The kinesin KIF1B β acts downstream from EglN3 to induce apoptosis and is a potential 1p36 tumor suppressor

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***VHL*, *NF-1*, *c-Ret*, and *Succinate Dehydrogenase Subunits B* and *D* act on a developmental apoptotic pathway that is activated when nerve growth factor (NGF) becomes limiting for neuronal progenitor cells and requires the EglN3 prolyl hydroxylase as a downstream effector. Germline mutations of these genes cause familial pheochromocytoma and other neural crest-derived tumors. Using an unbiased shRNA screen we found that the kinesin KIF1B β acts downstream from EglN3 and is both necessary and sufficient for neuronal apoptosis when NGF becomes limiting. *KIF1B β* maps to chromosome 1p36.2, which is frequently deleted in neural crest-derived tumors including neuroblastomas. We identified inherited loss-of-function *KIF1B β* missense mutations in neuroblastomas and pheochromocytomas and an acquired loss-of-function mutation in a medulloblastoma, arguing that *KIF1B β* is a pathogenic target of these deletions.**

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Developmental apoptosis of neuronal precursors is crucially important for determining the final number of terminally differentiated cells (Sommer and Rao 2002). Derivation of this process can cause disease (Zhu and Parada 2002). Paragangliomas and neuroblastomas are tumors of the sympathetic nervous system (paragangliomas arising in the adrenal medulla are called pheochromocytomas). During normal embryological development, neuronal progenitor cells, including cells capable of forming the sympathetic nervous system, compete with one another for growth factors such as nerve growth factor (NGF), with the losers undergoing apoptosis.

Germline *VHL*, *NF-1*, *c-Ret*, and *Succinate Dehydrogenase Subunits B* and *D* (*SDHB* and *SDHD*) mutations have been linked to the development of paragangliomas (Nakamura and Kaelin 2006). We reported recently that the products of these genes define a pathway that is activated upon NGF withdrawal, leading to apoptosis mediated by the EglN3 prolyl hydroxylase (Lee et al. 2005). This suggests that at least some paragangliomas arise due to failure to properly cull neuronal progenitor cells during development.

Results

To investigate the requirement of the proapoptotic prolyl hydroxylase EglN3 in neuronal apoptosis in a genetically defined system, we isolated primary sympathetic neurons from *EglN3*^{+/+}, *EglN3*^{+/-}, and *EglN3*^{-/-} mice. As

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expected, wild-type and heterozygous sympathetic neurons died after NGF withdrawal (Fig. 1A; Supplemental Fig. 1). In contrast, *EglN3*^{-/-} sympathetic neurons were remarkably resistant to apoptosis in this setting (Fig. 1A). Furthermore, *EglN3*^{-/-} cerebellar granular neurons (CGNs) were resistant to apoptosis induced by the neurotoxin cytosine arabinoside (Ara-C) compared with *EglN3*^{+/+} control CGNs (Fig. 1B). These results strengthen the earlier conclusion, reached with siRNAs and pharmacological agents, that EglN3 plays an important role in neuronal apoptosis (Lee et al. 2005).

Next, we infected different cell types with an adenovirus encoding EglN3. EglN3 killed SK-N-DZ and SK-N-SH neuroblastoma cells, PC12 rat pheochromocytoma cells, and SK-Mel28 melanoma cells, all of which are derived from neural crest progenitor cells (Fig. 1C,D). This effect was specific as EglN3 did not kill 786-O renal carcinoma cells, HK2 immortalized renal epithelial cells, or primary mouse embryo fibroblasts (Fig. 1C,D). EglN3 catalytic activity was required for killing since it could be partially rescued with the hydroxylase inhibitor dimethyl oxalylglycine (DMOG) (Fig. 1E). EglN3-induced apoptosis was not restricted to neural crest derivatives, however, because EglN3 also killed U2OS osteosarcoma cells, prostatic carcinoma cells (DU145 and PC3), H1299 lung carcinoma cells, and HCT116 colorectal carcinoma cells (Fig. 1C; Supplemental Fig. 2A).

p53 is a critical regulator of apoptosis and has been implicated in developmental cell death of sympathetic neurons (Aloyz et al. 1998). Isogenic HCT116 cells that are *p53*^{+/+} or *p53*^{-/-} were both killed by EglN3 (Supplemental Fig. 2A). Likewise, EglN3-induced cell death in neural crest-derived cells (SK-Mel28 melanoma cells) was not prevented by an effective p53 shRNA or by SV40

T antigen, which blocks p53 function (Supplemental Fig. 2B–D). Therefore, EglN3-induced apoptosis does not require p53.

To begin to understand how EglN3 induces apoptosis, we screened for shRNAs that can prevent EglN3-induced death. In pilot experiments, we determined the Ad-EglN3 titer required to kill all of the SK-Mel28 melanoma cells in subconfluent cultures (Fig. 1C; data not shown). Next, SK-Mel28 cells were infected with a previously described retroviral shRNA library (Berns et al. 2004) (or with empty retrovirus) prior to Ad-EglN3 infection (Fig. 2A). No survivors emerged among the control cells pretreated with the empty virus. In cells pretreated with the shRNA library, however, 12 surviving colonies emerged and were expanded for further analysis. Three died when rechallenged with Ad-EglN3 and were therefore considered false-positives, while nine remained resistant (Fig. 2B). The shRNA inserts from these latter colonies were isolated, sequenced, and retested for their ability to protect naive SK-Mel28 cells from EglN3-induced apoptosis. Sequence analysis of one of the two shRNAs that scored positively in this assay predicted that it targeted the β splice variant of KIF1B, a member of the kinesin 3 family (Nagai et al. 2000; Yang et al. 2001; Zhao et al. 2001). Down-regulation of endogenous KIF1B β , but not the alternative splice variant KIF1B α , was confirmed by Western blot analysis (Fig. 2C). KIF1B α and KIF1B β share an N-terminal motor domain but contain different C-terminal cargo domains. Protection against EglN3-induced cell death was conferred by two additional, independent, KIF1B β shRNAs, arguing that modulation of KIF1B β , rather than an off-target effect, was responsible for this protection (data not shown). Since *KIF1B* maps to 1p36 (Nagai et al. 2000; Yang et al.

Figure 1. Regulation of apoptosis by EglN3. (A) Percentage of DAPI-stained nuclei exhibiting apoptotic changes in primary mouse sympathetic neurons of the indicated genotypes in the presence of NGF (+NGF) or 48 h after (-NGF) NGF withdrawal. (WT) *EglN3*^{+/+}; (Het) *EglN3*^{+/-}; (KO) *EglN3*^{-/-}. (B) Immunoblot analysis of primary CGNs of the indicated genotypes 24 h after treatment with 100 μ M Ara-C. Crystal violet (C) and immunoblot analysis (D) of indicated cell lines after infection with adenovirus encoding EglN3 (Ad-EglN3) or Cre recombinase (Ad-Control). (E) Photomicrographs of SK-Mel-28 cells infected with the indicated amount of Ad-EglN3 (multiplicity of infection, MOI) in the presence or absence of 1 mM DMOG.

Figure 2. Recovery of KIF1B β shRNA. (A) Schema for identifying shRNAs that protect against EglN3-induced apoptosis. (B) Crystal violet staining of 12 isolated colonies that were retested for resistance of EglN3-induced death. (Neg. control) Naive SK-Mel28 cells. Clone found to contain KIF1B β shRNA is indicated. (C) Immunoblot and crystal violet staining of SK-Mel28 cells infected to produce recovered KIF1B β shRNA and subsequently infected with Ad-EglN3.

2001; Zhao et al. 2001), which is frequently deleted in multiple tumor types including nervous system tumors (Schwab et al. 1996), we hypothesized that it might function as a tumor suppressor gene through regulation of apoptosis in neuronal, and perhaps other, tissues.

To ask whether EglN3 and KIF1B β belong to the same pathway, PC12 cells were infected with Ad-EglN3. Induction of apoptosis, as determined by Caspase 3 cleav-

age, was accompanied by the induction of KIF1B β , but not its splice variant KIF1B α (Fig. 3A). Conversely, knockdown of human EglN3 in HeLa cervical carcinoma cells with two independent siRNAs decreased KIF1B β levels (Fig. 3B; Supplemental Fig. 3). Notably, an siRNA against EglN1, which regulates the HIF α transcription factor (Berra et al. 2003), did not affect KIF1B β , consistent with the earlier conclusion that regulation of apoptosis by EglN3 is HIF-independent (Lee et al. 2005). zKIF1B levels were also markedly attenuated in zebrafish treated with a morpholino oligonucleotide directed against zEglN3 (Fig. 3C).

During normal neuronal development, many cells undergo apoptosis as they compete for growth factors such as NGF (Sommer and Rao 2002). Pheochromocytomas are derived from sympathetic neuronal progenitor cells, and PC12 cells have been used extensively as a model to study the effects of NGF on neuronal differentiation and survival. Consistent with previous reports (Lipscomb et al. 1999; Lee et al. 2005), NGF withdrawal from PC12 cells caused the accumulation of EglN3, which coincided with the onset of apoptosis (Fig. 3D; data not shown). Importantly, KIF1B β was induced with similar kinetics. Similarly, NGF withdrawal induced both EglN3 and KIF1B β in primary rat sympathetic neurons (Supplemental Fig. 4). Moreover, the induction of KIF1B β by NGF withdrawal was prevented by DMOG in primary rat sympathetic neurons and did not occur in *EglN3*^{-/-} primary mouse sympathetic neurons (Fig. 3E,F). Likewise, KIF1B β was not induced by Ara-C in *EglN3*^{-/-} CGN (Fig. 3G). Collectively, these results indicate that EglN3 hydroxylase activity is necessary and sufficient for KIF1B β induction. Regulation of KIF1B β by EglN3 appears to be post-transcriptional (Supplemental Fig. 5). Whether KIF1B β is hydroxylated by EglN3 remains to be determined.

Introduction of KIF1B β into PC12 cells was, like EglN3 itself, sufficient to induce apoptosis, although apoptosis occurred more rapidly with KIF1B β (1–2 d vs. 3 d) (Fig. 4A; data not shown), which is consistent with KIF1B β acting downstream from EglN3. The percentage of apoptotic cells at any time point did not exceed 20%, however, because KIF1B β , like NGF withdrawal itself, killed asynchronously (Lee et al. 2005). KIF1B β also induced apoptosis in primary rat sympathetic neurons (Fig. 5B). Conversely, multiple KIF1B β shRNAs prevented apoptosis of primary rat sympathetic neurons following NGF withdrawal (Fig. 4B). Therefore, KIF1B β is both necessary and sufficient for apoptosis in this setting.

Neuroblastomas, like pheochromocytomas, are neural crest-derived tumors and frequently harbor deletions of chromosome 1p encompassing the *KIF1B* locus (Benn et al. 2000; White et al. 2005). *KIF1B* mRNA levels are decreased in neuroblastoma tumors with 1p36 deletions and are also decreased in advanced-stage disease (Caren et al. 2005; Wang et al. 2006; A. Nakagawara, pers. comm.). In contrast, KIF1B β protein levels appear to be highly variable across neuroblastoma lines and do not strictly correlate with the presence or absence of 1p de-

Figure 3. KIF1B β acts downstream from EglN3. (A–D) Immunoblot analysis of PC12 cells infected with Ad-EglN3 or control Ad-virus (A), HeLa cells transfected with the indicated siRNAs (B), zebrafish embryos injected with the indicated morpholino oligonucleotides (C), and differentiated PC12 cells subjected to NGF withdrawal (D). (E) Percentage of DAPI-stained nuclei exhibiting apoptotic changes in primary rat sympathetic neurons subjected to NGF withdrawal in the presence or absence of DMOG and immunoblotted for KIF1B β expression. (F) Immunofluorescent detection of KIF1B β (green) and propidium iodide-stained nuclei (red) of *EglN3*^{+/-} and *EglN3*^{-/-} primary mouse sympathetic neurons before (+NGF) and after (–NGF) NGF withdrawal. (G) Immunoblot analysis of KIF1B β induction in *EglN3*^{+/-} and *EglN3*^{-/-} primary mouse CGNs after treatment with Ara-C.

letions, possibly due to adaptation in culture as well as alternative mechanisms of KIF1B β down-regulation (data not shown). The neuroblastoma cell line NB-1 has an ~500-kb homozygous deletion at 1p36 that spans

KIF1B and five other known genes (Ohira et al. 2000; Yang et al. 2001; Krona et al. 2003). In contrast to SK-N-SH and CHP212 cells, NB-1 cells were resistant to EglN3-induced apoptosis but were, like other neuroblastoma lines, killed by wild-type KIF1B β (Fig. 4C–E). Notably, restoring the function of the other five 1p genes deleted in NB-1 cells has no effect (A. Nakagawara, pers. comm.). Similarly, LAN6 neuroblastoma cells and H460 lung carcinoma cells, both of which also produce low levels of KIF1B β , were resistant to EglN3 (Fig. 4C,D). Interestingly, H460 cells exhibit neuroendocrine features (Lee et al. 1992). Killing by KIF1B β in neuroblastoma cells appears to be independent of its kinesin motor function, since KIF1B β (600–1770), which lacks the KIF1B β motor domain, still induced apoptosis (Figs. 4E, 5A,B).

Next, we sequenced the 46 coding *KIF1B* exons in 111 neuroblastomas (including 44 with 1p loss of heterozygosity), 52 pheochromocytomas, and 14 medulloblastomas. The latter are derived from CGNs. We identified *KIF1B* missense variants in three neuroblastomas (E646V, T827I, and P1217S), two pheochromocytomas (S1481N and E1628K), and one medulloblastoma (S34L) (Fig. 5A; Table 1; Supplemental Fig. 6). None of these variants is a known polymorphism, nor were the variants detected among 270 controls of diverse ethnic backgrounds (Thorisson et al. 2005; data not shown). In addition, we repeatedly identified common polymorphic alleles that lead to a Y1087C or V1554M substitution (data not shown).

The relevant exons of the corresponding germline DNAs were, when available, also sequenced. Loss or retention of the wild-type *KIF1B* allele within the tumors was inferred by examination of DNA sequence tracing and by 1p36 copy number information from high-density single-nucleotide polymorphism (SNP) arrays or quantitative real-time PCR. In ambiguous cases, allele frequencies were determined by PCR amplification of the altered exon from tumor DNA, followed by subcloning and sequence analysis of multiple individual clones. The medulloblastoma patient was germline wt/wt while the tumor was wt/S34L (Table 1; Supplemental Fig. 6). The neuroblastoma and pheochromocytoma variants were, when evaluable, present in the germline (Table 1; Supplemental Fig. 6). In three tumors there was loss of the wild-type allele and in three tumors there was retention of the wild-type allele, including one in which there was low-level amplification of the mutant allele (Table 1). Interestingly, the S1481N variant was present in a 28-yr-old female who at 17 mo of age presented with a neuroblastoma and in adulthood developed a mature ganglioneuroma and bilateral pheochromocytoma. Her paternal grandfather harbored this allele and also developed bilateral pheochromocytoma (P.L. Dahia and P. Pigny, in prep.).

Next, primary rat sympathetic neurons were electroporated with plasmids encoding wild-type KIF1B β or these variants. The induction of apoptosis by all of the putative disease-causing variants (S34L, E646V, T827I, P1217S, S1481N, and E1628K) was clearly impaired relative to wild-type KIF1B β or the polymorphic variants

Figure 4. Induction of apoptosis by KIF1B β . (A) Percentage of PC12 cells exhibiting apoptotic changes, visualized with a GFP-histone marker, after cotransfection to produce either KIF1B β , wild-type EglN3, or catalytic-dead EglN3 H196A. (B) Primary rat sympathetic neurons were electroporated with the indicated pSuper-shRNA plasmids, subjected to NGF withdrawal, and analyzed for apoptotic DAPI-stained nuclei. (C,D) Crystal violet staining (C) and immunoblot analysis (D) of cell lines infected with Ad-EglN3. (E) Crystal violet staining of neuroblastoma cell lines transfected to produce the indicated KIF1B β variants.

Y1087C and V1554M (Fig. 5B,C; Supplemental Fig. 7). Comparable levels of protein production were confirmed by immunofluorescence and immunoblot analysis (Fig. 5C; Supplemental Fig. 7). These data argue that putative disease-causing variants are pathogenic rather than the result of benign polymorphisms or passenger mutations.

Discussion

The existence of one or more human tumor suppressor gene on chromosome 1p has been suspected for decades (Brodeur et al. 1977; Haag et al. 1981; Stoler and Bouck 1985). Our data suggest that one such tumor suppressor gene is *KIF1B*, and that this gene is relevant to certain tumors of neuronal origin. Nonetheless, we and others observed that the remaining *KIF1B* allele in 1p deleted tumors and cell lines is often wild-type, contrary to the Knudson Two-Hit scenario (Ohira et al. 2000; Yang et al. 2001; A. Nakagawara, pers. comm.; data not shown). Moreover, two of the variants we identified (S34L and S1481N) were not associated with the loss of the remaining wild-type allele. Perhaps *KIF1B* haploinsufficiency

is adequate for tumorigenesis in some contexts, especially when combined with the loss of other contiguous 1p genes such as *CHD5* (Bagchi et al. 2007). In this regard, we noted substantial protection against apoptosis with an shRNA that decreased KIF1B levels by ~50%, and the existence of multiple neuroblastoma and pheochromocytoma suppressor genes on 1p has been suggested before (Takeda et al. 1994; Cheng et al. 1995; Ichimiya et al. 1999; Benn et al. 2000; Opocher et al. 2003; Wang et al. 2006). At the same time, complete loss of KIF1B promotes (rather than inhibits) neuronal apoptosis (Zhao et al. 2001), as does nearly complete elimination of KIF1B using shRNAs (Supplemental Fig. 8).

Tumor development was, however, associated with loss of the remaining wild-type allele for the two germline neuroblastoma mutations E646V and P1217S and the pheochromocytoma mutation E1628K. We note that these mutations are not completely null, based on our apoptotic assay, and therefore loss of the remaining wild-type allele might still confer a survival advantage. The NB-1 line appears to be unusual insofar as it has a homozygous, rather than heterozygous, *KIF1B* deletion.

KIF1B β acts downstream from EglN3

Figure 5. Characterization of tumor-associated KIF1B β mutants. (A) KIF1B β schematic with the locations of mutations. (FHA) Forkhead-associated domain; (PH) Pleckstrin homology domain. (B) Percentage of DAPI-stained, GFP-positive, primary rat sympathetic neurons exhibiting apoptotic changes after electroporation to produce GFP along with the indicated KIF1B β variants. The values are mean $\pm$ SD from at least three individual experiments, and their statistical significance in comparison with the controls in a two-sample *t*-test with a pooled estimate of variance is indicated by asterisks. (*) $P < 0.0007$. (C) Immunofluorescent detection of Flag-KIF1B β variants (red) and GFP (green) in the cells shown in B. (D) Model linking familial pheochromocytoma genes to apoptosis when NGF becomes limiting during neuronal development.

Among several possibilities, these cells might harbor additional mutations that allow them to tolerate total loss of KIF1B β function.

Our findings have potential implications with respect to the pathogenesis of certain neural crest-derived tumors such as pheochromocytomas and neuroblastomas. Many cases of pheochromocytoma without a positive family history are nonetheless due to previously unsuspected germline mutations involving *VHL*, *c-Ret*, *NF1*, *SDHB*, or *SDHD*. We reported earlier that these genes, together with EglN3, define a pathway (Fig. 4I) that is responsible for the elimination of excess neuroblasts during normal embryological development when growth factors such as NGF become limiting. It is noteworthy that neuroblastomas frequently express an NGF receptor (Brodeur 1994), and both *NF1* and *VHL* mutations have been linked to this form of cancer also (Johnson et al. 1993; The et al. 1993; H. Greulich and M. Meyerson, unpubl.). In this report, we placed KIF1B β downstream

from EglN3 and identified loss-of-function germline KIF1B β mutations in some pheochromocytomas and neuroblastomas. Moreover, we obtained functional data consistent with the idea that partial loss of KIF1B β , such as might occur with the loss of one KIF1B allele, would protect neuroblasts from apoptosis in response to stimuli such as NGF withdrawal. We therefore suggest that some neuroblastomas, like pheochromocytomas, result from germline alterations that directly or indirectly compromise KIF1B β function and allow certain neuronal progenitor cells to escape developmental culling. This model is consistent with the prediction, based on epidemiological studies, that at least ~20% of pheochromocytomas and neuroblastomas involve a hereditary component (Knudson and Strong 1972; Knudson and Meadows 1976) and could account for previously described patients (Fairchild et al. 1979; Tatekawa et al. 2006) who, like our patient with the S1481N variant, developed both of these otherwise rare tumors as children or young adults.

Table 1. *KIF1B* variants identified in tumor samples

Tumor	AA change	Exon	Base pair change	1p36 status	N-MYC amp	Germline/ sporadic
Medulloblastoma	S34L	2	AAGGCAACT [C/T] GACCACTAT	Retention	N/A	Sporadic
Neuroblastoma	E646V	20	ACAAAAAGG [A/T] GAAGGAAGA	Loss	Yes	Germline
Neuroblastoma	T827I	24	GCGAAACCA [C/T] TGTGACTGG	Gain of mutant allele (2×)	No	Germline
Neuroblastoma	P1217S	33	GAACTGGAG [C/T] CTACAGGAG	Loss	No	Germline
Pheochromocytoma	S1481N	41	AATCCCTGA [G/A] CGACTCGTT	Retention	N/A	Germline
Pheochromocytoma	E1628K	44	CCAGCTGTG [G/A] AAACACCAT	Loss	N/A	Unavailable
Polymorphism	Y1087C	29	TCCCAGAGT [A/G] TGCAGATAT			
Polymorphism	V1554M	42	TTCAGCCAG [G/A] TGCACGGCA			

(N/A) Not applicable.

KIF1B α and KIF1B β are motor proteins implicated in anterograde transport of mitochondria and synaptic vesicle precursors, respectively (Nangaku et al. 1994; Zhao et al. 2001). The S34L mutation maps to the KIF1B β motor domain, although the motor domain is dispensable for KIF1B β -induced apoptosis (Figs. 4E, 5B). Conceivably, the S34L mutation affects the folding of KIF1B β or eliminates an important phosphorylation site. Clearly, additional studies are now needed to address how, mechanistically, KIF1B β regulates apoptosis and to determine how often it is deregulated, epigenetically or genetically, in cancer.

Materials and methods

Primary sympathetic neurons and cerebellar neurons

EglN3^{-/-} mice were a gift of Regeneron Pharmaceuticals. Sympathetic neurons from P4 rats or mice were isolated from the superior cervical ganglia (SCG) and were cultured in 20 ng/mL NGF (Harlan) as described previously (Palmada et al. 2002). Cerebellar granule neurons were isolated from 4-d-old mouse pups. Cerebella were removed and dissociated by trypsinization and plated on poly-L-ornithine-coated plates. Cells were cultured in neurobasal media (Gibco) supplemented with B27, 0.6% dextrose, 2 mM glutamine, 25 mM KCl, and penicillin/streptomycin.

Immunoblot analysis

Cell extracts were prepared in EBC buffer (50 mM Tris at pH 8.0, 120 mM NaCl, 0.5% NP-40) containing protease inhibitors, unless otherwise noted. Primary neurons were lysed in NP-40 lysis buffer (10% glycerol, 50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% NP-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μ g/mL leupeptin and aprotinin). For PARP assays, cells were lysed in 62.5 mM Tris HCl (pH 6.8), 6 M urea, 10% glycerol, 2% SDS, 5% β -mercaptoethanol, 0.000125% bromophenol blue, and protease inhibitors. Equal amounts of protein, as measured by the Bradford assay, were immunoblotted as described previously (Lee et al. 2005). Rabbit polyclonal anti-EglN3 sera were generously provided by Dr. Robert Freeman (specific to mouse, rat, and human) (Straub et al. 2003), and rabbit polyclonal antibody against HIF α has been described recently (Berra et al. 2003). Rabbit anti-KIF1B antibody specific for the β isoform (cross-reactive with mouse, rat, human, and zebrafish) (sc-28540) or the α form (sc-18739) were purchased from Santa Cruz Biotechnology. Antibody raised against cleaved Caspase 3 was pur-

chased from Cell Signaling (Asp715), and antibody raised against PARP was from Biomol International (P9055a).

Neuronal apoptosis assays

Sympathetic neurons from P4 rats or mice were cultured in 20 ng/mL NGF (Harlan) for 2 d, then rinsed twice in Ultraculture medium lacking NGF and once with Ultraculture medium containing anti-NGF (0.1 μ g/mL; Chemicon International), and then maintained in NGF-free media for 48 h, at which point cells were fixed in 4% paraformaldehyde (PFA) and stained with DAPI (Vector Laboratories). Approximately 70–100 nuclei were scored for apoptotic changes for each condition, as described before (Kenchappa et al. 2006). In some experiments, cells were pretreated with 1 mM DMOG for 6 h prior to and during NGF withdrawal.

Sympathetic neurons from P4 rat were isolated and transfected with a plasmid encoding GFP alone or cotransfected with pSuper plasmids using the Amaxa Nucleofactor device as described previously (Kenchappa et al. 2006). Neurons were maintained in 20 ng/mL NGF for 4 d and then subjected to NGF withdrawal as above. After fixation, GFP-positive neurons were evaluated for apoptosis as above.

Analysis of undifferentiated PC12 cells treated with NGF, followed by NGF withdrawal, was as described (Lee et al. 2005). For transfection experiments, undifferentiated PC12 cells were plated onto collagen-coated six-well plates 1 d before transfection with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Transfection mixes contained 500 ng of a plasmid encoding GFP-histone (a gift of Geoffrey Wahl) and 1–2 μ g of the plasmid of interest. Seventy-two hours later, ~400 GFP-positive cells were scored for the presence of apoptotic nuclei for each set of conditions.

Expression plasmids and siRNA

Adenovirus encoding EglN3 (Ad-EglN3) was a gift from Robert Freeman. The HA-EglN3 and HA-EglN3-H196A expression plasmids were described before (Lee et al. 2005). The NKI pRS hairpin library was described previously (Berns et al. 2004). A KIF1B β cDNA was PCR-amplified from a SK-Mel28 cDNA pool and ligated into pcDNA3 via KpnI and XbaI sites to make pcDNA3-Flag-KIF1B β . Site-directed mutagenesis to generate pcDNA3-Flag-KIF1B β -E646V, S34L, T827I, P1217S, S1481N, E1628K, Y1087C, and V1554M was performed using a QuikChange Site-Directed Mutagenesis kit (Stratagene) using the primers 5'-GGAGATCTTATACAAAAAGGTGAAGGAA GAAGCAGATCTT-3', 5'-TCATTGAGATGCAAGGCAACC TGACCAGTATTATTAACCC-3', 5'-CAAGACGAAAGCGA AACCATTGTGACTGGCAGCGATCCCT-3', 5'-TGAGATC

AGTGAAGTGGAGTCTACAGGAGAGTATATCCCA-3', 5'-AGCATCCCCAAATCCCTGAACGACTCGTTATCCCCCAGCC-3', 5'-TCAGATTGTCCAGCTGTGAAAACACCATATTTGGCCCA-3', 5'-AGTGGAACTCTCCAGAGTGTGCAGATATCTTCTGTCACT-3', and 5'-CAACAGAGAATTCAGCCAGATGCACGGCAGCGTCAGTGAC-3', respectively.

Retroviruses encoding human KIF1B β shRNAs were made using the pRetroSuper plasmid (Brummelkamp et al. 2002b) and the following sequences: (sh) #1, 5'-GGAGCCTCTTTACAGTAAC-3'; (sh) #3, 5'-GCAATGCCGTGTACCTAAA-3'; (sh) #7, 5'-CGAGAGCAGTGGCTATGAT-3'. pRS-shp53 has been described recently (Brummelkamp et al. 2002b).

Plasmids encoding shRNAs for rat EglN3 and rat KIF1B β were made using pSuper plasmid (Brummelkamp et al. 2002a) and the following sequences: EglN3, 5'-CAGGTTATGTTTCGTCATGT-3'; KIF1B β #1, 5'-AGAGCCACTCTCCAGTAAC-3'; KIF1B β #2, 5'-CAAGCTGGTTCGGGAGCTG-3'. siRNAs against human targets were EglN1, 5'-AGCUCCUUCUACUGCUGCA-3'; EglN3 #2, 5'-CAGGUUAUGUUGCCACGU-3'; and EglN3 #3, 5'-UUCUUCUGGUCAGAUUGUA-3'.

Cell culture and retroviral transduction

Undifferentiated PC12 cells were maintained in DMEM containing 5% fetal bovine serum (FBS) (Hyclone) and 10% horse serum (Sigma) in 10% CO₂ at 37°C. Human tumor cell lines were maintained in RPMI (neuroblastoma lines) or DMEM (other lines) containing 10% FBS (Hyclone) in the presence of 10% CO₂ at 37°C. The production of retroviruses and adenoviruses using Phoenix and 293A packaging cells, respectively, and subsequent infections was carried out as described (Peeper et al. 2002; Lee et al. 2005).

Morpholino injection in zebrafish

Zebrafish were maintained and bred as described (Westerfield 1993), and were staged according to Kimmel et al. (1995). Microinjections were performed on one-cell-stage embryos according to standard procedures (Westerfield 1993). Based on the published GenBank sequence for *zegln3* (gi:47086946), a translation-blocking morpholino was designed by GeneTools, Inc.: *zegln3*, GTGCTGAAGAAACGGCATTTTGTC. Zebrafish protein lysates were prepared from 100 3-d-old embryos by homogenizing in lysis buffer (1% NP-40, 0.1% SDS, 100 mM NaCl, 50 mM Tris at pH 7.5, 10 mM EDTA, 0.1% PMSF, supplemented with Roche complete protein inhibitor) using a micropestle, then centrifuged at 15,000 rpm in a microcentrifuge for 10 min at 4°C. The supernatant was transferred to a new tube and stored at -80°C.

Immunofluorescence staining

Primary sympathetic neurons were subjected to NGF withdrawal for 24 h as described above. The cells were then fixed in 4% PFA, permeabilized with 0.1% sodium citrate and 0.1% Triton X-100, blocked with 10% goat serum in PBS, and incubated with the KIF1B antibody (1:100 dilution) or EglN3 antibody (1:100) in PBS containing 0.1% Triton X-100. After incubation with anti-rabbit Alexa 488 (Molecular Probes) and staining with DAPI, images were acquired using a confocal laser imaging system (LSM 510; Carl Zeiss MicroImaging, Inc.) at 400 $\times$.

Tumor sample sequencing

Ninety-eight primary neuroblastoma tumor samples were identified from the Children's Oncology Group (COG) Neuroblas-

toma Nucleic Acids Bank. Samples were collected after obtaining parental informed consent, and institutional review board (IRB) guidelines were followed for the procurement of each sample. They were obtained at original diagnosis from patients who had received no previous treatment and immediately snap-frozen, and had a tumor cell content of >90% based on differential count, clonal hyperdiploid percentage in some tumors, and direct examination of H&E-stained tumor slides. All 98 patients met the COG criteria for having high-risk disease (Maris 2005). Patients were staged according to the International Neuroblastoma Staging System and histology was analyzed using the Shimada Pathology Classification (Shimada et al. 1984; Brodeur et al. 1993). Loss-of-heterozygosity (LOH) status was determined using conventional microsatellite markers and high-resolution SNP array analysis as described previously (George et al. 2007). DNA was extracted using conventional methods (Qiagen kit) and was sequenced by Agencourt, Inc., by automated sequencing. All 46 coding KIF1B β exons were PCR-amplified and sequenced in a duplicate, bidirectional manner. Sequence traces were analyzed to identify potential somatic mutations using the Mutation Surveyor software package (Soft-Genetics).

An additional 13 neuroblastoma tumors from the COG, as described above, and 14 medulloblastoma samples obtained at Children's Hospital in Boston under IRB approval were sequenced for KIF1B β at the Broad Institute. Briefly, DNA was extracted from the tumor and matched normal blood sample (Qiagen DNeasy kit), quantified using picogreen (Molecular Probes), and isothermally amplified using the Repli-g whole-genome amplification kit (Amersham). Five nanograms of DNA for each exon of KIF1B β were individually PCR-amplified (primer sequences available upon request) with the HotStar Enzyme (Qiagen) and the following cycling parameters: one cycle of 15 min at 95°C; followed by 35 cycles of 20 sec at 95°C, 30 sec at 60°C, and 1 min at 72°C; followed by a final extension of 3 min at 72°C. PCR products were sequenced in a duplicate, bidirectional manner. Sequence traces were analyzed to identify potential somatic mutations using an automated analysis pipeline comprised of the commercial software package Mutation Surveyor (SoftGenetics), PolyPhred 3.5 (Nickerson et al. 1997), and PolyDHAN (D. Richter, pers. comm.). The KIF1B β S34SL variant was confirmed by Sequenom mass spectrometric genotyping.

Fifty-two pheochromocytoma or paraganglioma samples were used to sequence the KIF1B β gene under an IRB-approved protocol. Fragments were obtained from the core of the tumor and contained >70% tumor cells. Samples with a clear adjacent cortical component were macrodissected. Specimens were snap-frozen at the time of surgical resection and stored at -70°C or in liquid nitrogen until processed. Diagnosis of pheochromocytoma and/or paraganglioma was confirmed by histology in every case. Eleven of these tumors came from individuals with hereditary disease (two MEN2A, one MEN2B, one VHL, two familial paraganglioma syndromes type 4-PGL4/SDHB, and five familial cases without an identifiable primary mutation in pheochromocytoma susceptibility genes). The remaining 41 tumors were sporadic or had an unknown familial history. Four tumors were recurrent or malignant (the latter were defined by the detection of metastasis at nonchromaffin sites), while the others were considered benign or had short follow-up.

Two approaches were used for sequencing these samples. Genomic DNA was isolated from 36 tumors using standard methods (Qiagen). Ten nanograms were used to amplify 50 amplicons spanning the 46 coding exons and exon-intron boundaries of the KIF1B β gene (primer sequences available upon request). For 16 tumors, only cDNA (prepared using Applied Biosystems

Reverse Transcription kit) was available. Twenty primer pairs spanning the entire coding region of the longest *KIF1B* transcript were used for PCR and sequencing of these samples.

PCR was performed using HotMaster Enzyme (Eppendorf). PCR conditions were as follows: one cycle of 5 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at 59°C, and 45 sec at 72°C; followed by a final extension of 5 min at 72°C. PCR products were purified and sequenced in both directions by Agencourt Bioscience using dye terminator technology. Sequence traces were analyzed using the commercial software Mutation Surveyor (SoftGenetics) and were manually verified. Variants were confirmed by the sequence of an independent sample.

The copy number of *KIF1B* was determined by real-time PCR. Pooled results from three reference housekeeping genes with distinct genomic locations ($\beta 2$ microglobulin, *Albumin*, and *TRIM43*) were used to calculate the copy number using the $\Delta\Delta C_t$ method as described previously. Primer sequences are available upon request.

Frequency of *KIF1B* S34L, E646V, T827I, P1217S, S1481N, E1628K, Y1087C, and V1554M in 270 controls of diverse ethnic backgrounds was determined by Sequenom mass spectrometric genotyping of the HapMap collection of normal DNA (Thorisson et al. 2005).

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The kinesin KIF1B β acts downstream from Egln3 to induce apoptosis and is a potential 1p36 tumor suppressor

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