

Analysis of Ret knockin mice reveals a critical role for IKKs, but not PI 3-K, in neurotrophic factor-induced survival of sympathetic neurons

M Encinas^{1,*}, EJ Rozen¹, X Dolcet², S Jain³, JX Comella^{1,8}, J Milbrandt^{4,5} and EM Johnson Jr^{4,6,7}

We analyzed the survival responses and downstream signaling elicited by GDNF on sympathetic neurons from different Ret knockin mice. Lack of tyrosine 1062, a multidocking site in Ret, completely prevented GDNF-mediated survival. Importantly, lack of tyrosine 981, although abrogating Akt phosphorylation, had no effect on neuronal survival, indicating that the PI 3-K/Akt pathway is not necessary for survival of sympathetic neurons. In contrast, silencing of B-Raf completely prevented not only GDNF-mediated but also NGF-mediated cell survival, independently of MEK-1/2. We identified IKKs as the main effectors of the protective effects of B-Raf. First, B-Raf interacted with and activated IKKs. Second, knockdown of IKKs reversed the protection afforded by a constitutively active form of B-Raf. Third, knockdown of IKKs prevented both NGF- and GDNF-mediated survival. In conclusion, our data delineate a novel survival pathway for sympathetic neurons linking B-Raf to IKKs, independently of both PI 3-K and MEK-1/2 pathways.

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The GDNF family ligands (GFLs) constitute a group of neurotrophic factors (GDNF, NRTN, PSPN and ARTN) that regulate several aspects of the developing nervous system. GDNF and NRTN were identified by virtue of their ability to promote the survival of cultured ventral mesencephalic dopaminergic neurons and sympathetic neurons, respectively. The spectrum of neuronal populations, which survive in response to GFLs has been broadened and now includes sensory, motor, parasympathetic and enteric neurons.¹

GFLs signal through a common tyrosine kinase receptor (Ret), and one of the four coreceptors known as GFR α 1–4. Ret is alternatively spliced to produce at least two variants of identical sequence up to residue 1063, followed by a unique C-terminal sequence composed of either 9 or 51 amino acids (Ret9 and Ret51) (reviewed in Airaksinen *et al.*²).

Ret has five important tyrosines, which serve as docking sites for downstream effectors. Tyr905 binds Grb10 and Grb7, whereas Tyr981 binds Src, Abl and SHB- β . Phospholipase C- γ and Grb2 bind to Tyr1015 and Tyr1096 (which is only present in Ret 51), respectively. Finally, Tyr1062 is a multidocking site, which binds Shc, IRS-1, FRS-2 and Dok4/5/6 (reviewed in Airaksinen and Saarma¹).

After phosphorylation of docking tyrosines and binding to the adequate adaptors, engagement of RTKs leads to the activation of signaling pathways that are ultimately responsible for the biological effects. Among them, the

phosphatidylinositol 3-kinase (PI 3-K)/Akt and the extracellular-related kinase 1 and 2 (ERK1/2) pathways have been shown to convey pro-survival signals in many different systems. Since Ret was an orphan receptor for years, most of our knowledge of its signaling properties comes from the analysis of its oncogenic forms. Thus, the relevance of the signaling pathways elicited by ligand-activated endogenous, wild-type Ret, in neuronal function is largely unexplored. On the other hand, the signaling pathways by which NGF, another neurotrophic factor for sympathetic neurons, promotes their survival is controversial, as contradicting reports on the role of the PI 3-K/Akt had been published.^{3–6}

We have previously generated knockin mice expressing point mutants of either Ret9 or Ret51 of human origin. These included tyrosine-to-phenylalanine mutations of residues 981, 1015 and 1062.⁷ In this work, we aim to elucidate the signaling pathways important for the survival of sympathetic neurons using such mutants and GDNF-mediated survival as readout. Our data reveal a critical role for tyrosine 1062, but not tyrosines 981 or 1015, in neuronal survival. Notably, lack of Ret tyrosine 981 impairs Akt phosphorylation but has no effect on cell survival, indicating that the PI 3-K/Akt pathway is not necessary for GDNF-mediated survival of sympathetic neurons. Instead, we find that a novel B-Raf/IKK pathway mediates the pro-survival effects of not only GDNF but importantly also NGF in sympathetic neurons.

¹Cell Signaling and Apoptosis Group, Departament de Medicina Experimental, Lleida 25198, Spain; ²Grup de Patologia Oncologica, Departament de Ciències Mèdiques Bàsiques, Institut de Recerca Biomedica de Lleida, Lleida 25198, Spain; ³Department of Medicine, Renal Division, St. Louis, MO 63110, USA; ⁴HOPE center for Neurological Disorders, St. Louis, MO 63110, USA; ⁵Department of Pathology, St. Louis, MO 63110, USA; ⁶Department of Molecular Biology and Pharmacology, St. Louis, MO 63110, USA and ⁷Department of Neurology, Washington University School of Medicine, St. Louis, MO 63110, USA

*Corresponding author: M Encinas, Laboratori d'Investigació HUAV/UdL, Hospital Universitari Arnau de Vilanova, 1a planta, Rovira Roure, 80, Lleida 25198, Spain; Tel/Fax: +34 973702213; E-mail: mario.encinas@mex.udl.cat

⁸Current Address: Institut de Neurociències, Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain.

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Abbreviations: GDNF, glial cell line-derived neurotrophic factor; NGF, nerve growth factor; PI 3-K, phosphatidylinositol 3-kinase; MEK, MAPK/ERK kinase; ERK, extracellular-signal regulated kinase; MAPK, mitogen activated protein kinase; IKK, I kappa B kinase; NF- κ B, nuclear factor kappa B; RTK, receptor tyrosine kinase
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Results

Neurons from mice expressing Ret9 or Ret51 display similar responses to GDNF. As mentioned above, two Ret splicing isoforms that differ at the carboxy terminus have been identified (Ret9 and Ret51). We wanted to analyze whether such differences affected Ret downstream signaling or bioactivity in sympathetic neurons. We first verified that neurons expressed the expected Ret isoforms by immunoprecipitating Ret from cultured sympathetic neurons from mice expressing either wild-type Ret9 or Ret51 (Figure 1a). We next checked the activation of the PI 3-K/Akt and Ras/MAPK pathways. Lysates from neurons stimulated with GDNF for 10 min were probed with phosphoantibodies specific for phosphorylated Ser473 Akt and dually phosphorylated ERK1/2. No differences between Ret^{RET9} or Ret^{RET51} knockin mice and their wild-type littermates were detected, despite the observation that mutant mice appeared to express slightly less Ret than

wild-type animals (Figure 1b). Finally, we analyzed the survival response prompted by GDNF on neurons from Ret^{RET9} and Ret^{RET51} mice. As shown in Figure 1c, GDNF promoted the survival of 80–90% of neurons from both Ret^{RET9} and Ret^{RET51} knockin mice, a response, which was similar to that of their wild-type littermates. This survival response was also comparable to NGF-dependent survival of both mutant and wild-type neurons. In summary, despite the differences between Ret9 and Ret51, the two splicing isoforms appear to elicit similar biological responses to GDNF in sympathetic neurons.

Akt and ERK1/2 activation are differentially affected by the mutation of tyrosines 981, 1015 or 1062 in the context of Ret9. To ascertain the role of Ret tyrosines 981, 1015 and 1062 in GDNF-mediated downstream signaling in sympathetic neurons, we checked the activation of both Akt and ERK1/2 after acute (10 min) stimulation with GDNF in

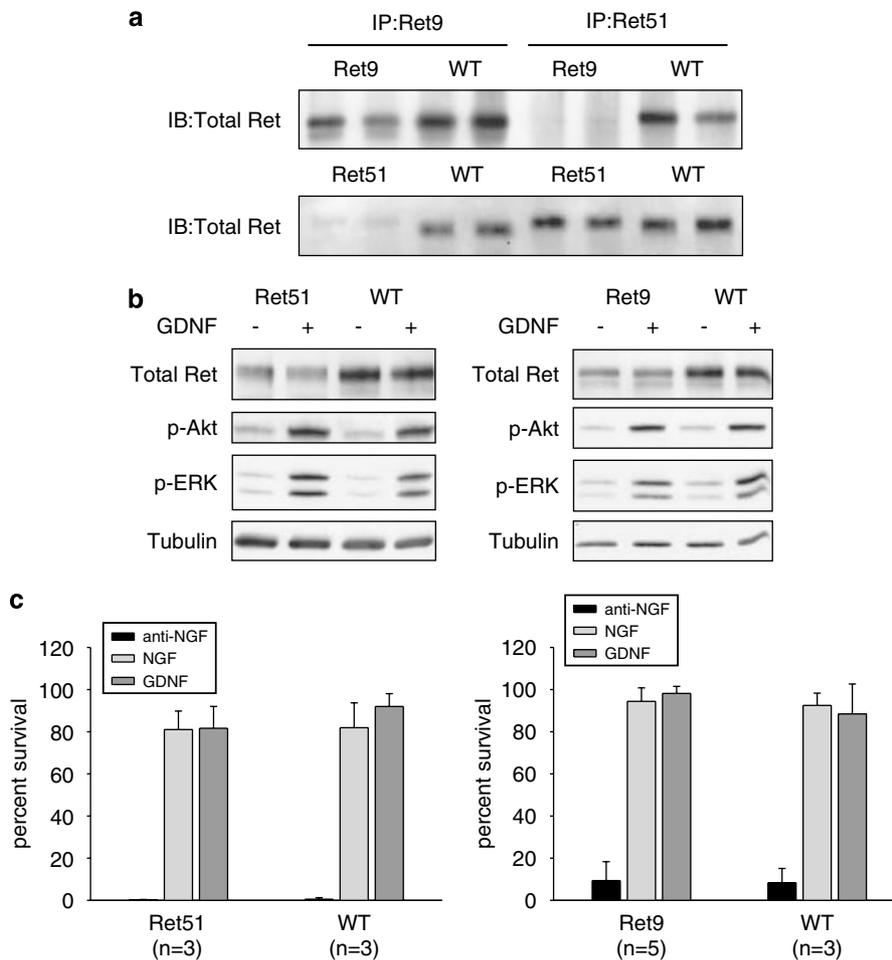


Figure 1 Neurons from knockin mice expressing only Ret9 or Ret51 display similar responses to GDNF. (a) Ret knockin mice express the expected splice isoforms. Lysates from sympathetic neurons from knockin mice expressing human, wild-type Ret9 or Ret51 were immunoprecipitated with the indicated isoform-specific antibodies and probed with a Ret antibody raised against the extracellular domain of the receptor (total Ret). Note the difference in electrophoretic mobility between Ret9 and Ret51. (b) Signaling from neurons expressing only Ret9 or Ret51 is indistinguishable from that of wild-type neurons. Cells were stimulated for 10 min with GDNF and lysates were probed with phosphospecific antibodies to phosphor-Ser473 Akt and dually phosphorylated ERK1/2. (c) GDNF fully rescues sympathetic neurons from both Ret^{RET9} and Ret^{RET51} knockin mice. Neurons from each genotype were cultured in NGF for 5 days and then incubated with media containing a blocking anti-NGF antibody, NGF or GDNF. Neuronal survival was scored 48 h later as described in Materials and Methods section. The number in parenthesis depicts the number of animals analyzed

lysates from mice lacking the above tyrosines, and compared mutant mice with their wild-type littermates. In the context of Ret9, lack of tyrosine 981 caused an almost complete abrogation of Akt phosphorylation together with a remarkable but less drastic reduction in ERK1/2 activation. Mutation of tyrosine 1015, on the other hand, decreased both Akt and ERK1/2 phosphorylation to approximately a half, whereas sympathetic neurons from Ret^{RET9(Y1062F)} mice showed no activation of either pathway above the baseline (Figure 2). Essentially, the same results were obtained when GDNF stimulation was performed for 24 h (Supplementary Figure 1). Surprisingly, none of the three mutations caused a significant change in the pattern of phosphorylation of either Akt or ERK1/2 in the context of Ret51 (data not shown and⁷).

These data, together with the observation that mice expressing either Ret51(Y1062F) or truncated Ret51 lacking

Tyr1096 develop normal kidneys, whereas Y1062F mutation introduced in Ret9 results in kidney agenesis,⁷ suggest that Tyr1096 plays a redundant role in Ret signaling. To characterize the effects of mutations of Ret tyrosines 981, 1015 and 1062 without the confounding effects of redundant signaling by the carboxy terminus of Ret51, we performed further studies only in the context of the Ret9 isoform.

Mutation of tyrosine 1062, but not 981 or 1015 abrogates GDNF-mediated survival.

Having shown that mutation of tyrosines 981, 1015 and 1062 in Ret9 decreased downstream signaling, we sought to analyze the effects of their absence on GDNF-mediated neuronal survival. Surprisingly, neurons from Ret^{RET9(Y981F)} mice did not show any reduction on GDNF-mediated survival (Figure 3, left column and Supplementary Figure 1), despite causing almost no phosphorylation of Akt. Neurons survived to levels

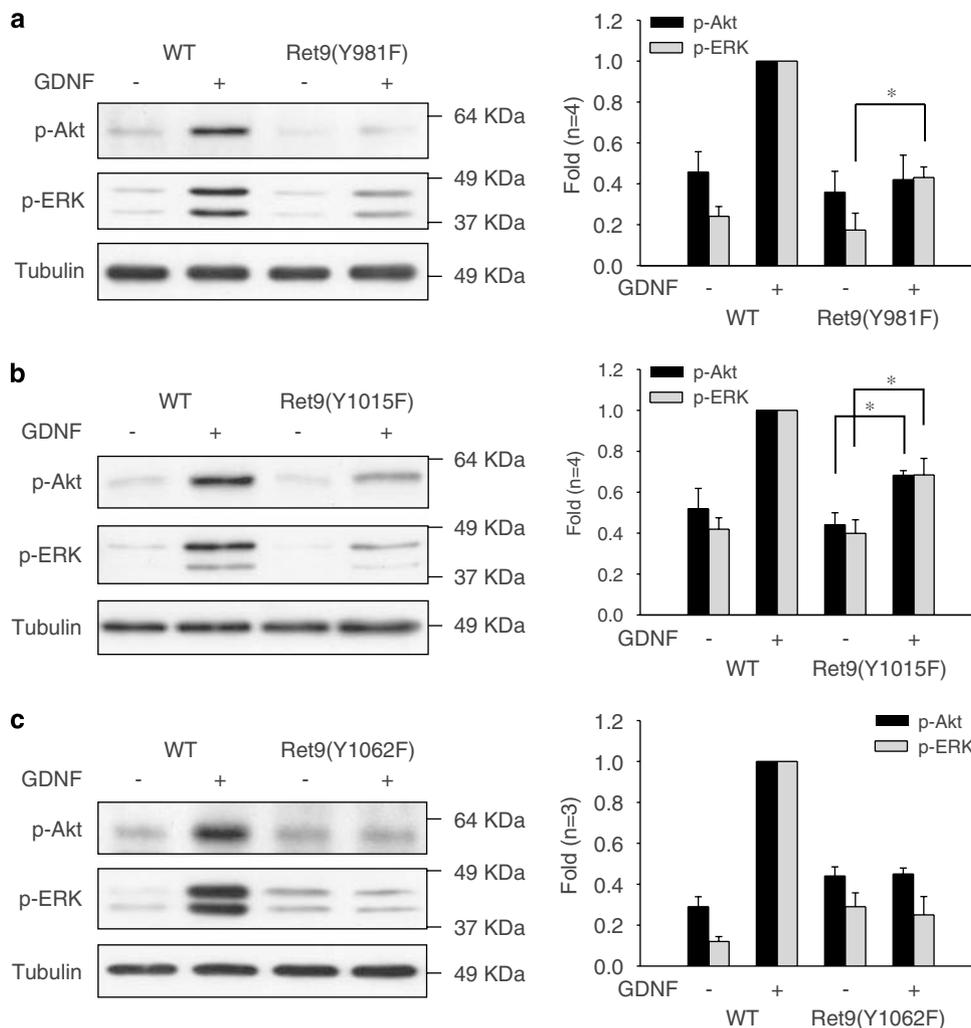


Figure 2 Mutation of Ret tyrosines 981, 1015 or 1062 differentially affects GDNF-mediated downstream signaling. Representative immunoblots of phospho-Ser473 Akt and dually phosphorylated ERK1/2 from sympathetic neurons from Ret^{RET9(Y981F)} (a), Ret^{RET9(Y1015F)} (b) or Ret^{RET9(Y1062F)} mice (c). For comparison, wild-type littermates are shown. Lack of tyrosine 981 caused an almost complete abrogation of Akt phosphorylation and reduced ERK phosphorylation to a lesser extent. In Ret^{RET9(Y1015F)} animals, both Akt and ERK phosphorylation was decreased approximately 50%, whereas mutation of tyrosine 1062 blocked both Akt and ERK phosphorylation to basal levels. Right panels show densitometric analysis of the indicated number of independent experiments. Data are expressed as mean \pm S.E.M. of fold induction. Asterisks depict $P < 0.05$ by two-tailed *t*-test

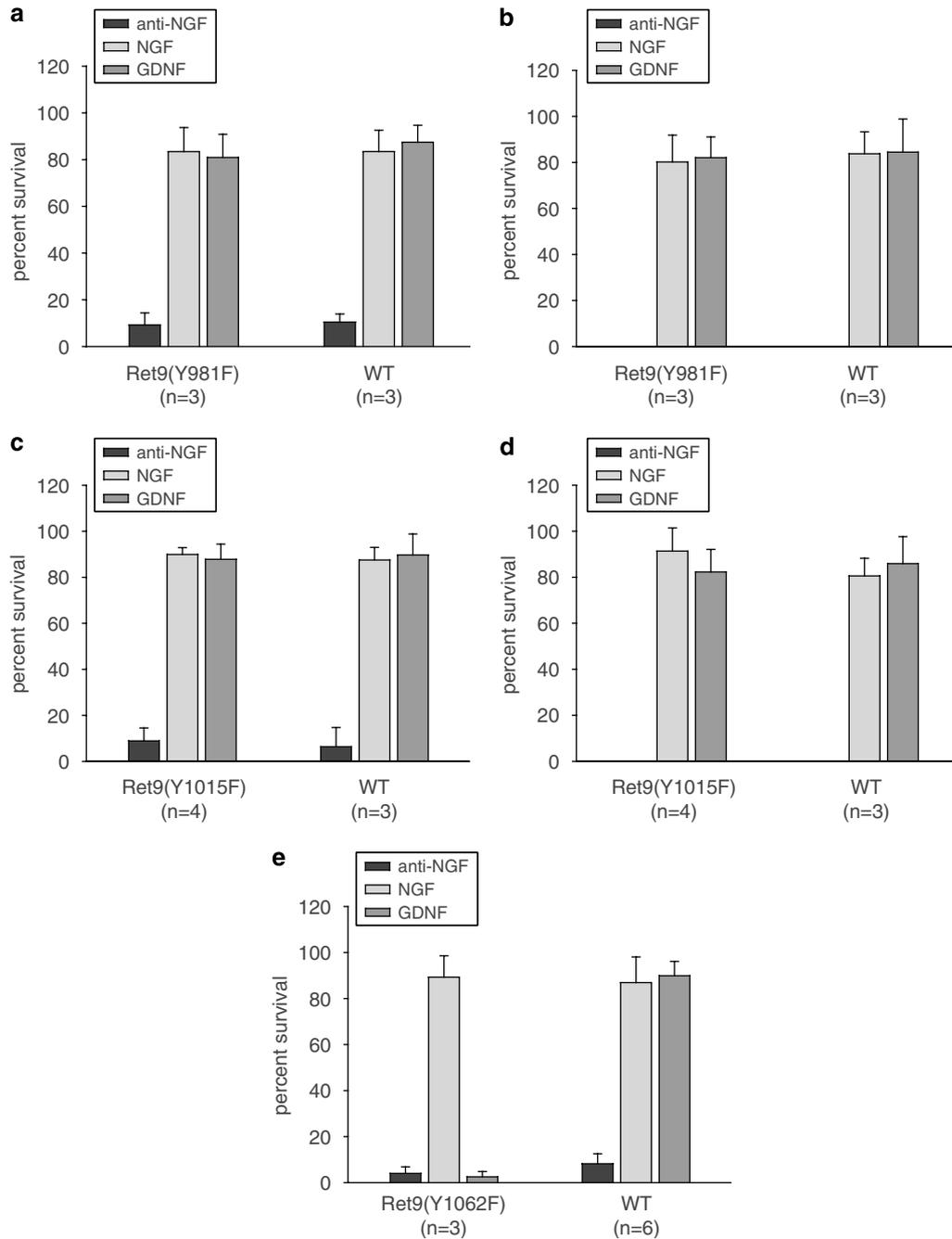


Figure 3 Only neurons from $Ret^{RET9(Y1062F)}$ mice fail to survive in the presence of GDNF. Quantification of the survival response to GDNF of sympathetic neurons from mice lacking tyrosine 981 (a and b), tyrosine 1015 (c and d) or tyrosine 1062 (e). The graphs on the left (a, c and e) depict survival 48 h after switching cells to GDNF, whereas the right panels (b and d) show the same cultures 1 week after switch. NGF-supported neurons were used as a control. The survival was compared to those of wild-type littermates. Number in parenthesis depicts the number of animals of each genotype analyzed

similar to those achieved by their wild-type littermates or by neurons from Ret^{RET9} mice. The survival response was similar to that elicited by NGF, and lasted at least up to 7 days, the last time point analyzed (Figure 3, right column and Supplementary Figure 2). Neurons from $Ret^{RET9(Y1015F)}$ mice showed a similar response, that is, full rescue by GDNF (Figure 3 and Supplementary Figure 2), whereas mutation of tyrosine 1062 completely prevented GDNF-mediated survival. Neurons expressing this mutation responded

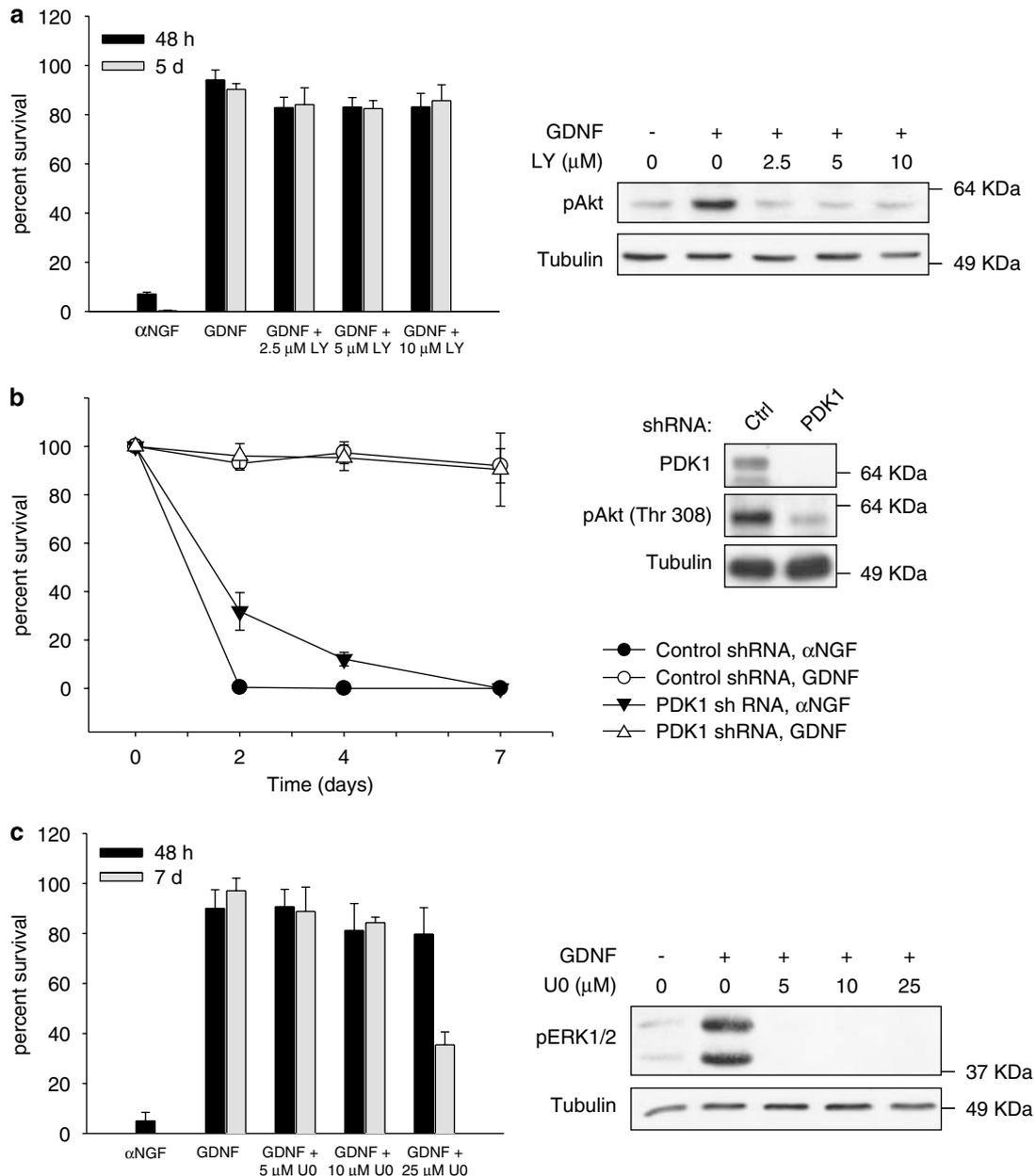
normally to NGF, demonstrating that the effect was specific for GDNF (Figure 3). Thus, although mutation of tyrosines 981 and 1015 diminished the activation of both Akt and ERK1/2, only mutation of tyrosine 1062 prevented GDNF-mediated survival of sympathetic neurons.

Neither the PI 3-K/Akt nor the MEK/ERK pathways are necessary for GDNF-mediated survival. Since the

reduction of Akt phosphorylation in neurons from *Ret^{RET9(Y981F)}* animals did not correlate with a decrease in cell survival, we directly tested the role of the PI 3-K pathway on GDNF-mediated survival of sympathetic neurons. Addition of increasing doses of LY294002, a selective inhibitor of PI 3-K, did not kill a significant number of wild-type neurons when compared to vehicle-treated cells. The same doses inhibited Akt phosphorylation to basal levels

suggesting a complete blockade of GDNF-mediated PI 3-K signaling (Figure 4a).

PDK1 is a 'master' kinase linking PI 3-K activation to its more relevant downstream effectors, namely Akt and other members of the AGC family of kinases (reviewed in Mora *et al.*⁸). Consistent with the results obtained with LY294002, lentiviral delivery of shRNA against PDK1 had no effect on cellular survival, further confirming that the PI 3-K/Akt



pathway is not necessary for GDNF-mediated survival of sympathetic neurons (Figure 4b).

Another candidate group of signaling intermediates that could be important for GDNF-mediated neuronal survival are the MAPK family members ERK1/2. We used U0126, a highly selective inhibitor of MEK1/2, the kinases upstream of ERK1/2. U0126 did not prevent the pro-survival effect of GDNF, at doses that effectively blocked phosphorylation of ERK1/2 (Figure 4c). Very high doses of U0126 (25 μ M) reduced cell survival after 1 week of treatment, suggesting a toxic effect of the drug. PD98059, the second MEK inhibitor used, also failed to block the survival of these neurons in the presence of GDNF (data not shown). Therefore, since two structurally unrelated MEK inhibitors did not prevent cell survival, it is unlikely that any of these kinases participate in the pro-survival effects of GDNF. In experiments using both PI 3-K and MEK1/2 inhibitors, we replaced the medium every 24 h to ensure sustained blockade of the pathways. As shown in Supplementary Figure 1, both inhibitors efficiently blocked activation of their respective targets after 24 h of stimulation.

The PI 3-K/Akt pathway supports cell growth. As sympathetic neurons mature in the presence of NGF, they progressively increase their size and become independent of NGF for survival. Thus, in mature neurons, NGF is required to stimulate the anabolic metabolism of the cell but not its survival,⁹ indicating that cell growth and cell survival are regulated independently in these neurons. Silencing of PDK1, although not causing cell death, resulted in a marked reduction in the soma diameter of these neurons, indicating that PI 3-K signaling stimulates cell growth (Figure 5a and b). Consistent with this observation, neurons from Ret^{RET9(Y981F)} animals increased their size over time in the presence of GDNF at a much slower rate than those from either Ret^{RET9} or Ret^{RET9(Y1015F)} mice (Figure 5c but see also Supplementary Figure 2). Therefore, as is the case for NGF,⁶ it appears that GDNF relies on the activation of the PI 3-K/Akt pathway to stimulate cell growth of sympathetic neurons.

Neurotrophic factor-induced neuronal survival of sympathetic neurons is mediated by B-Raf. The lack of phosphorylation of ERK1/2 in neurons from Ret^{RET(Y1062F)} mice correlated with the failure of GDNF to support survival of these sympathetic neurons, whereas in neurons from mice carrying either Ret Y981F or Y1015F this pathway, while diminished, was still active. Although experiments with U0126 ruled out a role of ERK1/2 in neuronal survival, it was still possible that apical elements of the pathway such as Ras or Raf could be involved in the pro-survival effects of GDNF, in a MEK-independent fashion. Lentiviral delivery of shRNAs against A-Raf, B-Raf and C-Raf effectively blocked the expression of each family member (Figure 6a–c). However, the protective effects of GDNF in cultured sympathetic neurons were only reversed by decreasing B-Raf levels. Importantly, knockdown of B-Raf killed sympathetic neurons also in the presence of NGF (Figure 6d). Finally, overexpression of a constitutively active form of B-Raf (V600E) was able to rescue trophic factor-deprived sympathetic neurons from death (Figure 7a).

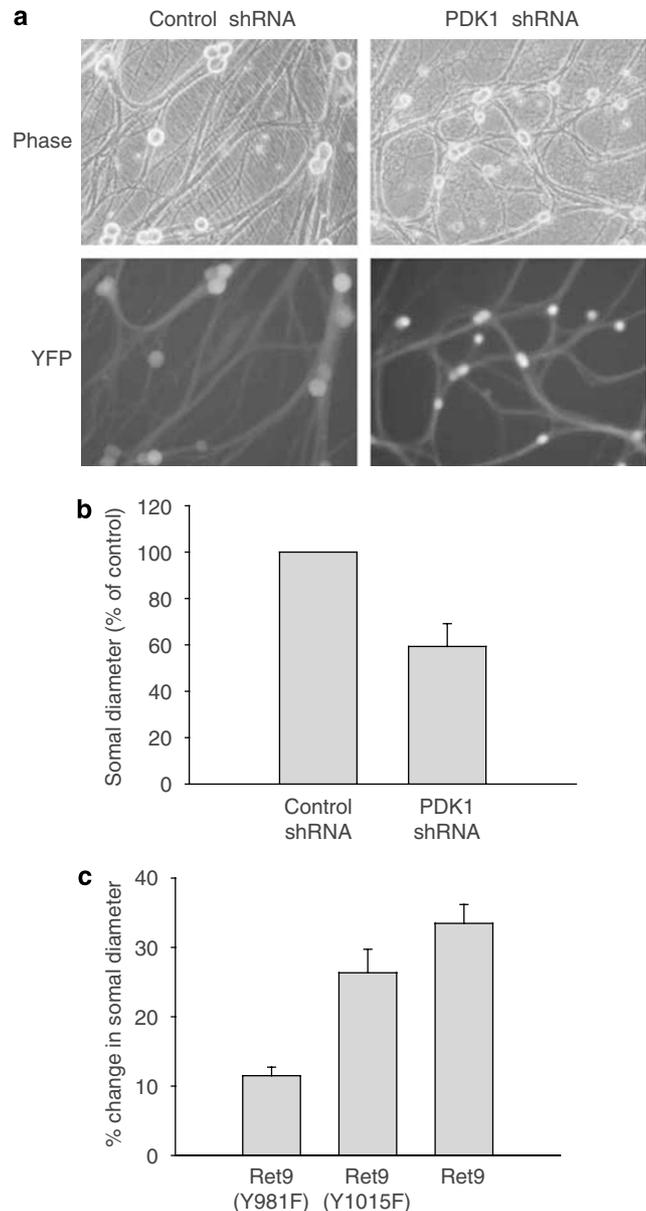


Figure 5 (a and b) Knockdown of PDK1 reduces cell growth. Wild-type mouse sympathetic neurons were infected with lentiviruses expressing shRNA to PDK1 as in Figure 6. Pictures were taken after 14 days in culture using a fluorescence microscope to show both the morphology and the degree of infection by visualizing expression of YFP. In panel b, the soma diameter was measured as described in Material and Methods. (c) Loss of Ret tyrosine 981 reduces cell growth. Sympathetic neurons from knockin mice expressing either Ret9, Ret9(Y981F) and Ret9(Y1015F) were kept in NGF for 5 days, then switched to GDNF and maintained for 7 additional days. Soma diameter was measured at the time of GDNF addition and 7 days after, and the percentage of increase in the soma size over this period of time was calculated

This observation is consistent with a critical role of B-Raf in neurotrophic factor-mediated survival of sympathetic neurons.

IKKs are downstream effectors of B-Raf in sympathetic neurons. Currently, it is unclear by which mechanism Raf kinases promote their biological effects independently of

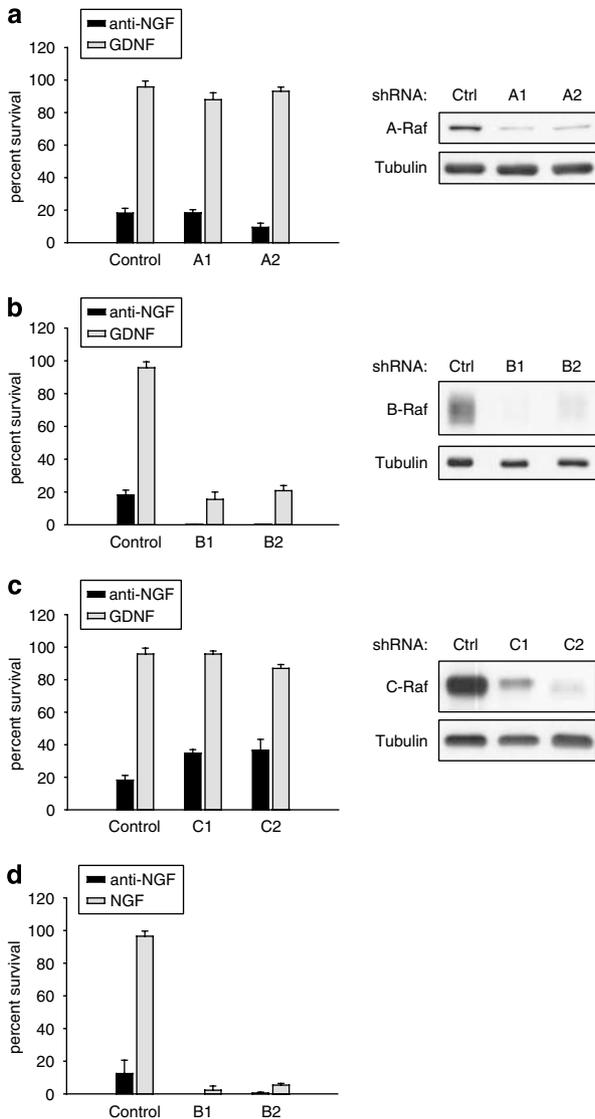


Figure 6 Knockdown of B-Raf, but not A-Raf or C-Raf, prevents GDNF- and NGF-mediated survival of wild-type mouse sympathetic neurons. (a, b and c) Left panels: neurons were infected with lentiviruses expressing shRNAs to A-Raf (A1 and A2), B-Raf (B1 and B2) and C-Raf (C1 and C2) 1 day after plating as described in Materials and Methods. Neurons were cultured in NGF for 5 days and then switched to GDNF. Survival was assessed after 48 h in the presence of GDNF. Right panels: the efficacy of the knockdown was assessed by immunoblot using isoform-specific Raf antibodies. In the case of B-Raf, cells were saved by the addition of the pan-caspase-inhibitor BAF 2 days before lysis. (d) Same as in panel b, but cells were treated with NGF instead of GDNF

MEK-1/2. Several putative Raf targets such as MTS-2, Rok- α , Bad and the NF- κ B pathway had been proposed (reviewed in Wellbrock *et al.*¹⁰ and Galabova-Kovacs *et al.*¹¹). We chose to test the latter, because the NF- κ B pathway has been reported to block apoptosis in sympathetic and sensory neurons.^{12,13} In the so-called canonical NF- κ B pathway, activated IKK complex formed by two catalytic subunits (IKK α and IKK β) and a regulatory subunit (IKK γ) phosphorylates the inhibitors of NF- κ B (I κ B), which become degraded by the proteasome. Degradation of I κ B frees cytoplasmic NF- κ B

dimers, which translocate to the nucleus to activate transcription of target genes. As shown in Figure 7b, simultaneous silencing of both IKK α and IKK β , but not the inhibition of PI 3-K or MEK-1/2, reversed the protection afforded by V600E B-Raf, suggesting that IKKs are downstream effectors of B-Raf. To further confirm this notion, we examined whether NGF or GDNF were able to activate IKKs in a B-Raf-dependent manner. Both neurotrophic factors induced phosphorylation of IKK α and IKK β in their activation loop (Figure 7c). Silencing of B-Raf prevented phosphorylation of IKKs, indicating that the activation of IKKs by NGF or GDNF requires B-Raf (Figure 7c). Finally, we tested whether B-Raf and IKKs form a complex in sympathetic neurons. As shown in Figure 7d, endogenous B-Raf coimmunoprecipitated with endogenous IKK α or IKK β independently of trophic factor stimulation, indicating that IKKs and B-Raf are constitutively associated in these neurons. Interestingly, our preliminary results indicate that such binding is retained in organs from newborn mice such as brain, heart or skin (data not shown), suggesting that the B-Raf/IKK pathway could operate in other cell types outside the nervous system. Taken together, these results place IKKs as downstream targets mediating the pro-survival effects of B-Raf.

IKKs are necessary for NGF- and GDNF-mediated survival of sympathetic neurons. Having shown that silencing of IKKs prevents the survival effects of V600E B-Raf, we next wanted to ascertain whether IKKs are necessary for neurotrophic factor-induced survival of sympathetic neurons. Silencing of IKKs in conjunction or individually (Figure 8a and b, respectively) killed sympathetic neurons maintained in the presence of either NGF or GDNF, indicating that both IKK α and IKK β are necessary for neuronal survival triggered by NGF or GDNF. Such phenomenon was not restricted to mouse sympathetic neurons, as rat sympathetic neurons or mouse trigeminal neurons also died when either IKKs or B-Raf were silenced (Supplementary Figure 3). Silencing of IKK β caused an increase in BimEL and PUMA levels in the presence of NGF, to levels comparable to those reached after NGF deprivation (Figure 7c). On the other hand, knockdown of Bax suppressed cell death caused by both NGF deprivation and lack of IKK β (Figure 7d). These results, therefore, suggest that cell death caused by loss of IKK signaling mimics trophic factor withdrawal-induced cell death.¹⁴ Finally, we checked the status of IKKs in sympathetic neurons from Ret^{RET(Y981F)} and Ret^{RET(Y1062F)} mice. As expected, mutation of tyrosine 1062 completely prevented activation of IKKs, whereas lack of tyrosine 981 had little or no effect (Figure 8e).

Discussion

Regulation of the PI 3-K/Akt and the ERK1/2 pathways by Ret tyrosines. Absence of tyrosine 1062 results in blockade of phosphorylation of both Akt and ERK1/2 to baseline. Importantly, B-Raf is the major MEK kinase in sympathetic neurons (Supplementary Figure 2), indicating that Ret9(Y1062F) is unable to activate B-Raf upon GDNF

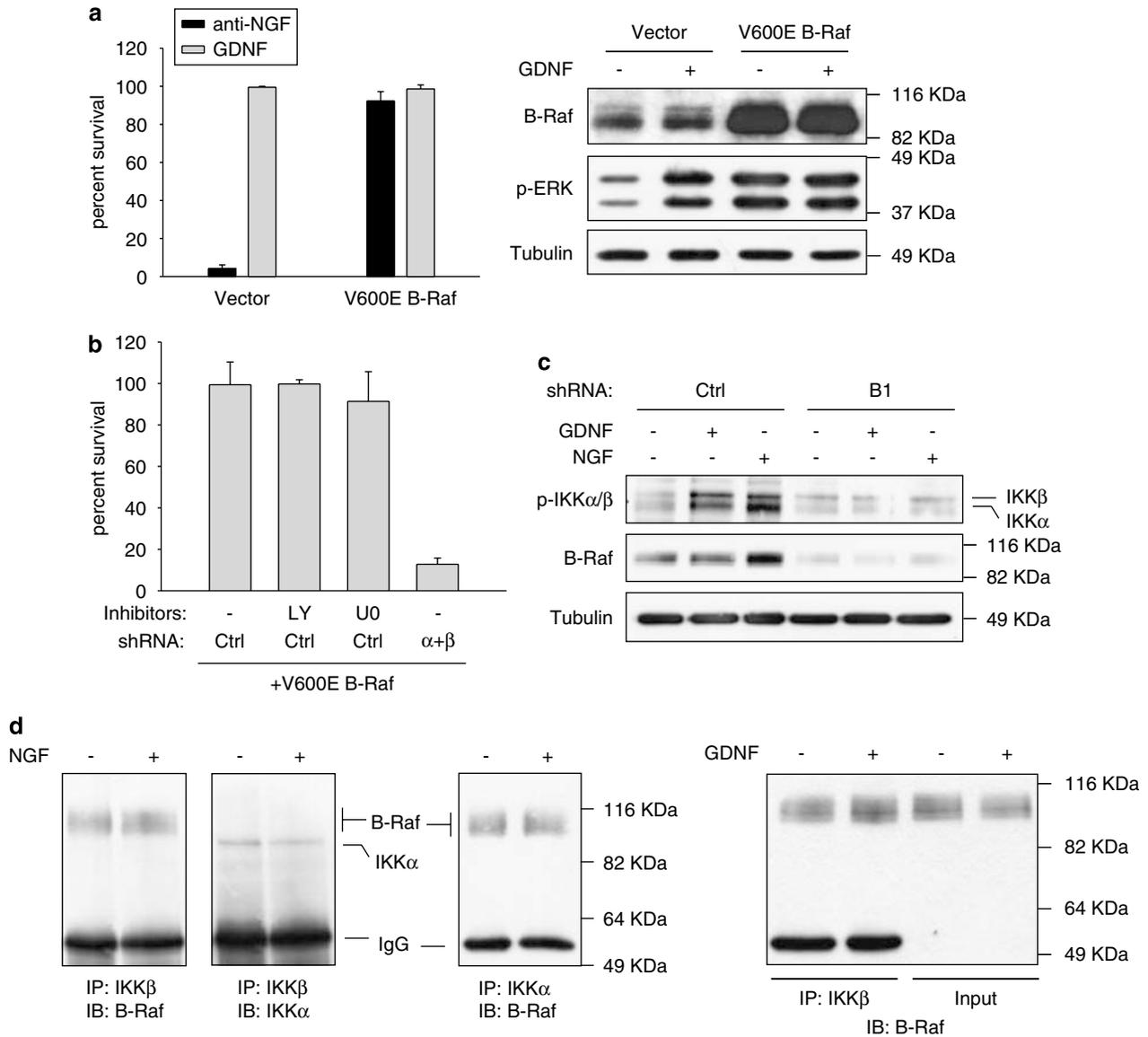


Figure 7 IKK α and IKK β are downstream effectors of B-Raf. (a) A constitutively active form of B-Raf (V600E) rescues trophic factor-deprived neurons. Left, cells were infected with a lentiviral construct coding for human V600E B-Raf or empty vector. Four days later, cells were switched to media containing a blocking anti-NGF antibody or GDNF. Survival was assessed after 48 h in the presence of GDNF. Right, V600E B-Raf mutant is constitutively active. Cells infected with V600E B-Raf or empty vector were stimulated with for 10 min with GDNF and lysates probed with antibodies to B-Raf and dually phosphorylated ERK1/2. (b) Silencing of IKKs, but not the inhibition of PI 3-K or MEK, reverses the protection afforded by V600E B-Raf. Cells were sequentially infected, as described in Materials and Methods, with V600E B-Raf, and either with control shRNAs or with a mixture of shRNAs to IKK α and IKK β ($\alpha + \beta$). Three days later cells were deprived and kept in the presence of the indicated inhibitors (10 μ M each) for two additional days, after which survival was evaluated. (c) B-Raf acts upstream of IKKs. Cells were infected with control shRNAs or a shRNA to B-Raf ('B1'). Four days after infection, cells were deprived overnight in the presence of 50 μ M BAF. Cells were stimulated for 10 min with the indicated factors and lysates probed against phospho-IKKs, B-Raf or tubulin. (d) Endogenous IKKs and B-Raf form a complex in sympathetic neurons. Neurons were kept in NGF for five days, deprived overnight and stimulated for 10 min with medium alone or containing the indicated factor. IKK β or IKK α were immunoprecipitated and lysates were immunoblotted (IB) with the indicated antibodies. In the right panel, 5% of the input lysate was resolved in parallel to IKK β immunoprecipitates

stimulation. Ret activates both the PI 3-K/Akt pathway and the Ras/ERK pathway through the recruitment of the adaptor protein Grb2 to phosphorylated Shc and/or FRS-2.^{15,16} Binding of Grb2 to Gab1/2 activates the PI 3-K/Akt pathway, whereas binding of Grb2 to Sos activates ERK1/2 through Ras-Raf-MEK. As mentioned earlier, both Shc and FRS-2 bind to phosphotyrosine 1062 and therefore, its absence would impair the phosphorylation of such adaptors

and its subsequent binding to Grb2. How Tyr981 regulates these pathways in the case of Ret signaling is unclear but many possibilities exist, such as phosphorylation of Dok6 through an Src family kinase,^{17,18} tyrosine phosphorylation of Akt by Src¹⁹ or inhibition of PTEN activity by an Src family kinase.²⁰ In any case, the mechanisms by which these tyrosines regulate downstream signaling need to be further explored.

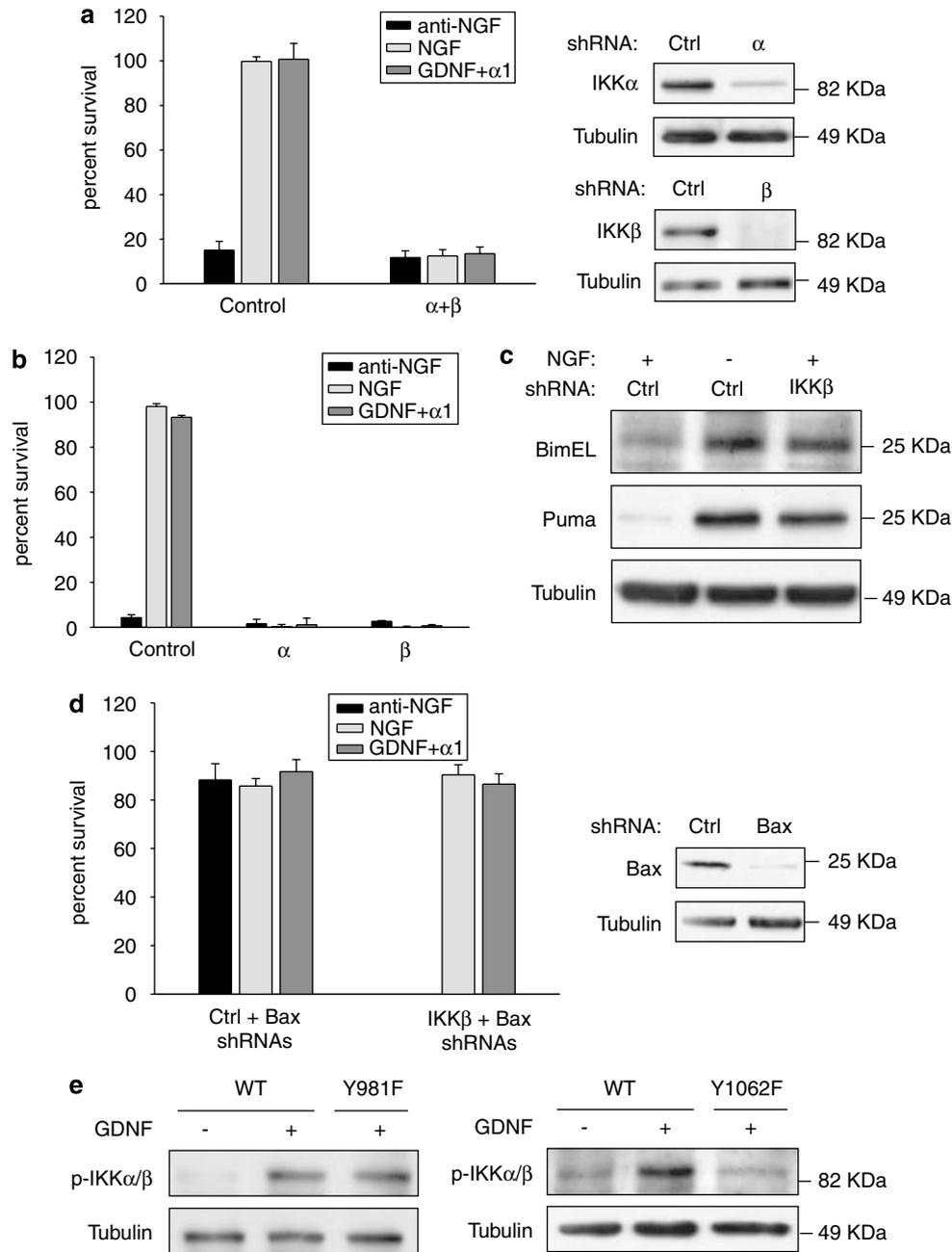


Figure 8 IKKs are necessary for NGF- and GDNF-mediated survival of sympathetic neurons. (a and b) Neurons were infected with shRNAs to IKKs in combination ($\alpha + \beta$) or alone (α or β) 2 days after plating. After 3 additional days in the presence of NGF, cells were switched to the indicated factors and survival was evaluated 48 h later. The right panel shows efficacy of the knockdown. (c) Silencing of IKKs induces BimEL and PUMA expression. On the second day after plating, cells were infected with control shRNAs or with an shRNA to IKK β and kept on NGF for 3 additional days. Cells were then deprived or kept in NGF for 24 h more as depicted, and BimEL and PUMA expression was assessed by western blot. (d) Silencing of Bax rescues cells from the knockdown of IKK β . Cells were infected with shRNAs to Bax, and subsequently infected with control or with shRNAs to IKK β as detailed in Materials and Methods. Cells were maintained in NGF for 3 additional days, and switched to media containing the indicated factors. Cell survival was assessed 48 h later. Right panels show efficacy of the knockdown. (e) Loss of tyrosine 1062, but not tyrosine 981, blocks GDNF-mediated phosphorylation of IKK α/β . Sympathetic neurons from the indicated genotypes were cultured in the presence of NGF for 5 days, deprived overnight and stimulated for 10 min with GDNF. Blots were probed with antibodies to phospho-IKKs. Note that owing to a higher acrylamide percentage, IKK α and IKK β were not resolved as in Figure 7c

The role of PI 3-K/Akt in neuronal survival. Our data do not support a significant role of the PI 3-K/Akt pathway in GDNF-mediated neuronal survival of sympathetic neurons. Although PI3-K has been shown to exert antiapoptotic effects in many paradigms, its role in the survival of sympathetic

neurons is controversial. The most thoroughly examined neurotrophic factor in this paradigm is NGF. Some authors have reported that blockade of PI 3-K function by different means prevents NGF-mediated survival,^{3,4} whereas others have shown the opposite.^{5,6} The reason for these

discrepancies is unclear, but it is probably related to both the culture conditions and methods for assessing survival. For example, evaluation of survival relying solely on metabolic assays such as MTT reduction could be misleading since the inhibition of PI 3-K reduces the cell size and metabolic activity of these cells (our data⁶). Likewise, the use of exceedingly high doses of LY294002, well above those needed to inhibit PI 3-K completely, might cause cellular toxicity or affect other targets than PI 3-K itself. Finally, blockade of PI 3-K does not trigger the apoptotic molecular events that take place in NGF-deprived SCG neurons such as Bax translocation to the mitochondria or release of cytochrome *c* to the cytosol,¹⁴ further indicating that inhibition of PI 3-K does not mimic the absence of trophic support completely.⁶

Control of cell growth by the PI 3-K/Akt pathway. Knockdown of PDK1, although not affecting cell survival, caused a marked reduction in cell size. Likewise, lack of Ret tyrosine 981, which resulted in an almost complete blockade of Akt phosphorylation, caused a reduction in cell size without affecting survival. Interestingly, mice expressing a hypomorphic mutation for PDK1 are viable and are fertile but have reduced body mass ultimately caused by a decrease in cell size but not in cell number.²¹ Moreover, fibroblasts from these animals undergo apoptosis at the same rate than those from wild-type animals.

We hypothesize that the control of cell size by PI 3-K is linked to the activation of mTOR, a kinase, which plays a well-known role in both nutrient sensing and stimulation of anabolism and cell growth. The connection between mTOR and the PI 3-K/Akt has been recently unveiled in the case of the insulin/IGF system by finding that Akt phosphorylates and inactivates the TSC1/TSC2 complex, an inhibitor of mTOR activity (reviewed in Martin and Hall²²). Thus, the inhibition of PI 3-K would result in repression of mTOR signaling, thereby reducing or stopping GDNF-mediated cell growth.

B-Raf as a key mediator of survival. Our data indicate that B-Raf is necessary for GDNF- and NGF-mediated survival in sympathetic neurons, independently of MEK. This is consistent with data from Wiese *et al.*,²³ who showed that targeted deletion of B-Raf prevented neurotrophin-mediated survival of embryonic motor and sensory neurons. In that work, the authors also show that lack of B-Raf causes a decrease in expression of IAPs, especially, XIAP. These observations are in agreement with those reported by Kato and colleagues,²⁴ who demonstrated that IAPs are essential for GDNF-mediated survival of embryonic motor neurons. Accordingly, we also observed a decrease in XIAP levels concomitant with silencing of B-Raf in our system (Supplementary Figure 4). However, sympathetic neurons from XIAP^{-/-} mice survive normally in the presence of NGF,²⁵ indicating that loss of XIAP expression by itself is not sufficient to preclude NGF-mediated survival of these neurons (see below). A recent report suggests that B-Raf is not necessary for the survival of sensory neurons but instead regulates axon growth *in vivo*.²⁶ We do not know whether these differences are cell specific or on the contrary are due to functional redundancy between family members, or

perhaps to incomplete excision of Raf at the onset of programmed cell death of DRG neurons.

Finally, it is worth noting that the central role of B-Raf as an effector of Ret signaling appears to be conserved outside the nervous system, as the RET–RAS–BRAF pathway has been identified as one of the major genetic alterations found in thyroid carcinomas, being its activation responsible for the proliferation and invasive phenotype of these cells.²⁷

How do IKKs regulate neuronal survival? One of the major findings of this paper is that IKKs are targets of B-Raf in sympathetic neurons, and that IKK activity is necessary for NGF and GDNF-mediated survival of these neurons. Although the role of IKKs in neuronal survival has not been addressed thoroughly, results obtained in other systems suggest a plethora of potential mechanisms by which IKKs could block apoptosis. First, antiapoptotic genes such as Bcl-2 family members A1/Bfl-1, Mcl-1 and Bcl-XL, or IAPs such as XIAP are found among transcriptional targets of NF- κ B.²⁸ Second, IKKs can block the JNK pathway through the activation of NF- κ B. It is well established that the activation of the JNK pathway is necessary for trophic factor withdrawal-induced death of sympathetic neurons.^{29–32} Blockade of the JNK pathway can be accomplished directly by inducing the expression of antagonists of the pathway such as Gadd45 β (which blocks the activity of MKK7).^{33,34} It can also be blocked indirectly by inducing the expression of antioxidants such as ferritin heavy chain,³⁵ that counteract the production of reactive oxygen species concomitant to some types of cell death, including that occurring in sympathetic neurons after NGF withdrawal.^{36,37} Third, IKKs may exert their protective effects independently of I κ B α degradation and NF- κ B activation. Thus, it has been shown that IKK β contributes to tumor development by directly phosphorylating and inducing ubiquitin-dependent degradation of the transcription factor FOXO3A.³⁸ Interestingly, it has been shown that Bim is under the transcriptional control of FOXO3A³⁹ (although Bim levels can be regulated through other transcriptional and post-transcriptional mechanisms), and that Bim deletion attenuates trophic factor withdrawal-induced death of sympathetic neurons.³⁹ The contribution of all these potential mechanisms to blockade of sympathetic neuronal cell death by IKKs is currently being investigated.

Materials and Methods

Antibodies and reagents. Antibodies to Ret9 (C-19G), Ret51(C-20G), A-Raf (C-20G) and B-Raf (F-7) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies to phospho-Akt (Ser473), dually phosphorylated ERK1/2 (Thr202/Tyr204) and phospho-IKK α (Ser180)/IKK β (Ser181) were from Cell Signaling (Beverly, MA). Anti-PDK1 and anti-C-Raf were purchased from BD Biosciences (San Jose, CA). Anti-IKK α and IKK β antibodies were from Calbiochem (Darmstadt, Germany), and anti-Bax was from Upstate (Lake Placid, NY). The Bim and XIAP antibodies were obtained from Stressgen (Victoria BC, Canada), and the anti- α -tubulin was from Sigma (St. Louis, MO). Anti-PUMA was from ProSci Inc. (Poway, CA). Finally, the antibody against the extracellular domain of Ret has been described previously.¹⁷ LY294002, PD98059 and U0126 were from Calbiochem. All other reagents were from Sigma.

Cell culture and assessment of neuronal survival. Mouse sympathetic neurons from the superior cervical ganglion of postnatal day 0–1 pups were dissected and cultured essentially as described.⁴⁰ Briefly, ganglia were sequentially digested in collagenase and trypsin (Worthington Lakewood, NJ),

dissociated by trituration and plated on rat tail collagen-coated dishes in the medium containing 50 ng/ml NGF (Alomone labs, Jerusalem, Israel), 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and anti-mitotics for 5 days (AMO medium). Subsequently, cells were washed three times with media and switched to media containing either anti-NGF, 50 ng/ml NGF (Alomone) or anti-NGF plus 50 ng/ml GDNF (Alomone) and 100 ng/ml Fc-GFR α 1 (R & D Systems, Minneapolis, MN). For survival experiments, one-tenth to one-fifth of a ganglion was plated in 24-well dishes. Neuronal survival was assessed by counting neurons of designated fields before and after treatments at the indicated time points. Typically, around 200–300 neurons per field were counted in triplicate wells. Results were expressed as a percentage of the initially counted cells. Experiments were repeated at least three times, the data was pooled and expressed as mean \pm S.D. For the measurement of soma diameter, pictures were analyzed with the AnalySIS software package (Soft Imaging System GmbH, Germany).

Immunoprecipitation and western blot. For western blot, cells were plated at higher density (typically 1–2 ganglia per well in 24-well or 12-well plates, respectively) and kept in NGF-containing media for 5–7 days. Then cells were washed three times with AMO and deprived overnight in media containing no NGF. Neurons were stimulated with 50 ng/ml GDNF plus Fc-GFR α 1 or 50 ng/ml NGF for 10 min. We added GFR α 1 to match the culture conditions in survival experiments, even though its presence did not alter the signaling pattern elicited by GDNF (data not shown). Cells were lysed in NP-40 containing buffer (150 mM NaCl, 50 mM Tris pH 8, 1 mM EDTA, 1 mM EGTA) freshly supplemented with a cocktail of protease inhibitors (Roche Applied Science), 1 mM sodium orthovanadate, 10 mM sodium fluoride and 50 mM β -glycerophosphate. Alternatively, cells were directly lysed in Laemmli's buffer. Proteins were resolved in SDS-PAGE, transferred to PVDF membranes (Millipore) and blocked in TBST containing 5% BSA. Primary antibodies were incubated overnight at 4°C, followed by peroxidase-conjugated secondary antibodies. Immunodetection was performed using ECL-Advance (Amersham, GE Healthcare). X-ray films were scanned with an EPSON Perfection 3200 PHOTO apparatus and acquired with Adobe Photoshop. Densitometric analysis was performed using the Quantity One software (Bio-Rad, Hercules, CA). For immunoprecipitation, NP-40 lysates were incubated with the indicated primary antibodies plus sepharose-bound protein A and/or protein G (Sigma). Immunocomplexes were washed with buffer and boiled in Laemmli's buffer.

Lentiviral delivery of short-hairpin RNAs (shRNAs). Lentiviral-based vectors for RNA interference-mediated gene silencing (FSVsi) consisted on a U6 promoter for expression of short-hairpin RNAs and the venus variant of YFP under the control of an SV40 promoter for monitoring transduction efficiency. Lentiviral particles were produced in 293T cells cotransfected by the calcium phosphate method with the above plasmid plus plasmids coding for the envelope and the packaging systems (VSV-G and Δ 8.9, respectively). The day after transfection, 293T cells were switched to AMO containing no antimetabolites and left for 2–3 days. Supernatants were then harvested, filtered through a 0.45 μ m filter, supplemented with NGF and antimetabolites and directly applied to sympathetic neurons. Typically, cells were infected overnight the day after or 2 days after dissection. In experiments aimed to assess the efficacy of gene knockdown by immunoblot, cells were typically kept for 5 days in NGF followed by 2 more days in GDNF, or alternatively 7 days in NGF. When gene silencing was deleterious for the cells, the pan-caspase inhibitor BAF (50 μ M) was added to the media 2–3 days before lysis. For overexpression of B-Raf, the human cDNA of B-Raf containing a V600E substitution was cloned in a lentiviral expression vector (FCMV), which contains the promoter of human cytomegalovirus. When sequential infections were needed, cells were infected overnight with the first set of viruses, allowed to recover the day after and infected the following night with the second set of viruses. After this second infection, neurons were kept in NGF for 3 more days before proceeding with the different experimental conditions. Viruses expected to promote beneficial effects on survival (i.e., V600E B-Raf and Bax shRNA) were infected first, whereas viruses expected to have deleterious effects were infected afterwards.

Sequences of shRNAs. The following sequences were used for generation of shRNAs:

PKD1: 5'-TGTTGAGGTCCTCCAACTGA-3', PKD1 control: 5'-GGGAATTTCC TAAGGACGC-3', A1: 5'-CCTATGTTGTTGTGCTCTAT-3', A2: 5'-TGCGTTGACA TGAGTACCAA-3', B1: 5'-GCTACCTATTCAAACATCAA-3', B2: 5'-GCTTACGA GAAATACACTGAA-3', C-Raf, C1: 5'-CCTATGTTGTTGTGCTCTAT-3', C2: 5'-TGCGTTGACATGAGTACCAA-3', Raf Control: 5'-GTCCGCTTCGTTTAGG

TCTT-3'; IKK α : 5'-CAGGCTCTTTCAGGGACAT-3'; IKK β : 5'-GCTGCACATT GAATCTGTAT-3'; IKK control: 5'-GCTCTCTGAAGGTTGATGA-3'; Bax 5'-GC AGCTGACATGTTTGTGCTAT-3', Bax control 5'-ACTGCCTTGACTGTGCTTT-3'.

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