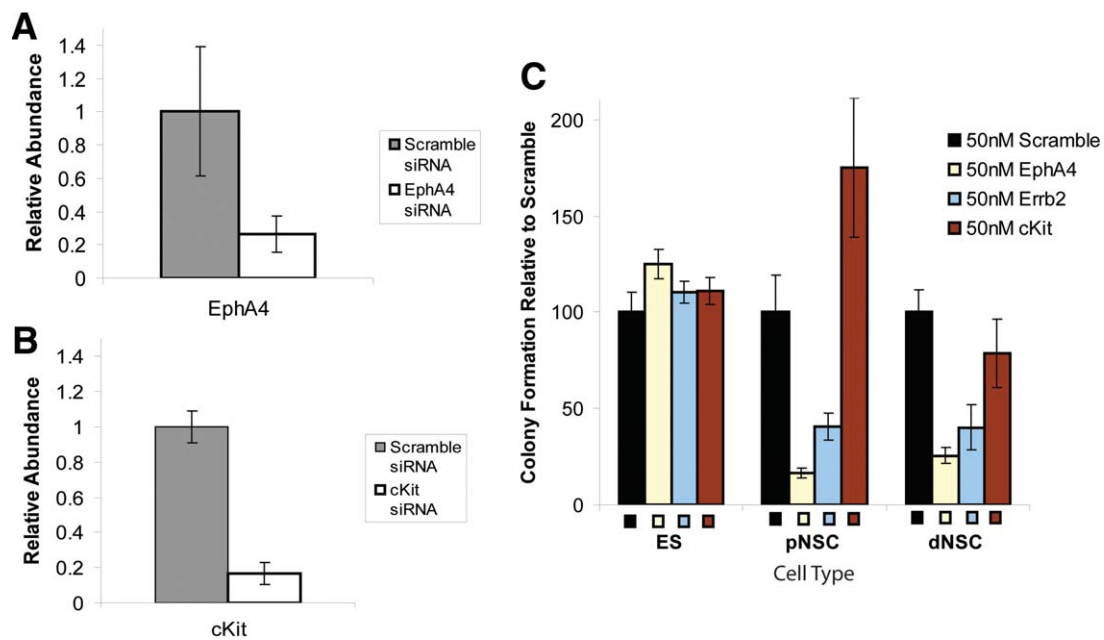




**Figure 4.** The ES-derived neural lineage predicts molecules that are functionally relevant for NSCs derived from adult mice. **(A):** Proteins found to be highly enriched in either pNSCs or dNSCs relative to ES cells were inhibited with Erb2 inhibitor II, cyclopamine (Hedgehog signaling; Hh), Gleevec (c-kit), or an EphA4 blocking peptide. ES cells were grown in serum and leukemia inhibitory factor (Lif), pNSCs in serum free media and Lif, and dNSCs in serum free media and FGF + B27 for this comparison. The frequency of 50  $\mu$ m colony formation was then compared between the different populations after normalizing to an appropriate vehicle control for each inhibitor. The normalized frequency of colony formation is depicted  $\pm$  SEM. An ANOVA was used to assess significance ( $F_{3,15} = 8.27, p < .05$ ). The Erb2 inhibitor blocked pNSC formation without impairing either ES or dNSC colony formation,  $*, p < .05$ . Cyclopamine blocked the formation of both pNSCs and dNSCs ( $*, p < .05$ ), as did the EphA4 inhibitor peptide  $*, p < .05$ . Conversely, Gleevec enhanced pNSC formation  $*, p < .05$  but did not affect colony formation of either ES or dNSCs. Notably, inhibitors which blocked the formation of ES-derived NSCs, also blocked the formation of adult mouse derived NSCs. The Erb2 inhibitor, Cyclopamine and the EphA4 inhibitor peptide all blocked aNSC formation  $*, p < .05$ . In the case of Gleevec, it impaired aNSC formation, in contrast to its lack of effect on ES-derived dNSCs and promotion of pNSC formation. **(B):** EphA4 and Hh have significant effects on NSC survival,  $*, p < .05$ . Viability after 24 hours is depicted  $\pm$  SEM. Viability in Erb2 inhibitor and Gleevec was unaffected. **(C):** pNSC colony size (depicted  $\pm$  SEM) was quantified in the presence of 4  $\mu$ M Gleevec and no difference was apparent relative to the vehicle control, indicating that the increase of pNSC colony formation in the presence of Gleevec is not the result of increased proliferation. Abbreviations: aNSC, adult mouse derived NSCs; dNSC, definitive neural stem cell; DMSO, Dimethyl sulfoxide; ES cell, embryonic stem cell; pNSC, primitive neural stem cell.

ventricular-derived dNSC proliferation [33]. We found that Hh inhibition reduced pNSC viability and prevented division, suggesting that Hh mediates both NSC viability and proliferation (Fig. 4B). EphA4 inhibition led to increased cell death of both pNSCs and dNSCs (Fig. 4B), suggesting that signaling through

EphA4 is required for NSC viability. Conversely, Erb2 inhibition did not have an effect on pNSC viability but limited division, indicating its signaling requirement for proliferation (Fig. 4B). Finally, c-kit inhibition did not have an appreciable effect on viability or the colony size of pNSCs (Fig. 4B, 4C),



**Figure 5.** siRNA validates identity of candidates implicated in NSC function. **(A, B):** Quantitative polymerase chain reaction (PCR) supports depletion of intended transcripts ( $p < .05$ , two-tailed Student's  $t$  tests). Relative abundance of EphA4 (A) and c-kit (B) transcripts in pNSC cultures following treatment with siRNAs targeting these respective transcripts (depicted  $\pm$  SEM). **(C):** siRNA confirmed the effects of targeting candidates with inhibitors. The candidates implicated in NSC function with inhibitors (Fig. 4) were targeted with siRNAs and assayed equivalently to confirm that the putative candidates were responsible for the observed effects. Treatments were normalized to a Scramble control as opposed to vehicle and depicted as the frequency of colony formation  $\pm$  SEM. siRNA targeting Erbb2 and EphA4 decreased pNSC formation, while c-kit depletion increased it ( $F_{3,11} = 28.03$ ,  $p < .05$ ). These effects and those of siRNA on dNSCs replicate the positive effects that small molecules had on NSCs (compare with Fig. 4A). Abbreviations: dNSC, definitive neural stem cell; ES cell, embryonic stem cell; pNSC, primitive neural stem cell; siRNA, Small interfering RNA.

suggesting that c-kit signaling regulates another property, perhaps pNSC specification or quiescence. Indeed, profiling by flow cytometry suggests that c-kit is present during the transition from ES cells to pNSCs before decreasing in abundance during NSC maturation (Supporting Information Fig. S2).

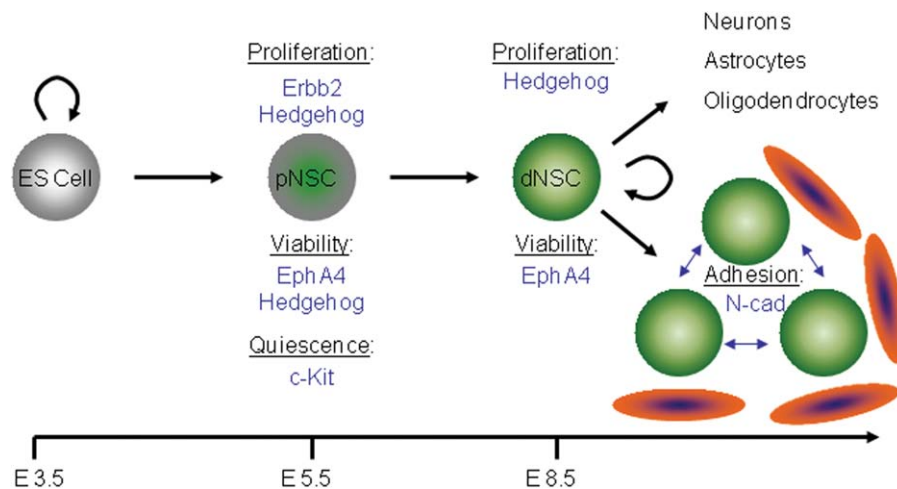
Next, we asked whether the molecules that affected the ES-derived NSCs were relevant to murine forebrain-derived populations. Using the same doses that affected ES-derived NSCs but did not impair ES colony formation, three of the four inhibitors blocked colony formation from forebrain derived NSCs, supporting the relevance of predictions made with the *in vitro* model to the *in vivo* case (Fig. 4A). Indeed, inhibition of c-kit by Gleevec—the lone inhibitor that did not produce an effect on aNSCs—enhanced colony formation of pNSCs from postnatal day 7 pups (unpublished data), supporting the relevance of this model, and suggesting that the effect is mediated through quiescence. While EphA4 and Hh are established mediators of NSCs *in vivo*, this work implicates c-kit in the biology of pNSCs and Erbb2 in the biology of adult mouse-derived NSCs.

To ensure that the proposed candidates were responsible for the effects on NSCs, we repeated the colony forming assays, targeting the candidates with siRNAs. Depletion of the transcripts of interest was confirmed by quantitative PCR (QPCR) (Fig. 5A, 5B). The effects on colony formation observed using inhibitors were confirmed with siRNA (Fig. 5C), supporting implication of the intended targets in NSC biology. An overall summary of these observations is provided in Figure 6.

## DISCUSSION

Direct glycoprotein profiling of NSCs bypassed the incongruity inherent in making inferences about protein abundance from transcriptional profiling [12] and revealed novel regulators in this lineage. The veracity of this model and profiling approach is demonstrated by markers presently used to enrich neural stem/progenitors from mice (prominin and CD24) being enriched in dNSCs relative to ES cells. This approach contributed to the identification of several molecules for which we have experimentally demonstrated biological roles in the earliest NSC populations, suggesting that the remainder of the profile is also likely to be rich in mediators of NSC behavior.

Our data support a model where N-cad is upregulated upon loss of Lif/Stat3 signal transduction, altering the adhesive profile of dNSCs such that they are excluded from adjacent ectoderm. N-cad is expressed in several tissues within the developing embryo, including the neural plate from approximately E7.5, consistent with the possibility that N-cad mediates segregation of early neural cell types from other lineages. The observation that pNSCs, cultured in SFM + Lif, maintain a comparable ability to colonize the ICM as ES cells indicate that loss of BMP plays a minor role, if any, in the upregulation of N-cad. Furthermore, while dNSCs cultured in FGF-containing media almost entirely lose the ability to colonize the ICM, addition of Lif results in a partial rescue of ICM compatibility. These data, combined with the observation that addition of Lif to dNSC culture does not maintain E-cad levels



**Figure 6.** Schematic summarizing the neural maturation model, with novel regulators implicated by this project in blue text. Abbreviations: ES cell, embryonic stem cell; N-cad, N-cadherin; pNSC, primitive neural stem cell.

equivalent to that of ES cells, suggests that Lif signal transduction is critical to maintaining the low N-cad levels required for ectoderm integration. That the Lif-based rescue of dNSC compatibility is only partial suggests that FGF and/or other signaling may also be involved in the adhesive profile transition. Indeed, the transition from ES to epiblast stem cells (EpiSCs) is characterized by Lif removal, FGF addition, and a corresponding decrease in ICM colonization [34, 35], while the combination of both Lif and BMP appears to induce ICM competence or reversion from EpiSCs [36]. EpiSC cultures are supplemented with Activin A to prevent neuralization and maintain pluripotency, distinguishing them from the default entry into the neural lineage exhibited by the pNSCs and dNSCs in this study [34, 35]. Our data suggest that Lif-mediated activation of JAK/STAT and/or MAPK signaling cascades is responsible for maintaining low N-cad expression that is compatible with ICM colonization.

Selective inhibition implicated several other candidates from the MS profiling in aspects of NSC function. In agreement with the independent conclusion that Ephrin B3 signaling through EphA4 promotes cell viability in the adult subependymal [19, 20], we found that EphA4 is required in adult-derived NSCs and ES-derived dNSCs for viability. In addition to these recapitulations, we report for the first time that EphA4 is required for viability of the early pNSC population. Conversely, c-kit inhibition increases the frequency of colony formation by pNSCs but neither ES-derived dNSCs nor forebrain-derived dNSCs. c-kit might promote specification of alternative cell fates, asymmetric divisions, or quiescence of pNSCs, perhaps as regulator of the population size. Erbb2 inhibition blocks colony formation of ES-derived NSCs as well as adult dNSCs. Erbb2 ligand Nrg-1 is required for proliferation of neural precursors [37], although the specific requirement for Erbb2, as opposed to another Nrg-1 receptor Erbb4, was not shown previously.

This study demonstrates the value of using CSC technology on viable cells and sequential, clonal precursor populations as a background to ascertain biologically relevant changes in cell-surface protein profiles during lineage maturation. We demonstrated functional roles for four proteins

showing increased abundance during the maturation of ES cells into NSCs. These represent a small fraction of the 112 peptides in this dataset that showed significant ( $p < .05$ ) enrichment (five times). Such confirmations, along with detection of high enrichment in proteins known to specifically mediate NSC functions such as EphA4 (31 times dNSC/ES) and Hh components (HhIP, Smo, and Ptch1 were all [mt]10 times dNSC/ES), suggest that the remainder of the dataset also will be rich in proteins relevant to NSC biology, and the earliest events in neural induction. Fruitful approaches to systematically exploring the biology of each cell type might include screening the list of candidates from this profile using antibody-based selection to test for enrichment of populations of interest from within the brain germinal zone populations or the use of siRNAs to determine molecules required for viability and proliferation. As such, this dataset is a step toward a higher resolution understanding of NSC biology.

#### ACKNOWLEDGMENTS

We thank Laurie Sellings and Lilian Doss for help with the statistics, Marina Gertsenstein for technical expertise as well as Andras Nagy for reagents. The work was funded by Canadian Institute for Health Research (CIHR) 'Cell Lineage and Brain Development' awarded to (D.v.d.K.), and 'Neural Plasticity and Repair' from the National Center of Competence in Research (NCCR) to (B.W.).

#### AUTHOR CONTRIBUTIONS

B.D.: conception, experimentation, and manuscript writing; D.B.F.: experimentation and manuscript writing; R.S., S.R., V.A., P.K., C.Y., H.S., and R.L.: experimentation; P.W.Z.: conception; B.W. and D.v.d.K.: conception, manuscript authoring, and funding.

#### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflict of interest.

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