RNA Helicase A Is a Downstream Mediator of KIF1Bβ Tumor-Suppressor Function in Neuroblastoma

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ABSTRACT

Inherited KIF1Bβ loss-of-function mutations in neuroblastomas and pheochromocytomas implicate the kinesin KIF1B as a 1p36.2 tumor suppressor. However, the mechanism of tumor suppression is unknown. We found that KIF1B isoform β (KIF1Bβ) interacts with RNA helicase A (DHX9), causing nuclear accumulation of DHX9, followed by subsequent induction of the proapoptotic XIAP-associated factor 1 (XAF1) and, consequently, apoptosis. Pheochromocytoma and neuroblastoma arise from neural crest progenitors that compete for growth factors such as nerve growth factor (NGF) during development. KIF1Bβ is required for developmental apoptosis induced by competition for NGF. We show that DHX9 is induced by and required for apoptosis stimulated by NGF deprivation. Moreover, neuroblastomas with chromosomal deletion of 1p36 exhibit loss of KIF1Bβ expression and impaired DHX9 nuclear localization, implicating the loss of DHX9 nuclear activity in neuroblastoma pathogenesis.

SIGNIFICANCE: KIF1Bβ has neuroblastoma tumor-suppressor properties and promotes and requires nuclear-localized DHX9 for its apoptotic function by activating XAF1 expression. Loss of KIF1Bβ alters subcellular localization of DHX9 and diminishes NGF dependence of sympathetic neurons, leading to reduced culling of neural progenitors, and, therefore, might predispose to tumor formation. Cancer Discov; 4(4); 1–18. ©2014 AACR.

See related commentary by Bernards, p. 392.

INTRODUCTION

During development of the peripheral nervous system, neural progenitor cells depend on and compete for growth factors, such as nerve growth factor (NGF). Mutations affecting NGF-dependent neuronal survival have been associated with sympathetic nervous system tumors such as neuroblastoma and later-developing malignancies of neural crest origin, such as paraganglioma and pheochromocytoma (1–5). Germline mutations associated with paragangliomas and pheochromocytomas (VHL, RET, NF1, and SDHB/C/D) are thought to define a pathway that is activated when NGF is limiting, leading to apoptosis mediated by the EGLN3 prolyl hydroxylase (4). Failure to properly cull the neuronal progenitor cells during development might predispose to neoplastic transformation (2, 6). Recently, we identified the KIF1B gene as a downstream mediator of the proapoptotic effects of the prolyl hydroxylase EGLN3 and demonstrated that KIF1B isoform β (KIF1Bβ) is necessary and sufficient for apoptosis when NGF is limiting. KIF1B is a member of the kinesin 3 family and encodes two alternatively spliced isoforms, KIF1Bα and KIF1Bβ (7–9). Both share an N-terminal motor domain but contain different C-terminal cargo domains. KIF1Bα and KIF1Bβ are motor proteins implicated in anterograde transport of mitochondria and synaptic vesicle precursors, respectively (10). However, the recently identified role of KIF1Bβ in NGF-mediated neuronal apoptosis implicates this kinesin as an important player during sympathetic neuron development. Moreover, KIF1B maps to chromosome 1p36.2, a chromosomal region that is frequently deleted in neural crest–derived tumors, including neuroblastomas (5). The identification in neuroblastomas and pheochromocytomas of inherited KIF1B missense mutations that remove KIF1Bβ’s ability to induce neuronal apoptosis (5, 11) suggests that KIF1Bβ is a pathogenic target of 1p36 deletion in these diseases. Therefore, we investigated the tumor-suppressive mechanism by which KIF1Bβ regulates apoptosis.

RESULTS

DHX9 Is a Binding Partner of the KIF1Bβ Apoptotic Domain

To investigate how EGLN3 regulates KIF1Bβ and how this promotes cell death, we mapped the domain of KIF1Bβ that is necessary and sufficient to induce apoptosis (Fig. 1A). We tested a series of N- and C-terminal truncated KIF1Bβ variants for apoptotic function by electroporating them into primary rat sympathetic neurons (Fig. 1B). Neurons were 4′,6-diamidino-2-phenylindole (DAPI) stained, and the nuclei of FLA-G-positive neurons were visualized for apoptotic changes. In addition, we transfected NB1 neuroblastoma cells with the KIF1Bβ variants and stained cells with crystal violet to determine cell viability (Fig. 1C). These results confirm earlier observations that both full-length and motor-deficient
Figure 1. DHX9 is a binding partner of KIF1Bβ apoptotic domain. A, schematic representation of KIF1Bβ deletion mutants and their ability to induce cell death, DHX9 binding, and DHX9 nuclear localization. FL, full-length. B, percentage of apoptosis in FLAG-positive, rat primary sympathetic neurons after electroporation with plasmids encoding FLAG-KIF1Bβ mutants as indicated. DAPI-stained nuclei were evaluated for apoptotic changes such as nuclear condensation and fragmentation. C and D, crystal violet staining to measure cell viability in NB1 and CHP212 cells after transfection with FLAG-KIF1Bβ plasmids as indicated. Transfected cells were selected with G418 (500 μg/mL) for several weeks. Empty vector (empty) served as negative control. E, immunoblot analysis of NB1 cells that were coinfected with lentivirus encoding Flag-KIF1Bβ mutants together with adenovirus encoding EGLN3 or coinfected with lentivirus encoding short hairpin targeting EGLN3 (shE3) or nontargeting control (SCR) as indicated. F, large-scale immunoprecipitation in NB1 cells that were infected with lentivirus encoding Stag-FLAG-KIF1Bβ600–1400. Silver-stained gel indicates (arrow) identified coimmunoprecipitated proteins and table listing peptides identified by mass spectrometry. G, immunoblot showing coimmunoprecipitation of endogenous DHX9 and KIF1Bβ in SK-N-SH cells that were immunoprecipitated with KIF1Bβ antibody. H, anti-DHX9 immunoblot analysis of NB1 cells transfected to produce Flag-KIF1Bβ protein and immunoprecipitated with anti-Flag antibody as indicated (arrow).
KIF1Bβ induce apoptosis when ectopically expressed (5, 12). The apoptosis-inducing region of KIF1Bβ was previously mapped to amino acids 637 to 1576 (12). Moreover, we identified the domain that retains apoptotic activity residing within the 600–1400 amino acid region, whereas the 600–1200 and the 1200–1600 domains failed to induce apoptosis (Fig. 1B and C). Therefore, we tested additional KIF1Bβ deletions and identified the domain-spanning amino acids 1000–1400 (KIF1Bβ1000–1400) as sufficient to induce apoptosis similar to full-length KIF1Bβ in NB1 and CHP-212 neuroblastoma cells (Fig. 1D). In contrast, internal deletion of amino acids spanning 1100–1300 abrogated the ability of KIF1Bβ600–1400a1100–1300 to induce apoptosis. Comparable levels of KIF1Bβ protein production were confirmed by immunoblot analysis (Supplementary Fig. S1A).

Because KIF1Bβ was previously identified downstream of the prolyl hydroxylase EGLN3 during apoptosis, we questioned whether the truncated KIF1Bβ variants are responsive to EGLN3. When ectopically expressed, the protein level of KIF1Bβ600–1400 domain that retains apoptotic activity was induced by EGLN3 (Fig. 1E, left). Conversely, knockdown of EGLN3 expression by lentivirus encoding short hairpin RNA (shRNA) markedly decreased ectopic KIF1Bβ600–1400 abundance (Fig. 1E, right), similar to what we observed with endogenous KIF1Bβ protein (Supplementary Fig. S1B). However, the proapoptotic variant KIF1Bβ1000–1400 was not regulated by EGLN3, in contrast with the nonapoptotic variant KIF1Bβ600–1200 (Fig. 1E). Therefore, regulation by EGLN3 and induction of apoptosis are mediated through distinct domains within the KIF1Bβ600–1400 region.

Next, we searched for proteins that interact specifically with the proapoptotic and EGLN3-responsive KIF1Bβ600–1400 domain. NB1 cells were transiently transduced with Stag-FLAG-KIF1Bβ600–1400 lentivirus and bound proteins were resolved by SDS-PAGE and analyzed by mass spectrometry (Fig. 1F). Among the peptides identified, 22 peptides from RNA helicase A (DHX9) were recovered (Fig. 1F). The interaction was specific, because DHX9 and DHX9 were confirmed in NB1 cells also confirmed the interaction with endogenous DHX9 (Fig. 1H). This interaction was specific, because DHX9 did not coprecipitate with nonapoptotic variant FLAG-KIF1Bβ600–1200 (Fig. 1H).

KIF1Bβ Requires DHX9 to Induce Apoptosis

Next, we asked whether DHX9 is necessary for KIF1Bβ apoptotic function. Knockdown of DHX9 in NB1 (Fig. 2A) and SK-N-SH cells (Fig. 2B) using lentiviral shRNA resulted in protection from apoptosis induced by ectopic expression of KIF1Bβ600–1400 as measured by crystal violet staining for viability. Cells transduced with nontargeting shRNA (shSCR) served as control. Protection from apoptosis was observed with multiple independent shRNAs targeting DHX9, indicating that the knockdown was “on-target” (Supplementary Fig. S2A). The same result was obtained using SK-N-SH cells that were engineered to induce KIF1Bβ upon tetracycline treatment (Fig. 2C). Cells treated with tetracycline died upon KIF1Bβ induction; however, transduction with shRNA against DHX9 resulted in resistance to KIF1Bβ-induced cell death (Fig. 2C). Conversely, coexpression of DHX9 together with KIF1Bβ600–1400 in CHP-212 cells had a synergistic effect on cell death, as measured by crystal violet staining for viability and cleavage of caspase-3 (Fig. 2D). This synergy was specific to the apoptotic domain of KIF1Bβ (KIF1Bβ600–1400), as coexpression of DHX9 with the nonapoptotic mutant KIF1Bβ600–1200 did not have the same effect (Fig. 2E).

In addition, we quantified apoptosis by scoring the nuclei of cells expressing GFP-histone fusion protein (Fig. 2F and Supplementary Fig. S2B). The nuclei of NB1 cells transfected to produce GFP-histone alone or together with WT-DHX9 were healthy and uniform. In contrast, cells transfected with full-length RFP-KIF1Bβ (Fig. 2F) or RFP-KIF1Bβ600–1400 (Supplementary Fig. S2B) displayed signs of apoptosis (nuclear condensation and fragmentation) in 22% and 30% of cells, respectively. However, this proportion increased synergistically to 68% and 72% when RFP-KIF1Bβ was coexpressed together with DHX9 (Fig. 2F and Supplementary Fig. S2B). Previous studies demonstrated that DHX9 functions in the nucleus as a transcriptional activator (13–18). To determine whether this function of DHX9 is required for KIF1Bβ-induced apoptosis, we used previously characterized DHX9 mutants (19, 20) that are defective in either nuclear transport (ANTD-DHX9) or transcriptional activation (TD-DHX9; Supplementary Fig. S2C). When tested, both DHX9 mutants, ANTD-DHX9 and TD-DHX9, failed to synergize with KIF1Bβ in apoptosis induction, implying DHX9 nuclear activity in the induction of apoptosis by KIF1Bβ (Fig. 2F and Supplementary Fig. S2B).

Promotes DHX9 Nuclear Localization

Motivated by our findings that nuclear localization of DHX9 is required to synergize with KIF1Bβ in apoptosis, we investigated DHX9 cellular localization upon KIF1Bβ expression. Endogenous DHX9 and exogenous eCFP-DHX9 were predominantly nuclear in 1p36-intact SK-N-SH neuroblastoma cells (KIF1Bβ+/+), in line with earlier reports describing nuclear DHX9 localization (Fig. 3A and B; refs. 13, 14, 16). However, in 1p36.2 homozygous-deleted NB1 cells (KIF1Bβ−/−), DHX9 localized mainly in the cytoplasm (Fig. 3A and B). To understand whether cytoplasmic DHX9 in NB1 cells is a consequence of KIF1Bβ loss, we restored KIF1Bβ by ectopically expressing RFP-KIF1Bβ and visualized endogenous DHX9 and exogenous eCFP-DHX9 (Supplementary Fig. S3A, S3C, and S3D). Nuclear localization of endogenous DHX9 in NB1 cells was restored in 89% of cells upon expression of RFP-KIF1Bβ or 80% of cells upon expression of RFP-KIF1Bβ600–1400 (Supplementary Fig. S3A). Similarly, ectopically expressed eCFP-DHX9 together with RFP-KIF1Bβ or RFP-KIF1Bβ600–1400 in NB1 cells resulted in nuclear localization of DHX9 in 92% and 80% of cells, respectively (Fig. 3C and D). In contrast, the nonapoptotic KIF1Bβ mutant (KIF1Bβ600–1200) that does not interact with DHX9 displayed significantly less nuclear localization of DHX9.
Figure 2. DHX9 is required for KIF1Bβ-induced apoptosis. A, crystal violet staining of NB1 cells and (B) SK-N-SH cells after infection with lentivirus encoding shRNA targeting DHX9 (shDHX9) or control virus (shSCR) and transfected with Flag-KIF1Bβ600–1400. Cells were selected with G418 (500 μg/mL) for several weeks. Bottom, corresponding immunoblot analysis. C, crystal violet staining of tetracycline-inducible SK-N-SH cells that were infected with lentivirus encoding shRNA targeting DHX9 (shDHX9) or control virus (shSCR) and treated with tetracycline (0.5 μg/mL) to induce Flag-KIF1Bβ. Bottom, corresponding immunoblot analysis. D, crystal violet staining of CHP-212 cells that were transfected with His-DHX9 plasmid as indicated and cotransfected with increasing amounts of Flag-KIF1Bβ600–1400 plasmid and selected with G418 (500 μg/mL) for several weeks. Bottom, corresponding immunoblot analysis. E, performed as D with addition of Flag-KIF1Bβ600–1200 plasmid as indicated (arrow). F, NB1 cells transfected with plasmid encoding GFP-histone along with plasmid encoding RFP-KIF1Bβ or WT-DHX9 (wild-type) alone, or RFP-KIF1Bβ in combination with WT-DHX9 or mutant DHX9 (ΔNTD-DHX9 or TD-DHX9) as indicated. Shown is the percentage of GFP-positive nuclei exhibiting apoptotic changes 48 hours after transfection (mean ± SD; n = 3; *, P < 0.05). G, corresponding immunofluorescence studies of F 24 hours after transfection with plasmids encoding RFP-KIF1Bβ (red) and eCFP-DHX9 (green).
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**Figure 3.** KIF1Bβ promotes DHX9 nuclear localization. A, left, anti-KIF1Bβ immunoblot analysis of NB1 and SK-N-SH cell lines. Right, immunofluorescence images of NB1 and SK-N-SH cells stained for DHX9 (green) and counterstained with Hoechst to visualize nuclei (blue). B, fluorescence studies of NB1 and SK-N-SH cells 24 hours after transfection with plasmid encoding eCFP-DHX9 (green) as indicated. C, fluorescence studies in NB1 cells 24 hours after transfection with plasmid encoding eCFP-DHX9 together with either RFP-KIF1Bβ-WT or RFP-KIF1Bβ mutants as indicated (arrow). D, graphical representation showing percentage of transfected cells with nuclear or cytoplasmic eCFP-DHX9 localization (mean ± SD; n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001). E, graphical representation of immunofluorescence images of NB1 cells (Supplementary Fig. S3D) transfected with Flag-KIF1Bβ mutants as indicated. Shown is the percentage of nuclear or cytoplasmic eCFP-DHX9 or both 24 hours after transfection (mean ± SD; n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001). F, anti-DHX9 (green) immunofluorescence staining of NB1 cells 24 hours after transfection with plasmids encoding Flag-KIF1Bβ wild-type (WT) or disease-causing variants as indicated (red). The percentage of transfected cells displaying nuclear DHX9 is shown in G (mean ± SD; n = 3; *, P < 0.05; **, P < 0.01; ***, P < 0.001).
as compared with RFP-KIF1Bβ and RFP-KIF1Bβ600–1400 (Fig. 3C and D).

In addition to NB1 cells, KIF1Bβ-inducible SK-N-SH cells (Tet-SK-N-SH) showed an enhanced nuclear accumulation of endogenous DHX9 24 hours after FLAG-KIF1Bβ induction by tetracycline (Supplementary Fig. S3B). Nuclear DHX9 in tetracycline-treated cells was concentrated in specific nuclear regions and colocalized with the nucleoli, visualized by anti-Fibrillarin counterstaining (Supplementary Fig. S3B).

Because DHX9 nuclear localization depends upon KIF1Bβ expression, we reasoned that silencing of KIF1Bβ in SK-N-SH cells (KIF1Bβ+/−) should result in the reverse. Knockdown of KIF1Bβ in SK-N-SH cells using lentiviral shRNA resulted in predominantly cytoplasmic DHX9 (Supplementary Fig. S3C). This was also achieved using shRNA targeting EGLN3, resulting in downregulation of KIF1Bβ protein and subsequent cytoplasmic localization of DHX9 (Supplementary Fig. S3C).

We next examined the ability of additional KIF1Bβ variants to influence eCFP-DHX9 localization. The proapoptotic domain 1000–1400, as well as the proapoptotic domain 1000–1600, significantly stimulated DHX9 nuclear localization in 40% and 52% of the cells, respectively, with 27% of the cells displaying partial DHX9 nuclear localization (Fig. 3E and Supplementary Fig. S3D). In contrast, the apoptotic-defective mutants 1200–1600 and 600–1400Δ1100–1300 were impaired in stimulating eCFP-DHX9 nuclear localization (Fig. 3E and Supplementary Fig. S3D), similar to what we observed for the apoptotic-defective mutant 600–1200 (Fig. 3D). However, although mutant 600–1200 is deficient in DHX9 binding, DHX9 nuclear localization, and apoptosis, the mutants 1200–1600 and 600–1400Δ1100–1300 retained the ability to bind to DHX9 despite being defective in apoptosis and DHX9 nuclear localization (Fig. 1A and Supplementary Fig. S3E). On the basis of the DHX9-binding data, we conclude that the region required for DHX9 binding resides on amino acids 1100–1200, as 1000–1400 localizes to amino acids 1300–1400, as 600–1400Δ1100–1300 retained the ability to bind DHX9, whereas 600–1200 did not (Fig. 1A and H and Supplementary Fig. S3E). In contrast, the binding site does not overlap with the DHX9 nuclear localization site. We conclude that the DHX9 nuclear localization site resides on amino acids 1100–1200, as 1000–1400 localizes DHX9 to the nucleus, whereas 1200–1600 and Δ1100–1300 do not (Figs. 1A and 3E). This indicates that DHX9 binding and DHX9 localization are dictated by two distinct adjacent sites. It further implies that both sites are required for KIF1Bβ-induced apoptosis, because mutants that either lack the DHX9 binding site (600–1200) or lack the DHX9 localization site (1200–1600 and Δ1100–1300) failed to induce apoptosis (Fig. 1A, C, and D). Collectively, these results suggest that additional KIF1Bβ-interacting partners/modifiers at the amino acid region 1100–1200 might be required to mediate DHX9 nuclear localization.

Next, we tested putative disease-causing KIF1Bβ variants that were identified in neuroblastomas and pheochromocytomas and were defective in apoptosis (5). The variants T827I and P1217S failed to relocate DHX9 to the nucleus (Fig. 3F and G). Moreover, ectopic expression of the variant E1628K displayed a significant reduction of nuclear DHX9 compared with wild-type FLAG-KIF1Bβ. However, the variants E646V and S1481N stimulated DHX9 nuclear localization similar to wild-type KIF1Bβ despite their impairment in apoptosis (Fig. 3F and G), indicating that DHX9 nuclear localization is necessary but not sufficient for KIF1Bβ to induce apoptosis, and additional mechanisms might be required for KIF1Bβ apoptotic function.

Exportin-2 Is Necessary for DHX9 Nuclear Localization and KIF1Bβ Apoptotic Function

To understand how KIF1Bβ regulates DHX9 nuclear localization, we investigated other KIF1Bβ-associated proteins that were identified by large-scale immunoprecipitation (Fig. 1F). In addition to DHX9, we identified Exportin-2 (XPO2) as a specific binding partner of the KIF1Bβ600–1400 apoptotic domain (Fig. 1F). XPO2 regulates nuclear import and export of cellular proteins via its ability to reexport Importin from the nucleus to the cytoplasm after imported substrates have been released into the nucleoplasm (21). Knockdown of XPO2 in SK-N-SH cells using two independent lentiviral shRNAs caused reduced nuclear localization of DHX9 (5%) compared with shSCR control (71%; Fig. 4A). XPO2 knockdown efficiency in these cells was verified by Western blot analysis (Fig. 4B and Supplementary Fig. S4). Because nuclear localization-deficient DHX9 (ΔNTD-DHX9; Fig. 2F and Supplementary Fig. S2B) failed to cooperate with KIF1Bβ in apoptosis, we tested whether silencing of XPO2 protects from KIF1Bβ-induced apoptosis. Coexpression of GFP-KIF1Bβ, together with plasmids encoding shRNAs targeting XPO2, demonstrated significant protection from KIF1Bβ-mediated apoptosis in NB1 cells (Fig. 4C). Together, these results demonstrate that XPO2 is required for nuclear localization of DHX9 and that activity is essential for KIF1Bβ-induced apoptosis.

DHX9 Nuclear Localization Induced by KIF1Bβ Stimulates Proapoptotic XAF1

Because transcription-dead mutant DHX9 (TD-DHX9) failed to cooperate with KIF1Bβ in apoptosis, we asked whether KIF1Bβ-mediated DHX9 nuclear localization results in activation of specific target genes. We performed RNA-seq to analyze gene expression in NB1 cells that were transduced with either shRNA targeting DHX9 (shDHX9) or control virus (shSCR) and subsequently transfected with plasmid encoding KIF1Bβ600–1400. Principal component analysis (PCA) of overall gene expression profiles revealed transcriptional changes in shSCR cells expressing KIF1Bβ600–1400 compared with pcDNA3 control (Fig. 5A). Although DHX9 knockdown in cells alone generally resulted in differences in gene expression compared with the shSCR control, there was minimal difference in gene expression upon expressing pcDNA3 and KIF1Bβ600–1400 in the context of DHX9 knockdown (Fig. 5A). Differential expression analysis using DESeq revealed 58 genes significantly upregulated (false discovery rate, FDR, < 0.05) by KIF1Bβ600–1400, and their expression is depicted as a heatmap (Fig. 5B). Twenty-three genes were upregulated at least 2-fold and, among those, 18 genes were dependent on DHX9 expression.
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**Figure 4.** Exportin-2 is necessary for nuclear localization of DHX9 and KIF1Bβ to induce apoptosis. 

A, anti-DHX9 (green) immunofluorescence staining of SK-N-SH cells stably expressing short hairpins targeting Exportin-2 (shXPO2) or nontargeting control (shSCR) as indicated. Right, percentage of corresponding cells with nuclear or cytoplasmic DHX9 or both: nuclear, percentage of cells with higher nuclear fluorescence intensity (green) compared with the cytoplasm; cytoplasmic, percentage of cells displaying higher cytoplasmic fluorescence intensity (green) compared with the nuclear region. Nuclear and cytoplasmic, percentage of cells displaying similar fluorescence intensity in the nucleus and cytoplasm. 

B, corresponding anti-XPO2 immunoblot analysis of stably infected cells with shRNA lentivirus targeting XPO2 that were displayed in A. NB1 cells were cotransfected with either empty pcDNA3 plasmid or GFP-KIF1Bβ600–1400 together with plasmids encoding short hairpin targeting XPO2 (pLKO-shXPO2) or nontargeting control (pLKO-shSCR) as indicated. Shown is the percentage of apoptotic cells at 96 hours after transfection determined by fluorescence-activated cell sorting (FACS) analysis using TMRE staining (mean ± SD; n = 3; ****, P < 0.0001). MOI, multiplicity of infection.
Figure 5. KIF1Bβ-driven nuclear DHX9 induces the expression of proapoptotic XAF1. A, PCA of overall gene expression profiles identified by RNA-seq analysis. RNA was obtained from NB1 cells stably expressing short hairpins targeting DHX9 (shDHX9) or nontargeting control (shSCR) that were transfected with plasmids encoding FLAG-KIF1Bβ600–1400 or pcDNA3 as indicated. PCA plot displaying three independent experiments. B, heatmap depicting expression of genes in A that were significantly upregulated (FDR < 0.05): green, lower expression; red, higher expression. C, list of genes identified by differential expression analysis using DESeq that were upregulated greater than 2-fold (FDR < 0.05) by KIF1Bβ600–1400 and dependent on DHX9 expression as displayed in A. D, relative mRNA levels of XAF1 determined by qRT-PCR (XAF1, corresponding to transcript variant 1; mean ± SD, n = 3; *, P < 0.05). E, anti-XAF1 immunoblot analysis of CHP-212 and NB1 cells transfected with plasmids encoding FLAG-KIF1Bβ600–1400 and FLAG-KIF1Bβ, respectively. F, anti-DHX9 (green) and anti-XAF1 (red) immunofluorescence analysis in SK-N-SH cells with tetracycline-inducible KIF1Bβ (-Tet, noninduced; +Tet, induced).
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( Supplementary Tables S1 and S2 and Fig. 5C). Most of the KIF1Bβ-induced, DHX9-dependent targets were IFN-induced or IFN-related, consistent with earlier reports implicating DHX9 in transcriptional regulation of IFN-α-inducible genes (22). Some of these genes are known NF-xB downstream targets (23–25), in line with earlier observations demonstrating a DHX9–NF-xB interaction resulting in the transcriptional activation of specific promoters (18, 26). In addition, the proapoptotic XIAP-associated factor 1 (XAF1) was identified (Fig. 5C). XAF1 functions as a negative regulator of members from the inhibitors of the apoptosis (IAP) family (27). Quantitative real-time PCR (qRT-PCR) confirmed that XAF1 mRNA is upregulated by KIF1Bβ in a DHX9-dependent manner (Fig. 5D).

Moreover, exogenous expression of KIF1Bβ600–1400 or full-length KIF1Bβ in CHF-212 and NB1 cells resulted in XAF1 protein induction (Fig. 5E). In addition, inducible KIF1Bβ neuroblastoma cells (Tet-SK-N-SH) resulted in enhanced DHX9 nuclear localization and XAF1 induction (Fig. 5F).

Loss of DHX9 Promotes Neuronal Survival in the NGF Signaling Pathway

We used differentiated PC12 cells to study the regulation of DHX9 during neuronal survival by NGF. Consistent with previous reports (5), NGF withdrawal from PC12 cells caused the induction of KIF1Bβ protein, followed by the induction of apoptosis (Fig. 6A and Supplementary Fig. S5A). We observed that, like KIF1Bβ, XAF1 protein was also induced with similar kinetics in PC12 cells after NGF deprivation and associated with increased XAF1 mRNA (Fig. 6A and Supplementary Fig. S5A and S5B). To determine whether induction of XAF1 depends on KIF1Bβ-mediated DHX9 nuclear accumulation, we first assayed endogenous DHX9 localization in PC12 cells followed by NGF withdrawal. NGF-deprived PC12 cells displaying apoptotic characteristics showed enhanced nuclear DHX9 (67% of cells), in contrast with cells maintained in NGF, which showed only 1% of cells with nuclear DHX9 (Fig. 6B). However, PC12 cells transduced with shRNA targeting KIF1Bβ prevented nuclear accumulation of DHX9 (Fig. 6C). Likewise, knockdown of EGLN3 reduced nuclear DHX9 and caspase-3 cleavage compared with shSCR control (Fig. 6D). Moreover, PC12 cells transduced with shRNA targeting KIF1Bβ abolished the induction of XAF1 upon NGF withdrawal (Fig. 6E). Furthermore, we observed induction of DHX9 protein upon NGF withdrawal in shSCR PC12 cells and in primary mouse sympathetic neurons (Fig. 6F, F, and G). Next, we asked whether loss of DHX9 blocks apoptosis in NGF-deprived PC12 cells. PC12 cells transduced with shRNAs targeting DHX9 were protected from apoptosis upon NGF withdrawal compared with control cells, as determined by cleaved caspase-3 quantification (Fig. 6H). Furthermore, silencing of DHX9 in NGF-deprived PC12 cells showed ablated XAF1 induction similar to that observed following KIF1Bβ knockdown (Fig. 6F). Silencing of DHX9 also abolished the induction of endogenous KIF1Bβ protein in NGF-deprived PC12 cells and SK-N-SH neuroblastoma cells, possibly by regulating the translation of KIF1Bβ mRNA (Fig. 6F and I and Supplementary Fig. S5C).

DHX9 Nuclear Localization Is Impaired in KIF1Bβ-Deficient Neuroblastoma Tumors

To investigate whether nuclear localization of DHX9 depends on KIF1Bβ in vivo, we studied the expression of DHX9 and KIF1Bβ in the mouse sympathetic nervous system when developmental apoptosis peaks, for example, around birth (28). In situ hybridization revealed that KIF1Bβ was highly and exclusively expressed in the sympathetic ganglia but not in non-neuronal tissue surrounding the ganglia, highlighting its specific role in the sympathetic nervous system (Fig. 7A and B). The identity of the superior cervical ganglia (SCG) was confirmed with immunohistochemistry for tyrosine hydroxylase, a marker for noradrenergic and adrenergic neurons (Fig. 7C). To investigate whether nuclear DHX9 localization coincides with KIF1Bβ expression in the SCG, DHX9 immunofluorescence studies were performed. We observed DHX9 in the nuclei of cells within the SCG, but in the cytoplasm of cell. DHX9 was observed in both the tissue that lack KIF1Bβ expression (Fig. 7D and Supplementary Fig. S6A). This is in accordance with our in vitro observation that DHX9 nuclear localization is dependent upon KIF1Bβ expression.

We next analyzed 13 primary neuroblastoma tumors for KIF1Bβ and DHX9 protein expression, determined DHX9 cellular localization, and sequenced the KIF1Bβ exome. Only two of 13 neuroblastomas, K14 and K33, were 1p36-intact (1p36+/−), whereas the remaining 11 samples harbored hemizygous 1p36 deletions (Supplementary Table S3). We observed a complete lack of KIF1Bβ protein expression in most of the 1p36-deleted tumors, in contrast with tumor K14 (1p36+/−) and the SK-N-SH cell line (1p36+/−; Fig. 7E). Also, tumor K33 (1p36+/−) did not express KIF1Bβ, and tumors K36 and K56 expressed faster migrating forms of KIF1Bβ protein, likely due to splicing alterations (Fig. 7E). Exome sequencing did not reveal any missense mutations in KIF1Bβ alleles, although polymorphic variants were identified in two tumors, K14 (V1554M) and K10 (M8071; Supplementary Table S4). The lack of KIF1Bβ protein expression in KIF1Bβ-hemizygous tumors might result from epigenetic silencing, translation, or splicing alterations. Notably, DHX9 protein expression varied across the different neuroblastoma tumors (Fig. 7E).

Next, we investigated DHX9 localization in these tumors in paraffin-embedded sections (Fig. 7F and Supplementary Fig. S6B and S6C). Specificity of the DHX9 staining in these paraffin-embedded sections was confirmed using the K11 tumor as negative control, because it lacks DHX9 protein expression (Supplementary Fig. S6C and Fig. 7E). In tumors that lacked KIF1Bβ protein expression (K10, K12, K33, K7, and K9), DHX9 was observed in both the cytoplasm and nucleus, with the majority in the cytoplasm (Fig. 7F and Supplementary Fig. S6B). However, the K14 tumor expressing KIF1Bβ protein displayed predominantly nuclear DHX9. These results accord with our observations in the mouse sympathetic nervous system and in vitro studies that nuclear localization of DHX9 depends on KIF1Bβ protein.

Collectively, our results demonstrate that DHX9 nuclear localization is impaired in KIF1Bβ-deficient neuroblastoma.
**Figure 6.** Induction of KIF1Bβ increases nuclear DHX9 and XAF1 expression during NGF withdrawal. **A,** anti-KIF1Bβ and anti-XAF1 immunoblot analysis of differentiated PC12 cells subjected to NGF withdrawal (24 hours; −NGF). Antibodies against DHX9 (green) and cleaved caspase-3 (red) were used in addition to Hoechst staining to visualize nuclei (blue). **B−D,** immunofluorescence images of differentiated PC12 cells that were subjected to NGF withdrawal (24 hours; −NGF). Antibodies against DHX9 (green) and cleaved caspase-3 (red) were used in addition to Hoechst staining to visualize nuclei (blue). **B,** right, percentage of PC12 cells exhibiting nuclear DHX9 and cleaved caspase-3 before (+) and after (−) NGF withdrawal at indicated times (mean ± SD; n = 3; *, P < 0.05). **C,** before NGF withdrawal, cells were infected with lentivirus encoding short hairpins targeting KIF1Bβ (shKIF1Bβ) or nontargeting control virus (shSCR). Right, corresponding percentage of cells displaying DHX9 nuclear accumulation (mean ± SD; n = 3; *, P < 0.05). **D,** before NGF withdrawal, cells were infected with lentivirus encoding short hairpins targeting rat-EGLN3 (shEGLN3). **E and F,** immunoblot analysis of differentiated PC12 cells before (+) and after (−) NGF withdrawal as indicated. Before NGF withdrawal, differentiated cells were infected with lentivirus encoding short hairpins targeting KIF1Bβ (shKIF1Bβ), DHX9 (shDHX9), or nontargeting control virus (shSCR). **G,** immunoblot analysis of primary mouse sympathetic neurons subjected to NGF withdrawal at indicated times. **H,** percentage of apoptosis in differentiated PC12 cells before (+) and after (−) NGF withdrawal that were transduced with lentivirus encoding short hairpins targeting DHX9 (shDHX9) or nontargeting control virus (shSCR). Apoptosis was scored by cleaved caspase-3 quantification (mean ± SD; n = 5; *, P < 0.05). **I,** immunoblot analysis of SK-N-SH cells infected with lentivirus encoding short hairpins targeting DHX9 (shDHX9) or control virus (shSCR). GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
RNA Helicase A Is Vital for KIF1Bβ Tumor Suppression

**Figure 7.** DHX9 is localized to the nuclei of developing mouse sympathetic neurons but not in human neuroblastoma tumors deficient in KIF1Bβ. A, in situ hybridization using a probe for KIF1Bβ on sagittal sections of wild-type mouse at embryonic day E17.5 showing KIF1Bβ expression in the SCG adjacent to the cochlea as indicated. B, in situ hybridization for KIF1Bβ at P2 using both antisense and sense probes in adjacent sections. C, in situ hybridization for KIF1Bβ in mouse SCG at P2 and immunohistochemistry for tyrosine hydroxylase showing expression in the same area. D, anti-DHX9 (red) immunofluorescence images in the SCG (1), non-neuronal surrounding tissue (2), and the cochlea (3) of mouse sagittal sections at postnatal day 1. E, immunoblot analysis of primary neuroblastoma tumors. 1p36 status has been characterized and is outlined in Supplementary Table S3 (1p36+/+, wild-type; 1p36+/−, 1p36 deletion). SK-N-SH cells (1p36-intact) served as positive control. F, immunohistochemistry for DHX9 on paraffin-embedded tissue sections of human neuroblastoma tumors counterstained with hematoxylin. K10, K12, K33, and K7 do not express KIF1Bβ but are positive for DHX9 based on the immunoblot analysis in E. K14 is positive for KIF1Bβ and DHX9 based on immunoblot analysis in E. The image in A was acquired using a x4 objective; images in F were acquired using a x20 objective, whereas higher magnification images of selected regions were acquired using a x100 objective. G, signaling model linking familial genetic lesions associated with sympathetic nervous system cancer (pheochromocytoma or neuroblastoma) to apoptosis when NGF becomes limiting during sympathetic neuronal development.
dependent apoptosis. However, silencing of DHX9 also decreased the expression of proapoptotic proteins, providing further evidence to suggest that KIF1Bβ is a neuroblastoma tumor suppressor (Supplementary Fig. S6D).

DISCUSSION

KIF1Bβ was previously characterized as a potential 1p36 tumor-suppressor gene that mediates neuronal apoptosis when NGF is limiting in the developing nervous system (5, 11, 12). Here, we provide a mechanistic understanding of KIF1Bβ-mediated tumor suppression. We identified the RNA helicase DHX9 as an interacting partner of KIF1Bβ and found that DHX9 is necessary for KIF1Bβ to induce apoptosis and is required for apoptosis when NGF is limiting.

DHX9 is a member of the DEAH-box DNA/RNA helicase family that catalyzes the ATP-dependent unwinding of double-stranded RNA and DNA–RNA complexes (14). Recently, DHX9 has been characterized in multiple cellular functions, including translation, RNA splicing, and miRNA processing. In addition, DHX9 localizes to both the nucleus and the cytoplasm and functions as a transcriptional regulator (13, 15–17). Here, we report that KIF1Bβ induces neuronal apoptosis by directing DHX9 nuclear accumulation, which leads to induction of proapoptotic XAF1. XAF1 has been reported as an antagonist of antiapoptotic XIAP and has been shown to convert XIAP into a proapoptotic protein to degrade survivin (27, 29). Inhibition of XIAP is necessary and sufficient for sympathetic neurons to acquire apoptotic competence during NGF withdrawal-induced apoptosis (30). Similarly, expression of survivin is strongly correlated with advanced stages of disease and unfavorable neuroblastoma outcomes (31).

Abnormal NGF signaling has been linked to nervous system tumors such as neuroblastoma, medulloblastoma, and pheochromocytoma (1–5, 32, 33). Our findings imply that alterations in NGF-mediated developmental apoptosis may play a role in these types of cancers. We found that DHX9 is induced when NGF is limiting, localizes and accumulates in the nucleus, and that nuclear accumulation is dependent upon KIF1Bβ expression. Furthermore, down-regulation of DHX9 diminished the expression of proapoptotic XAF1 and caused escape from NGF withdrawal-dependent apoptosis. However, silencing of DHX9 also abolished KIF1Bβ induction and, therefore, loss of DHX9 could also cause escape from apoptosis due to its effect on KIF1Bβ. Therefore, KIF1Bβ's function in apoptosis might involve additional mechanisms that account for NGF-dependent apoptosis. In this regard, we tested recently identified putative disease-causing KIF1Bβ mutants for their ability to regulate DHX9 localization. Indeed, variants T827I, P1217S, and E1628K—all of which are defective in apoptosis—failed to stimulate nuclear localization of DHX9, supporting our findings that nuclear DHX9 mediates KIF1Bβ apoptotic function. However, variants E646V and S1481N stimulated DHX9 localization to a comparable degree as wild-type KIF1Bβ despite their impaired apoptotic abilities. This indicates that DHX9 nuclear localization is necessary but not sufficient for KIF1Bβ to induce apoptosis, and that additional events are needed for KIF1Bβ to induce apoptosis.

In an attempt to mechanistically understand how KIF1Bβ regulates DHX9 nuclear localization, we examined other KIF1Bβ binding partners. In addition to DHX9, we found that XPO2 binds to the KIF1Bβ proapoptotic 600–1400 domain. XPO2 has been reported to regulate nuclear import and export of cellular proteins. Here, we show that loss of XPO2 impairs DHX9 nuclear localization and, consequently, impedes KIF1Bβ-induced apoptosis, further implicating DHX9 nuclear localization as a requirement for KIF1Bβ apoptotic function. XPO2 was previously implicated in regulating apoptosis induced by Pseudomonas exotoxin (34), and resistance to Pseudomonas exotoxin is phenocopied in Egl-9(−/−) worms (35), suggesting that XPO2 acts in the same apoptotic program mediated by EGLN3 and KIF1Bβ. Moreover, our results suggest that DHX9 binding and DHX9 nuclear localization are mediated by two distinct and adjacent sites, KIF1Bβ1300–1400 and KIF1Bβ1100–1200, respectively. Because both sites are required for KIF1Bβ apoptosis function, we concluded that additional KIF1Bβ binding partners on amino acid region 1100–1200 might participate in DHX9 localization. It was previously demonstrated that arginine methylation of DHX9 determines its subcellular localization (36). Indeed, we identified arginine methyltransferase PRMT5 in the large-scale KIF1Bβ600–1400 immunoprecipitation (data not shown). Therefore, PRMT5 might be such a modifier.

Finally, we demonstrate that the regulation of DHX9 by KIF1Bβ is relevant in neuroblastoma. Our in vivo studies in the mouse sympathetic nervous system demonstrate that DHX9 is specifically found in the nuclei of cells that express KIF1Bβ and that nuclear DHX9 is present in 1p36-intact tumors that express wild-type KIF1Bβ. In contrast, analysis of 1p36-deleted neuroblastomas with complete loss of KIF1Bβ protein expression showed impaired DHX9 nuclear localization. In addition to our findings that impair apoptosis of wild-type KIF1Bβ in neuroblastoma pathogenesis, other evidence points to dysfunction of RNA helicases in pediatric nervous system cancers. Medulloblastoma exome sequencing uncovered recurrent somatic missense mutations within RNA helicases in pediatric nervous system cancers. Medulloblastoma exome sequencing uncovered recurrent somatic missense mutations within RNA helicases in pediatric nervous system cancers. Medulloblastoma exome sequencing uncovered recurrent somatic missense mutations within RNA helicases in pediatric nervous system cancers.
In summary, we propose that loss of nuclear DHX9 due to impaired EGLN3 activity or loss of KIF1Bβ promotes neuronal survival during the NGF-dependent developmental culling of sympathetic neurons. On the basis of our studies, we propose that failure to properly cull neuronal progenitors during development predisposes to sympathetic nervous system tumors such as neuroblastoma. Alterations in developmental apoptosis due to dysfunction of EGLN3, KIF1Bβ, and DHX9 might play a role in the pathogenesis of these tumors by allowing neuronal progenitors to escape from developmental culling and thereby predisposing them to neoplastic transformation (Fig. 7G and Supplementary Fig. S7).

**METHODS**

**Cell Culture**

Human neuroblastoma cell lines (N18, CHP-212, and SK-N-SH) and PC12 cells were maintained as previously described (5). CHP-212, SK-N-SH, and PC12 cell lines were obtained from the American Type Culture Collection, and N18 cell lines were obtained from the Japanese Collection of Research Bioresources. The cell lines used were not further authenticated. Sympathetic neurons from P1 mice were isolated from the SCG and cultured as described previously (39).

**Plasmids**

FLAG-tagged KIF1Bβ plasmids and corresponding mutants were generated as described previously (5). RFP-KIF1Bβ, RFP-KIF1ββ(600–1400), and RFP-KIF1ββ(600–1200) were generated by cloning TagRFP into FLAG-KIF1ββ plasmids. RFP was cloned into the Xpn-1 digested 5′ region of FLAG-KIF1ββ pcDNA3.1 expression vectors. Lentivirus expressing RFP-KIF1ββ(600–1400) was generated as previously described using pLenti-Flag-KIF1ββ(600–1400). Stag was cloned into FLAG-KIF1ββ(600–1400) by annealing five phosphorylated oligonucleotides containing S-tag sequence and XbaI restriction site, followed by ligation with the XbaI-digested 5′ region of pLenti-Flag-KIF1ββ(600–1400). His-DHX9 and eCFP-DHX9 were purchased from Genocopoeia. eCFP-ΔNTD-DHX9, eCFP-TD-DHX9, and eCFP-R1166L-W332A, W339A, and W342A mutations at the MTAD domain of DHX9 lacking in NTD. eCFP-TD-DHX9 was created by introducing a premature STOP reverse: 5′-TCGAGAAGATCCATGTGTAAT-3′, (5′-CCAGAACATCCATGGCTGTTTT-3′), (5′-GGGCTATATC CATGGAATTT-3′). DHX9 (5′-GCCAAGGCUUAGAAGUGA-3′) and siDHX9(3) 5′-CAAGUCCCCAAUA-3′, siRNA targeting human XAF1 was purchased from Dharmacon under ON-TARGETplus SMARTpool siRNA. Cotransfections of siRNA with pcDNA3 plasmids were performed using DharmaFECT Duo Transfection Reagent according to the manufacturer’s recommendations.

**Viral Expression and Infection**

Adenovirus encoding rat EGLN3 (3M-20) was a gift from Robert Freeman (University of Rochester, Rochester, NY). Virus amplification and infection has been previously described (5). Lentiviral infection for gene silencing using shRNA was performed according to the manufacturer’s instructions (MISSION shRNA; Sigma).

**Immunoprecipitation and Mass Spectrometry**

A total of 160 × 10^6 NB1 cells were transduced with lentivirus expressing S-tag-KIF1ββ(600–1400). Two days later, cells were harvested with lysis buffer (150 mmol/L NaCl, 50 mmol/L Tris–HCl, 5 mmol/L EDTA, 0.1% CHAPS, and pH 7.4) and incubated for 2 hours at 4°C with rotation. The lysate was centrifuged at 20,000 × g for 20 minutes. The resulting supernatant was pre-cleared for 2 hours and, subsequently, S-protein agarose beads (Novagen) were added to lysates and incubated for 3 hours at 4°C with rotation. Samples were centrifuged and beads were washed five times in wash buffer (500 mmol/L NaCl, 50 mmol/L Tris–HCl, 5 mmol/L EDTA, pH 7.4). Bound protein complexes were eluted in Laemmli buffer containing 10 mmol/L Dithiothreitol and heated for 10 minutes at 95°C. Eluates were analyzed by SDS-PAGE, gels were subjected to silver stain, and bands of interest were excised for mass spectrometry identification. Mass spectrometry protocol was previously described (40).

**Coimmunoprecipitation of Endogenous Proteins**

One confluent p150 plate of SK-N-SH neuroblastoma cells was harvested with 1-mL immunoprecipitation (IP) buffer (20 mmol/L Tris, 150 mmol/L NaCl, 2 mmol/L EDTA, 10% glycerol, 0.1% CHAPS, and protease inhibitors, pH 7.4), incubated for 2 hours, and then centrifuged at 14,000 × g for 30 minutes. The resulting supernatant was pre-cleared with 40-μL Protein A agarose slurry for 3 hours and then centrifuged at 2,500 × g for 3 minutes. Either 5-μg rabbit polyclonal KIF1ββ antibody or rabbit immunoglobulin G (IgG) isotype control antibody (Cell Signaling Technology; DA1E, #3900) was added to 1-mL lysate overnight at 4°C. Subsequently, 40-μL Protein A agarose slurry was added and incubated on a rotator for 3 hours and then centrifuged at 2,500 × g for 30 seconds, and the resulting resin was washed with 1-mL IP buffer. The resin was washed three times in total, and reducing 2× sample buffer was added to the resin and boiled for 5 minutes and analyzed by immunoblotting. Coimmunoprecipitation of exogenous FLAG-KIF1ββ with Anti-FLAG M2 Affinity Gel was prepared according to the manufacturer’s instructions (Sigma).
Apoptosis Assays

Apoptosis was assessed using GFP-histone to quantify apoptotic nuclei as previously described (5). Alternatively, immunofluorescence staining for cleaved caspase-3 allowed for visualization and quantification of apoptotic cells via microscopy. Apoptosis in primary sympathetic neurons was scored by DAPI staining, to visualize apoptotic changes as previously described (5, 41). KIF1Bβ-induced apoptosis assays in XPO2-knockdown cells were performed by fluorescence-activated cell sorting (FACS) analysis using tetramethylrhodamine ethyl esters (TMRE; Invitrogen Corporation) 92 hours after transfection. Statistical analysis was performed by one-way ANOVA, followed by the Bonferroni posttest using the GraphPad Prism software (GraphPad Software Inc., version 6.00).

Tetracycline-Regulated Expression System

Tetracycline-responsive inducible KIF1Bβ expression in SK-N-SH cells was constructed using the T-REx System (Invitrogen). Tetracycline-inducible cells were plated in a 6-well plate and transduced with either shSCR or shDHX9 lentivirus. After 24 hours, cells were grown in selection medium (1 μg/mL puromycin) for 3 days before being replenished with fresh medium containing 0.5 to 1 μg/mL tetracycline. Fresh medium containing tetracycline and puromycin was replenished every 2 days. After 4 days of induction or upon reaching confluence, cells were transferred into 100 plates to undergo further selection and induction until resistant colonies were identified. Cells were maintained in blasticidin and zeocin at all times at concentrations previously mentioned.

Immunofluorescence and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde (PFA) and stained with 1 μg/mL Hoechst for 10 minutes at room temperature. Immunofluorescence was performed by fixation with 4% PFA for 15 minutes, followed by quenching with 10 mmol/L glycine for 20 minutes. Cells were then permeabilized with 0.1% Triton X-100, blocked with 5% goat serum, and incubated with primary antibodies in PBS containing 0.1% bovine serum albumin (BSA) overnight at 4°C. Secondary antibodies conjugated to fluorophores were incubated in PBS with 0.1% BSA at 1:1,000 for 1 hour. After washing with PBS or PBS with 0.1% BSA and performed at room temperature until resistant colonies were identified. Cells were maintained in blasticidin and zeocin at all times at concentrations previously mentioned.

Immunofluorescence and Immunohistochemistry

Cells were fixed with 4% paraformaldehyde (PFA) and stained with 1 μg/mL Hoechst for 10 minutes at room temperature. Immunofluorescence was performed by fixation with 4% PFA for 15 minutes, followed by quenching with 10 mmol/L glycine for 20 minutes. Cells were then permeabilized with 0.1% Triton X-100, blocked with 5% goat serum, and incubated with primary antibodies in PBS containing 0.1% bovine serum albumin (BSA) overnight at 4°C. Secondary antibodies conjugated to fluorophores were incubated in PBS with 0.1% BSA at 1:1,000 for 1 hour, followed by 1 μg/mL Hoechst for 10 minutes (Invitrogen). All steps were interspersed with two to three washes with PBS or PBS with 0.1% BSA and performed at room temperature until resistant colonies were identified. Cells were maintained in blasticidin and zeocin at all times at concentrations previously mentioned.

C57BL/6 mice were decapitated at postnatal day 1 (P1) and frozen on dry ice. Immunohistochemistry was performed on 12-μm cryostat sagittal sections. The sections were fixed with PFA and blocked with Mouse on Mouse blocking reagent, followed by incubation with 5% normal goat serum (NGS) in 0.3% Triton X-100 in PBS for 1 hour (Vector Laboratories). After blocking, the sections were incubated with mouse anti-DHX9 at 1:250 dilution (Novus Biologicals) and rabbit anti-tyrosoine hydroxylase at 1:1000 dilution (Pel-Freeze) in 5% NGS and Triton X-100 overnight at 4°C. The sections were subsequently washed in 0.1% Triton X-100 in PBS and incubated with anti-rabbit Alexa Fluor 488 and anti-mouse Alexa Fluor 546 secondary antibodies at 1:1,000 dilution (Invitrogen) for 1 hour at room temperature, washed, and coverslipped with Vectashield mounting medium containing DAPI (Vector Laboratories). Sections were analyzed using an LSM 5 Excenter confocal laser-scanning microscope (Zeiss). Alternatively, sections were incubated with a biotinylated goat anti-rabbit antibody at 1:500 dilution for 1 hour at room temperature, followed by the Vectastain ABC Kit and DAB visualization as above. The sections were counterstained with Mayer’s hematoxylin, dehydrated, cleared in xylene, coverslipped (Histolab), and viewed with a Nikon Eclipse E1000 microscope.

Microscopy

Immunofluorescence and fluorescent protein-tagged images were acquired and analyzed using Zeiss LSM 5 EXCITER or Zeiss LSM 510 META laser-scanning confocal microscopes together with Zeiss LSM 5 EXCITER or Zeiss LSM 510 software, respectively. Images were acquired using 63X Plan-Apochromat/1.4 NA Oil with DIC capability objective. The excitation wavelengths for TagRFP/Alexa Fluor 555, eCFP, GFP/Alexa Fluor 488, and DAPI/Hoechst were 543, 458, 488, and 405 nm, respectively. Images were captured at frame size: 1024, scan speed: 7, and 12-bit acquisition and line averaging mode: 8. Pinholes were adjusted so that each channel had the same optical slice of 1 to 1.2 μm. Image scaling was performed using the Photoshop CS6 “Place Scale Marker” tool, whereby the number of pixels was divided by the field size and multiplied by the desired distance to indicate the respective scales. Approximately 50 to 100 cells per sample were counted for quantification analysis. Images of neuroblasts paraffin-embedded tissue sections were acquired using ×20 and ×100 objectives mounted on a Nikon Eclipse E1000 microscope.

Antibodies

Rabbit polyclonal anti-Flag (F7425) and mouse monoclonal anti-Flag (F3165 and F1804) were purchased from Sigma. Mouse monoclonal anti-EGFLN3 was generously provided by Dr. Peter Ratcliffe (Oxford University, Oxford, UK). Mouse monoclonal anti-DHX9 was purchased from Novus Biologicals (3G7; H00001600-M01), XPO2 mouse monoclonal antibody (#610482; BD Transduction Laboratories) was used at a dilution of 1:1,000 for immunoblot analysis. Polyclonal KIF1Bβ-antibody was raised in rabbits against a synthetic peptide (GHYQHPLHGLGQELNSPPQPC) by Peptide Specialty Laboratories GmbH. Rabbit monoclonal anti-cleaved caspase-3 (D175; SAIE; #9664), rabbit monoclonal anti-Fibrillarin (C13C3; #2639), and rabbit polyclonal anti-His (#2365) were purchased from Cell Signaling Technology. Rabbit polyclonal anti-XAF1 (ab17204) was purchased from Abcam. Rabbit anti-tyrosoine hydroxylase was purchased from Pel-Freeze.

NGF Withdrawal

NGF withdrawal in primary sympathetic neurons and PC12 cell has been recently described (39).

RNA-seq Analysis

NB1 cells stably transduced with either shSCR or shDHX9 were transfected with either empty pcDNA3.1 plasmid or KIF1Bβ(600-1400) expression plasmid, in conjunction with pMACS K-βI plasmid in a 1:5 ratio (Milenyi Biotech). We made three biologic replicates for each condition. After 48 hours, cells expressing both the plasmid of interest and selection plasmid were isolated using the MACSelect Transfected Cell Selection System and performed as recommended by the vendor (Milenyi Biotech). RNA purification of isolated cells was carried out with the RNeasy Mini Kit according to the manufacturer’s instructions (Qiagen). Purified total RNA was sent for mRNA-seq.
RNA Helicase A Is Vital for KIF1Bβ Tumor Suppression


**RNA Helicase A Is Vital for KIF1Bβ Tumor Suppression**

RNA helicase A is vital for KIF1Bβ tumor suppression. This article discusses the role of RNA helicase A in the suppression of KIF1Bβ tumors and provides insights into the mechanisms underlying this effect. The study involves qRT-PCR analysis, in situ hybridization, and in vitro transcription. The authors also present a KIF1B exome sequencing analysis, which reveals significant findings.

**qRT-PCR Analysis**

qRT-PCR was performed on cDNA libraries previously created for RNA-seq using KAPA SYBR FAST Universal 2x qPCR Master Mix according to the manufacturer’s instructions. RNA was isolated from primary neuroblastoma tumors using the DNeasy Blood and Tissue Kit (Qiagen) and purified on an Agilent 2100 Bioanalyzer (Agilent Technologies).

**In Situ Hybridization**

In situ hybridization was performed using DIG RNA Labeling Kit (Roche). Oligonucleotides were designed to target the KIF1Bβ gene. Hybridization was performed at 70°C for 2 h, and detection was carried out using anti-DIG Fab fragments (Roche) and NBT/BCIP substrate (Roche).

**Mice**

C57BL/6J mice were necropsied at embryonic day 17.5 (E17.5) or P1, and were processed as described in the Materials and Methods section. The SCG was isolated from E17.5 and P1 mice and processed for immunohistochemistry and in situ hybridization.

**KIF1B Exome Sequencing**

Genomic DNA was isolated from primary neuroblastoma tumors and submitted for exome sequencing performed by Orogenetics. KIF1B exome mutation reports of primary neuroblastomas were based on custom target sequencing of human chromosome 1 from 10,270,764 to 10,441,661 using HiSeq2000 PE100 with minimum 80x coverage.

**Tumor Material**

All available neuroblastoma tumors were from the Swedish NB Registry. Tumors were staged according to the International Neuroblastoma Staging System (INSS; ref. 46) and INRG criteria (47). Ethical permission was granted by the local ethics committee. Tumor analysis has been previously described (48).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

Conception and design: Z.X. Chen, K. Wallis, P. Kogner, S. Schlisio

Development of methodology: Z.X. Chen, K. Wallis, V.R. Sobrado, S. Schlisio

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Z.X. Chen, K. Wallis, V.R. Sobrado, D. Ramsköld, U. Hellman, T. Martinson, J.I. Johnsen, P. Kogner, S. Schlisio

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Supervision of RNA-seq analyses: R. Sandberg

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**REFERENCES**


23. Chen et al.
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RNA Helicase A Is a Downstream Mediator of KIF1Bβ Tumor-Suppressor Function in Neuroblastoma

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