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Corina Anastasaki
Washington University School of Medicine in St. Louis

Sonika Dahiya
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David H. Gutmann
Washington University School of Medicine in St. Louis

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**KIR2DL5 mutation and loss underlies sporadic dermal neurofibroma pathogenesis and growth**

Corina Anastasaki¹, Sonika Dahiya² and David H. Gutmann¹

¹Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA
²Department of Pathology, Washington University School of Medicine, St. Louis, MO 63110, USA

**Correspondence to:** David H. Gutmann, email: gutmannd@wustl.edu

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**ABSTRACT**

Dermal neurofibromas (DNFs) are benign peripheral nerve sheath tumors thought to originate from Schwann cell progenitors. These tumors represent one of the hallmarks of the neurofibromatosis type 1 (NF1) tumor predisposition syndrome, where they can number in the thousands. While NF1-DNFs arise due to mutations in the NF1 gene, the vast majority of DNFs occur sporadically (sp-DNFs), where the genetic etiology is currently unknown. Herein, we employed whole-exome sequencing of sp-DNFs to identify a recurrent mutation in the KIR2DL5 gene, which codes for a protein suppressor of natural killer (NK) cell activity. While the function of KIR2DL5 outside of the immune system is unknown, we identified a KIR2DL5 N173D mutation in three of nine sp-DNFs, resulting in loss of KIR2DL5 protein expression. In contrast, two of these subjects had unrelated tumors, which retained KIR2DL5 protein expression. Moreover, loss of KIR2DL5 expression was demonstrated in 15 of 45 independently-identified sp-DNFs. Consistent with its potential role as a negative growth regulator important for neurofibroma maintenance, ectopic KIR2DL5N173D expression in normal human Schwann cells resulted in reduced KIR2DL5 expression and increased cell proliferation. Similarly, KIR2DL5 short hairpin RNA knockdown (KD) decreased KIR2DL5 protein levels and increased cell proliferation, as well as correlated with PDGFRβ and downstream RAS/AKT/mTOR hyperactivation. Importantly, inhibition of PDGFRβ or AKT/mTOR activity in KIR2DL5-KD human Schwann cells reduced proliferation to control levels. Collectively, these findings establish KIR2DL5 as a new Schwann cell growth regulator relevant to sp-DNF pathogenesis, which links sporadic and NF1-associated DNFs through RAS pathway hyperactivation.

**INTRODUCTION**

Dermal neurofibromas (DNFs) are common low-grade peripheral nerve sheath tumors [1], which are hypothesized to arise from Schwann cell progenitors [2–4]. While these tumors do not undergo malignant transformation, they can be painful or number in the thousands, as seen in some adults with the neurofibromatosis type 1 (NF1; OMIM: 613113) cancer predisposition syndrome. Nearly all adults with NF1 manifest DNFs, which result from bi-allelic inactivation of the NF1 tumor suppressor gene and loss of NF1 protein (neurofibromin) expression [5–7]. This bi-allelic NF1 loss reflects a combination of a germline NF1 gene mutation and the acquisition of a somatic NF1 mutation in Schwann cell lineage populations [3, 4]. As a RAS-GTPase-activating protein (RAS-GAP), neurofibromin loss in Schwann cells leads to increased RAS activation [8–10] and downstream mechanistic target of rapamycin (mTOR)-mediated hyperproliferation [11–14].

While NF1 mutations characterize NF1-DNFs, the majority of DNFs occurs in the absence of NF1. These sporadic DNFs (sp-DNFs) are histologically identical to their NF1-associated counterparts, and most often arise as solitary dermal tumors. Since these tumors are encountered in individuals lacking other features supportive of a
RESULTS AND DISCUSSION

In order to identify potential molecular etiologies for sporadic DNFs (sp-DNFs), we examined a cohort of 17 male patients (7 NF1-DNFs and 10 sp-DNFs) by whole-exome sequencing. Using this approach, NF1 gene mutations were detected in 5/7 NF1-DNFs, and surprisingly, 1/10 (Sp7) sp-DNFs where the identified point mutation is predicted to result in a splicing variant of the NF1 transcript (Figure 1A, 1B). The inability to detect mutations in two of the NF1-DNFs likely reflects the insensitivity of exome sequencing to identify intronic mutations or large-gene deletions, both common mutational types in NF1 [15, 16]. However, consistent with a clinical diagnosis of NF1 or the presence of a NF1 gene mutation, immunohistochemical staining confirmed loss of neurofibromin expression in the Sp7 tumor and in all NF1-DNFs (Figure 1C, Supplementary Figure 1). Subject Sp7 was lost to follow-up, and may have undiagnosed NF1 or harbored a tumor that underwent bi-allelic NF1 gene inactivation [17]. Conversely, all sp-DNFs without an identified NF1 mutation, as well as an independent cohort of 45 unrelated sp-DNFs retain neurofibromin expression (Supplementary Figure 1), consistent with exclusion of NF1 as a clinical diagnosis.

While 105 genes with exonic nucleotide variations (frameshift insertions/deletions, missense, and nonsense mutations; Supplementary Table 1) were identified in more than one DNF, eight genes were mutated in at least four DNFs (> 22.22% of the cohort; Figure 1D, Supplementary Figure 2). These mutated genes did not separate sporadic or NF1-associated DNFs into clear subgroups; however, the specific nucleotide alterations identified in 6 of the 8 genes were identical in all of the tumors examined (Figure 2). These mutated genes did not separate sporadic DNFs (> 22.22% of the cohort; Figure 1D, Supplementary Figure 1) from NF1-DNFs, suggesting that KIR2DL5 is likely a DNF-specific mutation. Moreover, in an independent cohort of sp-DNFs, 15/45 tumors were KIR2DL5-immunonegative (33%; Figure 2B, Supplementary Figure 3B), further supporting the hypothesis that KIR2DL5 expression loss is specific to sp-DNFs and likely is caused by the KIR2DL5N173D mutation.

To determine whether the KIR2DL5N173D mutation was responsible for the reduction in KIR2DL5 protein expression observed, normal human Schwann cells were infected with either KIR2DL5WT or KIR2DL5N173D viral expression constructs (Figure 2D). Whereas wild-type KIR2DL5 expression was easily detected by Western blot and promoted a minor increase in Schwann cell growth (1.18-fold increase compared to PKO-infected Schwann cells), KIR2DL5N173D mutant-expressing cells exhibited a 36.9% decrease in KIR2DL5 expression, a 1.88-fold increase in cell proliferation (BrdU incorporation) (Figure 2E), and a 1.5-fold increase in total cell numbers (Figure 2F) relative to KIR2DL5WT expressing cells. Together, these data demonstrate that the KIR2DL5N173D mutation functions in a dominant negative manner to regulate KIR2DL5 expression and Schwann cell growth.

KIRs were initially discovered on the surface of natural killer (NK) cells [26], where they have been reported to mediate the immune response of NK cells and a subset of T lymphocytes against infection and cancer [27–29]. While KIR2DL5 is an orphan receptor with no known ligands, its phosphorylation in NK cells recruits the Src homology region 2-containing protein tyrosine phosphatase-2 (SHP-2) molecule, resulting in activation of downstream signaling pathways. Since the role of KIR2DL5 has not been previously explored in non-immune system cells, we first demonstrated that KIR2DL5 is expressed in S100β+ Schwann cells within normal human peripheral (sural) nerve by immunofluorescence (Figure 2C), and in normal human Schwann cells by western blot (CTL; Figure 2D) and immunofluorescence (Supplementary Figure 4A). While the majority of the KIR2DL5 expression in both normal sural nerve and immunopositive sDNFs was found in the Schwann cell compartment, there was also expression in other cell types. In this regard, co-labelling of KIR2DL5+ cells with mast cell (tryptase, c-Kit) and fibroblast (vimentin)-specific antibodies (Supplementary Figure 4) revealed that the few remaining KIR2DL5-immunoreactive...
cells in the Sp4, Sp5 and Sp6 DNF tumor specimens were stromal elements.

As a complementary approach to expressing the mutant KIR2DL5 gene in normal human Schwann cells to reduce KIR2DL5 expression, endogenous KIR2DL5 expression was also decreased by RNA (shRNA) interference. Since KIR2DL5 is encoded by two genes, KIR2DL5A (OMIM: 605305) and KIR2DL5B (OMIM: 615727) that are > 99% sequence identical [25], three independently-generated short-

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**Figure 1:** NF1 gene mutation and neurofibromin expression stratify DNFs into two subgroups. (A) List of detected NF1 gene mutations and predicted protein alterations. (B) Schematic representation of NF1 gene mutations. (C) Representative images depicting decreased neurofibromin expression in tumors with NF1 mutations compared to sp-DNFs. Scale bar, 25 µm. (D) Genes recurrently mutated in more than four DNFs. (E) Recurrent mutations identified in more than four DNFs. Black boxes = mutation.
hairpin RNA molecules were employed to target both genes. Following Schwann cell infection, 76.78% (sh#1; TCTCTCCATGACTCACCCTAT), 88.29% (sh#2; CAGGAGCTCATTTGACATGTA) and 76.32% (sh#3; GAAACTCTTCAAGTAGTTCAT) KIR2DL5 knockdown was achieved (Figure 3A), leading to 2.2, 3.2- and 2.7-fold increases in cell number, respectively (direct cell counting; Figure 3B) relative to GFP-vector-infected cells (CTL). This increase in cell numbers represented a 2.7- (sh#1), 2.83- (sh#2), and 3- (sh#3) fold increase in proliferation (BrdU incorporation; Figure 3C), with no change in apoptosis (TUNEL staining; Figure 2).

**Figure 2: KIR2DL5N173D mutation reduces KIR2DL5 expression.** (A) 3/9 sp-DNFs harbor the KIR2DL5N173D mutation. (B) KIR2DL5 mutation is associated with decreased KIR2DL5 protein levels in sp-DNFs but not NF1-DNFs or non-DNF tissue. Tonsil and sural nerve (S.N.) were used as controls. (C) KIR2DL5N173D expression results in reduced KIR2DL5 levels, as demonstrated with a myc antibody immunoblot of human Schwann cells infected with a myc-tagged KIR2DL5WT or a KIR2DL5N173D construct. KIR2DL5N173D expression results in increased (D) cell proliferation and (E) cell number relative to KIR2DL5WT control-infected Schwann cells. PLKO-GFP-control (PLKO)-infected cells are shown for comparison. Data are represented as means of three independent infections/over-expression construct ± s.e.m. One-way ANOVA was used for statistical analysis. *p < 0.05; ***p < 0.001. Scale bar 50 μm.
Supplementary Figure 3C). Expression of control KIR2DL5 engineered so as not to be targeted by these shRNA constructs following shRNA knockdown restored KIR2DL5 expression, as well as cell proliferation and cell numbers (Supplementary Figure 3D–3F).

Since RAS-dependent signaling is enhanced in NF1-DNFs, the activation status of RAS (RAS-GTP) and RAS-regulated pathways were examined. Following KIR2DL5 knockdown (KIR2DL5A/B\textsuperscript{KD}), there was a 3.5- (sh#1), 4.7- (sh#2), and 2.9- (sh#3) fold increase in RAS activation (RAS-GTP; Figure 4A). While others have shown that NF1-DNF growth depends on ERK1/2 pathway activation [30, 31], KIR2DL5 reduction had no effect on ERK1/2\textsuperscript{Thr202/Tyr204} phosphorylation (activation; Supplementary Figure 5A–5D), but rather led to increased AKT activation (AKT\textsuperscript{Thr308} and AKT\textsuperscript{Ser473} phosphorylation; sh#1, 31.5-fold and 2.8-fold; sh#2, 19.6-fold and 5.9-fold; sh#3, 15.6-fold and 7.3-fold, respectively; \( n = 3 \)), as well as elevated mTOR activation (S6\textsuperscript{Ser240/244} phosphorylation; sh#1, 3.2-fold; sh#2, 4.9-fold; sh#3, 7.2-fold; \( n = 3 \)) (Figure 4A). Similarly, expression of the mutant KIR2DL5 protein (KIR2DL5 N173D) resulted in 2.24-fold and 1.78-fold increases in AKT and S6 phosphorylation, respectively (Supplementary Figure 3).

To demonstrate that KIR2DL5 regulates cell proliferation in an AKT/mTOR-dependent fashion, we initially employed an AKT-specific inhibitor (MK2206). Following a 4h MK2206 treatment of control- and shRNA-infected Schwann cells, AKT\textsuperscript{Ser473} phosphorylation (sh#1, 0.9; sh#2 0.95; sh#3 0.97-fold), as well as the phosphorylation of two of its downstream effectors S6 (sh#1, 0.4; sh#2 0.6; sh#3 0.5-fold) and PRAS40 (sh#1, 0.4; sh#2 0.6; sh#3 0.5-fold) and PRAS40 (sh#1, 0.4; sh#2 0.6; sh#3 0.5-fold) and PRAS40 (sh#1, 0.4; sh#2 0.6; sh#3 0.5-fold)

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**Figure 3: KIR2DL5 is a negative Schwann cell growth regulator.** (A) Co-localization of KIR2DL5 with S100β+ Schwann cells in normal human sural nerve. b. shRNA silencing of KIR2DL5A/B expression in normal human Schwann cells, where KIR2DL5A/BKD cell KIR2DL5 expression levels were calculated by normalizing the relative KIR2DL5/α-tubulin band intensity ratio to that of GFP-control-infected Schwann cells. (B) western blot. KIR2DL5A/BKD results in increased (C) cell number and (D) proliferation relative to GFP-infected serum-starved Schwann cells. Data are represented as means of three independent infections/shRNA construct ± s.e.m. One-way ANOVA with Bonferroni correction was used for statistical analysis. *** \( p < 0.001 \). Scale bar 25 μm.
0.7; sh#2, 1.3; sh#3, 1.2-fold), were reduced to control levels (Figure 4B). Additionally, AKT inhibition restored cell numbers (Figure 4C) and proliferation (Figure 4D) to normal levels. Next, to determine whether mTOR inhibition could similarly reverse the effects of KIR2DL5 KD, shRNA-infected Schwann cells were treated with rapamycin for 4h. Following rapamycin exposure, there was reduced AKT<sup>Ser473</sup> phosphorylation (sh#1, 0.7; sh#2 1.1; sh#3 1.2-fold) and S6 phosphorylation (sh#1, 0.8; sh#2 0.8; sh#3 0.8-fold) (Supplementary Figure 5E), as well as restoration of cell numbers (Figure 4E) and proliferation (Figure 4F) to control levels. Collectively,

Figure 4: KIR2DL5A/B is a negative regulator of RAS/AKT signaling. (A) shRNA silencing of KIR2DL5A/B in human Schwann cells results in increased RAS activity (RAS-GTP), as well as increased AKT<sup>Thr308</sup>, AKT<sup>Ser473</sup>, and S6<sup>Ser240/244</sup>, but not ERK1/2<sup>Thr202/Tyr204</sup>, phosphorylation relative to GFP-infected Schwann cells. (B) AKT<sup>Ser473</sup>, S6<sup>Ser240/244</sup> and PRAS40<sup>Thr246</sup> phosphorylation is restored to wild-type levels following MK2206 treatment of KIR2DL5A/B<sup>KD</sup> Schwann cells, 4 h MK2206 (C–D) or rapamycin (E–F) treatment restores (C, E) cell number and (D, F) proliferation of KIR2DL5A/B<sup>KD</sup> Schwann cells relative to GFP-infected controls. Experiments were repeated at least three times using independently-derived cell lysates. Data are represented as means ± s.e.m. Two-way ANOVA with Bonferroni correction was used for statistical analysis. ***<sup>p</sup> < 0.001.
these data establish that KIR2DL5-regulated Schwann cell growth activates RTK/AKT/mTOR signaling, raising the intriguing possibility that Sp-DNFs and NF1-DNFs converge on hyperactivation of the same mitogenic signaling pathway.

RAS/ AKT signaling is typically initiated by receptor tyrosine kinase (RTK) receptor engagement in Schwann cells [32–36]. In order to identify this hyperactivated RTK, a commercially-available human phospho-RTK array was employed, which recognizes 49 distinct RTKs (Figure 5A). Whereas the activity of most RTKs was not significantly altered (n = 48 RTK moieties), platelet-derived growth factor receptor beta (PDGFRβ) phosphorylation was elevated (sh#1, 3.5-; sh#2 3.9-; sh#3, 3.5-fold) in KIR2DL5A/BKD Schwann cells relative to controls. Independent validation of two of these activation-specific phosphorylation sites (PDGFRβ Tyr771: sh#1, 2-; sh#2, 4.5-; sh#3, 7.7-fold; and PDGFRβ Tyr1067: sh#1, 2-; sh#2, 5.4-; sh#3, 7.3-fold) confirmed receptor activation in KIR2DL5A/BKD Schwann cells (Figure 5B). Consistent with a dominant mechanism of action of the KIR2DL5 N173D mutation, there was a 1.84-fold activation of PDGFRβ in KIR2DL5 N173D-expressing Schwann cells (Supplementary Figure 3). To determine the necessity of PDGFRβ activation for KIR2DL5A/BKD-mediated Schwann cell growth, we employed two different tyrosine kinase inhibitors that target PDGFRβ activation (imatinib [37–42] and sunitinib [43]), both of which are currently in clinical trials for the treatment of NF1-plexiform neurofibromas (Figure 5C–5F). Incubation of KIR2DL5A/BKD Schwann cells for 16h with either inhibitor reduced PDGFRβ and AKT hyperactivation (Figure 5C–5D), as well as restored total cell numbers (Figure 5E) and cell proliferation (Figure 5F) to control levels.

While increased PDGFRβ activation was responsible for AKT-mediated hyperproliferation following KIR2DL5 loss, the mechanism by which KIR2DL5 regulates PDGFRβ is not clear. We hypothesize that KIR2DL5 binds PDGFRβ to restrict its activation. Consistent with this model, KIR2DL5 binds PDGFRβ in normal human Schwann cells by PDGFRβ antibody-mediated immunoprecipitation. This binding is ameliorated in KIR2DL5A/BKD Schwann cells, resulting in a 7.8-fold increase in PDGFRα phosphorylation binding to PDGFRα (sh#1, 5.2-; sh#2, 9.6-; sh#3, 8.7-fold increase; Figure 5G). As such, KIR2DL5 likely inhibits PDGFRβ through direct binding, which reduces PDGFR heterodimerization, PDGFRβ phosphorylation and downstream RAS/ AKT-mediated Schwann cell growth (Figure 5H).

Taken together, we identified the first mutation associated with Sp-DNFs. This KIR2DL5 N173D mutation is hypothesized to result in attenuated KIR2DL5 protein levels, and as such, KIR2DL5 functions as a negative regulator of Schwann cell proliferation. In this regard, KIR2DL5 reduction, resulting either from KIR2DL5 knockdown or KIR2DL5 N173D mutation, increases Schwann cell growth in a PDGFR/ AKT/mTOR-dependent mechanism. Moreover, these studies provide the first demonstration that KIR2DL5 can function outside the immune system. Finally, while the etiologic mechanisms underlying sp-DNF and NF1-DNF growth control are distinct, they share a common signaling pathway amenable to therapeutic targeting.

**MATERIALS AND METHODS**

**Tissue collection and processing**

De-identified 10µm-thick sections of formalin-fixed paraffin-embedded DNF tissue from 7 male patients with a confirmed NF1 diagnosis and from 10 male patients with sp-DNFs were collected from Washington University Surgical Pathology Department. Total genomic DNA was extracted following review by an experienced neuropathologist (S.D.) using a QIAamp DNA FFPE Tissue Kit (QIAGEN) following manufacturer’s instructions. Genomic DNA resuspended in elution buffer was sent for exome sequencing. Since this study was performed using de-identified dermal neurofibroma specimens and the patients could not be re-consented, we were unable to obtain blood for constitutional DNA analysis.

**Whole-exome sequencing and filtering**

Whole human exome sequencing was performed from total genomic DNA of 17 tumors by Otogenetics Co. Briefly, library preparation was performed using an Illumina platform. Pair-ended sequencing (100 bp) with a minimum average coverage of 30x or ~2.7–3Gb was performed on a HiSeq2500 platform (human exome V5). Sequence reads were mapped to human reference genome hg18. Variants were annotated in Annovar and were filtered in Microsoft Excel to include only the following criteria: (1) Exonic variations (2) Non-synonymous variations, stop loss/ stop gain, frameshift insertions and frameshift deletions (3) 1000 genome, ExAc and ESP6500 frequencies ≤ 0.01 (4) Deleterious variants according to SIFT, Polyphen and CADD predictions (5) Non-common polymorphism in Cosmic and ClinVar (Supplementary Table 1). Genes mutated more than twice per tumor or resulting in conservative amino acid changes were eliminated from further analysis. eBioportal for Cancer Genomics (www.ebiportal.org) was used to search recurrent identified mutations in other cancers. The complete exome sequencing data is in the process of being uploaded to dbGaP. The sequence reads were as follows:

<table>
<thead>
<tr>
<th>Sample</th>
<th>Size</th>
<th>Reads</th>
</tr>
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<tbody>
<tr>
<td>Sp1</td>
<td>1,016,288,144</td>
<td>9,587,624</td>
</tr>
<tr>
<td>Sp2</td>
<td>2,980,285,61</td>
<td>28,115,902</td>
</tr>
<tr>
<td>Sp3</td>
<td>4,023,566,444</td>
<td>37,958,174</td>
</tr>
</tbody>
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Immunohistochemistry and immunofluorescence

Paraffin-embedded tonsil, 4 normal peripheral nerves (sural nerve), 23 NF1-DNFs (13 males, 10 females) and 55 sp-DNFs (31 males, 24 females) were included in the immunohistochemical analyses. All DNFs were incubated with neurofibrinoma (Santa Cruz sc-67) antibodies, while and all DNFs, tonsil and normal sural nerve sections were incubated with KIR2DL5 (Abcam; ab175895) antibodies. Biotinylated secondary rabbit antibodies (Vector Laboratories) were used in combination with Vectastain Elite ABC development and hematoxylin counterstaining. Normal human tonsil was used as the reference control tissue for the KIR2DL5 antibody. Images were acquired on a Nikon Eclipse E600 microscope conjugated with a Nikon Plan Fluor 10x/0.30 DIC L objective and a Leica EC3 camera. Normal human sural nerve, sp-DNF sections, as well as primary cell cultures of normal human Schwann cells and HEK293T cells were analyzed by immunofluorescence using S100β (Millipore CB1040), c-Kit (Millipore; MAB1164), tryptase (Abcam; ab2378), vimentin (DSHB; AMF-17b) and KIR2DL5 (Abcam; ab129751) antibodies conjugated to appropriate Alexa Fluor secondary antibodies. Images were acquired on a Nikon Eclipse TE300 fluorescence microscope conjugated with a Nikon Plan Fluor 20x/0.45 ELWD objective and a Leica DFC3000G camera.

Normal human Schwann cell culture, lentiviral infection and pharmacological inhibitor treatments

Normal human Schwann cells (ScienCell Research Laboratories) were cultured on poly-L-lysine-coated plates in Schwann cell medium (ScienCell #1701) supplemented with Schwann cell growth factors, Pen/Strep and fetal bovine serum (FBS), unless otherwise stated and were maintained as per the manufacturer’s instructions. For serum-starvation experiments (Supplementary Figure 5), Schwann cells were plated on poly-L-lysine-coated plates in the presence of serum (FBS) for 16h, followed by a 36h period of serum deprivation during which cells were cultured only in Schwann cell medium supplemented with Schwann cell growth factors and Pen/Strep. 10% FBS was added to the cells for 2, 5, 15, 30 or 60 minutes at which time points cells were collected for western blotting analysis.

Three sets of lentiviral infection experiments were performed. Schwann cells were either infected once with lentivirus particles encoding (1) either a KIR2DL5WT control or KIR2DL5N173D to establish the effect of the identified mutation on normal Schwann cells (Figure 2), (2) either a GFP-control or shKIR2DL5 RNA to knock down KIR2DL5 expression (Figures 3–5), or were infected twice with lentiviral preparations encoding (3) both a GFP control and KIR2DL5CTL control or both shKIR2DL5 RNA and KIR2DL5CTL to confirm the specificity of the shKIR2DL5 RNA constructs (Supplementary Figure 4). For all lentiviral infections, HEK-293T cells were initially cultured in DMEM (Gibco) supplemented with 10% heat-inactivated FBS and 1% Pen/Strep solution (Gibco). Three micrograms of total DNA was transfected per well of a 6-well plate (1.5 µg KIR2DL5WT, KIR2DL5CTL, KIR2DL5N173D, shKIR2DL5 or pLKO-GFP control; 1.5 µg lentiviral packaging constructs) using Fugene HD reagent (Promega) following manufacturer’s instructions. 24h post transfection the media was replaced with fresh Schwann cell media supplemented with 10% FBS, Schwann cell growth factors and antibiotics. Viral preparations were collected at 48 and 72 h post-transfection, were filtered, supplemented with polybrene and used for direct infections of Schwann cells. The sequences of the siRNA constructs were:

- shKIR2DL5 #1: TCTCTCCATGACTCACCCTAT; shKIR2DL5 #2: CAGGAGCTCATTTGACATGTA; shKIR2DL5 #3: GAAACTCTTCAAGTAGTTCAT;
- CTL1 (Forward: 5′-GAAGGCACCATAGCAGTGG-3′); CTL2 (Forward: 5′-CCACTGCTTGTTTCTGTCACAGGAAAAG-3′).

Normal human Schwann cells were transfected with lentiviral particles in the presence or absence of the following reagents: HD reagent (Promega) following manufacturer’s instructions. 24h post transfection the media was replaced with fresh Schwann cell media supplemented with 10% FBS, Schwann cell growth factors and antibiotics. Viral preparations were collected at 48 and 72 h post-transfection, were filtered, supplemented with polybrene and used for direct infections of Schwann cells. The sequences of the siRNA constructs were:

shKIR2DL5 #1: TCTCTCCATGACTCACCCTAT; shKIR2DL5 #2: CAGGAGCTCATTTGACATGTA; shKIR2DL5 #3: GAAACTCTTCAAGTAGTTCAT; KIR2DL5WT cDNA cloned in a lentiviral vector was purchased from Origene and was either directly transfected into HEK293T cells, or was transfected following site-directed mutagenesis with QuikChange Site-Directed mutagenesis kit (Promega) following manufacturer’s instructions in order to generate KIR2DL5CTL (harboring six distinct base pair mutations within the short hairpin recognition sequences but resulting in identical amino acid sequence) or KIR2DL5N173D. All resulting constructs were subcloned and sequenced by Genewiz. The following primers were used to generate the expression constructs: KIR2DL5CTL (Forward: 5′-GAAGGCACCATAGCAGTGG-3′; Reverse: 5′-CCACTGCTTGTTTCTGTCACAGGAAAAG-3′); KIR2DL5N173D (Forward: 5′-GAAGGCACCATAGCAGTGG-3′; Reverse: 5′-CCACTGCTTGTTTCTGTCACAGGAAAAG-3′).
Figure 5: KIR2DL5A/B inhibits Schwann cell proliferation in a PDGFRβ/AKT-dependent manner. (A) PDGFRβ is hyperphosphorylated following silencing of KIR2DL5 in normal human Schwann cells as demonstrated with a human phospho-RTK array. (B) PDGFRβ<sup>Tyr771</sup> and PDGFRβ<sup>Tyr1009</sup> phosphorylation is increased following KIR2DL5A/B silencing. Inhibition of aberrant PDGFRβ and AKT phosphorylation following 16 h imatinib (C) or sunitinib (D) treatment restores cell numbers (E) and proliferation (F) to control levels. Data are represented as means ± s.e.m. All experiments were repeated at least three times. Two-way ANOVA with Bonferroni correction was used for statistical analysis. ***p < 0.001 compared to vehicle control, #p < 0.001 compared to vehicle sh#1, sh#2, sh#3, respectively. (G) KIR2DL5A/B silencing leads to increased PDGFRβ/PDGFRα binding, as assessed by immunoprecipitation. (H) The proposed model of KIR2DL5 growth regulation envisions that KIR2DL5 normally binds and sequesters PDGFRβ, such that KIR2DL5 loss leads to increased PDGFRα/β binding and RAS/AKT-mediated hyperproliferation.
GCTTCGGCTCGTTACATGATTCACCGTACGAGTG
GTCAGACCCGAGTG-3′, Reverse: 5′-CACTCGGTCTGACCACTCGTACGGTGAATCATGTAACGAGC
CGAAGCATGTGTAGG-3′); KIR2DL5N173D (Forward: 5′-CCTGGAATGTTCCATCGACGCTGGGCACTGC-3′; Reverse: 5′-GCAGTGCCCAGCGTCGATGGAACAT
CGAAGCATGTGTAGG-3′).

GFP control- or siRNA-infected Schwann cells underwent treatment with the following reagents: MK2206 (50nM in DMSO, 4 h; Selleckchem), rapamycin (10μM in DMSO, 4 h; Selleckchem), imatinib (10 μM in water, 16h; Sigma), sunitinib (10 μM in DMSO, 16 h; Sigma), or PD0325901 (10 nM in DMSO, 4h; Selleckchem).

Western blotting, immunoprecipitation, RTK arrays and RAS/RAS pathway activity assays

Western blotting was performed on Schwann cells lysed in buffer containing 1% NP-40 (nonyl phenoxethoxylethanol), supplemented with protease and phosphatase inhibitors using KIR2DL5 (Abcam), α–tubulin (Sigma), RAS (Millipore), phospho-AKTThr308, phospho-AKTSer473, AKT, phospho-S6Ser240/244, S6, phospho-ErK1/2Thr202/204, Erk1/2, phosphoPRAS40Thr246, PRAS40, phospho-PDGFRβTyr771, phospho-PDGFRβTyr1009, PDGFRβ, PDGFRα (Cell Signaling) primary antibodies, as well as HRP-conjugated secondary antibodies (Cell Signaling) and ECL (Fisher) chemiluminescence. Western signal band intensity was quantified using ImageJ Software (National Institutes of Health, USA, http://imagej.nih.gov/ij Java 1.7.0_67). KIR2DL5A/BKD cell KIR2DL5 expression levels (Figures 2A, 3D) were calculated by normalizing the relative KIR2DL5/α-tubulin band intensity ratio to that of GFP-control-infected Schwann cells. For the immunoprecipitation studies, 100 μg total Schwann cell protein lysate was pre-cleared for 1h in agarose protein G beads (Cell Signaling), immunoprecipitated with PDGFRβ overnight and collected with agarose protein G beads for 2 hours. For the human phospho-RTK array (human phospho-receptor tyrosine kinase array kit; R&D Systems) Schwann cells were lysed in the provided buffer and 80μg total protein was assayed using manufacturer’s instructions (RAS activation kit; Millipore). All experiments were repeated at least three times using independently derived cell lysates.

Proliferation and apoptosis assays

Schwann cell proliferation and TUNEL staining were assessed using the BrdU Cell Proliferation Colorimetric ELISA kit (Roche) or the TUNEL staining kit (Roche) following manufacturer’s instructions. For both assays, Schwann cells were seeded at 5000 cells/ well of a 96-well plate. For the proliferation assays, Schwann cells were serum-starved for 24 hours, if treated, incubated in vehicle or appropriate inhibitors for a total of 24 hours, labelled with BrdU for 18 h followed by 90min incubation in peroxidase-conjugated anti-BrdU antibody. The optical density of the cells was measured at 405 nm on a Bio-Rad spectrophotometer. For the TUNEL assay, cells were incubated in serum-containing media for 24 h before being fixed in 4% PFA, incubated in TUNEL substrate for 1 h and imaged using a fluorescent microscope.

Statistical analyses

All statistical tests were performed using GraphPad Prism 5 software. Student’s t-test, one-way or two-way analysis of variance (ANOVA) with Bonferroni post-test correction was employed for all experiments.

Authors’ contributions

C.A., S.D., and D.H.G. designed the experiments and wrote the manuscript. C.A. performed the experiments. S.D. provided neuropathological assessments and tumor specimens.

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CONFLICTS OF INTEREST

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