Electronic Supplementary Material (ESI) for Integrative Biology

Roccio et al. SUPPLEMENTARY MATERIALS AND METHODS

Protein PEGylation

Proteins were conjugated with a maleimide-PEG N-hydroxysuccinimide ester (MAL-PEG-NHS, mol.wt. 3.5 kDa, JENKEM TECH., USA). The PEG linker was dissolved in 0.1M Borate buffer at pH 8, rapidly added to the protein solution and incubated for 1 hour at room temperature. PEGylated proteins of interest were covalently immobilized to the PEG gel forming a stable 3-thiosuccinimidyl ether linkage with the PEG-SH precursor. When provided in amine containing buffers, proteins were dialyzed against PBS prior to PEGylation. Laminin1 (BD and Invitrogen), PLL (Sigma) and FITC-BSA (Sigma) were PEGylated with a ratio of 10:1 (PEG linker to molecule). Fibronectin fragment $FN_{9\text{-}10}$ (a generous gift of Dr. M. Martino) and Protein A (Biovision) were PEGylated in a ratio of 4:1.

Silicon wafer fabrication

All microfabrication work was carried out at the Center of MicroNanotechnology (CMI) at EPFL. Topologically structured silicon stamps were fabricated by employing standard photolithography. A photolithography mask was designed using CleWin (PHOENIX, Netherlands), written as an inverted pattern on a 5-inch square chrome blank and subsequently developed and etched in a chrome etch bath. A four inch silicon wafer was primed using HDMS prior to spin-coating of a 10 µm thick layer of AZ9260 photoresist on an automated photoresist processing cluster (EVG150, EV Group). The wafer was subsequently exposed to the previously produced mask on a mask aligner for 2 cycles of 15 seconds, followed by automated development of the photoresist. The silicon wafer was etched to a depth of 100 µm via DRIE (Alcatel601, AMMS). The topological features were confirmed on a surface profiler (Alpha-Step 500, Tencor). The structured silicon wafer was then post-

processed to remove the photoresist from the final silicon mold and surface activated using 30 seconds oxygen plasma treatment. Each silicon chip included 7 repeats of 288 pillars (12X24) with a diameter of 450 µm and 100 µm height, with a distance per well of 350 µm.

Protein printing

A robotic spotter (Qarray mini, Genetix, United Kingdom), equipped with an 8 pin head was utilized to dispense nanoliter quantities of proteins from a 384-well plate to the micropillars of the silicon stamp. PEG-conjugated recombinant proteins or Fc-Chimeric proteins were dissolved according to manufacturers' instructions and mixed with glycerol (30% final concentration) to allow optimal dispensing.

Isolation of neural stem/progenitor cells

Epidermal growth factor (EGF)-dependent neurosphere cultures were generated from subventricular regions of postnatal (p5) Hes5::GFP transgenic mice or WT siblings, as previously described [1, 2]. Briefly, subventricular regions were dissociated in 300 µL of a 1:1 papain to ovomucoid mix, at 37 °C for 45 min. The papain mix contained 30 U/µl papain (Sigma), 240 µg/ml cysteine and 40 µg/ml DNAse I (Sigma) in L15 medium (Invitrogen). The ovomucoid mix contained 1.125 mg/ml Trypsin inhibitor (Sigma), 0.5 mg/ml bovine serum albumin (BSA; Sigma) and 40 µg/ml DNAse I (Sigma) in L15. The cell suspension was centrifuged (5 min. 80g), dissociated, resuspended and cultured in suspension in neurosphere medium (DMEM/F12 + Glutamax, Invitrogen) containing 20 ng/mL EGF (R&D), and 1 x B27 supplement (Invitrogen) for 4 days at 37°C. Neurospheres were dissociated with 0.05% trypsin in Versene (Invitrogen) followed by mild mechanical trituration with a fire-polished pasteur pipette and expanded. Neurospheres were frozen at passage 2 and used at p3-p6 for experiments. For differentiation experiments, cells dissociated from spheres

were plated on PLL-coated plates in culture medium for one day, subsequently changed to low EGF containing medium (0.2ng/ml),for 10 days, fixed and immunostained.

Flow cytometry

NSC expanded as neurospheres were dissociated and either directly analyzed for GFP expression, or immunostained on ice for 30 minutes with primary antibodies against α6 integrin (R&D Systems) and CD133 (Ebioscience) diluted in PBS with 0.2% BSA, followed by incubation for 30 minutes on ice with the secondary fluorescently labeled antibodies anti-rat Alexa 647 and anti-mouse Alexa 647 (Invitrogen). Stainings with secondary antibodies only were used as control. Biotinylated EGF conjugated to Alexa Fluor 647 (Invitrogen) was incubated on ice for 30 minutes. Samples were analyzed using a CyAN ADPS (Beckman Coulter) or LSR II SORP (BD).

Time-lapse analyses of NSC proliferation

Following protein immobilization, hydrogel microwell arrays were extensively washed in PBS, UV-sterilized and incubated overnight in PBS at 4°C. Arrays were then equilibrated at 37°C with culture medium for at least one hour prior to cell seeding. Dissociated cells from neurosphere cultures (p3 to p6) were seeded at a density of $1x10^4$ per array (20 cm^2). Cells were trapped in microwells by gravitational sedimentation. For live-cell imaging, the slides were placed in the environmental chamber of an inverted microscope (Zeiss, Axio Observer.Z1) equipped with a motorized Zeiss scanning stage. The xyz-stage was programmed to repeatedly scan across the microwell array surface in a mosaic pattern. Brightfield images were acquired at 5x magnification for a period of 5 days and with one image taken every 3 hours. The resulting images were then automatically stitched and compiled into stacks

using the Metamorph software (Molecular Devices, USA). Microwells containing a single living cell at time point t = 0 were followed over time by visual inspection. Cells were scored as dead when they completely ceased to move and markedly shrunk in size. Cells continuously proliferating during the 5 days were scored as proliferating, while cells first dividing and subsequently dying were scored separately (proliferation>death). Quiescence was defined as absence of cell division within the entire duration of the experiment. At the end of each time-lapse experiment, arrays were fixed in 4% paraformaldehyde and immunostained.

Retrospective phenotyping by immunostaining

Cells grown on the arrays were fixed in 4% paraformaldehyde in PBS for 15 minutes at room temperature, permeabilized with 0.1% TX-100 for 5 minutes and incubated for 2 hours in blocking buffer (PBS containing 0.01% TX100, 2% BSA and 0.5 mg/ml human IgG) to saturate Protein A Fc-binding sites still available. The primary antibodies: mouse anti-Laminin1 (Sigma), rat anti-alpha 6 integrin (R&D), mouse anti-Nestin (BD Transduction Laboratories), rabbit anti-GFP (Abcam), rabbit anti-GFAP (Zymed), rabbit anti-βIII-tubulin (Abcam), mouse anti-βIIITubulin (R&D), mouse anti-O4 (R&D), mouse anti-Notch (Abcam) and mouse anti-Jagged-1(R&D) were incubated in blocking solution for 2 hours at room temperature. Arrays were washed in PBS and incubated for 1 hour at room temperature with Alexa-fluor conjugated secondary antibodies (1:500, Invitrogen). Arrays were incubated for 10 minutes with 10 µg/mL DAPI (Sigma) dissolved in PBS and finally washed 3 times in PBS before image acquisition.

Fluorescence image analysis and quantification

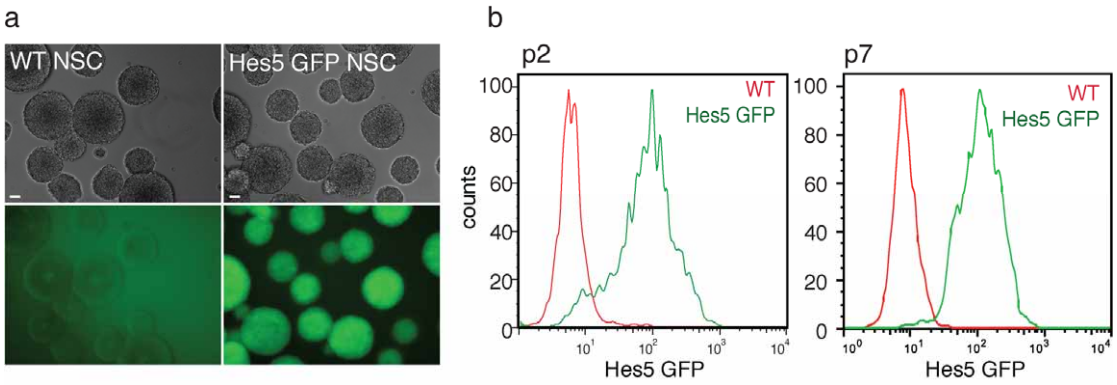
Arrays were scanned using a 10x objective with the scan slide mode of the Metamorph software and image reconstruction was then compared to the bright-field

images obtained during the time lapse. Only clones of cells derived from a single cell at day 0 were further quantified. The integrated area intensity of cells within each microwell was evaluated by thresholding a background-subtracted image. The number of cells per clone was measured by automatic counting the Dapi-positive cells using Metamorph software (Dapi count application) and data was logged in an Excel sheet. The integrated area intensity was then expressed relative to the number of Dapi counts for the same well, in order to obtain the integrated intensity per cell. The number of differentiated cells for each microwell positive for the neuronal marker βIII-tubulin was manually counted. Data was automatically logged in an Excel sheet.

1. O. Basak and V. Taylor, *The European journal of neuroscience*, 2007, **25**, 1006-1022.
2. C. Giachino, O. Basak and V. Taylor, *Methods Mol Biol*, 2009, **482**, 143-158.

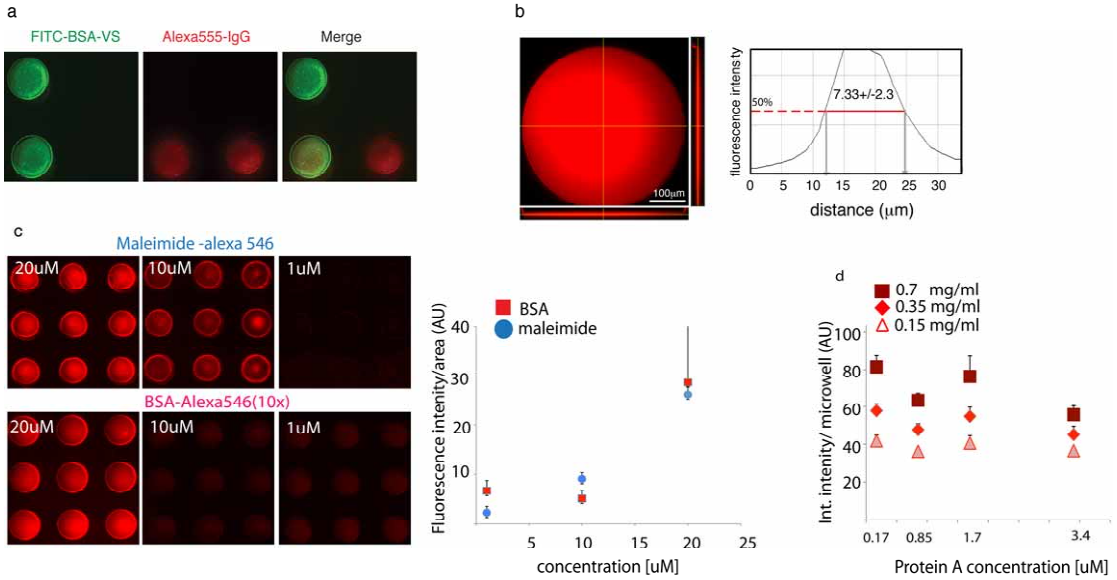
Supplementary Figures Roccio *et al.*

Supplementary Fig. S1:



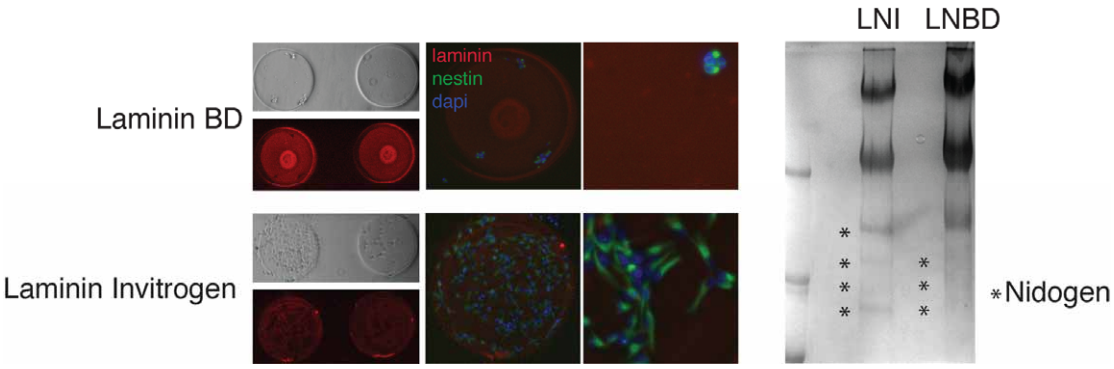
Supplementary S1. (a) Fluorescence pictures of WT and Hes5-GFP neurospheres in culture (scale bars = 100µm). (b) Flow cytometry analysis of Hes5-GFP cells after neurosphere dissociation at early (p2) and late (p7) passages (right).

Supplementary Fig. S2:



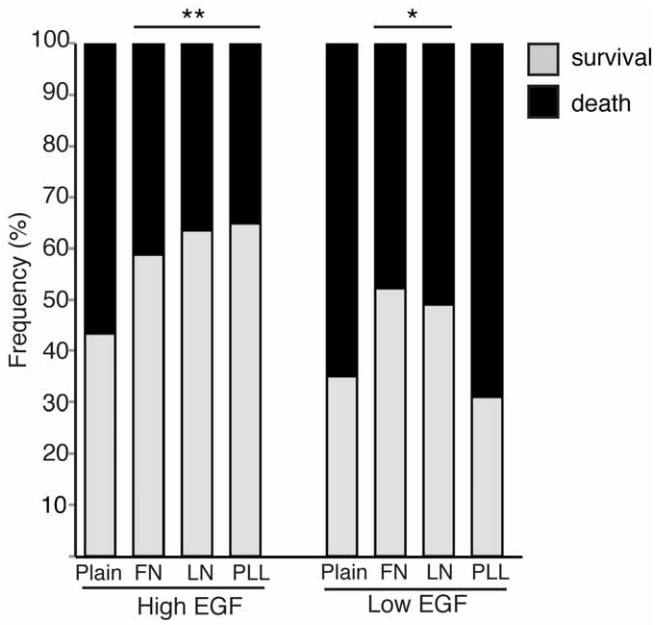
Supplementary S2. (a) PEG-modified FITC-BSA and Alexa555-conjugated hIgG are printed on a Protein A-containing gel orthogonally. (b) Confocal image of a microwell where alexa546-BSA-PEG has been spotted: cross-sections were used to evaluate thickness of the immobilized protein as distance between the 50% intensity values. Average thickness of thirty sections is indicated. (c) Alexa546-labeled Maleimide (Invitrogen) and Alexa546-labeled BSA-PEG were spotted on PEG gels at comparable molar concentration of the fluorescent dye. Average fluorescence intensity among 36 microwell per condition is reported in the graph. Examples at three different concentrations (20, 10 and 1 µm) is shown. (d) Alexa 555-labeled human IgG (sigma) spotted at a concentration of 0.7mg/ml, 0.35mg/ml and 0.15 mg/ml is immobilized to the same extent on gels containing different concentrations of Protein A (0.17µM, 0.85µM, 1.7µM and 3.4µM). The average integrated intensity among 10 microwells is shown.

Supplementary Fig. S3:



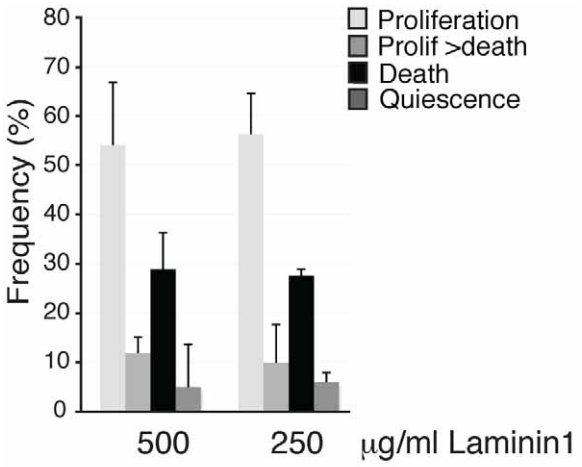
Supplementary S3. Two different commercially available Laminin1 sources (BD and Invitrogen) were immobilized on PEG gels and visualized by immunostaining. Cells shown in brightfield and by immunofluorescence grow as neurospheres (BD) or adherent (Invitrogen). SDS-PAGE of the two Laminin1 preparations was used to separate the Laminin1 chains. Bands were analyzed by mass spectrometry. In both cases lower molecular weight bands are identified as the Laminin binding protein Nidogen1.

Supplementary Fig. S4:



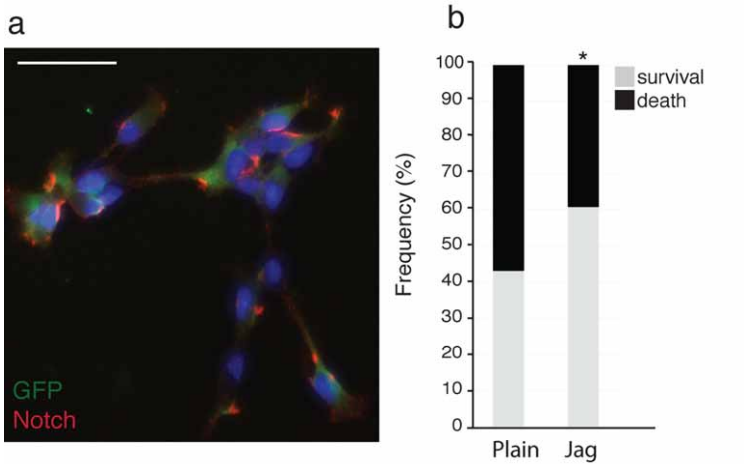
Supplementary S4. Survival (gray) and death (black) of cells growing in the presence of 20ng/ml EGF (left) and 0.2ng/ml EGF (right) on plain, $FN_{9\text{-}10}$, Laminin1 or PLL-functionalized microwell arrays, followed for 5 days by time-lapse microscopy (*$p<0.05$; ** $p<0.005$).

Supplementary Fig. S5:



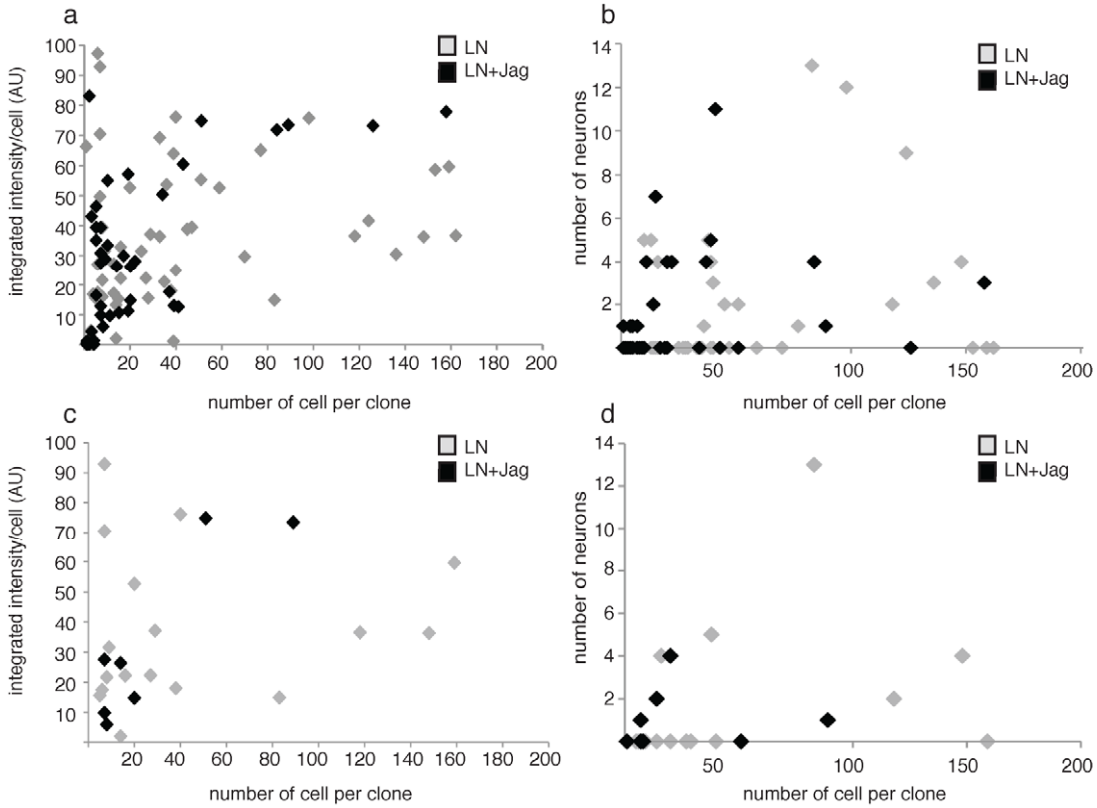
Supplementary S5. Cells growing in the presence of 20ng/ml EGF on Laminin1 arrays (500 or 250 µg/ml) followed by time-lapse microscopy for 5 days. Distribution of cells in four different categories (proliferation, proliferation>death, death, or quiescence (left to right order in the graph) are shown.

Supplementary Fig. S6:



Supplementary S6. (a) Hes5-GFP NSC plated on Laminin1 microwell arrays immunostained for GFP (green) and Notch1 (red). Nuclei stained with DAPI (blue). Scale bar is equal to 100µm.
(b) Survival (gray) and death (black) of cells growing in the presence of 20 ng/ml EGF on plain, or Jagged1 (200µg/ml)-functionalized microwell arrays followed for 5 days by time-lapse microscopy (*$p<0.05$).

Supplementary Fig. S7:



Supplementary S7. Retrospective immuno-staining of clones cultured on Laminin1 or Laminin1+Jagged 1 functionalized arrays. Arrays were followed by time lapse microscopy, fixed at day 5 and stained for βIIItubulin, Nestin and DAPI. Graphs show the integrated area intensity (Nestin) per cell plotted against the size of the same clone, for all microwells (a) and microwells with 1 cell at day 0 (c). The number of neurons for each clone is plotted against the clone size, for all microwells (b) or for microwells with 1 cell at day 0 (d).