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Midkine and Alk signaling in sympathetic neuron proliferation and neuroblastoma predisposition

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SUMMARY

Neuroblastoma (NB) is the most common extracranial solid tumor in childhood and arises from cells of the developing sympathoadrenergic lineage. Activating mutations in the gene encoding the ALK tyrosine kinase receptor predispose for NB. Here, we focus on the normal function of Alk signaling in the control of sympathetic neuron proliferation, as well as on the effects of mutant ALK. Forced expression of wild-type ALK and NB-related constitutively active ALK mutants in cultures of proliferating immature sympathetic neurons results in a strong proliferation increase, whereas *Alk* knockdown and pharmacological inhibition of Alk activity decrease proliferation. Alk activation upregulates *NMyc* and *trkB* and maintains *Alk* expression by an autoregulatory mechanism involving *Hand2*. The Alk-ligand Midkine (Mk) is expressed in immature sympathetic neurons and in vivo inhibition of Alk signaling by virus-mediated shRNA knockdown of *Alk* and *Mk* leads to strongly reduced sympathetic neuron proliferation. Taken together, these results demonstrate that the extent and timing of sympathetic neurogenesis is controlled by Mk/Alk signaling. The predisposition for NB caused by activating ALK mutations may thus be explained by aberrations of normal neurogenesis, i.e. elevated and sustained Alk signaling and increased NMyc expression.

KEY WORDS: Anaplastic lymphoma kinase, Sympathetic, Neuroblastoma, Neurogenesis, Chick

INTRODUCTION

Neurogenesis in sympathetic ganglia (SG) proceeds mainly by proliferation of immature but already differentiated neurons (DiCicco-Bloom et al., 1990; Rohrer and Thoenen, 1987; Rothman et al., 1978; Tsarovina et al., 2008). In contrast to other lineages in the peripheral and central nervous system (PNS and CNS), neuron differentiation is not linked to cell cycle withdrawal. Cell cycle exit and generation of postmitotic sympathetic neurons proceeds over a broad developmental period but the cellular and molecular mechanisms controlling proliferation in this lineage are not well understood.

NB, the major tumor of the developing PNS is restricted to the sympathoadrenal lineage, suggesting that predisposition and development of NB may be linked to the proliferation characteristics of sympathetic neuroblasts (Brodeur, 2003; Maris et al., 2007). Proliferation of precursors and immature sympathetic neurons are affected by the transcription factors Ascl1, Phox2b, Hand2, Insm1, Sox11 and Gata2/3 that were previously considered to control only initial specification and differentiation (Coppola et al., 2010; Hendershot et al., 2008; Morikawa et al., 2009; Pattyn et al., 1999; Potzner et al., 2010; Tsarovina et al., 2010; Wildner et al., 2008). Mutations in the *PHOX2B* gene were identified in familial cases of NB and result in tumor predisposition (McConville et al., 2006; Mosse et al., 2004; Trochet et al., 2004; van Limpt et al., 2004). NB *PHOX2B* mutations affect proliferation and differentiation of embryonic sympathetic neurons and the proliferation of NB cell lines (Raabe et al., 2008; Reiff et al., 2010).

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More recently, predisposing *ALK* mutations were identified in a large fraction of familial cases of NB (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). In addition, in sporadic cases of NB, a relatively high frequency of *ALK* mutations (6-10%) and *ALK* amplifications (1.7%) was observed (De Brouwer et al., 2010; Janoueix-Lerosey et al., 2010; Mosse et al., 2008; Schulte et al., 2011). Mutational hotspots in *ALK* are located in the tyrosine kinase domain affecting the residues F1174 and R1275, leading to constitutively active mutant proteins independent of ligand binding (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Constitutively active ALK receptors stimulate cell proliferation, as shown by ectopic expression of F1174 and R1275 ALK receptor in 3T3 fibroblasts (Chen et al., 2008) and murine lymphoid Ba/F3 cells (George et al., 2008). Importantly, in NB cell lines that express mutated ALK, shRNA-mediated ALK knockdown and pharmacological kinase inhibition lead to strongly reduced proliferation and increased cell death (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). ALK-dependent proliferation of NB cell lines with wild-type ALK implicates the presence of an activating ligand(s) (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Two potential Alk ligands, the growth factors Midkine (Mk) and Pleiotrophin (Ptn) have been identified with widespread functions in proliferation, differentiation and cell survival (Kadomatsu and Muramatsu, 2004; Muramatsu, 2010). Although *Alk* expression has been documented in developing SG (Hurley et al., 2006; Morris et al., 1997; Vernersson et al., 2006), the physiological role of Alk signaling in the sympathetic neuron lineage, as well as in other parts of the developing nervous system, is not well understood (Palmer et al., 2009).

In this study, an essential role for Alk signaling in sympathetic neuron proliferation was observed in culture and confirmed by *Alk* knockdown in vivo. The Alk ligand Mk was detected in SG and shown to be sufficient and required for neuron proliferation, which implicates Mk as physiological ligand in this lineage.

DEVELOPMENT

Activation of Alk signaling, in particular overexpression of ALK^{wt} and NB ALK mutants resulted in the upregulation of *NMyc* and *trkB*, which may contribute to continued proliferation and NB predisposition.

MATERIALS AND METHODS

Expression plasmids

PcDNA3.1 expression plasmids for human ALK^{wt}, ALK^{F1174L} and ALK^{R1275Q}, and pCAGGS expression plasmids for Hand2, $Phox2b^{wt}$ and $Phox2b^{K155X}$ have been described previously (Janoueix-Lerosey et al., 2008; Reiff et al., 2010).

shRNA constructs

The shRNA against *Gallus gallus Alk* and *Mk* were designed using BLOCK-iT RNAi Designer (Invitrogen, Karlsruhe, Germany) and target the following sequences: *shAlk*, 5′-AAU GGU UUC UCU CUA UGU CCA ACU C-3′; *shMk*, 5′-GAG CUG ACU GCA AGU ACA AGU UUG A-3′. Scrambled shRNAs (sc-shRNA, Invitrogen, Karlsruhe, Germany) served as controls.

In situ hybridization

Non-radioactive in situ hybridization on cryosections and preparation of digoxigenin labeled probes for chick *Phox2b* was carried out as described previously (Ernsberger et al., 1997; Stanke et al., 1999).

qPCR analysis

Equal amounts of RNA were used to synthesize cDNA with Oligo(dT)-primers and Superscript-III-reverse-transcriptase according to manufacturer's instructions (Invitrogen, Karlsruhe, Germany). The PCR was carried out using Abgene's Absolute Blue SYBR-Green qPCR Mix (Abgene, Epsom, UK) in a Stratagene Mx3000p Light Cycler (Stratagene, Waldbronn, Germany). All primers (MWG Biotech AG, Ebersberg, Germany) were designed to anneal optimally at 58°C with PerlPrimer (Marshall, 2004) (95°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds, repeated for 50 cycles and a subsequent dissociation curve). The primer pairs (see Table S1 in the supplementary material) were analyzed for efficiency (≥95%) and used for quantitative analysis. At least duplicates of every condition were performed in parallel. Data were normalized using *Gapdh* and *Islet1* as reference genes and evaluated by delta-delta C_t-method using Microsoft Excel. Experiments were repeated independently six times and statistically analyzed using paired two-tailed Student's *t*-test and ANOVA.

Primary sympathetic neuron culture preparation and transfection

Paravertebral lumbosacral sympathetic ganglia from E7 chicken embryos were dissociated (Rohrer and Thoenen, 1987; Zackenfels et al., 1995) and the Amaxa Nucleofector II was used to electroporate 200,000 cells [Program: Small Cell Number (SCN) #2, transfection efficiency ≈50%]. *pMAX-GFP* (0.5 μg) (Lonza, Cologne, Germany) was mixed with 0.5 μg of *empty vector/sc-shRNA* for controls or 0.5 μg of the appropriate *pcDNA3.1-Alk variants* or 0.5 μg of *shAlk* and *shMk*. Transfected cells were plated on poly-DL-ornithin/laminin-coated four-well culture dishes and cultured for 2 days as described (Reiff et al., 2010). NVP-TAE684 (Axon Medchem, Groningen, The Netherlands) was used to inhibit Alk kinase. Human recombinant MK (10 nM) (Biomol, Hamburg, Germany) was added to cultures where indicated. EdU was added to the culture medium 24 hours before fixation (1:1000, Invitrogen, Karlsruhe, Germany). For RNA isolation, 400,000 cells were electroporated, cultured for 2 days in vitro and harvested. RNA was isolated using the mRNEasy Mini Kit (Qiagen, Hilden, Germany).

Cell sorting by MACS

Cells from dissociated E7 SG were separated from cellular debris by centrifugation through a step gradient with 3% BSA in DMEM medium (20 minutes, 150 g). All centrifugation and incubation steps were carried out at 12°C. After incubation with mouse anti-Q211 antibodies (Zackenfels et al., 1995) (1:50, 15 minutes), unbound antibody was removed by dilution with 5 ml DMEM medium and the cells were pelleted by centrifugation through a 3% BSA step gradient. Resuspended cells were incubated with microbead-coupled anti-IgM (1:5, 15 minutes) (Miltenyi Biotec, Bergisch Gladbach, Germany), separated from unbound anti-IgM by BSA gradient centrifugation. Magnetically labeled cells were applied to MACS SM column separator (Miltenyi Biotec, Bergisch Gladbach, Germany). Unbound cells were eluted by three washing steps, magnetically labeled $Q211^{+}$ cells were eluted after removal of the column from the magnetic MACS separator using 3×500 μl of 0.1% BSA in DMEM medium. Both flow-through fraction and the magnetically labeled $Q211^{+}$ fraction underwent a second cycle of MACS column purification. The final cell suspensions were pelleted by centrifugation for RNA isolation and qPCR analysis. Before pelleting, aliquots of cell suspensions were plated onto poly-DL-ornithin/laminin-coated dishes and analyzed after short-term (3 hours) culturing by staining for Q211, TuJ1, TH, O4 and DAPI for the proportion of neurons, glial cells and fibroblast-like cells ($DAPI^{+}$, $Q211^{-}$, $TuJ1^{-}$, TH^{-}, $O4^{-}$).

Immunostaining

Cells were washed with PBS and fixed using 4% paraformaldehyde in 0.1 M sodium phosphate buffer for 15 minutes. After washing with PBS, cells were stained with the EdU labeling kit according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany, Click-It EdU Imaging Kit 594). Afterwards, primary antibodies were diluted 1:500 in staining buffer (PBS, 0.5% Triton X100 and 5% FCS) and incubated overnight (mouse, Th). After washing with PBS, appropriate secondary antibodies were used (1:500, goat anti-mouse Alexa 488, Invitrogen, Karlsruhe, Germany). Nuclei were stained with DAPI (Sanofi Aventis, Frankfurt, Germany). Stainings were covered with Aqua Polymount (Polysciences, Eppelheim, Germany) and analyzed at room temperature with a Zeiss Axiophot 2 microscope (Plan Neofluar Objective 40×, 0.75 Ph2). Transfected cells were identified by GFP fluorescence, whereas TH immunoreactivity served as a marker for immature noradrenergic neurons in non-transfected cells. GFP- or TH-positive neurons were analyzed for EdU staining and double-positive cells were counted in at least 20 visual fields per culture dish at a magnification of 40×. A Visitron Systems Spot RT3 camera was used for image acquisition and MetaVue (7.1.3.0) for digital image processing (adjustment of brightness and contrast). All results are given as mean±s.e.m. of six independent experiments and statistically analyzed with paired two-tailed Student's *t*-test.

TUNEL staining

TUNEL-positive neurons were detected on sympathetic neuron cultures with the in situ cell death detection kit (Roche Applied Sciences, Grenzach, Germany) according to the manufacturer's protocol followed by a DAB-reaction (Thermo Fisher, Schwerte, Germany). TUNEL staining on cryosections of chicken embryos was performed as described previously (Tsarovina et al., 2010). Total neuron number and TUNEL-positive neurons were counted in 20 visual fields per culture dish at a magnification of 40×. All results are given as mean±s.e.m. from six independent experiments and statistically analyzed with paired two-tailed Student's *t*-test.

RCAS short hairpin RNAi constructs, RCAS-Mk and virus concentrates

RCAS short hairpin RNAi constructs

Short hairpin RNAi viruses for specific knockdown of *Mk* and *Alk* were constructed as described previously (Das et al., 2006) with the following primer pairs (flanking primers W,Y) (see Das et al., 2006): *shAlk* forward, GAG AGG TGC TGC TGA GCG CCA CAA AGA AGA AAG TTG ACA GGT GAA GCC ACA GAT GTAAAG GGA GGG TGT GCG ATG; *shAlk* reverse, ATT CAC CAC CAC TAG GCA ACA CAA AGA AGA AAG TTG ACA GCA TCT GTG GCT TCA CT; *shMk* forward, GAG AGG TGC TGC TGA GCG CCA AAC TTG TAC TTG CAG TCA GCT CCG TGA AGC CAC AGA TGT A; *shMk* reverse, ATT CAC CAC CAC TAG GCA ACA AAC TTG TAC TTG CAG TCA GCT CCC ATC TGT GGC TTC ACT. Underlined sequence hybridizes with respective mRNA.

Gallus gallus Mk was amplified by PCR from E7 SG cDNA using the following primer pairs that add an additional Kozak sequence and *Cla*I (forward, TAATCGATACCACCATGCAGCCCCGGGGCCTCCTCCTC) and *Spe*I (reverse, TAACTAGTCTAGTCCTTCCCCTTGCCCTTCT-TTGC) restriction sites for RCAS cloning. DF1 chicken fibroblasts were

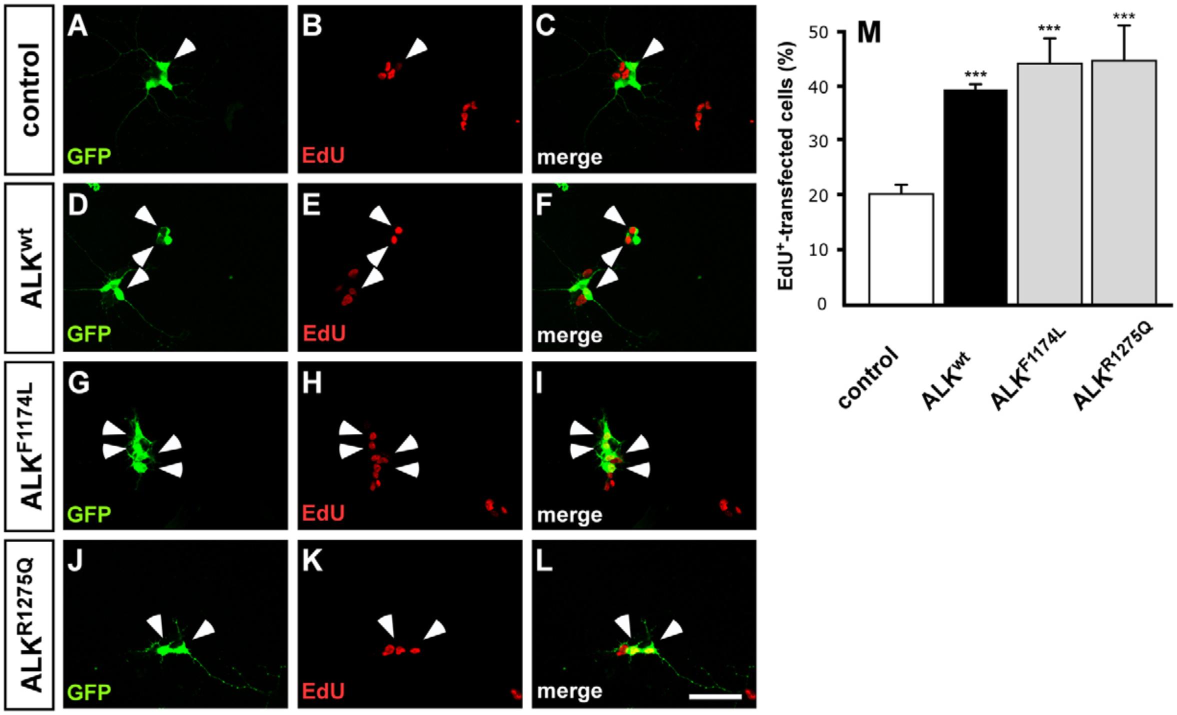

Fig. 1. Sympathetic neuron proliferation is increased upon overexpression of ALK^{wt} and NB-ALK mutations. (**A-L**) Immunofluorescence of GFP (A,D,G,J), EdU (B,E,H,K) and channel merge (C,F,I,L) of control-, ALK^{wt}-, ALK^{F1174L}- and ALK^{R1275Q}-transfected E7 sympathetic neurons. Arrowheads mark proliferating immature sympathetic neurons. Scale bar: 50 μm. (**M**) Proportion of EdU/GFP-double positive neurons 2 days in vitro. Data are mean+s.e.m. (*n*=6). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, significantly different from control.

transiently transfected with empty *RCAS(BP-B)*, *RCAS(BP-B)-Mk*, *RCAS(BP-B)-shMk* and *-shAlk (RCAS-shMk/RCAS-shAlk)*, and virus concentrate was prepared as described previously (Rüdiger et al., 2009).

Mk- and *Alk*-specific knockdown and *Mk* overexpression in vivo

Fertilized virus-free eggs (Charles River, Sulzfeld, Germany) were incubated and infected with empty *RCAS* virus for control or *RCAS-shMk*, *RCAS-shAlk* or *RCAS-Mk* virus concentrates as described (Rüdiger et al., 2009). Embryos were kept until stage 24 (E4) or E7, pulsed with EdU (400 μl of 10 mM EdU in PBS) for 3 hours, fixed in 4% paraformaldehyde and embedded. Cryosections (16 μm) were stained for EdU and TH, and analyzed with a Zeiss Apotome fluorescence microscope. The TH$^+$ area from infected SG was quantified with Axiovision 4.6 (Zeiss Apotome, 40× magnification). In situ hybridization for Phox2b was carried out as described previously (Rüdiger et al., 2009). Images of *Phox2b*$^+$-area were acquired by light microscopy (Zeiss Axiophot 2, 20× magnification) and analyzed with MetaVue 7.1.3.0 as described previously (Lucas et al., 2006; Tsarovina et al., 2004).

RESULTS

Forced expression of constitutively active ALK mutations, as well as ALK^{wt} overexpression lead to increased proliferation of sympathetic neurons

The function of Alk in normal sympathetic neuron development, which is implicated by the selective susceptibility of the sympathoadrenergic lineage to increased Alk signaling (Janoueix-Lerosey et al., 2010; Palmer et al., 2009) has remained elusive. Here, we study the role of Alk in cultures of proliferating immature neurons from embryonic (E7) chick SG, which contain proliferating cells virtually all of which harbor characteristic pan-neuronal and adrenergic markers and respond to proliferation stimulatory as well as antiproliferative signals (Reiff et al., 2010; Rohrer and Thoenen, 1987; Zackenfels et al., 1995). Glial cells and fibroblasts represent only 3±2% and 2±1% of E7 chick SG cells, respectively (Ernsberger et al., 1989). To investigate the effect of NB ALK mutations and mimic ALK locus amplification, cells were transfected with expression plasmids for two human NB ALK mutations (ALK^{F1174L} and ALK^{R1275Q}) and ALK^{wt}. Similar expression levels of *ALK*^{wt} and *ALK* variants were observed by quantitative PCR (qPCR) (data not shown). To pursue proliferation of transfected immature sympathetic neurons, an expression plasmid for GFP was co-transfected and the number of GFP$^+$ neurons that are positive for the proliferation marker EdU was counted. Overexpression of all three ALK variants led to a strong increase in the proportion of EdU$^+$ neurons compared with control (Fig. 1). To determine whether Alk is essential for basal proliferation in vitro, the effects of pharmacological Alk inhibition and shRNA-mediated knockdown of endogenous *Alk* were studied.

Pharmacological Alk kinase inhibition and Alk knockdown reduce sympathetic neuron proliferation

NVP-TAE684 is a potent and selective inhibitor of Alk signaling, which interacts specifically with the ATP-binding site (Galkin et al., 2007). In the presence of NVP-TAE684, sympathetic neuron proliferation was completely blocked, with an IC_{50} of 67.6±1.8 nM (Fig. 2A). As NVP-TAE684 also blocks insulin-like growth factor receptor (IGFR) at high concentrations (IC_{50} 1.2 μM), it was unclear whether the proliferation effects were entirely due to interference with Alk signaling (Galkin et al., 2007; Zackenfels et al., 1995). Thus, an *Alk*-specific shRNA-mediated knockdown was performed. A highly efficient shRNA (*shAlk*) was selected that resulted in a significant 71.3±3.8% (*n*=6) reduction in endogenous

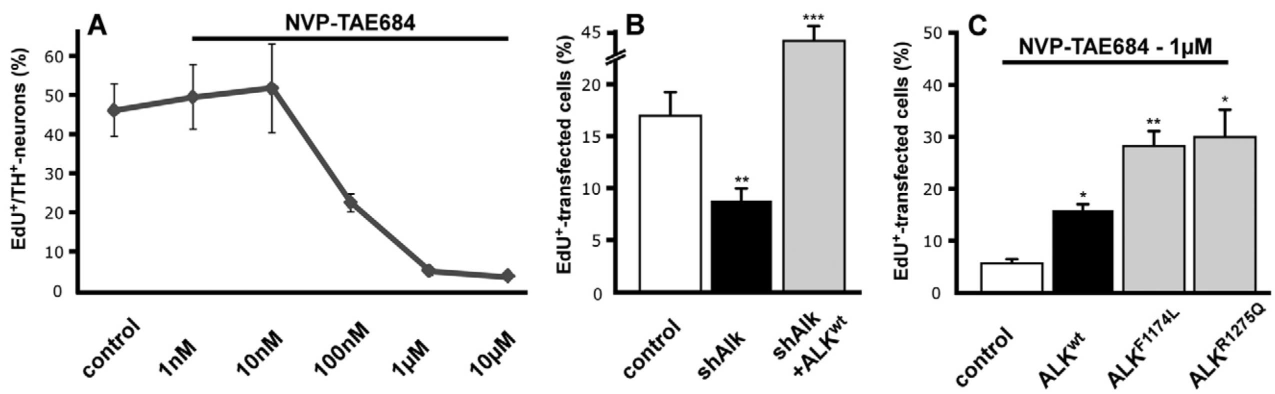

Fig. 2. Sympathetic neuron proliferation is decreased by NVP-TAE684- or shRNA-mediated *Alk* knockdown. (**A**) Sympathetic neuron cultures were treated with NVP-TAE684. After 2 days in vitro, the number of TH/EdU double-positive neurons was evaluated. (**B**) Cultures transfected with scrambled sc-shRNA for controls, Alk-specific shRNAs (*shAlk*) or *shAlk* and an ALK^{wt} expression plasmid were analyzed for the proportion of GFP^+/EdU^+ neurons. (**C**) Neurons transfected with empty expression vector (control) or plasmids encoding ALK variants and incubated with 1 μM NVP-TAE684. After 2 days in vitro, the proportion of GFP^+/EdU^+ neurons was determined. Cultures of untransfected cells display a higher proportion of EdU^+ cells (A) than do cultures of transfected cells (B,C). Data are mean±s.e.m. (*n*=6). **P*≤0.05, ***P*≤0.01, ****P*≤0.001, significantly different from control.

Alk mRNA levels 2 days after transfecting E7 sympathetic neurons. In *shAlk*-transfected sympathetic neuron cultures, a strong decrease in the number of proliferating EdU^+ cells was observed (Fig. 2B). As the proportion of transfected cells was not significantly altered (81.4±5.9% compared with control), side effects of *Alk* knockdown on cell survival are excluded. The anti-proliferative effects of *shAlk* and NVP-TAE684 were completely rescued by simultaneous overexpression of human ALK^{wt} and the ALK variants (Fig. 2B,C), excluding off-target effects of *shAlk*. The finding that the overexpression of the ligand-dependent ALK^{wt} rescues Alk inhibition and knockdown (Fig. 2B,C) and increases the number of proliferating cells (Fig. 1) suggested that a receptor-activating ligand is present in SG during development.

The Alk ligand Midkine is expressed in sympathetic ganglia during chicken embryo development

Mk and Ptn have previously been identified as Alk ligands that are able to elicit phosphorylation of Alk and the downstream kinase PI3K (Stoica et al., 2001; Stoica et al., 2002), although the role of Ptn as Alk ligand is controversial (Mathivet et al., 2007). To determine whether these ligands are expressed in developing SG, we performed qPCR for *Mk* and *Ptn* mRNA on cDNA of E7 SG cultures. The analysis revealed a strong *Mk* expression, whereas *Ptn* is present at a much lower level (46-fold; *n*=6). *Mk* levels in SG decrease from E6 until E12, whereas *Ptn* expression decreases until E10 and is subsequently upregulated to reach similar levels as *Mk* at E12 (Fig. 3A). *Alk* expression levels in SG are not changed significantly (Fig. 3B), which is in agreement with a previous analysis by in situ hybridization (Hurley et al., 2006).

To address the issue of whether Mk is derived from the neuronal or non-neuronal SG population, E7 SG were dissociated and neurons were immunopurified using the neuron-specific Q211 antibody (Rohrer et al., 1985; Rohrer and Thoenen, 1987; Zackenfels et al., 1995). MACS sorting resulted in a virtually pure neuronal population (98.42±0.28% $Q211^+$, 98.7±0.56% $Tuj1^+$; *n*=3) and a neuron-depleted flow-through fraction with >35-fold higher proportion of non-neuronal cells (27±19% $Q211^+$, 25±14% $Tuj1^+$, 37±10% $O4^+$; *n*=3). Analysis of Mk expression by qPCR revealed similar levels in the neuronal and neuron-depleted population [0.96-fold difference (± 0.08); s.e.m.; *n*=3]. Thus, Mk is expressed in both neuronal and non-neuronal cells. By contrast, Ptn is preferentially expressed [87.6-fold enriched (±30), s.e.m.; *P*<0.05; *n*=3] in the neuron-depleted population, i.e. in ganglion non-neuronal cells.

The selective NB development in SG when compared with parasympathetic ganglia suggests that proliferation of parasympathetic neuron progenitors is not regulated by Alk signaling. Indeed, much lower *Alk* expression levels (8.3±0.1-fold, s.e.m.; *P*<0.008; *n*=3) were detected by qPCR analysis in ciliary ganglia (E5) when compared with sympathetic ganglia (E7). This is in agreement with the differential *Alk* expression observed in a SAGE screen comparing E7 sympathetic and E5 ciliary ganglia (J. Stubbusch and H.R., unpublished). Importantly, neither *Mk* nor *Ptn* was detected by qPCR during neurogenesis in E5 ciliary ganglia (*n*=3).

Alk is expressed in SG not only during neurogenesis (Hurley et al., 2006) (present data) but up to stages where neuron proliferation has finished (Rothman et al., 1978). The timing of *Mk* expression suggests that sympathetic neuron proliferation is controlled by Mk and that neurogenesis may be terminated by decreased Mk availability. To address this issue, we investigated whether Mk is responsible for Alk activation and basal proliferation of cultured sympathetic neurons.

Midkine dependent Alk signaling controls proliferation of sympathetic neurons

Mk significantly increased the proportion of proliferating EdU^+/TH^+ neurons when applied to SG cultures (Fig. 4A). The moderate effect of exogenous MK may be explained by the high expression level of endogenous Mk that already stimulates sympathetic neurons close to the maximal proliferation rate (Reiff et al., 2010). The proliferation increase of sympathetic neurons in response to MK is also maintained at later stages when neurogenesis and endogenous *Mk* levels decrease (Fig. 4A), and is mediated by Alk, as revealed by *Alk* knockdown (Fig. 4B).

To address the role of endogenous Mk, a shRNA knockdown strategy was chosen. Knockdown of endogenous *Mk* mRNA levels by *shMk* to 27.4±2.9% of control levels resulted in a strong decrease of proliferating immature neurons (Fig. 4B) without

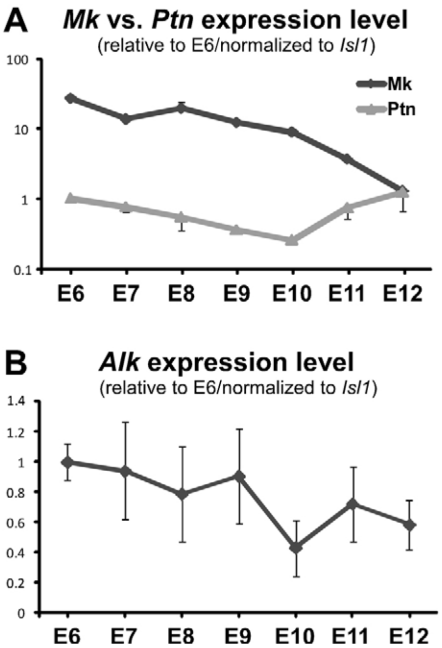

Fig. 3. *Mk* is expressed in early SG and its expression decreases during embryonic development. (**A**,**B**) SG were dissected from E6 to E12 and expression of *Mk*, *Ptn* and *Alk* was analyzed by qPCR and normalized to *Isl1* as a neuronal marker. Data are mean±s.e.m. (n=3). ANOVA: *Mk*, P=0.003; *Ptn*, P=0.7; *Alk*, P=0.7.

affecting cell survival (data not shown). The specificity of the *shMk*-mediated knockdown was demonstrated by the rescue through the addition of recombinant MK to the cell cultures (Fig. 4B). The *Mk* knockdown effect on proliferation was also rescued by overexpression of ALK^{F1174L} but not by ALK^{wt}. Thus, endogenous Mk is required for proliferation effects of transfected ALK^{wt} but not for increased proliferation by constitutively active ALK^{F1174L} (Fig. 4B,C). These findings show that Mk-dependent Alk signaling is essential for in vitro proliferation of immature sympathetic neurons and raise the question of to what extent sympathetic neurogenesis is controlled by Mk/Alk in the embryo.

In vivo effects of Alk and Mk knockdown and Mk overexpression in sympathetic ganglia

To investigate whether Alk signaling is involved in sympathetic neurogenesis in the embryo, an in vivo approach to knock down endogenous *Mk* and *Alk* was chosen. Premigratory neural crest cells were infected with high titers of RCAS-virus concentrates that target either *Mk* or *Alk* by specific shRNAs. Infection efficiency was monitored by in situ hybridization against the viral *reverse transcriptase* mRNA and showed no difference between *RCAS* controls, *RCAS-shAlk*, *RCAS-Mk* and *RCAS-shMk* (data not shown). The analysis of *RCAS-shAlk*- and *RCAS-shMk*-infected embryos revealed a massive reduction in SG size when measured by the area occupied by TH^+ and *Phox2b*$^+$ cells (Fig. 5A,B; see Fig. S1 in the supplementary material). Furthermore, sympathetic neuron proliferation is severely affected, as shown by the reduced percentage of $EdU^+/DAPI^+$ cells in the TH^+ ganglion area (Fig. 5A,C). By contrast, dorsal root ganglion (DRG) size (area of $TuJ1^+$ cells) and the proportion of EdU^+ cells in the DRG were not altered (see Fig. S2 in the supplementary material), although Alk is transiently expressed in developing DRG (Hurley et al., 2006). To analyze effects on cell survival, TUNEL-staining was performed on sections of *shAlk*- and *shMk*-infected embryos. As the number of $TUNEL^+$ cells in *shAlk*- and *shMk*-infected embryos (0.5±0.2 and 0.6±0.2 $TUNEL^+$ cells/1000 μm^2 TH^+ area, respectively) was not significantly increased above RCAS-infected controls (0.3±0.1 $TUNEL^+$ cells/1000 μm^2 TH^+ area) cell death is excluded as cause of reduced SG size. Ectopic Mk expression significantly increased E4 SG size, as well as the proportion of EdU^+ cells (Fig. 5B). Mk-overexpressing embryos could be maintained up to E7 but were growth retarded, most probably owing to impaired development of chorioallantoic blood vessels (not shown), which is in line with Mk functions in angiogenesis (van der Horst et al., 2008). The percentage of EdU^+ cells in E7 Mk expressing embryos was significantly increased when compared with E7 control infected embryos (16.5±1.98% versus 9.7±0.5%; n=3, P>0.01). However, increased proliferation was not paralleled by increased ganglion size [9001±438 μm^2 in controls when compared with 6858±832 μm^2 in Mk infections (n=3; P>0.05 n.s.)]. The higher proportion of EdU^+ cell in Mk-expressing growth-retarded embryos may thus be explained by increased proliferation in less mature ganglia, rather by a direct proliferation effect.

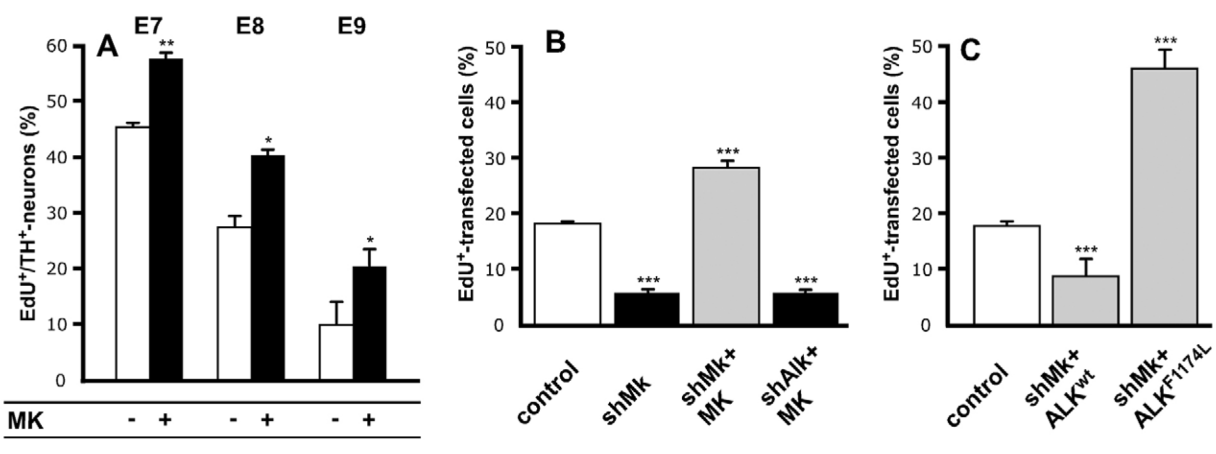

Fig. 4. Sympathetic neuron proliferation depends on Midkine (Mk) levels. (**A**) Sympathetic neurons from E7, E8 and E9 ganglia were cultured with or without 10 nM MK and analyzed for proliferation as in Fig. 1. (**B**,**C**) Sympathetic neurons transfected with sc-shRNA for controls, *shAlk, shMk* and expression plasmids for ALK variants and analyzed after 2 days in vitro as in Fig. 1. To rescue the *Mk* knockdown, recombinant MK (10 nM) was added. Data are mean+s.e.m. (n=6). *P≤0.05, **P≤0.01, ***P≤0.001, significantly different from control.

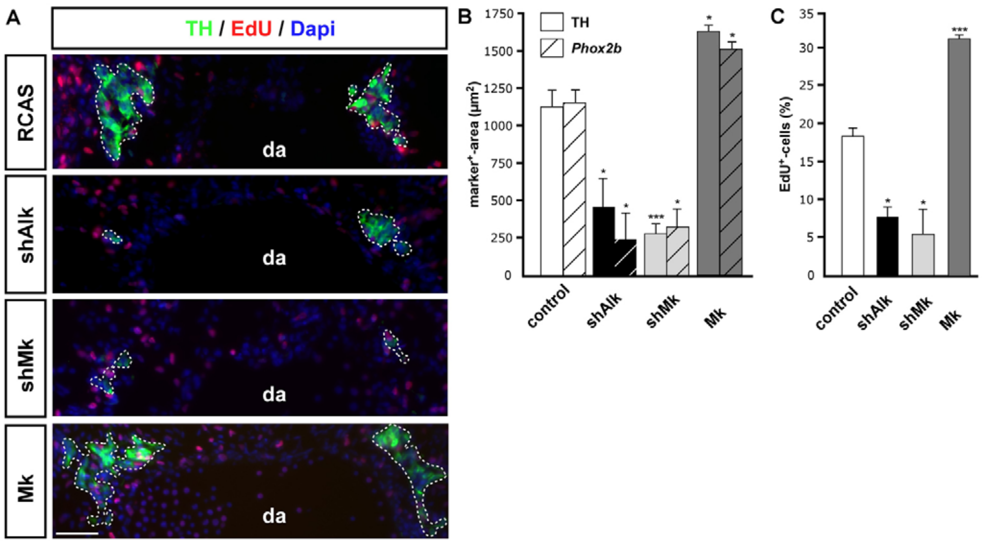

Fig. 5. Mk/Alk signaling controls SG proliferation in early chicken embryos. (**A**) Neural crest cells of E2 chicken embryos were infected in ovo with empty *RCAS*-virus (control, *n*=5), *RCAS-shAlk* (shAlk, *n*=3), *RCAS-shMk* (shMk, *n*=4) and *RCAS-Mk* (Mk, *n*=4). At stage 23, embryos were pulsed for 3 hours with EdU and fixed. After sectioning, slices were stained for TH, EdU and DAPI. dA, dorsal aorta; broken line surrounds SG. Scale bar: 50 μm. (**B**) Optical sections of the TH$^+$ area from infected SG were taken and quantified with Axiovision 4.6 (Zeiss Apotome, 40× magnification). Images of *Phox2b*$^+$ area were acquired with a Zeiss Axiophot 2 microscope at 20× magnification (see Fig. S1 in the supplementary material) and areas quantified with MetaVue 7.1.3.0. (**C**) The number of DAPI- and EdU-positive cells within the sympathetic ganglion defined by TH immunostaining was counted and results are given as proliferation rate (percentage of DAPI$^+$/EdU$^+$ double-positive neurons). *P≤0.05, **P≤0.01, ***P≤0.001, significantly different from control. Data are mean+s.e.m.

The knockdown of *Alk* and *Mk* seems to be compensated with time in vivo, as proliferation is no more reduced at E7 and ganglion size is partially recovered (see Fig. S3 in the supplementary material). Having confirmed the importance of Alk signaling for sympathetic precursor proliferation in vivo, the mechanisms that underlie these effects were studied.

Autoregulatory control of Alk expression in sympathetic neurons is mediated by Hand2

To identify potential downstream target genes of Alk signaling, E7 sympathetic neurons were treated by MK (10 nM) or transfected with *shMk* and subsequently analyzed for expression levels of candidate target genes by qPCR. The expression of the differentiation markers *DBH*, *TH*, *Phox2b*, *Gata2/3*, *trkA* and *trkC* was not significantly changed by Mk (data not shown). Interestingly, *Alk* expression was affected both by increasing and decreasing Mk levels, which indicates a positive autoregulatory control (Fig. 6A). A similar Mk-dependent expression was observed for the transcription factor *Hand2* (Fig. 6A). This finding, together with the previous demonstration that *Hand2* controls proliferation of sympathoadrenergic precursors and immature neurons in vitro and in vivo (Hendershot et al., 2008; Reiff et al., 2010; Schmidt et al., 2009) and the presence of *Hand2* expression in sympathetic ganglia (Fig. 6B), implicated *Hand2* in the regulation of *Alk* expression. It also suggested that the effects of *Hand2* on sympathetic neuron proliferation may be caused indirectly by controlling *Alk* expression levels. Indeed, we now demonstrate that forced Hand2 expression leads to significantly increased *Alk* expression (Fig. 6A) and that *shAlk* knockdown strongly reduces the proliferation stimulatory effects of Hand2 overexpression (see Fig. S4 in the supplementary material). By contrast, cell cycle genes *Cdk6* and *Ccnblip* identified as Hand2 target genes in developing heart tissue (Holler et al., 2010) were not affected in sympathetic neurons (not shown). As Phox2b has been implicated, together with Hand2, in the control of sympathetic neuron proliferation (Bachetti et al., 2010; Coppola et al., 2010; Reiff et al., 2010) we investigated whether wild-type Phox2b (Phox2b^{wt}) or a NB-related Phox2b variant (Phox2b^{K155X}), ectopically expressed in cultured immature sympathetic neurons, would affect *Alk* expression (Reiff et al., 2010). However, we were unable to detect effects of Phox2b^{wt} and Phox2b^{K155X} on *Alk* expression levels by qPCR (data not shown).

Alk signaling increases NMyc and trkB expression

Forced Hand2 expression also increased the expression of two additional proliferation regulators in sympathetic neurogenesis, *trkB* and *NMyc*. Activation of *trkB* through its ligand BDNF, as well as forced NMyc expression, lead to a strong increase in immature sympathetic neuron proliferation (Reiff et al., 2010; Straub et al., 2007). As the effects of Hand2 on sympathetic neuron proliferation are due to increased *Alk* expression, we investigated whether overexpression of ALK^{wt} and NB ALK variants may also lead to increased *NMyc* and *trkB* expression. Indeed, *trkB* and *NMyc* expression levels were elevated about twofold upon transfection of sympathetic neurons with expression vectors for ALK^{wt}, ALK^{F1174L} and ALK^{R1275Q} (Fig. 6C), taking the transfection efficiency (about 50%) into account.

DISCUSSION

The present work demonstrates that Alk signaling, stimulated by its ligand Mk, is essential for the proliferation of immature sympathetic neurons. Increased Alk receptor signaling is sufficient to enhance sympathetic neuron proliferation, which is accompanied by the upregulation of *Alk*, *NMyc* and *trkB*. Our findings uncover a novel mechanism that controls sympathetic neuron proliferation

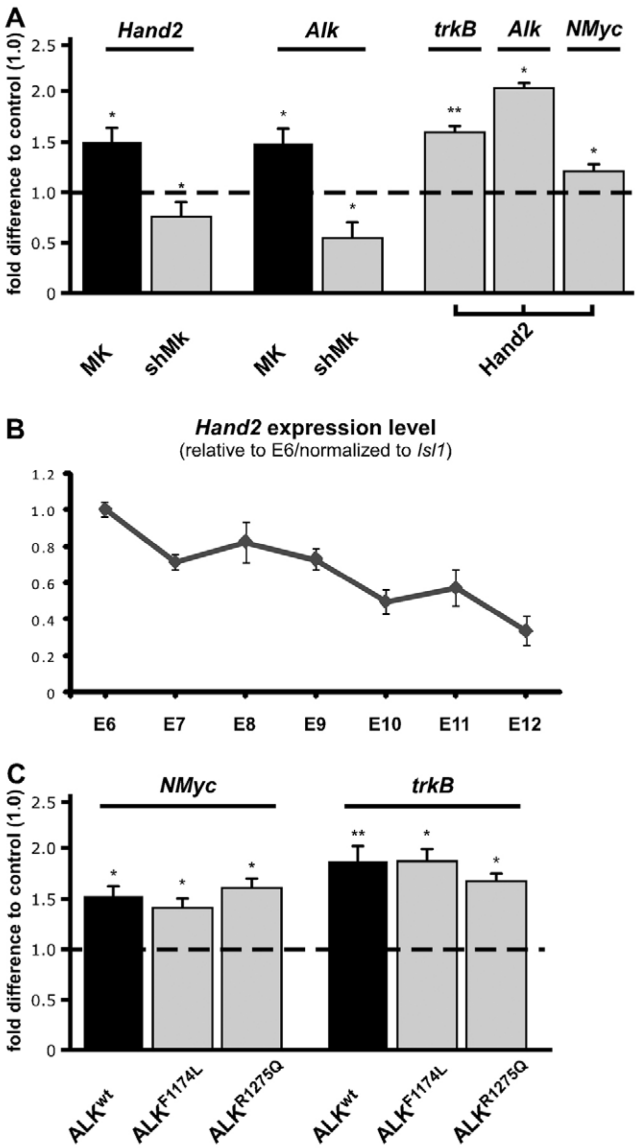

Fig. 6. Effects of Mk, Hand2 and ALK variants on E7 sympathetic neuron gene expression. (**A**,**C**) E7 sympathetic neurons were cultured with MK (10 nM) or transfected with *Alk* variants, *Hand2* or *shMk*. The mRNA was isolated after 2 days in vitro and analyzed by qPCR. Expression levels were normalized to *Gapdh* and referred to control transfections. (**B**) Lumbar SG were dissected and *Hand2* mRNA levels were analyzed by qPCR from E6 to E12. Data were normalized to *Isl1* and referred to as E6 values. (A) The effects of Mk on *Hand2* and *Alk* expression levels, and the effects of Hand2 overexpression on *trkB*, *Alk* and *NMyc* are shown. (C) Effects of ALK variants on *NMyc* and *trkB* expression are displayed. Data are mean±s.e.m. (n=6). (A,C) *$P\leq0.05$, **$P\leq0.01$, ***$P\leq0.001$, significantly different from control. (B) ANOVA: *Hand2*, P=0.004.

in vivo and in vitro, and provide insight into how activating ALK mutations may circumvent the termination of neurogenesis and predispose to NB.

Control of neurogenesis in sympathetic ganglia by Alk and Midkine

Neurogenesis in SG differs from other lineages, i.e. immature but already differentiated neurons proliferate (Rohrer and Thoenen, 1987; Rothman et al., 1978; Tsarovina et al., 2008). Thus, the expression of differentiation markers such as TH, Isl1 and TuJ1 is not linked to cell cycle exit; the mechanisms that lead to withdrawal from the cell cycle and the generation of postmitotic neurons remained largely unclear.

The Alk tyrosine kinase receptor is implicated in the control of sympathoadrenergic proliferation as gain-of-function mutations leading to constitutively active ALK were identified as a major cause for familial neuroblastoma (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). A function in proliferation was also supported by overexpression experiments in various cell lines (Chen et al., 2008; De Brouwer et al., 2010; George et al., 2008) and knockdown in ALK-expressing NB cell lines (Chen et al., 2008; George et al., 2008; Janoueix-Lerosey et al., 2008; Mosse et al., 2008). The present results confirmed the proliferation stimulatory role of NB ALK mutations in embryonic sympathetic neuron cultures – the potential tumor founder cells. Interestingly, Alk signaling is not only sufficient but is also essential for normal sympathetic neuron proliferation in vitro and in vivo, as revealed by pharmacological inhibition and *Alk* knockdown. Our finding that forced expression of normal ALK^{wt} increased proliferation of sympathetic neurons, whereas ALK^{wt} had no effect in NIH3T3 and Ba/F3 cells (Chen et al., 2008; George et al., 2008) suggested the presence of (an) endogenous ligand(s) in sympathetic neuron cultures available for activation of the ectopically expressed receptor.

Two potential ligands for the Alk receptor are known, Mk and Ptn (Ardini et al., 2010; Palmer et al., 2009), with similar expression patterns in rodents (Mitsiadis et al., 1995). However, besides Alk (Stoica et al., 2002), a number of additional receptor types have been identified for Mk, including PTPζ (Maeda et al., 1999), integrins (Muramatsu et al., 2004), Notch2 (Huang et al., 2008), LDL receptor-related protein (Muramatsu et al., 2000) and neuroglycan C (Ichihara-Tanaka et al., 2006). Both MK and PTN are expressed at high levels in neuroblastoma, but only MK expression correlates with aggressive NB (Fiegel et al., 2008; Nakagawara et al., 1995). Mk-deficient mice have not been analyzed specifically for defects in SG development (Nakamura et al., 1998). A reduction in ganglion size may have remained inconspicuous with respect to mouse behavior under normal animal housing conditions. In our study, Mk was identified as the physiologically relevant Alk activator for sympathetic neurons because: (1) *Mk* is expressed at much higher levels in SG when compared with *Ptn*; (2) Mk stimulates sympathetic neuron proliferation in vitro and in vivo; (3) the effect of MK is mediated by Alk, as shown by Alk knockdown; and (4) *Mk* knockdown results in a strong decrease in sympathetic neuron proliferation in vitro and in vivo. Mk is expressed both in immature sympathetic neurons and in the ganglion non-neuronal population, and may act in both an autocrine and paracrine manner, as previously shown for IGF-II (Zackenfels et al., 1995). It remains to be shown whether the activation of the Alk receptor involves direct binding of Mk or the PTPζ signaling pathway (Maeda et al., 1999; Perez-Pinera et al., 2007).

In vivo knockdown of *Mk* and *Alk* initially leads (E4) to a massively reduced number of proliferating EdU^{+} immature sympathetic neurons and to smaller ganglion size. The proliferation of Sox10^{+}/TH^{-} sympathetic neuron progenitors may also depend on Mk/Alk signaling and contribute to the effect on ganglion size. The finding that E7 sympathetic ganglia of *Mk/Alk* knockdown embryos show partially recovered ganglion size and a normal proportion of EdU^{+} cells suggests that interruption of Alk signaling may be compensated over time by other signals like IGF-I and IGF-II (Zackenfels et al., 1995). It should be noted, however, that normal neuron proliferation at E7 depends on Alk signaling, as shown by our acute gain- and loss-of-function experiments in vitro. The recent observation in *Drosophila* CNS that neuroblast lineages devoid of Alk signaling fail to produce the full complement of neurons and display a reduced proportion of $BrdU^+$ cells demonstrate an evolutionary conserved role for Alk in the control of neurogenesis (Cheng et al., 2011).

The mechanisms that lead to the termination of neurogenesis in sympathetic ganglia are unsolved and may either involve an increased probability of cell cycle exit or a regulated process, e.g. by the accumulation of cell cycle inhibitors or the downregulation of positive regulators such as Mk. It is unclear to what extent the decrease in *Mk* expression between E6 and E12 contributes to the strong reduction in neuron proliferation during this period (Rothman et al., 1978) as the effects of Mk overexpression could not be analyzed beyond E7. A tight control of *Mk* expression levels and/or availability would be required in order for Mk to function in controlling the extent and timing of neurogenesis. Several explanations for the decrease in *Mk* expression can be provided: (1) downregulation by extrinsic signals such as TGFβ, which is known to interfere with proliferation (Rahhal et al., 2004); (2) reduction by an intrinsic timing mechanism that determines when precursors withdraw from the cell cycle, similar to the reduction of Id4 in oligodendrocyte precursors (Kondo and Raff, 2000); (3) as *Mk* expression is regulated by a HIF responsive element, downregulation of HIF-2α protein may also lead to decreased *Mk* levels (Favier et al., 1999; Reynolds et al., 2004).

Autoregulatory control of Alk expression and function involves Hand2

What is the link between Alk and other signals also known to be involved in the proliferation control of sympathoadrenergic precursor cells and immature neurons? *Hand2* and *Alk* have been identified as genes affected by raised extracellular Mk levels and its knockdown. Elevated *Alk* expression in response to increased Alk signaling suggests a positive autoregulation of Alk. As Alk receptors may be limited in SG, this autoregulatory loop will ensure proliferation of all cells with access to Mk. Notably, higher levels of *ALK* mRNA were observed in NB samples harboring ALK mutations when compared with ALK^{wt} (Caren et al., 2008; Mosse et al., 2008; Schulte et al., 2011). The recent observation that NB with high-level ALK^{wt} expression display similar unfavorable phenotypes and clinical courses as do NB with activating ALK mutations demonstrates the important role of ALK^{wt} and its ligand in individuals with NB with elevated *ALK* expression (Schulte et al., 2011). The autoregulatory expression of Alk has also been shown for glioblastoma and may confer a proliferative advantage to the cells (Powers et al., 2002).

Hand2 is not only increased in parallel but is responsible for the upregulation of *Alk* and previously described proliferation effects of Hand2 (Reiff et al., 2010) are due to increased Alk expression and signaling. Supporting this notion, a function for Hand2 downstream of Alk has been demonstrated in *Drosophila* midgut visceral muscle development (Varshney and Palmer, 2006). It is presently unclear how *Alk* expression is initiated in primary SG and whether Hand2 is involved in this initial process.

What are potential mechanisms that underlie NB predisposition caused by *ALK* mutations and amplification?

High level expression of constitutively active ALK were shown to transform 3T3 fibroblasts and Ba/F3 lymphoid cells in vitro, which involves the PLCγ, PI3K/AKT, RAS/MAPK and JAK/STAT pathways (Chen et al., 2008; George et al., 2008). In addition, in vivo forced expression of ALK^{F1147L} in TH- or DBH-expressing embryonic mouse sympathetic neurons is able to induce NB in 4- to 6-month-old mice (J. Schulte, personal communication). The mouse model displays the incomplete penetrance and delayed tumor development of human ALK germline mutations, i.e. some carriers of human ALK mutations in familial NB remain asymptomatic and other mutation carriers in affected families do not develop detectable tumors during embryonic life but during postnatal months and years (Janoueix-Lerosey et al., 2008; Mosse et al., 2008). Delayed tumor development results from cells of solid tumors, such as NB, accumulating several 'driver' mutations in cancer genes and genomic aberrations in order to achieve a neoplastic status (Bozic et al., 2010; Parsons et al., 2011; Vogelstein and Kinzler, 2004). NB can also be elicited in mice by forced *NMYC* expression under the control of the *TH* promoter (Weiss et al., 1997). Interestingly, in this mouse model of NB, there is also a large delay between tumor-mediated initiation of low level *NMYC* expression and overt tumor generation, which suggests that additional mutations and chromosomal aberrations are accumulated during this period (Hansford et al., 2004). Our finding that sustained and increased Alk signaling leads to increased *NMyc* expression, together with the conclusion from the TH-NMYC mouse that elevated NMYC levels are sufficient to elicit NB development (Alam et al., 2009; Hansford et al., 2004; Weiss et al., 1997) implicates NMyc as an important component of the ALK-induced NB predisposing mechanism. Interestingly, *NMyc* amplification occurs postnatally in the TH-NMYC mouse and is also associated with the ALK F1174 mutation in human NB (De Brouwer et al., 2010; Hansford et al., 2004). Elevated *trkB* levels may contribute by increased proliferation of sympathetic progenitors (Reiff et al., 2010; Straub et al., 2007).

The differential *Mk* and *Alk* expression supports our notion that the restriction of NB development to sympathoadrenergic cells when compared with parasympathetic, enteric and sensory precursors is caused by different modes of neurogenesis and cell cycle control (Reiff et al., 2010; Tsarovina et al., 2008). The present data suggest that Alk-dependent normal neurogenesis defines the lineage and the developmental time period when *ALK* mutations are effective for tumor predisposition.

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