The kinesin KIF1Bβ 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 KIF1Bβ acts downstream from EglN3 and is both necessary and sufficient for neuronal apoptosis when NGF becomes limiting. KIF1Bβ maps to chromosome 1p36.2, which is frequently deleted in neural crest-derived tumors including neuroblastomas. We identified inherited loss-of-function KIF1Bβ missense mutations in neuroblastomas and pheochromocytomas and an acquired loss-of-function mutation in a medulloblastoma, arguing that KIF1Bβ is a pathogenic target of these deletions.

[Keywords: Apoptosis; kinesin; neuroblastoma; pheochromocytoma; prolyl hydroxylase]

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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). De-regulation 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...
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 naïve 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 KIF1Bβ, but not the alternative splice variant KIF1Bα, was confirmed by Western blot analysis [Fig. 2C]. KIF1Bα and KIF1Bβ 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, KIF1Bβ shRNAs, arguing that modulation of KIF1Bβ, 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.
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 belong to the same pathway, PC12 cells were infected with Ad-Egln3. Induction of apoptosis, as determined by Caspase 3 cleavage, was accompanied by the induction of Kif1b, but not its splice variant Kif1bo (Fig. 3A). Conversely, knockdown of human Egln3 in HeLa cervical carcinoma cells with two independent siRNAs decreased Kif1b levels [Fig. 3B, Supplemental Fig. 3]. Notably, an siRNA against Egln1, which regulates the HIFs transcription factor (Berra et al. 2003), did not affect Kif1b, consistent with the earlier conclusion that regulation of apoptosis by Egln3 is HIF-independent (Lee et al. 2005).

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 was induced with similar kinetics. Similarly, NGF withdrawal induced both Egln3 and Kif1b in primary rat sympathetic neurons [Supplemental Fig. 4]. Moreover, the induction of Kif1b by NGF withdrawal was prevented by DMOG in primary mouse sympathetic neurons (Fig. 3E,F). Likewise, Kifb 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 induction. Regulation of Kif1b by Egln3 appears to be post-transcriptional [Supplemental Fig. 5]. Whether Kif1b is hydroxylated by Egln3 remains to be determined.

Introduction of Kif1b into PC12 cells was, like Egln3 itself, sufficient to induce apoptosis, although apoptosis occurred more rapidly with Kif1b (1–2 d vs. 3 d) [Fig. 4A; data not shown], which is consistent with Kif1b acting downstream from Egln3. The percentage of apoptotic cells at any time point did not exceed 20%, however, because Kif1b, like NGF withdrawal itself, killed asynchronously [Lee et al. 2005]. Kif1b also induced apoptosis in primary rat sympathetic neurons [Fig. 5B]. Conversely, multiple Kif1b shRNAs prevented apoptosis of primary rat sympathetic neurons following NGF withdrawal [Fig. 4B]. Therefore, Kif1b 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 protein levels appear to be highly variable across neuroblastoma lines and do not strictly correlate with the presence or absence of 1p de-
KIF1Bβ and five other known genes (Ohira et al. 2000; Yang et al. 2001; Krona et al. 2003). In contrast to SK-N-SH and CHF212 cells, NB-1 cells were resistant to EglN3-induced apoptosis but were, like other neuroblastoma lines, killed by wild-type KIF1Bβ (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 KIF1Bβ, were resistant to EglN3 [Fig. 4C,D]. Interestingly, H460 cells exhibit neuroendocrine features (Lee et al. 1992). Killing by KIF1Bβ in neuroblastoma cells appears to be independent of its kinesin motor function, since KIF1Bβ(600–1770), which lacks the KIF1Bβ motor domain, still induced apoptosis (Figs. 4E, 5A,B).

Next, we sequenced the 46 coding KIF1Bβ exons in 111 neuroblastomas (including 44 with 1p loss of heterozygosity), 52 pheochromocytomas, and 14 medulloblastomas. The latter are derived from CGNs. We identified KIF1Bβ 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 KIF1Bβ or these variants. The induction of apoptosis by all of the putative disease-causing variants (S34L, E646V, E1628K, P1217S, S1481N, and E1628K) was clearly impaired relative to wild-type KIF1Bβ or the polymorphic variants.

Figure 3. KIF1Bβ 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 KIF1Bβ expression. (F) Immunofluorescent detection of KIF1Bβ[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 KIF1Bβ 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 KIF1Bβ down-regulation (data not shown). The neuroblastoma cell line NB-1 has an ∼500-kb homozygous deletion at 1p36 that spans
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 KIF1Bβ, and that this gene is relevant to certain tumors of neuronal origin. Nonetheless, we and others observed that the remaining KIF1Bβ 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 KIF1Bβ 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 KIF1Bβ 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 KIF1Bβ promotes (rather than inhibits) neuronal apoptosis [Zhao et al. 2001], as does nearly complete elimination of KIF1Bβ using shRNAs [Supplemental Fig. 8].

Tumor development was, however, associated with loss of the remaining wild-type allele for the two germ-line 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, KIF1Bβ deletion.
Among several possibilities, these cells might harbor additional mutations that allow them to tolerate total loss of KIF1Bβ 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 \textit{VHL}, \textit{c-Ret}, \textit{NF1}, \textit{SDHB}, or \textit{SDHD}. We reported earlier that these genes, together with \textit{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.

For more information, you can refer to the following references:
- Knudson and Strong (1972)
- Knudson and Meadows (1976)
- Fairchild et al. (1979)
- Tatekawa et al. (2006)

In this report, we placed KIF1Bβ downstream from \textit{EglN3} and identified loss-of-function germline KIF1Bβ mutations in some pheochromocytomas and neuroblastomas. Moreover, we obtained functional data consistent with the idea that partial loss of KIF1Bβ, 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 KIF1Bβ 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 \sim20\% of pheochromocytomas and neuroblastomas involve a hereditary component.

For a more detailed understanding, you can refer to the following figure and data from our study.
KIF1Bα and KIF1Bβ 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 KIF1Bβ motor domain, although the motor domain is dispensable for KIF1Bβ-induced apoptosis [Figs. 4E, 5B]. Conceivably, the S34L mutation affects the folding of KIF1Bβ or eliminates an important phosphorylation site. Clearly, additional studies are now needed to address how, mechanistically, KIF1Bβ 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% dextran, and KIF1Bα and KIF1Bβ have been described recently (Berra et al. 2003). QuikChange Site-Directed Mutagenesis kit (Stratagene) using the primers 5'-GGAGATCTTATACAAAAAGGTGAAGGAA and 5'-TCATTCAGATGCAAGGCAACC. cDNA was PCR-amplified from a SK-Mel28 cDNA pool and ligated into pcDNA3 via KpnI and XbaI sites to make 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 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.

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 µg/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 above (Kenchappa et al. 2006). In some experiments, cells were 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 apoptotic changes 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 µg 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 KIF1Bβ cDNA was PCR-amplified from a SK-Mel28 cDNA pool and ligated into pcDNA3 via KpnI and XbaI sites to make 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 apoptotic changes as above.

Expression plasmids and siRNA

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 µg/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 antibody against HIFα was 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 purchased from Cell Signaling [Asp715], and antibody raised against PARP was from Biomol International [P9055a].

Immunoblot analysis

Table 1. KIF1Bβ 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] | GACCAGTAT   | Retention   | N/A Sporadic      |
| Neuroblastoma  | E646V     | 20   | ACAAAGAGG [A/T] | GAGGGAAGA   | Loss        | Yes Germine       |
| Neuroblastoma  | T927I     | 24   | GGCAAACAAC [C/T] | TGTGACTCGG  | Gain of mutant allele (2x) | No Germline |
| Neuroblastoma  | P1217S    | 33   | GAACTGGGAC [C/T] | CTACAGGAG   | Loss        | No Germine        |
| Pheochromocytoma | S1481N | 41   | ATTCCTTGA [G/A] | CGACTCTGT  | Retention   | N/A Germine       |
| Pheochromocytoma | E1628K | 44   | CACGCTGTTG [G/A] | AAACACCAT  | Loss        | N/A Unavailable   |
| Polymorphism   | Y1087C    | 29   | TCCAGAGAT [A/G] | TGCAGATAT   |             |                   |
| Polymorphism   | V1554M    | 42   | TTCAGCCGAG [G/A] | TGCACGGCA   |             |                   |

([N/A] Not applicable.)

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 µg/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 apoptotic changes 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 µg 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.
AGTGAACTGGAGTCTACAGGAGAGTATATCCCA-3’; 5’-AGCATCCCAAACTCCGACAGCTTATATCCCCCAC-3’; 5’-TCAGAGTTCTCCACAGCTGAAACAACCATTTGGAGCCGA-3’; 5’-AGTGAAACCTCTCAAGTGTGAGATATCTCTCTGTGACT-3’; and 5’-CAACAGAAATTACGGCACTGCGGACCTCACTGAC-3’, respectively.

Retroviruses encoding human KIF1Bβ shRNAs were made using the pRetroSuper plasmid (Brummelkamp et al. 2002b) and the following sequences: [sh] #1, 5’-GGAGGCCTTTATCTTTAGAATG TAC-3’; [sh] #3, 5’-GCAATGCGCTTGAATCCTAAA-3’; [sh] #7, 5’-CGAGACGGCTGTGCTATGAT-3’. pRS-shp53 has been described recently (Brummelkamp et al. 2002b). Plasmids encoding shRNAs for rat EglN3 and rat KIF1Bβ were made using pSuper plasmid (Brummelkamp et al. 2002a) and the following sequences: EglN3, 5’-CAGGTATGTTCTGCTATG-3’; KIF1Bβ #1, 5’-AGAGCCACTCTCAAGTAA-3’, KIF1Bβ #2, 5’-CAAGCTGTTGGCAAGCTG-3’. siRNAs against human targets were EglN1, 5’-AGCUCCUCUCUCAGCUGCAG-3’; EglN3 #2, 5’-CAGGUUAUGUUCGCAGCAG-3’, and EglN3 #3, 5’-UUCCUGGUGCUAGCUAGUA-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 (Peeler 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 zebgn3 (g:47086946), a translation-blocking morpholino was designed by GeneTools. Inc.: zebgn3, GTGCAGAAGGACTCAGATTTGCC. 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 Microlmaging, Inc.) at 400x.

Tumor sample sequencing

Ninety-eight primary neuroblastoma tumor samples were identified from the Children’s Oncology Group [COG] Neuroblastoma 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 KIF1Bβ 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 [SoftGenetics].

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 KIF1Bβ 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 KIF1Bβ 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 PolyDAN [D. Richter, pers. comm.]. The KIF1Bβ S43SL variant was confirmed by Sequenom mass spectrometric genotyping.

Fifty-two pheochromocytoma or paraganglioma samples were used to sequence the KIF1Bβ 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 macródissected. 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 500 ampiclons spanning the 46 coding exons and exon–intron boundaries of the KIF1Bβ 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 KIF1Bβ 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 Agenourt 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 KIF1Bβ was determined by real-time PCR. Pooled results from three reference housekeeping genes with distinct genomic locations (β2 microglobulin, Albumin, and TRIM43) were used to calculate the copy number using the ΔΔCt method as described previously. Primer sequences are available upon request.

Frequency of KIF1Bβ 534L, 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 (Thorsson et al. 2005).

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Johnson, M.R., Look, A.T., DeClue, J.E., Valentine, M.B., and Lowy, D.R. 1993. Inactivation of the NFI gene in human melanoma and neuroblastoma cell lines without impaired regulation of GTP.Ras. Proc. Natl. Acad. Sci. 90: 5539–5543.

References

Aloyz, R.S., Bamiji, S.X., Pozniak, C.D., Toma, J.G., Atwal, J., Kaplan, D.R., and Miller, F.D. 1998. p53 is essential for developmental neuron death as regulated by the TrkA and p75 neurotrophin receptors. J. Cell Biol. 143: 1691–1703.

Bagchi, A., Papazoglou, C., Wu, Y., Capurso, D., Brodt, M., Francis, D., Bredel, M., Vogel, H., and Mills, A.A. 2007. CHD5 is a tumor suppressor at human 1p36. Cell 128: 459–475.

Benn, D.E., Dwight, T., Richardson, A.L., Delbridge, L., Bambach, C.P., Stowasser, M., Gordon, R.D., Marsh, D.J., and Robinson, B.G. 2000. Sporadic and familial phaeochromocytomas are associated with loss of at least two discrete intervals on chromosome 1p. Cancer Res. 60: 7048–7051.

Berns, K., Himans, E.M., Mullenders, J., Brummelkamp, T.R., Velds, A., Heimerikx, M., Kerkhoven, R.M., Madiredjo, M., Nijkamp, W., Weigelt, B., et al. 2004. A large-scale SNP screen in human cells identifies new components of the p53 pathway. Nature 428: 431–437.

Berra, E., Benizri, E., Ginouves, A., Volmat, V., Roux, D., and Pouyssegur, J. 2003. HIF prolyl-hydroxylase 2 is the key oxygen sensor setting low steady-state levels of HIF-1α in normoxia. EMBO J 22: 4082–4090.

Brodeur, G.M. 1994. Molecular pathology of human neuroblastosomas. Semin. Diagn. Pathol. 11: 118–125.

Brodeur, G.M., Sekhon, G., and Goldstein, M.N. 1977. Chromosomal aberrations in human neuroblastomas. Cancer 40: 2256–2263.

Brodeur, G.M., Pritchard, J., Berthold, F., Carlsten, N.L., Castel, V., Castelberry, R.P., De Bernardi, B., Evans, A.E., Favrot, M., Hedborg, F., et al. 1993. Revisions of the international criteria for neuroblastoma diagnosis, staging, and response to treatment. J. Clin. Oncol. 11: 1466–1477.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002a. A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 505–503.

Brummelkamp, T.R., Bernards, R., and Agami, R. 2002b. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2: 243–247.

Caren, H., Ejekar, K., Fransson, S., Hesson, L., Latif, P., Sjoberg, R.M., Krona, C., and Martinsson, T. 2005. A cluster of genes located in 1p36 are down-regulated in neuroblastomas with poor prognosis, but not due to CpG island methylation. Mol. Cancer 4: 10. doi: 10.1186/1476-4598-4-10.

Cheng, N.C., Van Roy, N., Chan, A., Beitsma, M., Westerveld, A., Speelman, F., and Versteege, R. 1995. Deletion mapping in neuroblastoma cell lines suggest two distinct tumor suppressor genes in the 1p36 region, only one of which is associated with N-myec amplification. Oncogene 10: 291–297.

Fairchild, R., Kyner, J., Hermreck, A., and Schimke, R. 1979. Neuroblastoma, phaeochromocytoma, and renal cell carcinoma. Occurrence in a single patient. JAMA 242: 2210–2211.

George, R.E., Attiyeh, E.F., Li, S., Moreau, L.A., Neuberg, D., Li, C., Fox, E.A., Meyerson, M., Diller, L., Fortina, P., et al. 2007. Genome-wide analysis of neuroblastomas using high-density single nucleotide polymorphism arrays. PLoS ONE 2: e255. doi: 10.1371/journal.pone.0000255.

Haag, M.M., Soukup, S.W., and Neely, J.E. 1981. Chromosome analysis of a human neuroblastoma. Cancer Res. 41: 2959–2999.

Ichimiya, S., Nimura, Y., Kageyama, H., Takada, N., Sunahara, M., Shishikura, T., Nakamura, Y., Sakiyama, S., Seki, N., Ohira, M., et al. 1999. p75 at chromosome 1p36.3 is lost in advanced stage neuroblastoma but its mutation is infrequent. Oncogene 18: 1061–1066.

Johnson, M.R., Look, A.T., DeClue, J.E., Valentine, M.B., and Lowy, D.R. 1993. Inactivation of the NFI gene in human melanoma and neuroblastoma cell lines without impaired regulation of GTP.Ras. Proc. Natl. Acad. Sci. 90: 5539–5543.

Kenchappa, R.S., Zampieri, N., Chao, M.V., Barker, P.A., Teng, H.K., Hemppstead, B.L., and Carter, B.D. 2006. Ligand-dependent cleavage of the P75 neurotrophin receptor is necessary for NRIIF nuclear translocation and apoptosis in sympathetic neurons. Neuron 50: 219–232.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., and Schilling, T.F. 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203: 253–310.

Kudson Jr., A.G. and Meadows, A.T. 1976. Developmental genetics of neuroblastoma. J. Natl. Cancer Inst. 57: 675–682.

Kudson Jr., A.G. and Strong, L.C. 1972. Mutation and cancer: Neuroblastoma and phaeochromocytoma. Am. J. Hum. Genet. 24: 514–532.

Krona, C., Ejekar, K., Abel, F., Kogner, P., Bjelke, J., Bjork, E., Sjoberg, R.M., and Martinsson, T. 2003. Screening for gene mutations in a candidate region in chromosome 1p; mutation and stage-specific expression in UBE4B/UFD2. Oncogene 22: 2343–2351.

Lee, M., Draoui, M., Zia, F., Gazard, A., Oie, H., Bepler, G., Bellot, F., Tarr, C., Kris, R., and Moody, T. 1992. Epidermal growth factor receptor monoclonal antibodies inhibit the
growth of lung cancer cell line. *J. Natl. Cancer Inst. Monogr.* 13: 117–123.

Lee, S., Nakamura, E., Yang, H., Wei, W., Linggi, M.S., Sajan, M.P., Farese, R.V., Freeman, R.S., Carter, B.D., Kaelin Jr., W.G., et al. 2005. Neuronal apoptosis linked to EglN3 prolyl hydroxylase and familial pheochromocytoma genes: Developmental culling and cancer. *Cancer Cell* 8: 155–167.

Lipscomb, E., Sarmiere, P., Crowder, R., and Freeman, R. 1999. Expression of the SM-20 gene promotes death in nerve growth factor-dependent sympathetic neurons. *J. Neurochem.* 73: 429–432.

Maris, J.M. 2005. The biologic basis for neuroblastoma heterogeneity and risk stratification. *Curr. Opin. Pediatr.* 17: 7–13.

Nagai, M., Ichimiya, S., Ozaki, T., Seki, N., Mihara, M., Furuta, S., Ohira, M., Tomioka, N., Nomura, N., Sakiyama, S., et al. 2000. Identification of the full-length KIAA0591 gene encoding a novel kinesin-related protein which is mapped to the neuroblastoma suppressor gene locus at 1p36.2. *Int. J. Oncol.* 16: 907–916.

Nakamura, E. and Kaelin Jr., W.G. 2006. Recent insights into neuroblastoma suppressor gene locus at 1p36.2. *Clin. Endocrinol.* (Oxf.) 73: 117–123.

Peeper, D.S., Pramil, C., and Amler, L.C. 1996. Genomic instability in 1p and human malignancies. *Genes Chromosomes Cancer* 16: 211–229.

Schwab, M., Pramil, C., and Amler, L.C. 1996. Genomic instability in 1p and human malignancies. *Genes Chromosomes Cancer* 16: 211–229.

Shimada, H., Chatten, J., Newton Jr., W.A., Sachs, N., Hamoudi, A.B., Chiba, T., Marsden, H.B., and Misugi, K. 1984. Histopathologic prognostic factors in neuroblastic tumors: Definition of subtypes of ganglioneuroblastoma and an age-linked classification of neuroblastomas. *J. Natl. Cancer Inst.* 73: 405–416.

Sommer, L. and Rao, M. 2002. Neural stem cells and regulation of cell number. *Prog. Neurobiol.* 66: 1–18.

Stoler, A. and Bouck, N. 1985. Identification of a single chromosome in the normal human genome essential for suppression of hamster cell transformation. *Proc. Natl. Acad. Sci. U.S.A.* 82: 570–574.

Straub, J.A., Lipscomb, E.A., Yoshida, E.S., and Freeman, R.S. 2003. Induction of SM-20 in PC12 cells leads to increased cytchrome c levels, accumulation of cytchrome c in the cytosol, and caspase-dependent cell death. *J. Neurochem.* 85: 318–328.

Takeda, O., Homma, C., Maseki, N., Sakurai, M., Kanda, N., Schwab, M., Nakamura, Y., and Kaneko, Y. 1994. There may be two tumor suppressor genes on chromosome arm 1p closely associated with biologically distinct subtypes of neuroblastoma. *Genes Chrom. and Cancer* 10: 30–39.

Tatekawa, Y., Muraji, T., Nishijima, E., Yoshida, M., and Tsugawa, C. 2006. Composite pheochromocytoma associated with adrenal neuroblastoma in an infant: A case report. *J. Pediatr. Surg.* 41: 443–445.

The, I., Murthy, A.E., Hannigan, G.E., Jacoby, L.B., Menon, A.G., Gusella, J.F., and Bernards, A. 1993. Neurofibromatosis type 1 gene mutations in neuroblastoma. *Nat. Genet.* 3: 62–66.

Thorisson, G.A., Smith, A.V., Krishnan, L., and Stein, L.D. 2002. The International HapMap Project Web site. *Genome Res.* 15: 1592–1593.

Wang, Q., Diskin, S., Rappaport, E., Attiyeh, E., Mosse, Y., Shue, D., Seiser, E., Jagannathan, J., Shusterman, S., Bansal, M., et al. 2006. Integrative genomics identifies distinct molecular classes of neuroblastoma and shows that multiple genes are targeted by regional alterations in DNA copy number. *Cancer Res.* 66: 6050–6062.

Westerfield, M. 1993. *The zebrafish book.* University of Oregon Press, Eugene, OR.

White, P.S., Thompson, P.M., Gotoh, T., Okawa, E.R., Igarashi, J., Kok, M., Winter, C., Gregory, S.G., Hogarty, M.D., Maris, J.M., et al. 2005. Definition and characterization of a region of 1p36.3 consistently deleted in neuroblastoma. *Oncogene* 24: 2684–2694.

Yang, H.W., Chen, Y.Z., Takita, J., Soeda, E., Piao, H.Y., and Hayashi, Y. 2001. Genomic structure and mutational analysis of the human KIF1B gene which is homozygously deleted in neuroblastoma at chromosome 1p36.2. *Oncogene* 20: 5075–5083.

Zhao, C., Takita, J., Tanaka, Y., Setou, M., Nakagawa, T., Takeda, S., Yang, H.W., Terada, S., Nakata, T., Takej, Y., et al. 2001. Charcot-Marie-Tooth disease type 2A caused by mutation in a microtubule motor KIF1Bβ. *Cell* 105: 587–597.

Zhu, Y. and Parada, L.F. 2002. The molecular and genetic basis of neurological tumours. *Nat. Rev. Cancer* 2: 616–626.
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