Experimental

Construction and expression of Histidine-tagged DM-GRASP

The cDNA encoding the extracellular domain of DM-GRASP was obtained by polymerase chain reaction using oligonucleotides AGCTAAGCTTTTGTATACAGTAAATGCAG (sense primer) and CCACCTCGAGGCTTTGCCTGGTCATTAACC (antisense primer). Sense and antisense primers contain restriction sites (underlined) for HindIII and XhoI, respectively, used for ligation of the PCR product into the eukaryotic expression vector pSecTag2b (Invitrogen, USA) containing a Histidine tag consisting of six consecutive histidine residues. In addition, a signal sequence that targets nascent polypeptide chains to the ER is attached to the amino-terminal end of the extracellular domain of DM-GRASP. The accuracy of the generated pSecTag-His-DMGRASP was confirmed by DNA sequencing. To obtain cell lines stably secreting Histidine-tagged DM-GRASP, HEK293 cells were stably transfected with Zeocin-selectable pSecTag-His-DM-GRASP using Lipofectamine transfection reagent (Invitrogen), basically as described before.[1] In brief, HEK293 cells maintained in DMEM (supplemented with 10% foetal bovine serum) were plated in 100 mm dishes and having reached 50% confluence transfected with 10 µg of DNA and 20 µl of Lipofectamine in 6.4 ml of serum-free OptiMEM medium (Invitrogen) for 4 hours. Two days after transfection, HEK293 cells were passaged for clonal selection and cultured for additional 10–14 days in presence of 500 µg/ml Zeocin (Invitrogen) to obtain stably DM-GRASP-secreting cells. DM-GRASP was purified from the culture supernatant by nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen, Germany). Integrity of the fusion protein and purity of the preparation was ascertained by Western blotting using a polyclonal DM-GRASP-specific antibody[2] as well as by protein staining (Coomassie Brilliant Blue, Serva, Germany).

Generation of nanopatterned surfaces

Large area (approx. 400 mm^2) gold nanodot patterns from diblock copolymer micelles were prepared as described[3] The molecular characteristics of polystrene-block-poly(2-vinylpyridine) (PS-b-P2VP), diblock copolymer PS(500)-b-P2VP(270), and diblock copolymer PS(990)-b-(385) were described previously.[3] Immobilizing linear polyethylene glycol (PEG, CH3-(O-CH2-CH2)17-NH-CO-NH-CH2-CH2-CH2-Si(OEt)3) to glass interfaces on coverslips (Roth, Germany) was performed by first chemically activating the substrates (H_2SO_4/H_2O_2=3/1) followed by substrate immersion in 1 mM PEG solution in dry toluene (Merck, Germany) under nitrogen atmosphere and incubation in oil bath at 80°C for 24 hours. Substrates were then rinsed intensively with methanol and ethyl acetate (Aldrich, USA), blow dried with nitrogen, and used directly for biofunctionalisation. Nanodot patterns were created with interspacings of 53 nm and 78 nm and diameters of 5 and 6 nm, respectively; quality of the gold nanodot pattern was verified by scanning electron microscopy.

Biofunctionalisation of nanopatterned surfaces

To functionalize the gold nano-dots with NTA moieties, PEG-modified substrates were immersed for 4 hours in a ethanolic solution of a NTA-thiol which has been described previously.[4] Samples were washed with MilliQ-water, shortly incubated with 10 mM Ni^{2+} in Hank's buffered saline (HBSS), washed with phosphate-buffered saline (PBS), and incubated with 1 µg/ml DM-GRASP in PBS over night at 4°C. Biofunctionalisation was verified by immunofluorescence staining. For this, nanopatterns were incubated with a polyclonal DM-GRASP-specific antibody (1:500) over night at 4°C, for 1 hour with Tetramethyl Rhodamine Iso-Thiocyanate (TRITC)-conjugated anti-rabbit antibody (1:100, Sigma), and visualised employing a Leica Fluorescence Microscope.

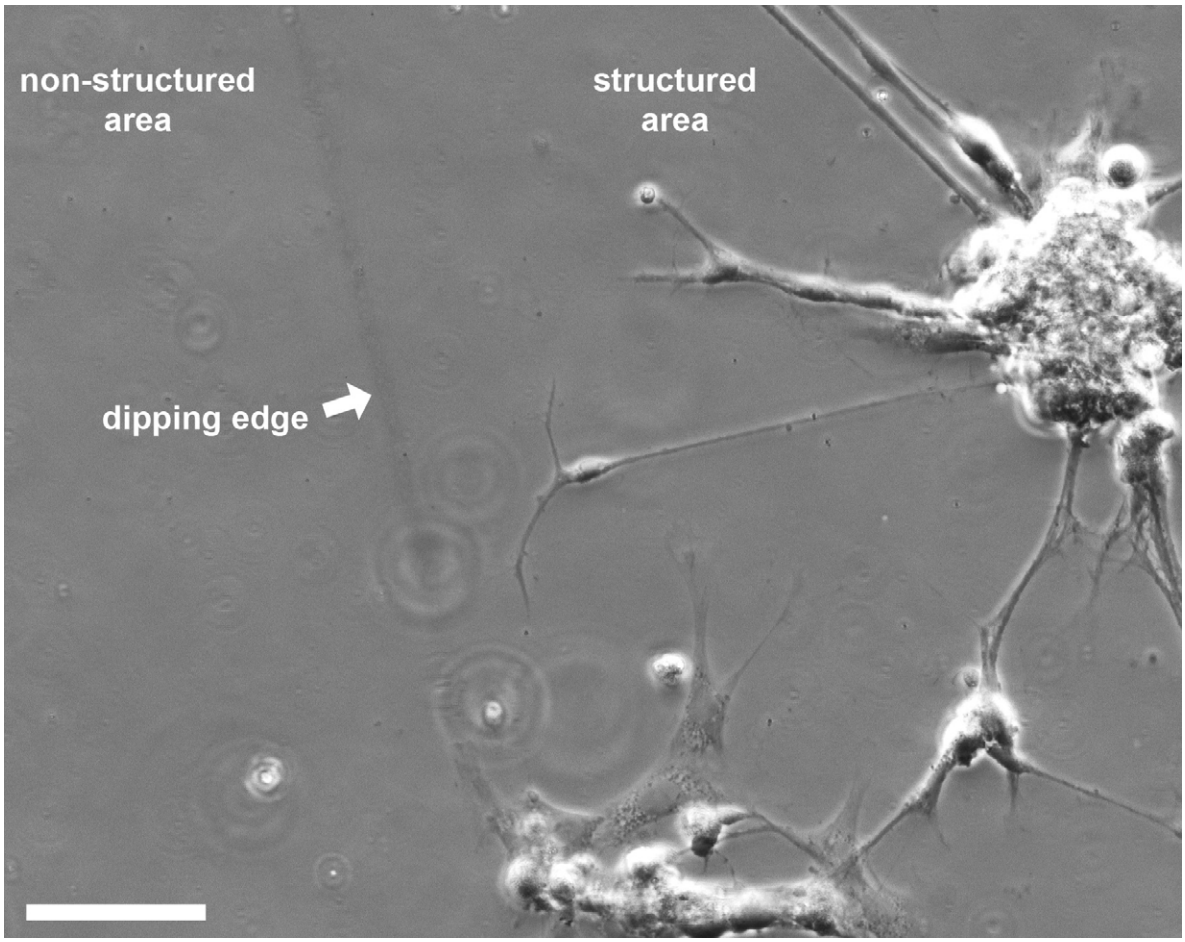
Cell culture and stainings

Neuroblastoma N2a cells were maintained in DMEM supplemented with 10% foetal bovine serum, penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37°C. To establish cell lines stably expressing DM-GRASP, N2a cells were co-transfected with pMIW-DM-GRASP and the neomycin-selectable pcDNA3 expression plasmid using Lipofectamine (Invitrogen). Transfected N2a cells were passaged for clonal selection into culture medium supplemented with 500 µg/ml geneticin (PAA, Germany) and cultured for 10–14 days. Then 18 geneticin-resistant clones were screened for DM-GRASP expression by Western blot analysis. The stably DM-GRASP-expressing N2a cell lines were maintained in culture medium supplemented with 250 µg/ml geneticin. Dorsal root ganglia were dissected from chick embryos of embryonic day 10. For single cell culture, DRGs were treated with 1 mg/ml trypsin (Worthington, USA) in HBSS for 30 minutes at 37°C. Dissociated cells were seeded with a density of $3*10^4$ cells per 3.5 cm dish (containing one nano-patterned substrate) and grown at 37°C, 5% CO_2 in DMEM/F-12 supplemented with 1% foetal bovine serum, 50 ng/ml nerve growth factor, and antibiotics. After 30 hours, cells were fixed by adding pre-warmed 8% paraformaldehyde (PFA) in DMEM/F12 (final concentration 4% PFA) for 25 minutes

before replacing the medium / fixative mixture with 4% PFA in PBS for another 30 minutes. For immunofluorescence stainings, cells were permeabilized with 0.1% Triton-X100 in PBS for 10 minutes. Proteins were stained by indirect immunofluorescence as described.[5] Tubulin β3 was detected using monoclonal antibody Tuj1 (Covance, USA), followed by a secondary antibody goat-anti-mouse Fluorescein Isothiocyanate (FITC, Dianova, Germany). Actin was visualised using Cy3-conjugated Phalloidin (Sigma, Germany). Nuclear stainings (4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), Hoechst, Germany) were performed to determine cell numbers and to support identification of cell types in cell clusters (neurons: DAPI-bright, small, round nuclei; mesenchymal cells: large, ellipsoid nuclei). 20 randomly chosen optic fields (0.14 mm^2/field) per experiment were evaluated; images were taken using an inverted fluorescence microscope (Axiovert 200M, Zeiss) equipped with a digital camera (AxioCam, Zeiss), and annotated with Adobe Photoshop 7.0.

1. L. K. Needham, K. Thelen and P. F. Maness, *J Neurosci*, 2001, **21**, 1490-1500.
2. G. E. Pollerberg and T. G. Mack, *Dev Biol*, 1994, **165**, 670-687.
3. M. Arnold, E. A. Cavalcanti-Adam, R. Glass, J. Blummel, W. Eck, M. Kantlehner, H. Kessler and J. P. Spatz, *Chemphyschem*, 2004, **5**, 383-388.
4. A. Tinazli, J. Tang, R. Valiokas, S. Picuric, S. Lata, J. Piehler, B. Liedberg and R. Tampe, *Chemistry*, 2005, **11**, 5249-5259.
5. G. E. Pollerberg, C. Kuschel and M. Zenke, *J Neurosci Res*, 1995, **41**, 427-442.

Supplemental Figure



Adhesion of DRG cells and neurite growth of DRG neurons is restricted to the nanostructured area functionalized with edDM-GRASP; no cells are attached to the non-structured area. Note the borderline ("dipping edge") separating both areas (indicated by arrow). Scale bar: 25 µm