Adult Schwann cells (SCs) possess an inherent plastic potential. This plasticity allows SCs to acquire repair-specific functions essential for peripheral nerve regeneration. Here, we investigate whether stromal SCs in benign-behaving peripheral neuroblastic tumors adopt a similar cellular state. We profile ganglioneuromas and neuroblastomas, rich and poor in SC stroma, respectively, and peripheral nerves after injury, rich in repair SCs. Indeed, stromal SCs in ganglioneuromas and repair SCs share the expression of nerve repair-associated genes. Neuroblastoma cells, derived from aggressive tumors, respond to primary repair-related SCs and their secretome with increased neuronal differentiation and reduced proliferation. Within the pool of secreted stromal and repair SC factors, we identify EGFL8, a matricellular protein with so far undescribed function, to act as neuritogen and to rewire cellular signaling by activating kinases involved in neurogenesis. In summary, we report that human SCs undergo a similar adaptive response in two patho-physiologically distinct situations, peripheral nerve injury and tumor development.
Schwann cells (SCs) are the principal glia of the peripheral nervous system and evolve in close contact with neurons into peripheral nerve fibers. Reciprocal signaling between SCs and neurons regulates the survival, fate decisions, and differentiation of both cell types, but also influences their behavior in regenerative and pathological conditions\textsuperscript{1-9}. Hence, understanding the molecular mechanisms underlying SC-neuron interaction is of utmost interest to develop effective treatment strategies for injuries and pathologies of the peripheral nervous system.

Despite being necessary for correct nerve development, SCs earned recognition because of their plasticity that allows differentiated SCs, further called adult SCs, to transform into a dedicated repair cell after peripheral nerve injury. The process is referred to as adaptive cellular reprogramming and includes profound transcriptional and morphological changes\textsuperscript{27-37}. This phenotypic switch is mediated by dedifferentiation causing the regain of immature/precursor SC properties followed by re-differentiation into a repair-specific state\textsuperscript{10}. The resulting repair SC phenotype is characterized by the re-expression of markers known to be upregulated in SCs during development, and by distinct repair functions and repair-associated ligands distinguishing repair SCs from adult SCs or developing SCs\textsuperscript{11-13}. Those repair functions comprise the degradation of myelin debris, attraction of phagocytes, the formation of regeneration tracks for axon guidance, and the expression of cell surface proteins and trophic (neuroprotective and neuritogenic) factors promoting axon survival and re-growth\textsuperscript{9,10,14-16}. We have recently provided a comprehensive transcriptomic and proteomic characterization of human repair SCs demonstrating that SCs isolated from excised peripheral nerves adopt the same repair-related phenotype and function in culture as in nerve tissue explants. These included the expression of master transcriptional regulators, such as JUN, as well as myelinophagy, phagocytosis, and antigen processing and presentation via MHC-II\textsuperscript{17}. Importantly, transcriptomic signatures of primary repair-related SC cultures indicated the expression of a variety of neurotransphins and neurotigenoids and, thus, present an ideal in vitro model to study processes involving nerve repair and neuronal differentiation\textsuperscript{17}.

Interestingly, a prevalent stromal SC population is found in usually benign-behaving subtypes of peripheral neuroblastic tumors\textsuperscript{18,19}. Peripheral neuroblastic tumors originate from trunk neural crest-derived sympathetic neuroblasts\textsuperscript{20,21} and are categorized in neuroblastomas (NBs), ganglionneuroblastomas (GNBs), and ganglioneuromas (GNs) that represent a spectrum from NBs, the most aggressive form, to GNs, the most benign form, and GNBs, which exhibit various elements of both\textsuperscript{20,22-24}. NB and GN subtypes are associated with distinct genomic alterations and strikingly different morphologies\textsuperscript{20,22}. In general, NBs consist of un- or mostly poorly differentiated tumor cells and cancer-associated fibroblasts\textsuperscript{25}, whereas GNs are composed of differentiated, ganglionic-like tumor cells scattered within a dominant SC stroma\textsuperscript{19,26}. The content of SC stroma was early recognized as a valuable prognostic factor as it correlates with the degree of tumor cell differentiation and a favorable outcome\textsuperscript{19}. The ganglionic-like tumor cells also extend numerous neuritic processes that form entangled bundles surrounded by ensheathing stromal SCs\textsuperscript{26}. This ganglion-like organoid morphology was assumed to arise from a bi-potent neoplastic neuroblastic precursor cell capable to differentiate along a neuronal and glial lineage\textsuperscript{27}. Hence, an active role of stromal SCs in peripheral neuroblastic tumors has been neglected due to their supposed neoplastic origin.

Of note, we and others provided evidence for a non-tumor background of stromal SCs\textsuperscript{1,28}. In a detailed immunohistochemical study, it was shown that the earliest appearance of stromal SCs is confined to the tumor blood vessels and connective tissue septa and not intermingled within the tumor as a clonal origin would imply\textsuperscript{28}. Furthermore, we demonstrated the absence of numerical chromosomal aberrations in stromal SCs, while adjacent ganglionic-like tumor cells possessed a typical aneuploid genome\textsuperscript{129,36}. These surprising findings argue against the hitherto presumed model of GNB/GN development based on a bi-potent neoplastic cell and support that the tumor cells are able to attract adult SCs from the nervous environment to the tumor.

In detaching the origin of stromal SCs in GNB/GN from a neoplastic cell, we realized how little we know about their nature. What is the cellular state of stromal SCs? How do they affect GNB/GN development? And why are they not manipulated by the tumor cells to support tumor progression but are associated with a benign tumor behavior? We and others have shown that the aggressiveness of NB cell lines, derived from high-risk metastatic NBs, can be reduced upon exposure to SCs and their secreted factors\textsuperscript{31-35}. Accordingly, a mouse study comparing intra- or extra-fascicularly grown tumor xenografts confirmed that NBs within the nervous environment were infiltrated by SCs and developed a less aggressive tumor phenotype\textsuperscript{38}. However, a comprehensive analysis to assess the origin and functional characteristics of stromal SCs in tumors is still missing.

Based on the inherent plasticity of adult SCs and the yet unresolved nature of SC stroma, we speculate that GNB/GN development could be the result of a reactive/adaptive response of SCs to peripheral neuroblastic tumor cells similar to injured nerve cells. Thus, we here compared the cellular state of stromal SCs in GNs to repair SCs in injured nerves by transcriptome profiling of human GN and human injured nerve tissues. Moreover, we analyzed the effect of human primary repair-related SCs and their secreted factors on genetically diverse NB cell lines in co-culture studies and identified a promising candidate factor of therapeutic potential for aggressive NBs and peripheral nerve injuries.

**Results**

**Transcriptome profiling revealed that ganglioneuromas contain stromal Schwann cells with a nerve repair-associated gene expression signature.** To assess the cellular state of stromal SCs, we performed a comprehensive transcriptomic analysis involving human tissues of SC stroma-rich GNs, SC stroma-poor NBs, and repair SC-containing injured nerves, alongside with cultures of primary human repair-related SCs and human NB cell lines (Supplementary Table 1). Immunofluorescence stainings of respective tissue sections for SC marker S100B determined a prevalent SC population of about 84% in injured nerves (Fig. 1a, Supplementary Fig. 1a), and of about 76% in GNs (Fig. 1b) as well as the almost complete absence of SCs in NBs (Fig. 1c, Supplementary Fig. 1a). Co-staining with neurofilament heavy polypeptide (NF200), an intermediate filament protein associated with mature neurons\textsuperscript{37}, marked axons in injured nerves that have mostly disintegrated after the degeneration period of 7 days (Fig. 1a). NF200 also stained ganglionic-like tumor cells with abundant neuritic processes in GNs (Fig. 1b). In line with the un- or poorly-differentiated state of tumor cells in NBs, hardly any NF200 signals were detected in NB tumor samples (Fig. 1c).

Human repair-related SC cultures have been isolated according to our established protocol\textsuperscript{38} and were positive for S100B, and Human repair-related SC-containing injured nerves, alongside with cultures of primary human repair-related SCs and human NB cell lines (Supplementary Fig. 1a). Co-staining with neurofilament heavy polypeptide (NF200), an intermediate filament protein associated with mature neurons\textsuperscript{37}, marked axons in injured nerves that have mostly disintegrated after the degeneration period of 7 days (Fig. 1a). NF200 also stained ganglionic-like tumor cells with abundant neuritic processes in GNs (Fig. 1b). In line with the un- or poorly-differentiated state of tumor cells in NBs, hardly any NF200 signals were detected in NB tumor samples (Fig. 1c). Human repair-related SC cultures have been isolated according to our established protocol\textsuperscript{38} and were positive for S100B, and showed the typical parallel alignment (Fig. 1d). Cultured NB cell lines highly expressed the neuronal ganglioside GD2 (Fig. 1e) that is characteristicly found on tumor cells in NBs (Supplementary Fig. 1b) and only on some ganglionic-like tumor cells in GNs (Supplementary Fig. 1c).

Hierarchical clustering and principal component analysis of obtained RNA-seq data showed that biological samples derived...
from the same tissue or cell type cluster together and that primary SCs and SC-containing tissues, i.e. injured nerves and GNs, differ from NB cell lines and NB tumors (Fig. 1f). To further confirm tissue/cell identity, we validated the expression of genes associated with either NBs, such as the miRNA suppressor LIN28B and the transcription factor MYCN\(^{39,40}\), or the SC lineage, such as S100B and transcription factor SOX10\(^{10}\). Indeed, expression of LIN28B was significantly higher in NBs and NB cell lines, and the MYCN expression level reflected the presence or absence of MYCN amplifications in NB cell lines and tumors (Fig. 1g, Supplementary Table 2&3). Of note, amplification of the MYCN oncogene is associated with an aggressive NB tumor behavior and poor
outcome. The SC specific genes S100B and SOX10 were significantly and strongly expressed in primary SCs, injured nerves, and GNS (Fig. 1h). Immunofluorescence stainings on tissue sections acknowledged that SOX10 positive cell nuclei corresponded to S100B positive repair SCs in injured nerves (Fig. 1i, Supplementary Fig. 2a) and stromal SCs in GNS (Fig. 1j, Supplementary Fig. 2b). Moreover, the elevated level of SOX10 mRNA found in NB-TU 50 could be ascribed to a high proportion of infiltrating stroma containing S100B and SOX10 positive SCs (Fig. 1k, Supplementary Fig. 2c), while the sections analyzed from other NB tumors such as NB-TU 49 lacked S100B and SOX10 positive cells and mRNA (Fig. 1l, Supplementary Fig. 2d).

We next defined the characteristic expression signatures of GNS and injured nerves, that both possessed a predominant SC content (Supplementary Fig. 1a), by selecting for genes significantly up-regulated (q-value > 0.05; |log2FC| >1) in GNS versus NBs, and injured nerves versus NBs. In this way, we excluded genes also present in NBs and enriched for genes characteristic for repair SCs in injured nerves and stromal SCs in GNS. Then, we compared the identified expression signatures associated with stromal SCs and repair SCs, which showed an overlap in 2755 genes (q-value > 0.05; |log2FC| >1) (Fig. 2a). Functional annotation analysis of these stromal/repair SC genes revealed pathways and gene ontology terms that could be grouped into distinct functional competences. Importantly, these functions reflected the main tasks of human repair SCs involving axon guidance, lipid/myelin degradation/metabolism, basement membrane formation/ECM coating and transport, protein transport and binding, as well as acetylation and protein N-linked glycosylation (Supplementary Fig. 6b,c). In turn, genes characteristic for injured nerves not shared with GNS were assigned to gene ontology terms for the endoplasmatic reticulum, the Golgi apparatus, vesicle coating and transport, protein transport and binding, as well as inflammation. The expression signature of HMC-II is not the sole result of tissue resident immune cells, but indeed attributed to repair and stromal SCs, we stained respective tissue sections for HLA-DR and S100B. The images showed that injured nerves and GNS were highly positive for HLA-DR (Supplementary Fig. 3a,b), whereas HLA-DR staining signals were mainly restricted to the stromal portion and only scattered within the tumor cell portion of NBs (Supplementary Fig. 3c,d). Indeed, HLA-DR was expressed by S100B+ repair SCs in injured nerves (Supplementary Fig. 3a) as well as stromal SCs in GNS (Supplementary Fig. 3b) in addition to HLA-DR+/S100B+ immune cells (Supplementary Fig. 3a-d).

A possible repair-associated cell state of stromal SCs should be reflected by key signatures of both, developing/dedifferentiated SCs and repair-specific SCs. Accordingly, the repair/stromal SC enriched gene set included genes characteristic for SCs during development and after injury such as transcription factors JUN, SOX2, ZEB2, and RUNX2 (Supplementary Fig. 4a), and receptors NGFR, ERBB3, GFRα1, and CADH19. Notably, we also detected significant levels of GDNF, LIF, SHH, CLCFL1, BTC, CCL2, and UCN2 (Fig. 2c) that were reported to be exclusively expressed by repair SCs and not by adult or developing SCs[11-13] (Supplementary Fig. 4b). JUN is the key transcription factor determining the repair identity of SCs by up-regulating repair-specific target genes such as SHH and GDNF. Hence, we performed immunofluorescence stainings for SOX10 and JUN on nerve and GNS tissue sections, which confirmed that SOX10+/ JUN+ nuclei of both, repair SCs and stromal SCs were positive for JUN (Fig. 2d-f, Supplementary Fig. 5a,b). In line with the transcriptomic data, JUN was also expressed by SOX10+ cells such as ganglionic-like tumor cells in GNS (Fig. 2g, Supplementary Fig. 5b) and tumor cells in NBs (Fig. 2h, Supplementary Fig. 5c,d).

Functional annotation analysis of GNS characteristic genes that were not shared with injured nerves revealed an enrichment of gene ontology terms implicated in innate immunity, inflammation as well as T- and B-cell receptor signaling pathways (Supplementary Table 5). Immunofluorescence stainings for CD3 and S100B confirmed the presence of CD3+ T-cells within the S100B+ stroma in GNS (Supplementary Fig. 6a), while CD3+ T-cells were only sparsely detected in the tumor cell portion of NBs (Supplementary Fig. 6b,c). In turn, genes characteristic for injured nerves not shared with GNS were assigned to gene ontology terms for the endoplasmatic reticulum, the Golgi apparatus, vesicle coating and transport, protein transport and binding, as well as acetylation and protein N-linked glycosylation (Supplementary Table 6). Those annotations suggest an active protein modification and transport machinery in repair SCs.

Taken together transcriptome profiling demonstrate that the expression signature shared by stromal SCs in GNS and repair SCs in injured nerves contain distinct nerve repair-associated genes and functions. Direct contact to repair-related Schwann cells promotes alignment and neurite out-growth of neuroblastoma cells. Since we identified a repair SC-associated gene expression signature in stromal SCs, we used a co-culture model to analyze how NB cells react to repair-related SCs in vitro (Fig. 3a). Therefore, we used human primary SCs cultures as a model, as these have been shown to reflect all major characteristics of repair SCs. SC cultures (passage 1) characterized by the expression of S100B, SOX10, and the intermediate filament vimentin (VIME) were used for experimentation (Fig. 3b). SCs were co-cultured with a well established human NB cell line (CLB-Ma) and short-term cultured patient-derived NB cells (STA-NB-6) alongside controls of SCs and NB cells cultured alone for 11 days. As a qualitative read-out, we established an immunofluorescence staining panel, which identified NB cells by GD2 expression and SCs by S100B expression. After 11 days, CLB-Ma and STA-NB-6 cell controls showed their typical morphology of clustered cell bodies with short, randomly extended neuritic processes (Fig. 3c,d). However, in the co-cultures with SCs, NB cells had aligned along the bipolar SC extensions and increased the length of neuritic
processes, predominantly in close contact with the SC surface (Fig. 3e,f,g,h arrows). Quantification of neurite length and alignment confirmed a significant increase of the mean neurite length (Fig. 3i) and neurite alignment (Fig. 3j) in co-cultures. These results suggest that the contact to human repair-related SCs induces a directed neuritic out-growth of NB cells in vitro.

Repair-related Schwann cells induce neuronal differentiation of neuroblastoma cells independent of direct cell–cell contact. We next aimed to dissect the effect of repair-related SCs on NB cells and distinguish signaling effects between cell bound and secreted molecules. Therefore, we refined the co-culture setting and used flow cytometry as a quantitative read-out. NB cells were either seeded in
direct contact with SCs or in a trans-well insert placed above SC cultures allowing diffusion of soluble molecules and reciprocal signaling. The refined co-culture set-up is illustrated in Fig. 4a.

In order to functionally validate whether isolated repair-related SCs reenact their key ability of regulating neuronal differentiation on NB cells in vitro, three well-established human NB cell lines (SH-SY5Y, IMR5, CLB-Ma) and two short-term NB cell cultures (STA-NB-6, STA-NB-10) covering the genetic spectrum of NBs, were co-cultured with the neuronal differentiation marker NF200 (gating strategy Supplementary Fig. 7a). We found that NF200 expression was significantly upregulated in the MYCN non-amplified STA-NB-6 and SH-SY5Y NB cells after 16 days of direct contact to repair-related SCs (Fig. 4b). Of note, all NB cell lines, except STA-NB-10, showed a significant increase in NF200 expression at day 16 when co-cultured in the trans-wells without direct contact (Fig. 4b). We also noticed that the presence or absence of MYCN amplification in the analyzed NB cells correlated with their responsiveness to SCs (Fig. 4b). The mean fluorescence intensity histograms of NF200 further revealed that the basal NF200 expression level varied among the analyzed NB cells from low, as in CLB-Ma cells (Fig. 4c, CTRL), to highest in STA-NB-6 cells (Fig. 4d, CTRL). They also demonstrated that the increase in NF200 expression after co-culture was either due to the occurrence of a NF200+ subpopulation, e.g. in CLB-Ma cells (Fig. 4c, co-cultured), or an overall elevated expression, e.g. in STA-NB-6 cells (Fig. 4d, co-cultured). These findings were confirmed by qualitative assessment of NF200 expression by immunofluorescence stainings of co-cultures compared to controls of CLB-Ma cells (Fig. 4e,f) and STA-NB-6 cells (Fig. 4g,h).

To analyze whether the increase in neuronal differentiation is SC specific, we co-cultured STA-NB-6 and SH-SY5Y cells, which showed the strongest response to SCs, with immortalized human fibroblasts (iFBs) and cancer associated FBS (CAFAs). After 16 days, the NF200 expression of both NB cell cultures was either unaffected or even significantly decreased upon direct and indirect contact with iFBs (Supplementary Fig. 8a) or CAFAs (Supplementary Fig. 8b).

Taken together, the results demonstrate that primary repair-related SCs and/or their secreted factors are sufficient to induce neuronal differentiation of aggressive NB cell lines and primary NB cultures in vitro.

### Repair-related Schwann cells impair proliferation and increase apoptosis of neuroblastoma cells.

As cellular differentiation is accompanied by cell cycle arrest, we next determined the proliferation rate of NB cells by EdU incorporation in combination with DNA content analysis after direct and trans-well co-culture with SCs (gating strategy Supplementary Fig. 7b). Notably, after 16 days of direct SC contact the number of NB cells in the S-phase was strongly reduced in all tested NB cell cultures (Fig. 5a). The proliferation rate of trans-well co-cultures was also significantly decreased in all NB cells, except STA-NB-10, but less pronounced as upon direct contact (Fig. 5a). The strongest anti-proliferative effects were detected in MYCN non-amplified STA-NB-6 and SH-SY5Y cells, as well as MYCN amplified IMR5 cells (Fig. 5a). Representative FACS plots illustrated the reduction of proliferation in CLB-Ma cells (Fig. 5b) and almost absent proliferation in STA-NB-6 cells (Fig. 5c) after 16 days of co-culture. This was also visualized by immunofluorescence stainings of co-cultures compared to controls of CLB-Ma cells (Fig. 5d,e) and STA-NB-6 cells (Fig. 5f,g) including the proliferation marker Ki67. In contrast, direct or indirect co-cultures with iFBs and CAFAs did not influence the proliferation rate of STA-NB-6 and SHSY5Y cells (Supplementary Fig. 7c,d).

In addition to increased differentiation and impaired proliferation, also cell death contributes to the decrease of tumor cells during GN development. Hence, we performed a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in combination with immunofluorescence staining for GD2 and S100B to detect apoptotic NB cells in control and co-cultures (Supplementary Fig. 9a,b). Quantitative evaluation showed that the apoptosis rate of both, MYCN non-amplified STA-NB-6 and MYCN amplified CLB-Ma cells, was increased about 10% at day 11 after direct co-culture (Supplementary Fig. 9c).

These findings show that direct and/or indirect contact to repair-related SCs decreased proliferation and elevated apoptosis of NB cells. As observed for neuronal differentiation, the MYCN amplification status correlated with the responsiveness of NB cells to SCs and revealed STA-NB-6 as the strongest and STA-NB-10 as the weakest SC-responsive NB cell cultures tested.
regeneration but with yet unknown function.\textsuperscript{17} Other neurotrophic factor transcripts, such as PTN, highly expressed in stromal but not in repair SCs, and CNTF, expressed in repair but not in stromal SCs, were included in the panel of candidate factors as transcripts of their putative receptors were present in NBs (Supplementary Fig. 10).

In order to validate the effect of a set of 8 candidate factors, the recombinant proteins NGF, BDNF, GDNF, CNTF, PTN, FGF7, IBP6 and EGFL8 were added to the SC-weakly-responsive STA-NB-10 and SC-strongly-responsive STA-NB-6 cells. Proliferation and neuronal differentiation were monitored by flow cytometry after 16 days of exposure to respective factors. As suspected, the factors had less impact on the SC-weakly-responsive STA-NB-10 cells, however, NGF and EGFL8 caused a significant anti-proliferative effect (Fig. 6b). In contrast, the SC-strongly-responsive STA-NB-6 cells were significantly impaired in
proliferation and showed increased neuronal differentiation after treatment with either NGF, EGFL8, BDNF, CNTF, PTN or GDNF (Fig. 6c). Notably, the effect of EGFL8 was concentration dependent and comparable to NGF, one of the most potent neurotrophins known so far (Fig. 6d). EGFL8 also acted pro-differentiating on CLB-Ma and SH-SY5Y cells, while an anti-proliferative effect was only observed in the latter (Fig. 6e). Phase contrast images illustrated the reduction of cell number and longer neuritic processes (Fig. 6f). Compared to untreated controls, STA-NB-6 cells showed a significant increase of neurite length after NGF- and EGFL8-treatment (Fig. 6g).

These findings demonstrate that EGFL8, a protein so far only described in thymocyte development, has a neurotrophic function able to enhance neuronal differentiation and/or to impair proliferation of aggressive NB cells.

**EGFL8 gene expression level in neuroblastomas correlates with increased patient survival.** As EGFL8 exerted anti-tumor activity on NB cells in vitro, we next assessed whether EGFL8 expression levels in peripheral neuroblastic tumors may correlate with the clinical outcome. Analysis of the overall patient survival according to EGFL8 gene expression was performed using the R2: Genomics Analysis and Visualization platform. Two different datasets, comprising 649 and 283 tumor specimens, respectively, demonstrated an over 90% and 70%, respectively, 5-year overall survival probability for patients with high EGFL8 expression, but less than 60% and 40%, respectively, for patients with low EGFL8 expression (Fig. 6h & Supplementary Fig. 11 a,b). Information about the stromal SC content of the included tumor specimens was not available. These data show that EGFL8 expression correlates with increased patient survival, which could be due to its neurotrophic effect on peripheral neuroblastic tumor cells.

The EGFL8 protein is significantly elevated in ganglioneuromas compared to neuroblastomas and expressed by repair Schwann cells and stromal Schwann cells. To verify whether the high EGFL8 gene expression detected in GNs is reflected by the EGFL8 protein level, we performed high-resolution mass spectrometry analysis of SC stroma-rich GNs, SC stroma-poor NBs, as well as primary NB cultures and evaluated this data set together with our existing proteomic data set comprising repair SC-containing injured nerve tissue as well as primary repair-related SC cultures. The results demonstrated a significantly higher abundance of the EGFL8 protein in injured nerves and GNs when compared to NBs (Fig. 7a). In addition, the protein levels of EGFL8 in primary cells matched their respective tissue of origin (Fig. 7a). This was confirmed by immunostaining illustrating SOX10 and EGFL8 co-expression by repair SCs in injured nerve tissue (Fig. 7b) and stromal SCs in GNs (Fig. 7c), while EGFL8 was absent on tumor cells in GN and NB primary tumors (Fig. 7d,e). The images further showed that EGFL8 was also expressed in S100+ cells, e.g. in the perineurium and blood vessel-like structures (Fig. 7a,b).

Hence, mass spectrometric analyses and immunostaining confirmed that the EGFL8 protein is highly abundant in stromal SCs in GNs and repair SC in injured nerve tissues as well as primary repair-related SC cultures.

**EGFL8 protein is secreted by repair-related Schwann cells and rewires kinase-mediated signaling in neuroblastoma cells in vitro.** As EGFL8 is a predicted secreted factor and recombinant EGFL8 was able to induce neuronal differentiation, we next determined whether SC-produced EGFL8 is indeed secreted and investigated its mode-of-action in NB cells. First, we co-stained primary repair-related SC cultures for EGFL8 and membranous nerve growth factor receptor (NGFR), a marker associated with immature/repair SCs. EGFL8 showed an intracellular staining pattern with accumulation of positive signals in clusters of different sizes (Fig. 7f). 3D analysis illustrated EGFL8 positive vesicular structures embedded within the cytoplasm beneath the NGFR+ SC membrane (Supplementary Movie 1). In addition, we performed WB analysis for EGFL8 on cell lysates of human primary SCs, STA-NB-6 and SH-SY5Y cells, and conditioned culture medium (supernatants) of respective cultures (Fig. 7g, Supplementary Fig. 12). A GST-tagged recombinant EGFL8 protein was used as positive control. EGFL8 has an expected mass of 32 kDa, accordingly, the antibody detected the GST-tagged (GST corresponding to 26 kDa) recombinant EGFL8 protein at around 58 kDa in positive controls. In all four SC whole cell lysates, two bands were visible at around 32 and 37 kDa and three SC samples showed an additional band at around 55 kDa. In three out of four analyzed SC supernatants, prominent bands were detected at 37 kDa, which could indicate that the secreted EGFL8 protein underwent posttranslational modifications.

Second, we addressed the down-stream signaling of EGFL8 in NB primary cultures. As no data currently exist on EGFL8 receptor or signaling in human or any other mammalian cells, we employed an unbiased global- and phospho-proteomics approach in EGFL8-responsive STA-NB-6 versus non-responsive STA-NB-10 NB cells in a time-resolved manner. In total we identified 6385 and 6122 proteins expressed by STA-NB-6 and STA-NB-10 cells, respectively (Supplementary data 1). 6.408 and 6.133 sites were found phosphorylated corresponding to 1.851 and 1.820 proteins in proteins expressed by STA-NB-6 and STA-NB-10 cells, respectively (Supplementary data 1). 6.408 and 6.133 sites were found phosphorylated corresponding to 1.851 and 1.820 proteins in STA-NB-6 and STA-NB-10, respectively (Supplementary Data 2). While STA-NB-6 showed a clear trajectory in the phospho-correlation network (Supplementary Fig. 11a), a more diffuse dynamics was observed in STA-NB-10 (Supplementary Fig. 11a). As the most pronounced change was evident after 15 min, we focused on this time point and performed kinase enrichment analysis (KSEA), revealing a significant activation (enrichment z-score ≥ 1, p ≤ 0.05, substrate cutoff ≥ 3) of 11 kinases in STA-NB-6 and 18 kinases in STA-NB-10 (Fig. 7h,
**Fig. 4** Neuronal differentiation analysis of neuroblastoma cell lines in response to repair-related SCs in vitro.

(a) Refined SC/NB cell co-culture set up for FACS analysis. Three NB cell lines and two NB cell short-term cultures were co-cultured with primary repair-related SCs and NF200 expression levels were analyzed by flow cytometry (FACS) and immunofluorescence (IF). Bar diagrams show the normalized mean fluorescence intensity (MFI) of NF200 ± SD in GD2+/S100B- NB cells upon direct co-cultures (STA-NB-6, p = 0.033; SH-SY5Y, p = 0.363; CLB-Ma, p = 0.048; IMR5, p = 0.146; STA-NB-10, p = 0.666) and trans-well co-cultures (STA-NB-6, p = 0.042; SH-SY5Y, p = 0.816; CLB-Ma, p = 0.331; IMR5, p = 0.331; STA-NB-10, p = 0.988) with SCs at day 8 as well as direct co-cultures (STA-NB-6, p = 0.001; SH-SY5Y, p = 0.049; CLB-Ma, p = 0.132; IMR5, p = 0.762; STA-NB-10, p = 0.713) and trans-well co-cultures (STA-NB-6, p = 0.017; SH-SY5Y, p = 0.041; CLB-Ma, p = 0.023; IMR5, p = 0.050; STA-NB-10, p = 0.242) with SCs at day 16. STA-NB-6: day 8 direct co-culture n = 7, trans-well n = 4, day 16 direct co-culture n = 5, trans-well n = 4; SH-SY5Y: n = 4; CLB-Ma: n = 5; IMR5: n = 3; STA-NB-10: direct co-culture n = 4; trans-well n = 3. A paired two-tailed Student’s t-test comparing against the control was performed. n refers to the number of independent experiments; * p ≤ 0.05; ** p ≤ 0.01; *** p ≤ 0.001; n.s. not significant. Representative FACS histograms show the unstained controls (light grey) and the MFI of NF200 in control and co-cultured (e) CLB-Ma and (d) STA-NB-6 cells (dark grey) at day 16. FACS gating strategy is detailed in Supplementary Fig. 7a. Representative IF images of co-cultured CLB-Ma cells (e) and STA-NB-6 (g) cells stained for NF200, S100B, GD2 and DAPI at day 11 of direct co-culture and respective NB cell controls stained for GD2, NF200, and DAPI (f, h); arrows indicate long neuritic processes of NB cells strongly positive for NF200 in co-cultures. Stainings were performed on NB cell controls and corresponding co-cultures with SCs derived from three independent donors.
Kinases activated in STA-NB-6 and counter- or not regulated in STA-NB-10, such as HIPK1, p38β/MAPK11, ERK5/MAPK7, SGK1 and TLK2, and their substrates, e.g. PML, PAK2 or NDRG2, present key components of the EGFL8-induced signaling network (Fig. 7h, Supplementary Data 3 and 4, Supplementary Fig. 13-15).

In line with the predicted secretion of EGFL8, we here show that EGFL8 is present in vesicular structures within the cytoplasm and released in the medium of cultured human repair-related SCs. Further, we demonstrate that EGFL8 addition leads to a rapid (within 15 min) and specific phosphorylation of substrates of e.g. HIPK1, p38β/MAPK11, ERK5/MAPK7 only in EGFL8-responsive STA-NB-6, but not in the non-responsive STA-NB-10 short-term NB cell cultures, providing evidence for dynamic changes in the kinome associated with neuronal differentiation triggered by EGFL8.

Discussion
This study presents a comparative analysis of human repair SCs in injured nerves and stromal SCs in GNs that builds upon previous efforts to delineate the role of SCs in nerve regeneration and the tumor microenvironment. By investigating human tissues and primary cultures with deep RNA-sequencing, high-resolution imaging, and functional assays, we have identified key signaling pathways and putative secreted factors that are differentially regulated in response to injury and differentiation stimuli. These findings have implications for understanding the cellular and molecular mechanisms driving nerve repair and regeneration.

Supplementary Data 3, Supplementary Fig. 15). Kinases activated in STA-NB-6 and counter- or not regulated in STA-NB-10, such as HIPK1, p38β/MAPK11, ERK5/MAPK7, SGK1 and TLK2, and their substrates, e.g. PML, PAK2 or NDRG2, present key components of the EGFL8-induced signaling network (Fig. 7h, Supplementary Data 3 and 4, Supplementary Fig. 13-15).
resolution mass spectrometry, and confocal imaging, we reveal a similar cellular state and functional competences of repair SCs and stromal SCs. Our comprehensive approach identified EGFL8 as a neuritogenic factor expressed by repair SCs and stromal SCs, which highlights matricellular proteins as tissue active components involved in regenerative and pathological responses of SCs in the peripheral nervous system. Focusing on the interaction of tumor cells and SCs, we developed a co-culture model combined with a flow cytometry-based read-out demonstrating that NB cells react to repair-related SCs in a similar fashion as peripheral neurons upon injury. Moreover, the established co-culture model is broadly applicable and contributes to the ongoing research in
Fig. 6 Neuronal differentiation and anti-proliferative effects of secreted factors shared by stromal and repair SCs. a Expression levels of chosen candidate factors NGF, EGFL8, BDNF, GDNF, IGFBP6, FGF7, CNTF and PTN shown for primary repair-related SCs (SC, n = 5 biological replicates from 4 donors), repair SC rich injured nerve fascicle tissue (SC-IN, n = 3 biological replicates), SC stroma rich GN tissue (SC-GN, n = 6 biological replicates), NB tissue (NB-TU, n = 15 biological replicates) and NB short-term cell cultures (NB-CL, n = 5 biological replicates from 3 donors). Empty symbols indicate MYCN non-amplified NB-TUs and NB-CLs. Data are depicted as mean ± SD; ** p < 0.01, * p < 0.05. b–e FACS analyses of neuronal differentiation (NF200 MFI) and proliferation (EdU incorporation) of NB cells treated with recombinant candidate proteins compared to untreated NB cell controls (CTRL) after 16 days of culture; data are shown as normalized mean values ± SD; n refers to the number of independent experiments; * p ≤ 0.05. Proliferation levels (green) of (b) SC-responsive weak-repair SC-NB-10 exposed to recombinant candidate proteins NGF (p = 0.107), EGFL8 (p = 0.042), BDNF (p = 0.636), CNTF (p = 0.390), PTN (p = 0.026), GDNF (p = 0.671), FGF7 (p = 0.128), and IBP6 (p = 0.486) compared to CTRLs (all n = 4) and (c) SC-strong responsive STA-NB-6 exposed to recombinant candidate proteins NGF (p = 0.009), EGFL8 (p = 0.030), BDNF (p = 0.197), CNTF (p = 0.099), PTN (p = 0.033), GDNF (p = 0.042), FGF7 (p = 0.935), and IBP6 (p = 0.082) compared to CTRLs (all n = 7, except EGFL8 n = 4) at concentrations as indicated. Indications levels (magenta) of (b) SC-responsive weak-repair SC-NB-10 exposed to recombinant candidate proteins NGF (p = 0.567), EGFL8 (p = 0.099), BDNF (p = 0.252), CNTF (p = 0.783), PTN (0.153), GDNF (p = 0.335), FGF7 (p = 0.934), and IBP6 (p = 0.926) compared to CTRLs (all n = 4) and (c) SC-strong responsive STA-NB-6 exposed to recombinant candidate proteins NGF (p = 0.222), EGFL8 (p = 0.049), BDNF (p = 0.050), CNTF (p = 0.014), PTN (p = 0.025), GDNF (p = 0.100), FGF7 (p = 0.015), and IBP6 (p = 0.232) compared to CTRLs (all n = 8, except EGFL8 n = 5). d Proliferation levels (green) of STA-NB-6 cells exposed to 25 ng/ml EGFL8 (n = 3, p = 0.978), 50 ng/ml EGFL8 (n = 3, p = 0.196), and 100 ng/ml EGFL8 (n = 4, p = 0.030) compared to CTRLs (n = 4). Differentiation levels (magenta) of STA-NB-6 cells exposed to 25 ng/ml EGFL8 (n = 4, p = 0.087), 50 ng/ml EGFL8 (n = 3, p = 0.106), and 100 ng/ml EGFL8 (n = 5, p = 0.049) compared to CTRLs (n = 5). e–k Statistical test: One way ANOVA or mixed effects model and adjustments for multiple testing was performed. e Proliferation (green) and differentiation (magenta) levels of SH-SY5Y cells after treatment with 100 ng/ml EGFL8 (proliferation: p = 0.059, differentiation: p = 0.050) compared to CTRLs (both n = 4, paired, two-tailed Student’s t-test) and CLB-Ma cells after treatment with 100 ng/ml EGFL8 (proliferation: p = 0.116, differentiation: p = 0.046) compared to CTRLs (both n = 5, paired, two-tailed Student’s t-test); f Representative bright field images of STA-NB-6 cells at day 16 cultured in the absence (CTRL) or presence of 100 ng/ml EGFL8 or 20 ng/ml NGF (n = 3). Enlargements illustrate the neuritic processes of STA-NB-6 cells in CTRL as well as EGFL8- and NGF-treated cultures. Data are depicted as mean ± SD; *** p < 0.001, ** p < 0.01, * p ≤ 0.05. g Quantiﬁcation of neurite length (STA-NB-6 cells treated with 20 ng/ml NGF (p = 0.004) or 100 ng/ml EGFL8 (0.006) compared to untreated CTRLs. Data are depicted as mean normalized neurite length ± SD (n = 6 images per treatment over 3 independent biological replicates); Statistical test: repeated measures ANOVA and Dunnett’s multiple comparison test; *p-value ≤ 0.05. h Kaplan-Meier survival plot show the overall survival (OS) probability of patients grouped according to high and low EGFL8 expression in primary tumors at diagnosis. Data were derived from the Kocak dataset (GSE45547) of the R2 Genomics Analysis and Visualization platform (https://r2.amc.nl) (see also Supplementary Fig. 11).
aggressive high-risk NBs, to human primary repair-related SCs. Both, the direct contact to SCs and the in-direct contact to the SCs’ secretome, were sufficient to induce neuronal differentiation and to impair proliferation of NB cells. Of note, this anti-tumor effect could be replicated by replacing SCs with recombinant neurotrophic factors discovered within the repair/stromal SC secretome.

In addition to their influence on neuroblastic tumor cells, stromal SCs also hold a considerable potential to modulate the tumor microenvironment. We found that stromal SCs express MHC-II, which is in line with other studies that reported the capacity of SCs to express MHC-II in (auto-) inflammatory or infectious neuropathies. We also discovered that stromal SCs express potent chemokines and confirmed the presence of macrophages and T-cells in GNs, which is in accordance with the increasing reports about the immunomodulatory potential of SCs. Furthermore, the shared expression signature of...
stromal/repair SCs contained basement membrane components and ECM remodelers such as metalloproteinases and matricellular proteins. Stromal SCs could therefore recruit and interact with immune cells, as well as execute tissue remodeling functions in the tumor environment with the original goal to rebuild an organized nerve structure similar to repair SCs upon nerve injury.

Taken together, the nerve repair-like phenotype equips stromal SCs with different strategies to influence their environment. Stromal SCs could either directly induce neuronal differentiation of peripheral neuroblastoma tumor cells or indirectly manipulate the tumor microenvironment via immunomodulation and ECM remodeling responsible for a favorable tumor development.

Here, we introduce the matricellular protein EGFL8 as neuritogen. EGFL8 shares similar domains and molecular weight with EGFL7, which was described to induce neural stem cell differentiation. We demonstrated high expression of the EGFL8 protein by repair SCs and stromal SCs and its secretion by repair-related SCs in vitro. Moreover, EGFL8 expression in peripheral neuroblastoma tumors correlated with an increased patient survival. We provide evidence for a neurotogenic function of human EGFL8, a protein of so far unknown function, as its recombinant form was sufficient to induce neuronal differentiation of NB cells at similar efficiency as NGF. Further, our comprehensive map of the activated kinome at baseline and upon EGFL8 stimulation delineates the down-stream signaling dynamics in NB cells. EGFL8 addition leads to specific phosphorylation of HIPK1, p38/MAPK- and ERK5/MAPK7-substrates only in the sensitive cell line STA-NB-6, but not in the insensitive STA-NB-10. While ERK and MAPK are well established key nodes transmitting neurotrophic/neuritogenic signals, HIPK1, SGK1 and TLR2 have not been implicated in peripheral neuronal differentiation yet. Interestingly, the wiring of cellular signaling by EGFL8 converged at known regulators of neurogenesis, such as PML, NDRG2 and PAK2, corroborating the role of EGFL8 as neuritogenic factor. It will be interesting to elaborate the common and unique roles of EGFL8 in the concert of neurotrophic factors.

The discovery of EGFL8 as neuritogen underlines the plasticity of adult SCs in the repair state. EGFL8 shares similar domains and molecular weight with EGFL7, which was described to induce neural stem cell differentiation. We demonstrated high expression of the EGFL8 protein by repair SCs and stromal SCs and its secretion by repair-related SCs in vitro. Moreover, EGFL8 expression in peripheral neuroblastoma tumors correlated with an increased patient survival. We provide evidence for a neurotogenic function of human EGFL8, a protein of so far unknown function, as its recombinant form was sufficient to induce neuronal differentiation of NB cells at similar efficiency as NGF. Further, our comprehensive map of the activated kinome at baseline and upon EGFL8 stimulation delineates the down-stream signaling dynamics in NB cells. EGFL8 addition leads to specific phosphorylation of HIPK1, p38/MAPK- and ERK5/MAPK7-substrates only in the sensitive cell line STA-NB-6, but not in the insensitive STA-NB-10. While ERK and MAPK are well established key nodes transmitting neurotrophic/neuritogenic signals, HIPK1, SGK1 and TLR2 have not been implicated in peripheral neuronal differentiation yet. Interestingly, the wiring of cellular signaling by EGFL8 converged at known regulators of neurogenesis, such as PML, NDRG2 and PAK2, corroborating the role of EGFL8 as neuritogenic factor. It will be interesting to elaborate the common and unique roles of EGFL8 in the concert of neurotrophic factors.

The discovery of EGFL8 as neuritogen underlines the increasingly recognized impact of matricellular proteins in injury response and pathological conditions. Stromal SCs and repair SCs also shared the expression of other matricellular proteins such as SPARC, SPP1 (osteopontin) and CCN3 (NOV). Notably, SC stroma-derived SPARC was previously reported to suppress NB progression by inhibiting angiogenesis and introducing changes in the ECM composition, suggesting that stromal/repair SCs are a source of various matricellular proteins that foster neuronal differentiation.

The plastic potential of adult SCs is a double-edged sword. While essential for nerve repair, recent studies point out its adverse effect in neuropathies and epithelial cancer progression. Here, we demonstrate a favorable impact of SC plasticity on peripheral neuroblastoma tumor cells as it manifests in SC stroma during the development of benignly behaving GNB/GN. The cellular similarities between stromal SCs and repair SCs suggest that stromal SCs are able to exert nerve repair-associated functions in the tumor microenvironment. Exploiting the strategies repair/stromal SCs use to generate a neuronal (re-)differentiation supporting environment could therefore hold a valuable therapeutic potential.

The prerequisite for a possible treatment approach is the susceptibility of aggressive NBs to SCs. We and others have previously investigated the effect of SCs and their secreted factors on aggressive NB cell lines. These studies confirmed that SCs are able to induce neuronal differentiation and impair the growth of NB cells, which were derived from SC-stroma poor high-risk NBs. The confirmation that aggressive NB cells, although lacking the ability to attract SCs, are still responsive to SCs, offers essentially two therapeutic options. 1) including SC-derived factors as anti-tumor agents and 2) the induction of NB stroma in aggressive NBs. Furthermore, identifying how the repair SC state can be sustained is also of high value for the field of regenerative medicine, since one of the main reasons for axonal regeneration failure after injury is the deterioration of repair SCs over time. Thus, the more detailed knowledge about the molecular processes involved in GNB/GN development and nerve regeneration is promising to enrich treatment approaches for both nerve repair and aggressive NBs. In conclusion, our study demonstrates that the cellular state of stromal SCs in GNS shares key features with repair SCs in injured nerves. This finding provides essential insight into GNB/GN development as it suggests that the inherent plasticity allows adult SCs to react to peripheral neuroblastoma tumor cells in a similar way as to injured neurons. As a consequence, stromal SCs could exert repair-associated functions that shape an anti-tumor
microenvironment and induce neuronal differentiation of tumor cells responsible for a benign tumor behavior. Among the factors released by SCs, we identified the matricellular protein EGFL8 and report its neurotrophic effect on neuroblastic tumor cells. EGFL8 mediated neuronal differentiation through broad kinase activation including and beyond p38/ MAPK and ERK signaling, might hold considerable treatment possibilities for the therapy of aggressive NBs and patho-physiological conditions compromising peripheral nerve integrity.

Methods

Human material. The collection and research use of human peripheral nerve tissue from human tumor specimens was conducted according to the guidelines of the Council for International Organizations of Medical Sciences (CIOMS) and World Health Organisation (WHO) and has been approved by the Ethikkommission Medizinische Universität Wien (EK2281/2016 and 1216/2018). Informed consent has been obtained from all patients or parents/guardians/legally authorized representatives in this study, in accordance with the informed consent for the surgery for reconstructive surgery or amputations or research use of left over materials from medically necessary procedures participating in this study. The consent for the CCRI Biobank covers the use of left over materials from medically necessary procedures. This material, which, after completion of routine diagnostic procedures, is biobanked (EK1853/2016) and available for research purposes, including genetic analysis, that are further specified in EK1216/2018: to conduct genetic, proteomic, imaging analysis and cell cultivation.

Neuroblastoma cell lines and primary cultures are available upon request. Primary Schwann cell cultures and tumor tissues are limited materials and therefore cannot be provided.

Human peripheral nerve explants and primary Schwann cell cultures. Human peripheral nerves were collected during reconstructive surgery, amputations or tumor resection. For the ex vivo degeneration period of 8 days in SCEM (MMEM, GIBCO) and αMEM + 1% (g-e), 150 U/ml Dispase II, 0.125% Collagenase type IV and 3 mM Mucin. The fascicles were prepared from nerve explants and digested overnight using 1.25 U/ml pepsin and 100 U/ml collagenase on ice. The digested peripheral nerve tissue covers the space filled with materials from medically necessary procedures. The collection and research use of human peripheral nerve tissue was conducted according to the guidelines of the Council for International Organizations of Medical Sciences (CIOMS) and World Health Organisation (WHO) and has been approved by the Ethikkommission Medizinische Universität Wien (EK2281/2016 and 1216/2018); the informed consent for the CCRI Biobank covers the use of left over materials from medically necessary procedures participating in this study. The consent for the CCRI Biobank covers the use of left over materials from medically necessary procedures. This material, which, after completion of routine diagnostic procedures, is biobanked (EK1853/2016) and available for research purposes, including genetic analysis, that are further specified in EK1216/2018: to conduct genetic, proteomic, imaging analysis and cell cultivation. To generate cell cultures containing 95% purity, as determined via immunostaining analyses of cryosections using a Zeiss Axiovert 40C with the pixelink application version AL/A6XX.

Neuroblastoma/ganglioneuroma tissue, neuroblastoma cell lines and patient-derived short-term cultures. Tumor specimen from diagnostic NB tumors and GN tumors have been collected during surgery or biopsy for diagnostic purposes and left-overs were cryopreserved until analysis. Cryosections of GN tissue were analyzed for SC stroma rich areas identified by H–E-staining, immunofluorescence staining for SC marker S100B, and confirmed by a pathologist. The corresponding tumor region was excised using a scalpel and cryopreserved until RNA and protein extraction. The immunostaining analyses of cryosections were performed on three independent specimen per analyzed tissue, e.g. GN cryosections derived from three patients were stained for S100B, SOX10 and DAPI. The SCs and NB cell lines are derived from biopsies or surgical resection of aggressively behaving NB tumors of patients suffering from high-risk metastatic NBs. In-house established, short-term cultured primary NB cells STA-NB-6, STA-NB-10, IMRS, and CLB-Ma were cultured in MEM GlutaMAX™, 1% αMEM and 1% m-sodium pyruvate, 10 ng/mL fetal calf serum (FCS) and 1% human heat inactivated serum (HIS) (ROTH). The cell proliferation was measured using a Cell Titer Glo assay (Promega). Samples were then incubated with appropriate secondary antibodies for 1h. Samples were then again washed, and incubated with appropriate secondary antibodies for 1h. Samples were then incubated with appropriate secondary antibodies for 1h. Samples were then incubated with primary antibodies against extracellular targets, washed and incubated with appropriate secondary antibodies for 1h. Samples were then incubated with primary antibodies against extracellular targets, washed and incubated with appropriate secondary antibodies for 1h. Samples were then incubated with primary antibodies against extracellular targets, washed and incubated with appropriate secondary antibodies for 1h. Samples were then incubated with primary antibodies against extracellular targets, washed and incubated with appropriate secondary antibodies for 1h. Finally, samples were incubated with 2 μg/mL DAPI in 1x PBS for 2 min, washed and embedded in Fluoromount-G mounting medium (SouthernBiotech). Images were acquired with a confocal laser scanning microscope (Leica Microsystems, TCS SP8X) using Leica application suite X version 1.8.1.13759 or the LAS AF Lite version 4.0 software (Leica). Confocal images are shown as maximum projection of total z-stacks and brightness and contrast are adjusted in a homogeneous manner using the Leica LAS AF software (Leica Microsystems).

Quantiﬁcation of neurite length and alignment. The ImageJ plugin NeuronXML was used to quantify the mean length of extended neurites by NB cells either on phase contrast images or immunofluorescence images between treated NB cells (co-culture with SCs, or exposure to EGFL8) and untreated NB cell controls (n = 3); at least two images were analyzed per condition. To evaluate the orientation (alignment) of NB cells after co-culture with SCs compared to NB cell cultured alone (n = 3), three GD2 stained immunofluorescence images per condition were analyzed with the ImageJ plugin Orientation/Measure function [http://bigwww.epfl.ch/demo/orientation/], that calculates a distribution of pixels orientations (varying from -90 to 90 degrees) per image. In order to merge information of all images, the calculated distributions of orientations were mean-normalized resulting in a mean cell orientation of 0 degrees. To obtain a measure distinguishing the NB cell alignment between control and co-cultures, the

Proliferation and differentiation FACS panels. All antibody details are listed in Supplementary Table 3. If not stated otherwise, all steps of the staining procedures were performed on ice. The following antibodies have been conjugated to fluorochromes using commercially available kits according to the manufacturer’s instructions: anti-S100B has been conjugated to FITC (FLUKA) using Iliustra NHS conjugate kit (GE Healthcare) and anti-D2 (ch14-18, kindly provided by Professor Rupert Handgretinger, Department of Hematology/Oncology, Children’s University Hospital, Tübingen, Germany) has been conjugated to AF546 using the AlexaFluor® 546 protein labeling kit (Molecular probes) and anti-NF200 has been conjugated to AF647 using the AlexaFluor® 647 protein labeling kit (Molecular probes).

Untreated SC and NB cell cultures, co-cultures or NB cultures stimulated with recombinant neurotrophic factors (Supplementary Table 3) were detached using Accutase (LifeTechnologies) and washed with FACS-buffer (1x PBS containing 0.1% BSA and 0.02% EDTA). For the differentiation FACS panels, cultures were incubated with GD2-AF468 for 6 days at 4 °C washed, and blocked with Cytofix/Cytoperm (BD Biosciences) in the dark for 20 min. After washing with 1x perm/wash (BD), cells were stained with anti-S100B-FITC and NF200-AF647 for 20 min. Cells were washed in 1x perf/wash and analyzed immediately at the Beckman Coulter flow cytometer equipped with flow cytometry equipped with 5 lasers (535, 408, 488, 561 and 640 nm) and the FACSDivisio and FACSFortessa.

Immunofluorescence staining and confocal image analysis. All antibody details are listed in Supplementary Table 3. If not stated otherwise, the staining procedure was performed on RT and each washing step involved three washes with 1x PBS for 5 min. Primary antibodies against extracellular targets were diluted in 1x PBS containing 1% BSA and 1% serum; primary antibodies against intracellular targets were diluted in 1xPBS containing 1% BSA, 0.1% TritonX-100 and 1% serum. Briefly, thawed tissue cryosections or frozen SC/NB cell co-cultures were fixed with Roti-Histo Fix 4% (ROTH) for 1h at 4 °C, washed, and blocked with 1x PBS containing 1% BSA and 3% serum for 30 min. Cells and tissue sections were incubated with primary antibodies against extracellular targets, washed and incubated with appropriate secondary antibodies for 1h. Samples were then again fixed with Roti-Histo Fix 4% for 10 min. After washing, cells were permeabilized and imaged with the Leica TCS SP8X (Leica Microsystems, TCS SP8X) with the appropriate secondary antibodies for 1h. Samples were then again fixed with Roti-Histo Fix 4% for 10 min. After washing, cells were permeabilized and imaged with the Leica TCS SP8X (Leica Microsystems, TCS SP8X) with the appropriate secondary antibodies for 1h. Staining was performed with 1 μM DAPI for 20 min, washed and embedded in Fluoromount-G mounting medium (SouthernBiotech). Images were acquired with a confocal laser scanning microscope (Leica Microsystems, TCS SP8X) using Leica application suite X version 1.8.1.13759 or the LAS AF Lite version 4.0 software (Leica). Confocal images are shown as maximum projection of total z-stacks and brightness and contrast are adjusted in a homogeneous manner using the Leica LAS AF software (Leica Microsystems).
variability of the merged distributions was calculated (zero variability would reflect a perfect alignment). For each pair of measurements (control and co-culture), a t-test was performed. Values were given as means ± SD of at least 3 biological replicates. Statistical analyses were performed using GraphPad Prism8 and conventional methods. For the interpretation of phosphoproteomics data, a kinase-substrate enrichment analysis of class I phospho-Sites (p > 0.75 utilizing PhosphositePlus and NetworKIN was performed, applying a NetworKIN score cutoff of 2, p-value cutoff of 0.05 and substrate count cutoff 3×10^4. For the integration of phosphoproteomics data, a kinase-substrate enrichment analysis of class I phosphosites (p > 0.75 utilizing PhosphositePlus and NetworKIN was performed, applying a NetworKIN score cutoff of 2, p-value cutoff of 0.05 and substrate count cutoff 3×10^4. For the visualization of enriched kinases in context of the global kinome, the application Coral was used62.

Statistical analyses. If not mentioned otherwise, Excel 2016 and GraphPad Prism 8 was used for statistical analysis. Values were given as means ± SD of at least 3 independent biological samples or independent biological replicates. For paired analyses a Student’s t-test, for parametric analysis of multiple conditions one-way ANOVA and Tukey’s multiple comparisons post-hoc test was performed. p-values ≤ 0.05 were considered significant.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. All data sets produced and used in this study are available in public repositories as listed in Supplementary Table 4. RNA-sequencing datasets were uploaded to the gene expression omnibus (GEO) repository (https://www.ncbi.nlm.nih.gov/geo/) with the dataset identifiers GSE96711, GSE94035, GSE147635, the Kocab dataset GSE45547 and NRC dataset GSE85047 are publicly available. The mass spectrometry global and phospho-proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org) via the PRIDE partner repository106 with the dataset identifiers PXD019267 and PXD022217 and are publicly available. Source data are provided with this paper.

Code availability. No custom codes have been developed in this study.
References

1. Ambros, I. M. et al. Role of ploidy, chromosome 1p, and Schwann cells in the maturation of neuroblastoma. N. Engl. J. Med. 334, 1505–1511 (1996).
2. Mirsky, R. et al. Schwann cells as regulators of nerve development. J. Physiol. Paris 96, 17–24 (2002).
3. Furlan, A. & Adameyko, I. Schwann cell precursor: a neural crest cell in disguise? Dev. Biol. 444, S25–S35 (2018). Suppl 1.
4. Monk, K. R., Feltri, M. L. & Taveggia, C. New insights on Schwann cell development. Glia 63, 1376–1393 (2015).
5. Nave, K. A. Myelination and the trophic support of long axons. Nat. Rev. Neurosci. 11, 275–283 (2010).
6. Syroid, D. E. et al. Cell death in the Schwann cell lineage and its regulation by neuregulin. Proc. Natl Acad. Sci. USA 93, 9229–9234 (1996).
7. Jessen, K. R., Mirsky, R. & Lloyd, A. C. Schwann Cells: Development and Role in Nerve Repair. Cold Spring Harb. Perspect. Biol. 7, a200487 (2015).
8. Ambros, P. F. et al. Regression and progression in neuroblastoma. Does neuroblastoma disguise? Cancer Res. 62, 7357–7363 (2002).
9. Weiss, T., Taschner-Mandl, S., Ambros, P. F. & Ambros, I. M. Detailed Protocols for the Isolation, Culture, Enrichment and Immunostaining of Primary Human Schwann Cells. Methods Mol. Biol. 1739, 67–86 (2018).
10. Lastowska, M. et al. Comprehensive genetic and histopathologic study reveals three types of neuroblastoma tumors. J. Clin. Oncol. 19, 3080–3090 (2001).
11. Molemaa, J. J. et al. Sequencing of neuroblastoma identifies chromothripsis and defects in neurotigogenes genes. Nature 483, 589–593 (2012).
12. Brodeur, G. M. Molecular basis for heterogeneity in human neuroblastomas. Eur. J. Cancer 31A, 505–510 (1995).
13. Bunge, M. B., Williams, A. K. & Wood, P. M. Neurom-Schwann cell interaction in basal lamina formation. Dev. Biol. 92, 449–460 (1982).
14. Quintes, S. et al. Zeb2 is essential for Schwann cell differentiation, myelination and nerve repair. Nat. Neurosci. 19, 1050–1059 (2016).
15. Yu, L. M. et al. Zeb2 recruits HDAC-1 to inhibit Notch and controls Schwann cell differentiation and remyelination. Nat. Neurosci. 19, 1060–1072 (2016).
16. Hung, H. A., Sun, G., Keles, S. & Saven, J. Dynamic regulation of Schwann cell enhancers after peripheral nerve injury. J. Biol. Chem. 290, 6937–6950 (2015).
17. Cadoni, A., Zicca, A. & Mancardi, G. L. Schwann cell expression of HLA-DR membrane and neuropathic pain through MHC class II. Do they have a role in antigen presentation? J. Neuroimmunol. 86 (2018).
18. Subhan, F. et al. Epidermal growth factor-like domain 8 inhibits the survival and differentiation of neuroblastoma. Cancer Lett. 385, 331–337 (1994).
19. Bergsteinsdottir, K., Kingston, A., Mirsky, R. & Jessen, K. R. Schwann cells promote post-traumatic nerve inflammation and neuropathic pain through MHC class II. Sci. Rep. 7, 12518 (2017).
60. Ydens, E. et al. The neuroinflammatory role of Schwann cells in disease. Neurobiol. Dis. 55, 95–103 (2013).
61. Meierink, G. V., Shabani, G., V. K., Keokinov, A. A., Runimovich, Y. L. & Shurin, M. R. Schwann cells shape the neuro-immune environment and control cancer progression. Cancer Immunol. Immunother. 68, 1819–1829 (2019).
62. Fitch, M. J., Campagnolo, L. & Kuhnert, F. Stuhlmann H. Egfl7, a novel epidermal growth factor-domain gene expressed in endothelial cells. Dev. Dyn. 239, 316–324 (2014).
63. Schmidt, M. H. et al. Epidermal growth factor-like domain 7 (EGFL7) modulates Notch signalling and affects neural stem cell renewal. Nat. Cell Biol. 11, 873–880 (2009).
64. Chao, M. V. Neurotrophins and their receptors: a convergence point for many signalling pathways. Nat. Rev. Neurosci. 4, 299–309 (2003).
65. Yu, J. H. et al. Restoration of promyelocytic leukemia protein-nuclear bodies in neuroblastoma cells enhances retinoic acid responsiveness. Cancer Res. 64, 928–933 (2004).
66. Shin, E. Y. et al. Phosphorylation of p85 beta PIX, a Rac/Cdc42-specific guanine nucleotide exchange factor, via the Ras/ERK/PAK2 pathway is required for basic fibroblast growth factor-induced neurite outgrowth. J. Biol. Chem. 277, 44417–44430 (2002).
67. Takahashi, K., Yamada, M., Ohta, H., Honda, K. & Yamada, M. Ndrg2 promotes neurite outgrowth of NGF-differentiated PC12 cells. Neurosci. Lett. 388, 157–162 (2005).
68. Allford, A. I. & Hankenson, K. D. Matricellular proteins: Extracellular matrix regulators of bone development, remodeling, and regeneration. Bone 38, 749–757 (2006).
69. Chiodoni, C., Colombo, M. P. & Sangaletti, S. Matricellular proteins: from homeostasis to inflammation, cancer, and metastasis. Cancer Metastasis Rev. 29, 295–307 (2010).
70. Murphy-Ullrich, J. E. & Sage, E. H. Revisiting the matricellular concept. Matrix Biol. 37, 1–14 (2014).
71. Bornstein, P. & Sage, E. H. Matricellular proteins: extracellular modulators of cell function. Curr. Opin. Cell Biol. 14, 608–616 (2002).
72. Chlenski, A. et al. SPARC expression is associated with impaired tumor growth, inhibited angiogenesis and changes in the extracellular matrix. Int J. Cancer 118, 310–316 (2006).
73. Deborde, S. & Wong, R. J. How Schwann cells facilitate cancer progression in nerves. Cell Mol. Life Sci. 74, 4405–4420 (2017).
74. Crawford, S. E. et al. Pigment epithelium-derived factor (PEDF) in cancer. Nat. Rev. Cancer 8, 9–19 (2008).
75. Meijering, E. et al. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytom. A 58, 167–176 (2004).
76. Ollik, I. Contributions to Probability and Statistics: Essays in Honor of Harold Hotelling. Stanford University Press (1980).
77. Rolfabegovic, F. et al. Neuroblastoma cells undergo transcripomic alterations upon dissemination into the bone marrow and subsequent tumor progression. Int J. Cancer 142, 297–307 (2018).

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Author contributions
T.W. and S.-T.M. planned experiments, performed research, analyzed and interpreted data and wrote the manuscript; H.S., A.B., I.I. and F.R. performed research and analyzed data; F.K. analyzed data; C.F. and M.K. developed bioinformatics tools and analyzed data; C.G. provided high-throughput sequencing and microarray studies. Nucleic Acids Res. 43, e47 (2015).

Competing interests
The authors declare no conflict of interest.
Additional information

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