Epigenetic induction of the Ink4a/Arf locus prevents Schwann cell overproliferation during nerve regeneration and after tumorigenic challenge

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The number of Schwann cells is fitted to axonal length in peripheral nerves. This relationship is lost when tumorigenic stimuli induce uncontrolled Schwann cell proliferation, generating tumours such as neurofibromas and schwannomas. Schwann cells also re-enter the cell cycle following nerve injury during the process of Wallerian degeneration. In both cases proliferation is finally arrested. We show that in neurofibroma, the induction of Jmd3 (jumonji domain containing 3, histone lysine demethylase) removes trimethyl groups on lysine-27 of histone-H3 and epigenetically activates the Ink4a/Arf-locus, forcing Schwann cells towards replicative senescence. Remarkably, blocking this mechanism allows unrestricted proliferation, inducing malignant transformation of neurofibromas. Interestingly, our data suggest that in injured nerves, Schwann cells epigenetically activate the same locus to switch off proliferation and enter the senescence programme. Indeed, when this pathway is genetically blocked, Schwann cells fail to drop out of the cell cycle and continue to proliferate. We postulate that the Ink4a/Arf-locus is expressed as part of a physiological response that prevents uncontrolled proliferation of the de-differentiated Schwann cell generated during nerve regeneration, a response that is also activated to avoid overproliferation after tumorigenic stimuli in the peripheral nervous system.

Keywords: cellular biology; nerve injury; nerve regeneration; Schwann cells; neuroscience

Introduction

The plasticity of the Schwann cell lineage contributes to the successful regeneration of peripheral nerves. After injury, axotomized myelinating Schwann cells de-differentiate and proliferate, generating an environment that stimulates the growth of axons from the proximal stump and helps to direct them back towards target tissues. It has been suggested that this process depends on...
expression of the AP-1 transcription factor c-Jun (Guerin et al., 2005; Jessen and Mirsky, 2008; Parkinson et al., 2008). Re-entry of growing axons into the distal segment induces Schwann cell differentiation and remyelination, eventually leading to successful nerve repair. However, if for some reason the axons cannot reach the distal stump, the Schwann cells will remain in a de-differentiated but non-proliferative state (Scherer et al., 1994; Shy et al., 1996).

Schwann cell de-differentiation and proliferation are also hallmarks of PNS tumours (Carroll and Ratner, 2008). In type I neurofibromatosis (produced by mutations in Nf1 gene) loss of function mutations chronically activate the RAS/RAF/ERK pathway contributing to uncontrolled Schwann cell proliferation and tumour development (Harrisingh et al., 2004; McClatchey, 2007; Parrinello and Lloyd, 2009). We have recently shown that the neuronal over-expression of a specific isoform of neuregulin (type III-β, also known as SMDF) activates the ERK pathway and induces Schwann cell hyperproliferation and neurofibroma development (Gomez-Sanchez et al., 2009). The benign nature of neurofibromas is probably due to the eventual cessation of proliferation. This occurs for no obvious reason and despite the persistence of the tumorigenic stimulus. Here we show that, in these benign tumours, Schwann cell proliferation is limited by the activation of the oncogene-induced senescence programme, a fail-safe mechanism that prevents uncontrolled growth after pathological activation of oncogenes that signal through the RAS/RAF/ERK pathway (Collado and Serrano, 2010). As happens with other non-malignant tumours (Agger et al., 2009; Agherbi et al., 2009; Barradas et al., 2009), neurofibroma oncogene induced senescence is promoted by the epigenetic induction of the Ink4a/Arf locus mediated by the histone H3 demethylase Jmjd3, which through the p19Arf/p53 and p16ink4a/Rb pathways, blocks Schwann cell proliferation. We also show that expression of this locus and the demethylase Arf locus mediated by the histone H3 demethylase Jmjd3, which through the p19Arf/p53 and p16ink4a/Rb pathways, blocks Schwann cell proliferation. We also show that expression of this locus and the demethylase Jmjd3 is induced in the distal stump of injured nerves. Interestingly loss of function of this signalling pathway (in the Ink4a/Arf−/− or p53−/− mice) provokes an increased cell proliferation rate after nerve injury, suggesting that, akin to neurofibromas, the epigenetic induction of the Ink4a/Arf locus prevents uncontrolled Schwann cell proliferation in distal nerve segments when axon regeneration is delayed or precluded.

Materials and methods

Antibodies

The antibodies and primers are listed in Table 1.

Plasmids

pCMV-Jmjd3 was obtained from K. Helin (Agger et al., 2007) (Addgene plasmid 24167). pEYFP was from Clontech.

Animal studies

All animal work was conducted according to EU guidelines and with protocols approved by the ‘Comité de Bioética y Bioseguridad del

Human tissues

All the procedures were performed according to the EU guidelines and approved by the hospital ethics committee. Patients were diagnosed according to the accepted standard NF1 diagnostic criteria. They were informed about the study, and consent was obtained from all. Neurofibromas were obtained from seven patients after surgery. Samples from six tumours were sectioned, deparaffinized and submitted to antigen retrieval by using the citrate method. Samples were prepared for immunofluorescence, as described below, and incubated with the indicated antibodies. One neurofibroma was used to obtain RNA. Healthy adult human peripheral nerves were obtained from diagnostic biopsies and submitted to the same protocol described for the tumours.

Cell cultures

Schwann cells were cultured from sciatic nerves of neonatal rats as described previously by Brockes et al. (1979). All the procedures were performed following EU and institutional guidelines. Cell cultures were expanded in Dulbecco’s modified Eagle medium supplemented with 3% foetal bovine serum, 5 μM forskolin and 50 nM GST-Nrg1. Where indicated, cells were transfected with plasmid DNA using Nanojuice® transfection reagent (Merck) following the manufacturer’s recommendations.

Messenger RNA detection and quantification by quantitative reverse transcription polymerase chain reaction

To detect and quantify gene expression, animals were euthanized, the sciatic nerve was dissected, and total RNA isolated using PureLink® Micro-To-Midi™ kit according to the instructions of the manufacturer (Invitrogen). Genomic DNA was removed by incubation with RNase free DNase I (Fermentas), and RNA was primed with random hexamers and retrotranscribed to complementary DNA with SuperScriptTII reverse transcriptase (Invitrogen). Control reactions were performed omitting retrotranscriptase. Quantitative real-time PCR was performed using the Applied Biosystems 7500 Real Time PCR System and Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). To avoid genomic amplification, PCR primers were designed to fall into separate exons flanking a large intron when possible. Reactions were performed in duplicates of three different dilutions, and threshold cycle values were normalized to the housekeeping gene 18S. The specificity of the products was determined by melting curve analysis and gel electrophoresis. The ratio of the relative expression for each gene to 18S was calculated by using the 2^ACT formula.
### Table 1 Antibodies and primer sequences

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### Primer sequences

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Immunofluorescence and electron microscopy studies

For immunofluorescence, mice were sacrificed and sciatic nerves dissected, embedded in O.C.T. (TissueTek) and frozen on dry ice. Longitudinal or transverse nerve sections (10 μm) were fixed in 4% paraformaldehyde, blocked for 1 h in 10% horse serum and 0.1% Triton™ X-100 in PBS. Primary antibodies were diluted in blocking solution and incubated overnight at 4°C (human tissues were incubated for 36 h at 4°C). Sections were then washed with PBS, and detection was performed applying the appropriate fluorescent secondary antibodies (Alexa Fluor® 594 anti-mouse, Alexa Fluor® 488 anti-rabbit and anti-chicken, Alexa Fluor® 555 anti-rat: 1:1000; Invitrogen) for 1 h. Nuclei were counterstained with bisbenzimide (Hoechst nuclear stain) in PBS. Samples were mounted in Fluoromount™ G (Southern Biotechnology Associates). Anti-Krox 20 immunofluorescence was performed as described by Le et al. (2005). Images were obtained using a confocal ultraspectral microscope (Leica TCS SP2). For ultrastructural images of the malignant tumours, symptomatic mice were anaesthetized by intraperitoneal injection of 40 mg/kg ketamine and 30 mg/kg xylazine and then intracardially perfused with 2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M phosphate buffer, pH 7.4. Tissues were dissected and immersed in the same fixative solution at 4°C overnight, washed in phosphate buffer, post-fixed in 1% osmium tetroxide, dehydrated in graded ethanol series, and embedded in epoxy resin (Durcupan). Semi-thin sections were cut with a glass knife at 1–3 μm and stained with toluidine blue to check the quality of the tissue before the electron microscopy studies. For electron microscopy, ultrathin sections (70–90 nm) were cut on an ultramicrotome (Reichert Ultracut E; Leica) and collected on 200-mesh nickel grids. Staining was performed on drops of 1% aqueous uranyl acetate, followed by Reynolds’s lead citrate. Ultrastructural analyses were performed in a Philips TECNAI 12 electron microscope.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis and immunoblotting

Nerves or cultured Schwann cells were homogenized at 4°C in radioimmunoprecipitation assay buffer (PBS, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and 5 mM EGTA) containing protease inhibitors (Complete MINI tablets; Roche) and, where necessary, phosphatase inhibitors (Phospho STOP tablets, Roche). Protein concentrations were determined by the bicinchoninic acid method (Pierce). Total protein (10–50 μg) of was subjected to SDS-PAGE and blotted onto Protran nitrocellulose membrane (Whatman). Membranes were blocked and incubated overnight at 4°C with the indicated primary antibody, washed and incubated with secondary antibodies, and developed with ECL Plus (GE Healthcare). The secondary antibodies were conjugated with horseradish peroxidase (1:2000; Sigma).

Chromatin immunoprecipitation assay

The chromatin immunoprecipitation (ChiP) assay was a modification of the method described by Jang et al. (2006). Briefly, nerves from seven post-natal Day 20 mice (NSE-SMDF+/− mutants and wild-type littermates) were removed, chopped into small pieces and incubated in PBS/1% paraformaldehyde for 25 min at room temperature. Tissue was harvested by centrifugation (1000g for 3 min) and washed with PBS. Pellet was resuspended in 1.2 ml of buffer A (150 mM NaCl, 10% glycerol, 0.3% Triton, 50 mM Tris-HCl pH8 and protease inhibitors), homogenized and sonicated (20 pulses of 20 s separated by 40 s on ice between each pulse) to ‘high power’ in the Bioruptor (Diagenode). Chromatin was clarified by centrifugation at 21 000g for 30 min at 4°C. Protein concentration in the supernatant was quantified by the bicinchoninic acid method (BCA, Pierce). An aliquot was saved as input. The volume corresponding to 60–100 μg of protein was incubated with the corresponding antibody (anti-H3K27me3 or control IgG) overnight at 4°C to form immunocomplexes. Protein A sepharose (CL-4B, GE Healthcare) was resuspended in distilled water and pelleted by centrifugation (~500g). Resin was resuspended in water with 0.5 mg/ml of bovine albumin and 0.2 mg/ml of sonicated DNA (her-ring sperm, Sigma). This slurry (40 μl) was added to the immunocomplex and incubated for 1 h at 4°C. Immunocomplexes were centrifuged (500g, 3 min) and washed twice with 1 ml of ‘low salt buffer’ (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.1, 150 mM NaCl, Complete protease inhibitors, Roche). Then washed once with 1 ml of ‘high salt buffer’ (the same as the low but with 500 mM NaCl) and washed three times with 1 ml of LiCl buffer (0.25 M LiCl, 1% IGEPAL, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8.1, protease inhibitors). Chromatin from immunocomplexes and input was eluted with 300 μl of 1% SDS, 0.1 M NaHCO3, 200 mM NaCl and incubated at 65°C for 6 h (to break the DNA–protein complexes). DNA was purified using a column purification kit (GE healthcare) and submitted to SYBR® green quantitative PCR with the indicated primers.

Microarray analysis

Total RNA was extracted from the sciatic nerves of three matched NSE-SMDF+/− mice and three wild-types from two different litters. The RNA from each mouse was hybridized to GeneChip® Mouse Gene 1.0 ST arrays according to the manufacturer’s protocol (Affymetrix). The microarray data were then analysed using GeneSpring GX 11 (Agilent Technologies, Inc). Robust Multichip Average algorithm was used for data normalization. Values show fold increase. P-value for each gene is shown. Complete microarray data are available on request.

Results

Schwann cell proliferation is halted in neurofibromas from adult NSE-SMDF+/− mutant mice

We have previously shown that overexpression of the SMDF neur-egulin isoform [in the NSE-SMDF+/− mice] induces tumour development in peripheral nerves. Intriguingly, after an initial period of growth (from post-natal Days 5 to 14) Schwann cell proliferation stops in the nerves of these mice. Consequently, cellularity remains increased (about three times) but stable during the whole life of the mutant animals. We initially reasoned that proliferation was halted because of a decrease in the expression of the transgene in adult animals, however, the transgene is clearly expressed in adult animals (Supplementary Fig. 1A–C), ruling out this possibility. Then, we looked for a putative downregulation of neuregulin receptors in the mutant mice but as shown in Supplementary Fig. 1D, erbB2 and erbB3, the unique erbB
receptors in Schwann cells, are normally expressed in mutant nerves. In fact, we consistently observed an increase in the expression of erbB3 in the sciatic nerves of the NSE-SMDF+/− mice. We then explored, at different ages, the activation status of the ERK signalling pathway, which is pivotal for neuregulin-induced Schwann cell proliferation (Birchmeier and Nave, 2008). To this end we determined the phosphorylation status of ERK1/2. As shown in Supplementary Fig. 1E, the ERK signalling pathway remains activated even when proliferation in the mutant nerves has halted (Supplementary Fig. 1F). Neuregulin promotes cell proliferation by inducing cyclin D1, a positive regulator of the G1/S transition (Kim et al., 2000; Atanasoski et al., 2001; Yang et al., 2008). Therefore, the possibility existed that the proliferation arrest was the consequence of a failure to express this pivotal gene. Surprisingly, we found that cyclin D1 was not downregulated, but notably increased in the adult transgenic nerves (Supplementary Fig. 1G). Taken together, our data show that, despite neuregulin signalling remaining hyperactivated in the NSE-SMDF+/− transgenic nerves, Schwann cell proliferation stops after post-natal Day 14.

**Proliferation of Schwann cells is restricted by the activation of the replicative senescence programme in the NSE-SMDF+/− neurofibromas**

It has been established that over-activation of intracellular signalling pathways by oncogenes is necessary, but not sufficient, for cancer development (Collado et al., 2007; Collado and Serrano, 2010; Kuliman et al., 2010). Tumour overgrowth is prevented when cells detect an aberrant situation and activate a genetic programme (Oncogene Induced Senescence) that blocks the cell cycle. This programme is primarily mediated by the Arf/p53 and p16Ink4a/Rb pathways (Matheu et al., 2008; Gorgoulis and Halazonetis, 2010). The establishment of oncogene induced senescence depends on ERK activation downstream of RAS and RAF. As outlined previously, in NSE-SMDF+/− nerves Schwann cell proliferation is halted after post-natal Day 14 despite the persistence of ERK pathway activation. To test if this is mediated by the activation of the oncogene induced senescence programme, we looked for the expression of p19Arf in transgenic nerves. As is shown in Fig. 1A, a clear nuclear expression of p19Arf was detected in post-natal Day 20 and 150 mutant nerves. In contrast, practically no expression could be detected in the PNS of wild-type littersmates. This result was confirmed by western blot (Fig. 1B). Interestingly, p19Arf was exclusively expressed by the non-myelinating Schwann cells, as demonstrated by the almost complete absence of co-localization with Krox-20 (Fig. 1C). The4a/Arf locus encodes for both p19Arf (p14Arf in humans) and p16Ink4a. Although working through different pathways, both proteins are known to activate the senescence programme. As shown (Fig. 1D), p16Ink4a expression is also increased in mutant nerves and co-localizes with p19Arf, suggesting that the p16Ink4a/Rb pathway contributes to control Schwann cell over-proliferation in the neurofibromas developed by the NSE-SMDF+/− mice. The absence of p19Arf expression in some p16Ink4a positive cells is probably consequence of the p53 capacity to block the former transcript through a previously described feedback loop, that has no effect on p16Ink4a (Gil and Peters, 2006). To obtain further insight we measured the expression levels of p19Arf and p16Ink4a messenger RNAs at different ages by quantitative PCR. As is shown in Fig. 1E and F, both transcripts were clearly increased in transgenic nerves from post-natal Days 20 to 720.

To obtain a more comprehensive view we performed high throughput gene expression analysis of the mutant nerves using Affymetrix Genechip Mouse Gene 1.0ST Array. Sciatic nerves of post-natal Day 20 NSE-SMDF+/− mice and wild-type littersmates were removed, and total RNA extracted. RNA from three animals per genotype was separately hybridized to the chip. To validate the array approach we first looked for myelination markers. In agreement with what we found previously (Gomez-Sanchez et al., 2009), most messenger RNAs for myelin proteins (like CNPase, MBP or MAG) were downregulated in the mutant nerves. A similar result was obtained for cholesterol biosynthetic enzymes. This is a consequence of the increased number of non-myelinating Schwann cells that over-populate mutant nerves and is not generated by expansion of the peri/epineural compartment (Gomez-Sanchez et al., 2009). In support of this, we did not observe an increase in markers for this compartment (such us Resistin, Lpl or Acpr30) in the microarray data. Thus far, these results confirm our previous findings and validate the microarray approach for our subsequent analysis.

Next we analysed the expression of genes involved in oncogene induced senescence. As shown (Table 2), not only Ink4a/Arf but also Ink4b (p15) is upregulated in mutant nerves, suggesting that both loci collaborate in the establishment of the senescence programme. Interestingly, it has been shown that the expression of three genes (p15ink4b, p16ink4a and p19Arf) is highly coordinated in other systems (Gil and Peters, 2006). We also found increased levels of p53 messenger RNA and, interestingly, Wnt16 messenger RNA, a recently described new marker of cellular senescence (Binet et al., 2009). In contrast cell cycle inhibitors like p27kip1 and (intriguingly) p21cip were downregulated.

**Histone demethylase Jmjd3 contributes to the activation of the Ink4a/Arf locus in neurofibroma Schwann cells**

It has recently been shown that Jmjd3 epigenetically controls the expression of the Ink4a/Arf locus after oncogenic activation of RAS in primary fibroblasts (Agger et al., 2009; Agherbi et al., 2009; Barradas et al., 2009). To establish if a similar mechanism mediates activation of the Ink4a/Arf locus in the NSE-SMDF+/− mice, we checked the expression of Jmjd3 in mutant nerves. First we performed immunofluorescence analysis with a polyclonal anti-Jmjd3 antibody. As shown (Fig. 2A), Jmjd3 immunoreactivity was significantly increased in mutant nerves. Measuring messenger RNA expression levels for this gene by quantitative PCR confirmed this result (Fig. 2B). The increase was specific, as demonstrated by the unchanged expression of the related demethylase Utx (Fig. 2B and data from the microarray analysis in Table 2). Secondly, we explored the activation/repression status of the Ink4a/Arf locus by +/− ChiP. Using methylation-sensitive specific antibodies,
Figure 1 Ink4a/Arf locus products are expressed in the peripheral nerves of the NSE-SMDF^{+/−} mice. (A) p19Arf is highly expressed in the neurofibromas developed by the mutant mice (TG) but barely in the nerves of wild-type (WT) littermates. Longitudinal sections of mutant and wild-type nerves (P20 and P150) were incubated with anti-p19Arf rat monoclonal antibody. A clear nucleolar labelling (red) was observed in many of the neurofibroma cells under the confocal microscope. In contrast, p19Arf was barely detected in wild-type nerves. Quantification shows that the number of p19Arf^+ cells increases slightly in wild-type (as expected) and remains high in transgenics with age (three mice per condition were used; a mean of 2752 ± 650 cells per mice were counted; t-test, **P < 0.001). (B) p19Arf upregulation was confirmed by western blotting. Protein extracts of wild-type and transgenic sciatic nerves were submitted to SDS-PAGE and blotted with the anti-p19Arf antibody. β-actin was used as loading control. This experiment was repeated twice. A representative western blot is shown. (C) Only cells that do not enter the myelinating programme (i.e. those that are negative for Krox-20) become senescent (positive for p19Arf), as supported by the practically absence of co-localization between Krox-20 and p19Arf expression. (D) The other product of the Ink4a/Arf locus (p16Ink4a) is also upregulated in transgenic nerves. A high degree of co-localization between p16Ink4a and p19Arf was observed (bottom) suggesting a coordinated expression of both products (see text). These experiments were repeated three times. More than three sections per sample were analysed with similar results. A typical image is shown. Scale bars = 50 μm.
we immunoprecipitated H3K27me3 and performed quantitative PCR with specific primers for different promoter regions of the Ink4a=Arf locus (Barradas et al., 2009). Our data show that there is less H3K27me3 bound to the promoters of p19Arf and p16Ink4a in transgenic than in wild-type nerves (Fig. 2C), suggesting that they are transcriptionally active. Indeed, when we enforced the expression of Jmjd3 in cultured rat Schwann cells (by transfecting the pCMV-Jmjd3 vector) the expression of p19Arf was induced. Taken together, our data suggest that the activation of the senescence programme is mediated by the recruitment of Jmjd3 on Ink4a=Arf promoter regions and its de-repression by the demethylation of the H3K27me3.

Oncogene-induced senescence in human plexiform neurofibromas

NF1 patients develop plexiform neurofibromas, nerve enlargements caused by mixed cell tumours that although initially benign, can degenerate (10%) to form malignant peripheral nerve sheath tumours (Cichowski et al., 1999; Evans et al., 2002; McClatchey, 2007). To determine whether the oncogene-induced senescence programme is also activated in human neurofibromas we collected samples from human NF1-associated plexiform neurofibromas and explored the expression of senescence markers. As is shown in Fig. 3, p14Arf (the human homologue for p19Arf) is highly expressed in the human plexiform neurofibromas analysed, but not in normal peripheral nerves. A similar result was observed for p16Ink4a, suggesting that as in mice, malignant proliferation of Schwann cells in human plexiform neurofibroma is prevented by the activation of the senescence programme, mediated by expression of the Ink4a=Arf locus. We observed overexpression of this locus in 6 of 6 human NF1 plexiform neurofibomas analysed (Fig. 3 and Supplementary Fig. 2).

Malignant progression of neurofibromas is associated with the loss of p19Arf expression

We have previously shown that despite the development of neurofibromas, most NSE-SMDF+/- mice show a normal external phenotype and life expectancy (Gomez-Sanchez et al., 2009). Nevertheless, ~15% of them develop neurological deficits (starting at 8 months), which are associated with the local growth of some nerve roots and/or dorsal root ganglia. In the histopathological analysis of these tumours (Fig. 4A) we observed high proliferation of atypical fusiform and round cells in short
fascicles and focal whorled arrangement, as well as pleomorphic nuclei with high mitotic rate and focal necrosis, all of them consistent with the histology of human malignant peripheral nerve sheath tumours. To discover if this local growth is produced by resumption of Schwann cell proliferation in these neurofibromas, we explored the level of phospho-histone H3, a marker of cell proliferation, and compared it with levels in neurofibromas where proliferation was halted. As shown in Fig. 4B, the phospho-histone H3 labelling is dramatically increased, suggesting an augmented proliferation rate in these tumours due to re-entry into the cell cycle of some previously arrested Schwann cells. We reasoned that transformation of these non-malignant neurofibromas into malignant tumours could be related to the loss of the capacity of some cells to enter (or to stay) the senescence programme. To explore this point, we determined the messenger RNA expression levels for p19Arf and p16Ink4a in 10 highly proliferative tumours developed by the NSE-SMDF+/- mice between post-natal Days 240 and 720. As shown in Fig. 4C, seven of these tumours showed a reduced expression of p19Arf, suggesting that their malignant transformation is mediated by loss of the oncogene-induced senescence programme. In contrast, we found high variability in levels of p16Ink4a expression (Fig. 4D), implying that the loss of the senescence programme is mainly a consequence of a failure in signalling mediated by p19Arf. To verify this hypothesis,
we decreased the capacity to activate the senescence programme through the p19Arf/p53 pathway by obtaining NSE-SMDF+/-; p53+/- double heterozygotes. Strikingly, the Kaplan-Meier survival curve (Fig. 5A) demonstrated that elimination of a single copy of p53 increases up to 100% the incidence (and decreases significantly the age) of malignant tumour development. As in the case of the NSE-SMDF+/- simple heterozygotes, most tumours developed on nerve roots and dorsal root ganglia, but, as shown in Fig. 5B, some highly proliferative tumours also developed on sciatic nerve neurofibromas, and could be distinguished easily by the increased proliferation rate (pH3). When analysed by transmission electron microscopy they showed aberrant structures including huge deposits of extracellular collagen and dissociated Schwann cells (Fig. 5C), resembling malignant peripheral nerve sheath tumours.

**Ink4a/Arf locus is induced in Schwann cells during Wallerian degeneration**

After nerve injury, the myelinating Schwann cells of the distal stump de-differentiate in a process dependent on the transcription factor c-Jun (Parkinson et al., 2008). If the growing axons of the proximal segment can reach the distal stump, Schwann cells will differentiate and remyelinate generating a functional peripheral nerve. However, if the axons cannot enter the distal stump, Schwann cells will not differentiate but will remain in a non-proliferative state that can last up to 2 months (Scherer et al., 1994; Shy et al., 1996). The cause of proliferation arrest in the distal stumps is unknown. It could be explained by both the washing out of some mitogens and/or by the induction of some mechanism that actively blocks proliferation. To check whether, as in neurofibroma, the Ink4a/Arf-mediated replicative senescence contributes to halting Schwann cell proliferation after nerve injury, we performed anti-p19Arf immunofluorescence studies of the distal stump of transected nerves. As shown in Fig. 6A, in the Schwann cells of the distal stump p19Arf is expressed from the fourth to the 24th day post-injury. p19Arf-positive cells were identified as non-myelinating Schwann cells as they express S100B but fail to express Krox-20 (Fig. 5B). To unambiguously identify the p19Arf immunoreactivity, we performed transection experiments in Ink4a/Arf knock-out mice. As shown in Fig. 6C, no nucleolar labelling could be observed in the distal stumps of Ink4a/Arf-/- mice, confirming the specificity of the immunolabelling in the wild-types. Interestingly Jmjd3 was also upregulated soon after injury (Fig. 6D), suggesting that histone H3K27 demethylation also contributes to de-repressing this locus in the distal stumps of transected nerves. As expected, cell proliferation (evaluated with anti-phospho histone H3 antibody) is very low after 24 days (Fig. 6E).

Schwann cells are highly plastic and can de-differentiate and re-differentiate multiple times in vivo (Monje et al., 2010). To determine if they can also revert the senescence programme we induced nerve injury by crushing the sciatic nerve with forceps. This strategy produces total axotomy, but the epineuria remains intact, allowing proximal segment axons to grow into the distal stump facilitating rapid nerve repair. We found that p19Arf is upregulated in crushed nerves (Fig. 6F), but remarkably, as soon as the re-entry of axons induces the differentiation programme in Schwann cells, the expression of p19Arf is downregulated (Fig. 6G). Thus far, our data suggest that the Ink4a/Arf-mediated senescence programme in the injured PNS is reversible, and can be switched off when Schwann cells re-enter the myelination programme.

**Ink4a/Arf-mediated senescence contributes to growth arrest during Wallerian degeneration**

To investigate the role of the Ink4a/Arf locus in the control of cell proliferation after PNS injury, we performed sciatic nerve transections in Ink4a/Arf-/- mice. Four and 12 days after injury proliferation rate was calculated by estimating the number of Ki67-positive cells in relation to the number of total cells in defined areas of the distal stumps. As a control, we performed the same experiment in wild-type littermates. As is shown in Fig. 7A, we found a significantly increased proliferation rate in the distal stumps of mutant mice. This increased proliferation was still observed up to 12 days post injury, when it had reached ~30%. Thus far, our data suggest...
that the gene expression products of the Ink4a/Arf locus contribute to the proliferation arrest in the distal stumps of transected nerves.

As we have shown previously, the stabilization of p53 by p19Arf is needed to block Schwann cell proliferation in the neurofibromas developed by the NSE-SMDF+/− mice. To determine the contribution of the p19Arf/p53 pathway to Wallerian degeneration growth arrest, we performed nerve transection experiments in p53 knockout mice. As shown in Fig. 7B, proliferation rate after injury was found increased in the p53−/− nerves, suggesting that, as in neurofibromas, the stabilization of p53 by p19Arf contributes to the prevention of over-proliferation during the initial steps of peripheral nerve regeneration.

Discussion

Control of Schwann cell numbers in the PNS is a tightly regulated process that fits the number of glial cells to the axon length, guaranteeing adequate myelination and nerve conduction velocity (Sherman and Brophy, 2005). To this aim, axons produce signalling molecules that critically control the proliferation of the Schwann cell lineage during development (Salzer and Bunge, 1980; Salzer et al., 1980). One of these axon-derived signals is thought to be a product of the NRG1 gene (Jessen and Mirsky, 2005). We have recently shown that axonal over expression of the type III-β neuregulin-1 induces Schwann cell hyper-proliferation and nerve enlargement.

Figure 4 Loss of the senescence programme induces malignant transformation of neurofibromas. (A) Histopathological aspect of fast-growth tumours developed by ~15% of the NSE-SMDF+/− mice. Left: Proliferation of atypical fusiform and round cells, in short fascicles and focal whorled arrangement. Right: Pleomorphic nuclei with high mitotic rate and focal necrosis, all consistent with malignant peripheral nerve sheath tumour. (B) Proliferation rate is notably increased in the malignant tumours developed in the PNS by the NSE-SMDF+/− mice, as demonstrated by anti-phospho-histone H3 staining. Sections of fast growing tumours and NSE-SMDF+/− sciatic nerves were incubated with anti-phospho-histone H3 antibody (green) and nuclei counterstained with Hoechst (blue). As is shown, many cells were positive for phospho-histone H3 in the tumours but not in neurofibromas. The experiment was repeated three times with similar results. A representative experiment is shown. Scale bars = 50 μm. (C) Total RNA was obtained from 10 fast-growing tumours developed by the NSE-SMDF+/− mice and the messenger RNA for p19Arf quantified by reverse transcription quantitative PCR. Results were normalized against 18S ribosomal RNA. p19Arf messenger RNA was plotted against the levels of wild-types and transgenics. As is shown, in seven of these tumours p19Arf messenger RNA was downregulated to the levels of wild-type nerves. (D) By contrast the variability in expression levels of p16ink4a messenger RNA in the tumours is high, and there is no clear downregulation.
Surprisingly, after an initial period of active cell proliferation, nerve growth stops with the tissue acquiring most of the macroscopic and histological traits of neurofibromas. Interestingly, the growth of plexiform neurofibromas in NF1 patients is also limited, despite the persistence of the tumorigenic stimulus (Harrisingh et al., 2004; McClatchey, 2007; Carroll and Ratner, 2008). Our data suggest that the stop in the growth of both types of neurofibromas is a consequence of induction of the Ink4a=Arf locus which activates the oncogene-induced senescence programme. But, how is the Ink4a=Arf locus induced? In physiological conditions this locus is silenced by the trimethylation of the Lys27 on histone H3, a modification imposed by the polycomb group (PcG) proteins (Bracken et al., 2007). It has been shown that in RAS transformed fibroblasts induction the Ink4a=Arf locus is mediated by the demethylase Jmjd3 (Agger et al., 2009; Agheri et al., 2009; Barradas et al., 2009). The activity of Jmjd3 removes the methyl groups on the K27 of histone H3, reverting the silencing effects of the PcG proteins. Remarkably, we found increased levels of Jmjd3 in mice neurofibromas. Using ChIP assay we show evidence that this demethylase is recruited to the p19Arf and p16Ink4a promoters, where it removes the trimethyl groups of the lysine 27 in histone H3 (Fig. 2C) de-repressing this locus. One question that remains is how Jmjd3 itself is induced. Interestingly there is a binding site for AP-1 in the promoter of Jmjd3 (Ameyar-Zazoua et al., 2005).

Figure 5 Loss of p19Arf/p53 pathway function induces malignant transformation of the neurofibromas developed by NSE-SMDF+/− mice. (A) Elimination of a single copy of p53 (NSE-SMDF+/−; p53+/− double heterozygotes) increases dramatically the incidence (up to 100%) of malignancies and decreases the age of malignant tumour formation. Kaplan-Meier survival plots are shown (the exact number of animals per genotype is indicated in the graph). (B) Low magnification field of a highly proliferative tumour developed by the NSE-SMDF+/−; p53+/− mice. The origin of the malignant tumour can be identified as a localized region of the neurofibroma with increased proliferation rate, which gives rise to a highly proliferative mass of tissue attached to the nerve. Sections of the tissue were incubated with anti-phospho-histone H3 antibody (green) and nuclei counterstained with Hoechst (blue). Scale bar = 600 μm. (C) Malignant tumours developed by the double mutants show dramatically increased collagen-rich extracellular matrix with dissociated Schwann cells (arrows) intermingled with normally myelinated axons (arrowheads). Transmission electron microscopy images from a malignant tumour developed by a NSE-SMDF+/− mice (right) and from the sciatic nerve of a wild-type littermate (left). Similar findings were obtained in two different tumours. Scale bar = 5 μm.
Figure 6  Nerve injury induces the expression of the Ink4a/Arf locus in Schwann cells. (A) After complete sciatic nerve transection, expression of p19Arf (arrows) is induced in the distal stump. Expression of this protein is maintained up to 24 days post-injury. The re-expression of c-Jun denotes the presence of de-differentiated Schwann cells in the injured nerve segment. (B) p19Arf is only expressed by non-myelin forming Schwann cells as demonstrated by its co-localization with S100β and the absence of colocalization with Krox2. (C) The specificity of the nucleolar p19Arf labelling was demonstrated by the absence of any staining in the injured nerves of the Ink4a/Arf−/− mice. (D) Jmjd3 is also upregulated suggesting that epigenetic mechanisms activate the senescence programme after injury. (E) As expected, cell proliferation (phospho-histone H3 immunoreactivity) decreases with time in the distal stump. (F) p19Arf expression is also induced in crushed (but not transected) nerves. At 12 days post-crush, regrowing axons trigger the re-expression of Krox-20 and subsequently the downregulation of c-jun. Despite this, c-Jun and p19Arf can be detected in Schwann cells. (G) By Day 24 post-crush levels of c-Jun were almost undetectable and levels of p19Arf were found to be reduced to those present in non-injured nerves. Longitudinal sections of the distal stump of transected or crushed sciatic nerves (and the contralateral non-injured nerves) from adult wild-type mice were incubated with the indicated antibodies and nuclei counterstained with Hoechst. Three or more animals were used per condition. A representative image is shown. Scale bars = 50 μm.
We have found increased c-Jun expression in both, human and mouse neurofibromas (Supplementary Fig. 2 and 3). We also found upregulated c-Fos, the main c-Jun partner (Table 2). Therefore, it is possible that the c-Jun/c-Fos dimer stimulates Jmjd3 expression. Functional AP-1 binding sites have also been described in the p14Arf promoter (Ameyar-Zazoua et al., 2005). It is thus possible that the activity of Jmjd3 makes the Arf promoter accessible to the AP-1 complex which subsequently promotes Arf expression. We found other genes involved in the establishment of replicative senescence also upregulated in mouse neurofibromas. One of them p15Ink4b (Table 2) is located next to the Ink4a = Arf locus and their expression is usually coordinated (Gil and Peters, 2006). We also observed a slight upregulation of p53, a downstream effector of p19Arf. Surprisingly, messenger RNA for p21cip is decreased, which could seem to contradict the proposed model. However, it has been shown that p21^{c-/-} fibroblasts can enter the senescence programme (Pantoja and Serrano, 1999). Moreover, the role of p21^{c-/-} in the biology of Schwann cells is more complex than being a purely inhibitory molecule for the cell cycle (Atanasoski et al., 2006). Another senescence gene that is remarkably upregulated is Wnt16 (Table 2). Notably, this gene is over-expressed and secreted from cells undergoing oncogene-induced senescence, both in vitro and in the in vivo murine model of KRasV12-induced senescence (Binet et al., 2009).

It has been shown that a negative feedback signalling network contributes to the oncogene-induced senescence in human fibroblasts with decreased neurofibromin expression (Courtois-Cox et al., 2006). This is mediated by the inhibition of the Ras/PI3K pathway through the Sprouty family of proteins, and can impact on the senescence machinery through HDM2 and FOXO. Although we found no changes in Hdm2 and Foxo gene expression, sprouty homolog 4 was found upregulated in the mouse neurofibromas (data from the microarray analysis). Therefore the possibility exists that this mechanism contributes to block cell cycle in mouse neurofibroma Schwann cells. In summary, our results suggest that a complex array of mechanisms leading to senescence is activated in response to tumorigenic stimuli in the PNS.

The malignant peripheral nerve sheath tumours risk in NF1 is ~10% during life time (Evans et al., 2002). Akin to human NF1 patients, ~15% of peripheral nerve tumours developed by the NSE-SMDF^{+/} mice progress to malignancy. Here we show that 70% of these malignant tumours have a decreased expression of p19Arf. By contrast, p16Ink4a expression is quite variable and appears to be normal (or even increased) in most. These data suggest that the p19Arf/p53 pathway plays a more relevant role in maintaining the senescence programme in neurofibromas. In support of this, we found that elimination of a single copy of p53 increases dramatically the incidence of malignancies (up to 100%) and decreases the age of tumour initiation (Fig. 5A). These fast growing tumours are histologically similar to the malignant peripheral nerve sheath tumours developed by some human NF1 patients (Fig. 4A).

In agreement with our results with the NSE-SMDF^{+/} mice, it has been shown by others that Nf1^{+/}; p53^{+/} heterozygotes develop soft tissue sarcomas, including malignant peripheral nerve sheath tumours (Vogel et al., 1999). Moreover, the inactivation of p14Arf and p16Ink4a has been associated with a bad prognosis in human malignant peripheral nerve sheath tumours (Endo et al., 2011). Taken together, these data support the view that a failure in the establishment of the senescence programme in plexiform neurofibromas underlies their transformation into highly aggressive malignant tumours.

In addition to tumourigenesis, differentiated Schwann cells also re-enter cell cycle during Wallerian degeneration. Thus, after injury
Figure 7 Ink4a/Arf locus contributes to proliferation arrest in injured nerves. (A) Cell proliferation is increased in the injured nerves of *Ink4a/Arf* ^−/−^ mice. The number of Ki-67-positive cells was counted in the distal stumps of *Ink4a/Arf* ^−/−^ and wild-type littermates. Proliferation rate was calculated by dividing the number of Ki-67-positive cells by the total nuclei. As is shown, the density of proliferating cells was significantly increased in the *Ink4a/Arf* ^−/−^ mice (*n* = 3; **P*-value = 0.016). (B) Proliferation rate was also increased in the injured nerves of *p53* ^−/−^ mice (*n* = 3; **P*-value = 0.012). (C) Graphical summary: the senescence programme contributes to controlling the proliferation of Schwann cells in the distal stump of injured nerves. If the re-growing axons can reach the distal stump, c-Jun is downregulated and Krox-20 upregulated, inducing Schwann cells to go into the myelination programme. In contrast, when the axons are prevented from entering the distal stump, the *Ink4a/Arf* locus remains activated maintaining Schwann cells in the replicative senescence programme. dpT = days post-transaction; ns = no significant.
factors are released that induce Schwann cell de-differentiation and proliferation (Guertin et al., 2005). It has been elegantly shown that this process is controlled by the re-expression of the transcription factor c-Jun in the Schwann cell (Parkinson et al., 2008; Arthur-Farraj et al., 2012). Later, contact of Schwann cells with regrowing axons downregulates c-Jun and upregulates Krox-20, instructing them to myelinate and regenerate a full-blown and functional nerve. However, if the regrowing axons cannot reach the distal segment (as happens in some complete nerve transactions), c-Jun expression remains high for long periods of time. Intriguingly, despite this, Schwann cell proliferation stops after few days (Scherer et al., 1994; Shy et al., 1996). Here we show that this stop in proliferation is associated with p19Arf upregulation. Interestingly we also observed strong immunoreactivity for Jmjd3 in these nerves, suggesting that this demethylase derepresses the Ink4a/Arf locus in the distal stumps of injured nerves. By using genetically modified mice we show that Ink4a/Arf loss of function increases the cell proliferation rate in distal stumps, proving that replicative senescence contributes to the arrest of proliferation after injury. We found a similar effect in the distal stumps of p53 knock-out mice suggesting that as in neurofibromas, the p19Arf/p53 pathway mediates this arrest. However, in this case the p16Ink4a/Rb pathway is also involved, as shown by the increased proliferation of Schwann cells in the injured nerves of the p16Ink4a knock-out mice (Atanasoski et al., 2006). Proliferation arrest eventually occurs in the Ink4a/Arf−/− and p53−/− injured nerves, suggesting that other pathways (potentially involving p15InK4b and/or Wnt16) may also contribute to block proliferation.

In summary, our data support the tenet that a physiological response is activated to control Schwann cell overproliferation after nerve injury. But, why is the control of Schwann cell proliferation biologically important in the distal stumps of injured nerves? Schwann cell plasticity is central for nerve regeneration (Jessen and Mirsky, 2005). To this end, the fully differentiated myelinating Schwann cells completely de-differentiate to an immature-like stage after injury. These immature-like cells are sensitive to mitogens and proliferate in response to factors like neuregulins or TGF-β (Ridley et al., 1989; Levi et al., 1995). It has been shown that they can also synthesize and release neuregulins and other trophic factors, which hypothetically can stimulate autocrine proliferation (Carroll et al., 1997; Rosenbaum et al., 1997). Also, in addition to this, soluble neuregulins are present in the serum (Shibuya et al., 2010) and can theoretically activate erbB receptors on Schwann cells, as the blood nerve barrier is broken in injured nerves. Therefore the plasticity of the Schwann cell lineage is a double-edged sword. To guarantee re-innervation it is needed to generate de-differentiated Schwann cells, which are highly sensitive to mitogens and oncogenic stimuli, and exposes the organism to a dangerous situation. The risk is particularly high if axons cannot finally reach the distal stumps and Schwann cells have to remain de-differentiated for long periods of time. Interestingly, we also observed that the expression of p19Arf disappears when the contact with axons induces Schwann cell re-differentiation in regenerating nerves (Fig. 6G). Although we cannot rule out that some p19Arf expressing cells could be cleared by phagocytosis, most of them probably re-differentiate to myelinating Schwann cells, in what could be another example of the remarkable Schwann cell lineage plasticity. Experiments to unambiguously address this point are currently ongoing in our laboratory.

Our data suggest that senescence is a general mechanism for the control of Schwann cell proliferation. An emerging idea proposes that some types of tumours arise when oncogenic stimuli activate persistently the repair programme of a tissue (Dvorak, 1986; Beachy et al., 2004). Thus tumour growth may represent the continuous activity of an unregulated state of tissue repair that fails to come back to the quiescence that normally follows regeneration. In the PNS, it has been reported that nerve injury fosters neurofibroma development (Riccardi, 1981; Parrinello and Lloyd, 2009; Napoli et al., 2012) suggesting a link between nerve regeneration and tumorigenesis. Our microarray analysis shows that many genes that are re-expressed after nerve injury by Schwann cells (Sox2, Notch1, Id2, Krox-24, Egr3 and Tnc) (Fruttiger et al., 1995; Jessen and Mirsky, 2008) are also highly expressed during neurofibroma development (Table 2). Importantly, we show (Supplementary Fig. 2 and 3) that c-jun, a master gene that drives Schwann cell de-differentiation during Wallerian degeneration (Parkinson et al., 2008; Arthur-Farraj et al., 2012) is highly expressed in human and mice neurofibromas. This suggests that the same programme that drives Schwann cell de-differentiation and proliferation after injury is activated by oncogenic stimuli to induce tumours in the PNS. We also show that a common mechanism, mediated by the epigenetic induction of the Ink4a/Arf locus, is used to avoid Schwann cell over-proliferation after injury and after oncogenic challenge. Together our results substantiate the idea of a link between nerve regeneration and tumour development, and support the concept that peripheral nerve tumours can be produced by the misactivation of persistent state of repair.

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Supplementary material

Supplementary material is available at Brain online.
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