Interaction of Brn3a and HIPK2 mediates transcriptional repression of sensory neuron survival

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The Pit1-Oct1-Unc86 domain (POU domain) transcription factor Brn3a controls sensory neuron survival by regulating the expression of Trk receptors and members of the Bcl-2 family. Loss of Brn3a leads to a dramatic increase in apoptosis and severe loss of neurons in sensory ganglia. Although recent evidence suggests that Brn3a-mediated transcription can be modified by additional cofactors, the exact mechanisms are not known. Here, we report that homeodomain interacting protein kinase 2 (HIPK2) is a pro-apoptotic transcriptional cofactor that suppresses Brn3a-mediated gene expression. HIPK2 interacts with Brn3a, promotes Brn3a binding to DNA, but suppresses Brn3a-dependent transcription of *brn3a*, *trkA*, and *bcl-x*$_L$. Overexpression of HIPK2 induces apoptosis in cultured sensory neurons. Conversely, targeted deletion of HIPK2 leads to increased expression of Brn3a, TrkA, and Bcl-x$_L$, reduced apoptosis and increases in neuron numbers in the trigeminal ganglion. Together, these data indicate that HIPK2, through regulation of Brn3a-dependent gene expression, is a critical component in the transcriptional machinery that controls sensory neuron survival.

Introduction

Programmed cell death (apoptosis) is controlled by highly conserved mechanisms and plays important roles in the nervous system during development and in pathological conditions (Yuan and Yankner, 2000; Yuan et al., 2003). Among many effectors that contribute to apoptosis, signaling mechanisms transmitted through neurotrophin receptors regulate the decision between survival and cell death during the development of sensory neurons (Kaplan and Miller, 2000; Chao, 2003; Huang and Reichardt, 2003). Failure to acquire an adequate amount of neurotrophins leads to elimination of neurons through apoptosis. Interestingly, the expression of neurotrophin receptors follows a dynamic pattern during sensory neurogenesis. For instance, p75NTR is expressed in the migrating neural crest cells, but its expression is rapidly down-regulated in differentiated sensory neurons. In contrast, expression of TrkA, TrkB, and TrkC sharply increases in dorsal root and trigeminal ganglia when active neurogenesis occurs (Farinas et al., 1998; Huang et al., 1999a; Rifkin et al., 2000). Loss of neurotrophins or Trk receptors leads to a dramatic increase in apoptosis in selective population of sensory neurons (Farinas et al., 1998; Huang et al., 1999a).

In contrast to the well-characterized role of Trk receptors during sensory neuron development, much less is known about the transcriptional mechanisms that control the expression of Trk receptors. Emerging evidence indicates that the cis-regulatory element in the *trkA* gene is enriched with highly conserved DNA binding sequences that can potentially recruit transcription factors to regulate the spatial and temporal expression of TrkA (Ma et al., 2000). Indeed, analyses of mice with a targeted deletion in Pit1-Oct1-Unc86 domain (POU domain) transcription factor Brn3a shows that Brn3a is essential for survival of sensory neurons (McEvilly et al., 1996; Huang et al., 1999b). Loss of Brn3a results in a progressive down-regulation of TrkA expression, leading to a dramatic increase in apoptotic cell death (Huang et al., 1999b). Consistent with this, Brn3a binding sites have been identified in the *trkA* enhancer that are required to drive transgene expression in sensory ganglia (Ma et al., 2003). Together, these data provide

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Abbreviations used in this paper: EGFP-HIPK2, EGFP-tagged HIPK2; ES, embryonic stem; HDAC, histone deacetylase; HIPK2, homeodomain interacting protein kinase 2; HSV, herpes simplex virus; LacZ, β-galactosidase; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection; POU domain, Pit1-Oct1-Unc86 domain; qRT-PCR, quantitative RT-PCR.

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convincing evidence for a direct involvement of Brn3a in the regulation of TrkA expression.

In addition to regulating Trk expression, Brn3a has also been reported to regulate the expression of Bcl-2, Bcl-x_L, and Bax in vitro, providing another possible mechanism through which Brn3a mediates neuronal survival (Smith et al., 1998, 2001; Budhram-Mahadeo et al., 1999; Sugars et al., 2001). Targeted disruption of *bax* eliminates programmed cell death in the developing nervous system, resulting in an increased number of neurons in selected populations (Deckwerth et al., 1996; White et al., 1998). In contrast, deletion of *bcl-x_L* leads to global and excessive apoptotic cell death in the central and peripheral nervous systems (Motoyama et al., 1995). Despite the critical roles of Bcl-x_L and Bax in neuronal survival, however, the mechanisms by which Brn3a regulates their expression under physiological conditions have not been fully characterized. Brn3a responsive elements have been identified in the promoter sequences of *bcl-2*, *bcl-x_L*, and *bax* (Smith et al., 1998, 2001), yet it is unclear if expression of these Bcl-2 members is altered in the sensory ganglia of *Brn3a*$^{-/-}$ mutants.

Several lines of evidence indicate that additional cofactors may regulate Brn3a-dependent transcription. First, although Brn3a expression is detected in sensory ganglia as early as E9.5, cell death and down-regulation of Brn3a target genes are detected only after E13.5, suggesting that Brn3a may not be fully functional in early sensory ganglia (Fedtsova and Turner, 1995; McEvilly et al., 1996; Huang et al., 1999b). Second, *brn3a* enhancer element-regulated β-galactosidase (LacZ) expression is higher in the sensory ganglia of *Brn3a*$^{-/-}$ mutants than in wild-type mice, suggesting that Brn3a may negatively regulate its own expression directly or via recruitment of transcriptional corepressors (Trieu et al., 2003).

Here, we provide evidence that interaction of Brn3a and homeodomain interacting protein kinase 2 (HIPK2) regulates the transcription of a common set of prosurvival genes, including TrkA and Bcl-x_L. Whereas HIPK2 interacts with the POU homeodomain of Brn3a and promotes Brn3a binding to DNA, HIPK2 suppresses Brn3a auto-regulation and Brn3a target genes. As a consequence, overexpression of HIPK2 leads to neuronal apoptosis in cultured sensory neurons. Conversely, *HIPK2* null mutants show up-regulation of Brn3a and Brn3a target genes in the trigeminal ganglion, which leads to reduced apoptosis and increased neuron number in this sensory ganglion.

Results

Identification of HIPK2 as an interacting partner for Brn3a

The important roles of Brn3a in regulating its own expression and the expression of Trk receptors and members of the Bcl-2 family motivated us to investigate additional components that regulate Brn3a-mediated gene expression. Using the yeast two-hybrid screen and a cDNA library prepared from mouse E10-11 embryos (Hollenberg et al., 1995), we isolated several independent clones that interacted with full-length Brn3a, one of which encoded protein sequence between amino acids 752 and 897 of HIPK2, a Ser/Thr kinase that interacts with homeodomain transcription factors of the Nkx family (Fig. 1; Kim et al., 1998). Further characterizations showed that full-length

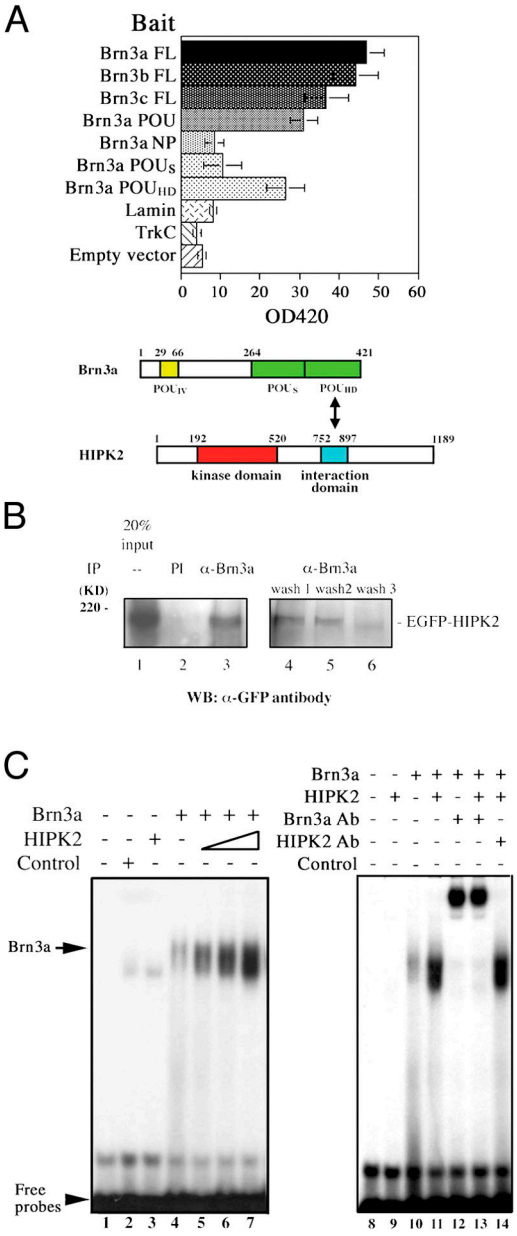

Figure 1. **HIPK2 interacts with Brn3a and promotes Brn3a binding to DNA elements.** (A) Isolation of HIPK2 by yeast two-hybrid screen. The domain between amino acids 752 and 897 of HIPK2 interacts with full-length Brn3a, Brn3b, and Brn3c. Interaction between Brn3a and HIPK2 is mediated by the POU homeodomain (Brn3a POU$_{HD}$), not the POU specific domain (POU$_S$) or non-POU domain (Brn3a NP). Data represent mean ± SEM (n = 3). A schematic diagram of Brn3a and HIPK2 interaction. (B) Brn3a and HIPK2 are present in a protein complex that can be coimmunoprecipitated with Brn3a antibody, but not preimmune sera (PI). Interaction between Brn3a and HIPK2 is reduced by increasing wash stringency. Whereas detergents NP-40 (1%; wash 1) and sodium deoxycholate (0.5%; wash 2) preserve Brn3a–HIPK2 interaction, the presence of ionic detergent SDS (0.1%; wash 3) significantly reduces such interaction. (C) Electrophoretic mobility shift assays for Brn3a and HIPK2. HIPK2 enhances Brn3a binding to consensus DNA element (b3s1). Routinely, 2 μl of in vitro translated Brn3a protein is added (lanes 4–7) and increasing amount of HIPK2 (1, 2, and 5 μl) is added to the reaction (lanes 5–7). Although Brn3a antibody produces a supershift, the addition of HIPK2 antibody does not (lanes 10–14).

HIPK2 also interacted with Brn3a (not depicted) and that HIPK2 interacted with the POU homeodomain (POU_{HD}) of Brn3a, but not POU specific (POU_S) or non-POU domain (Fig. 1, A and B). Not surprisingly, given the high sequence homology in the POU domain among members of the Brn3 family (>95%; Ryan and Rosenfeld, 1997), HIPK2 also interacted with Brn3b and Brn3c (Fig. 1 A).

To further investigate the interaction between Brn3a and HIPK2 in vivo, we performed coimmunoprecipitation in COS cells that expressed Brn3a and EGFP-tagged HIPK2 (EGFP-HIPK2). Cell lysates from COS cells were immunoprecipitated with Brn3a antibody and probed with anti-GFP antibody in Western blots. Our data showed that HIPK2 was present in protein complexes immunoprecipitated by Brn3a antibody, but not by preimmune serum (Fig. 1 B, lanes 2 and 3). Interestingly, the stability of Brn3a and HIPK2 protein complex appeared to be influenced by increasing wash stringency, as the addition of 0.1% SDS significantly reduced protein complex formation between Brn3a and HIPK2 (Fig. 1 B, lanes 4–6).

The effect of wash stringency on the protein complex formation between Brn3a and HIPK2 suggested that the interaction of these two proteins might be transient or require additional components, similar to the interaction between HIPK2 and homeodomain transcription factors in the Nkx family (Kim et al., 1998; Choi et al., 1999). To further characterize this interaction, we determined the effects of HIPK2 on Brn3a binding to the consensus DNA sequence b3s1 (Trieu et al., 1999). Using in vitro translation products of full-length Brn3a and HIPK2 proteins, we showed that Brn3a formed a stable protein complex with this sequence (Fig. 1 C, lane 4). HIPK2 enhanced Brn3a binding, but caused no further shifting in the Brn3a–DNA complex (Fig. 1 C, lanes 5–7). Furthermore, whereas addition of a Brn3a antibody produced a supershift of the complex (Fig. 1 C, lanes 12 and 13), addition of a polyclonal antibody that recognized the COOH terminus of HIPK2 did not (Fig. 1 C, lane 14). These results were consistent with those from coimmunoprecipitation and again suggested that the interaction between Brn3a and HIPK2 may be transient or may require additional factors (Fig. 1 B). However, we cannot completely rule out the possibility that the HIPK2 antibody may not be fully capable of recognizing Brn3a–HIPK2 complex due to steric hindrance.

HIPK2 suppresses Brn3a auto-regulatory activity and expression of Brn3a downstream targets

The pro-survival effect of Brn3a depends on its auto-regulatory activity and the ability of Brn3a to up-regulate several prosurvival genes, including Trk receptors and members of the Bcl-2 family (Huang et al., 1999b; Smith et al., 2001; Ma et al., 2003; Trieu et al., 2003). Indeed, the mRNA levels of TrkA and Bcl-x_L, as determined by quantitative RT-PCR (qRT-PCR) assays, showed progressive down-regulation in the trigeminal ganglia of *Brn3a*$^{-/-}$ mutants at E13.5 and E14.5, preceding the drastic increase in cell death at E15.5 to E17.5 (Fig. 2 A; Huang et al., 1999b). In contrast, expression of Bcl-2 and Bax in the trigeminal ganglion of *Brn3a*$^{-/-}$ mutants at the same stages remained unchanged (Fig. 2 A). Consistent with the qRT-PCR results, protein levels of TrkA and Bcl-x_L showed significant reductions in the trigeminal ganglion of *Brn3a*$^{-/-}$ mutants at E15.5, whereas Bax remained unchanged (Fig. 2 B). To determine if the effect of HIPK2 on Brn3a-mediated gene expression was direct, we used luciferase constructs that contained three synthetic tandem repeats of Brn3a binding sites identified in the *brn3a* enhancer element (Prox3), which has been shown to be a reliable reporter for Brn3a activity. Consistent with previous data (Trieu et al., 1999), Brn3a activated the Prox3 reporter in a dose-dependent fashion, whereas HIPK2 by itself did not affect the baseline level of Prox3 luciferase activity (Fig. 2 C). Interestingly, addition of HIPK2 suppressed Brn3a-mediated activation of Prox3 (Fig. 2 C). To determine if expression of HIPK2 in sensory neurons leads to down-regulation of Brn3a in a more physiological setting, we used herpes virus vectors to introduce EGFP-HIPK2 into cultured sensory neurons (Coopersmith and Neve, 1999), achieving almost 100% infection efficiency at 50–100 multiplicity of infection (MOI). Subsequent to infection, total RNA was extracted and mRNA converted to cDNA. qRT-PCR assays were used to measure Brn3a mRNA levels, which were standardized to the endogenous GAPDH level. Our results indicated that, expression of EGFP-HIPK2 leads to a progressive down-regulation of Brn3a mRNA in sensory neurons. By 36 h after infection, Brn3a mRNA was reduced to <40% of that in control neurons infected with LacZ or EGFP virus ($P < 0.01$; Fig. 2 D).

The suppressive effect of HIPK2 on Brn3a suggests that HIPK2 may inhibit Brn3a auto-regulation, which may in turn lead to down-regulation of Brn3a target genes. However, it is equally possible that HIPK2 may directly suppress the ability of Brn3a to activate its targets. To test latter hypothesis, luciferase constructs containing *trkA* (two tandem repeats of 5′ Brn3a binding site) or *bcl-x$_L$* (a single Brn3a binding site) enhancer sequences were used (Smith et al., 2001; Ma et al., 2003). Similar to the Prox3 reporter, Brn3a significantly up-regulated TrkA luciferase activity, whereas HIPK2 suppressed the ability of Brn3a to activate TrkA luciferase activity (Fig. 2 E). Brn3a also activated Bcl-x_L luciferase activity and the addition of HIPK2 suppressed Brn3a-mediated activation of Bcl-x_L luciferase (Fig. 2 G). Consistent with the luciferase data, addition of Brn3a to cultured sensory neurons up-regulated the expression of TrkA and Bcl-x_L mRNA by >12- and 3-fold, respectively ($P < 0.01$, $n = 3$; Fig. 2, F and G). In contrast, expression of HIPK2 in sensory neurons reduced TrkA mRNA to ~7% and Bcl-x_L mRNA level to ~20% of control EGFP-expressing neurons (Fig. 2, F and H). Together, these data support the notion that HIPK2 is a transcriptional corepressor that can suppress the expression of Brn3a and Brn3a downstream targets TrkA and Bcl-x_L in sensory neurons.

HIPK2 induces apoptosis in sensory neurons

In previous work, we showed that loss of Brn3a led to a dramatic loss of trigeminal neurons due to increased apoptosis (Huang et al., 1999b). These results lead to the prediction that suppression of Brn3a by HIPK2 should mimic Brn3a loss-of-function phenotype and result in neuronal apoptosis. To test this

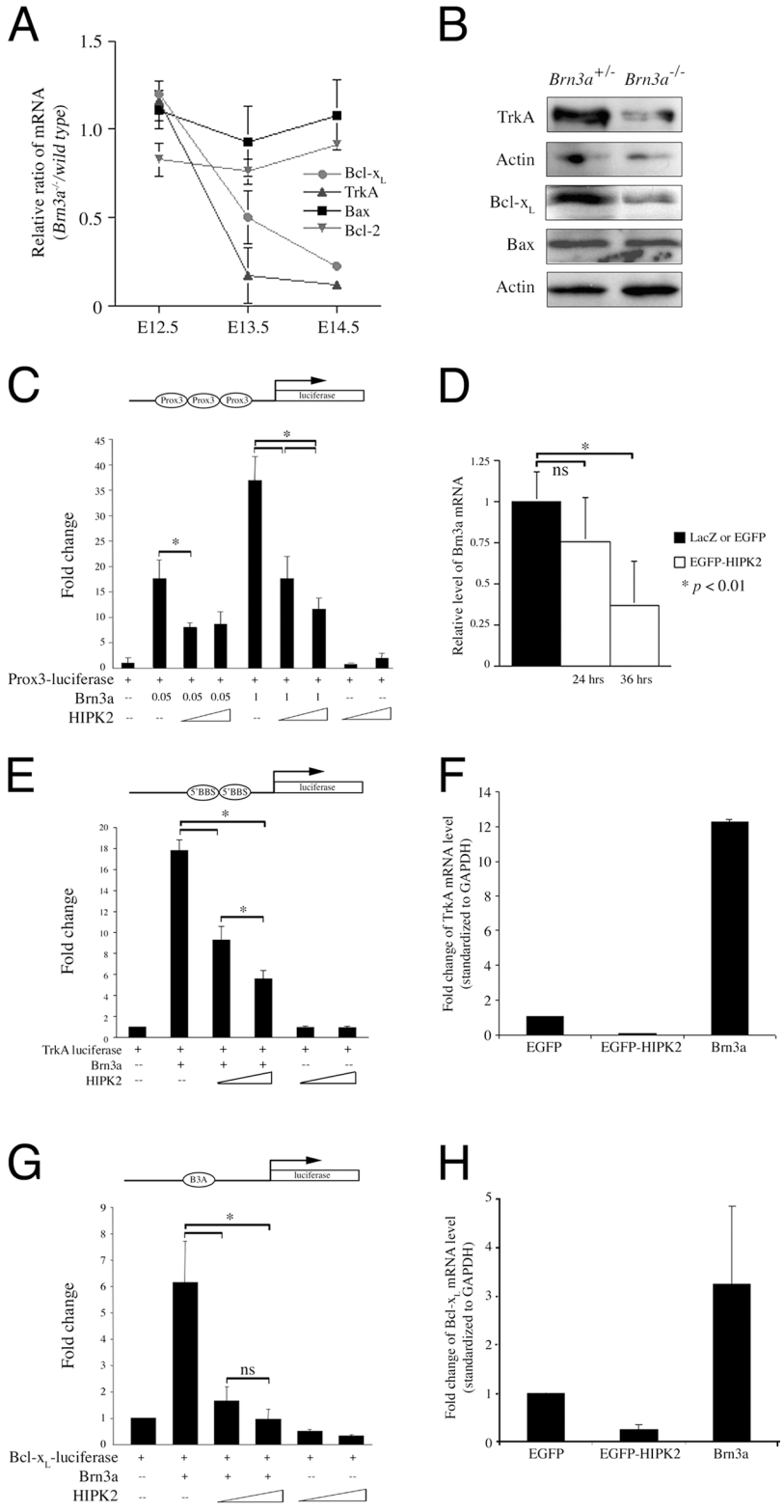

Figure 2. **HIPK2 suppresses Brn3a auto-regulatory activity and Brn3a downstream target genes.** (A) Progressive down-regulation of TrkA and Bcl-x_L, but not Bcl-2 or Bax, in trigeminal ganglion of *Brn3a*$^{-/-}$ mutants. The mRNA levels of TrkA, Bcl-2, Bcl-x_L and Bax are determined in the trigeminal ganglion of wild-type and *Brn3a*$^{-/-}$ embryos from E12.5 to14.5 using qRT-PCR assays. Although the expression levels of these genes in *Brn3a*$^{-/-}$ are comparable to those in wild type at E12.5, mRNA levels of TrkA and Bcl-x_L show significant down-regulation at E13.5. By E14.5, <20% of TrkA and Bcl-x_L mRNAs are detected in the trigeminal ganglion of *Brn3a*$^{-/-}$. In contrast, mRNA levels of Bcl-2 and Bax in *Brn3a*$^{-/-}$ are comparable to those in wild type at the same stages. Data represent mean ± SEM (n = 3). (B) Consistent with the qRT-PCR results, the amounts of TrkA and Bcl-x_L protein show significant reductions in the trigeminal ganglion of *Brn3a*$^{-/-}$ at E15.5. In contrast, no reduction in Bax is detected. (C) HIPK2 inhibits Brn3a-mediated activation of luciferase construct Prox3, which contains synthetic DNA binding elements from *brn3a* enhancer. The luciferase activities of Prox3 are presented as mean ± SEM (n = 3, * indicates $P < 0.01$, t test). The amounts of DNA added to each reaction are indicated below each graph. All luciferase activities are normalized to Renilla luciferase reporter activity. (D) Consistent with its ability to suppress the luciferase activity of Prox3, expression of EGFP-HIPK2 in cultured trigeminal neurons using HSV leads to a progressive down-regulation of Brn3a mRNA. In contrast, expression of LacZ or EGFP in neurons has no effect on Brn3a mRNA level. Data represent mean ± SEM (n = 3, ns indicates no significant difference and * indicates $P < 0.01$, t test). (E) HIPK2 inhibits its Brn3a-mediated activation of the TrkA luciferase activity. TrkA luciferase construct contains two Brn3a binding sites identified in the 5′-end of *trkA* promoter (Ma et al., 2003). TrkA luciferase activities, measured 24 h after transfection, are expressed as fold change compared with control. Values are expressed as mean ± SEM (n = 3). * Indicates $P < 0.01$ using t test. (F) HIPK2 down-regulates TrkA mRNA level to <10% of that in neurons expressing EGFP. In contrast, Brn3a up-regulates the mRNA of TrkA in cultured sensory neurons by at least 12-fold. Changes in the level of TrkA mRNA are determined using qRT-PCR assays and standardized to GAPDH. Neurons are collected for RNA extraction 48 h after infection. qRT-PCR assays are done in triplicates and at least three samples from each treatment are examined. Data represent mean ± SEM. (G) HIPK2 also inhibits Brn3a-mediated activation of Bcl-x_L luciferase activity. Data represent mean 6 SEM (n = 3, * indicates $P < 0.01$, t test). (H) Expression of EGFP-HIPK2 down-regulates the endogenous level of Bcl-x_L mRNA in cultured sensory neurons to <20% of that in neurons expressing EGFP. In contrast, expression of Brn3a increases Bcl-x_L mRNA by more than threefold. Data represent mean ± SEM (n = 3).

hypothesis, a gain-of-function approach was used to determine the effect of HIPK2 on the survival of cultured sensory neurons. Although herpes simplex virus (HSV)–mediated expression of EGFP did not affect the survival of sensory neurons (Fig. 3, A–D), expression of EGFP-HIPK2 induced apoptosis, indicated by positive TUNEL staining, in a dose-dependent manner (Fig. 3, E–I). Ectopically expressed EGFP-HIPK2 could be detected either diffusely in the nucleus of trigeminal neurons or in structures resembling nuclear bodies (Fig. 3 E, inset). Neurons expressing EGFP-HIPK2 showed TUNEL-positive staining as early as 24 h after infection (Fig. 3, E–H, and J), by 96 h >80% of these neurons were dead as assessed by morphological criteria (Fig. 3 J). In contrast, expression of EGFP had no effect on survival of infected neurons (Fig. 3, I–J). To determine if the

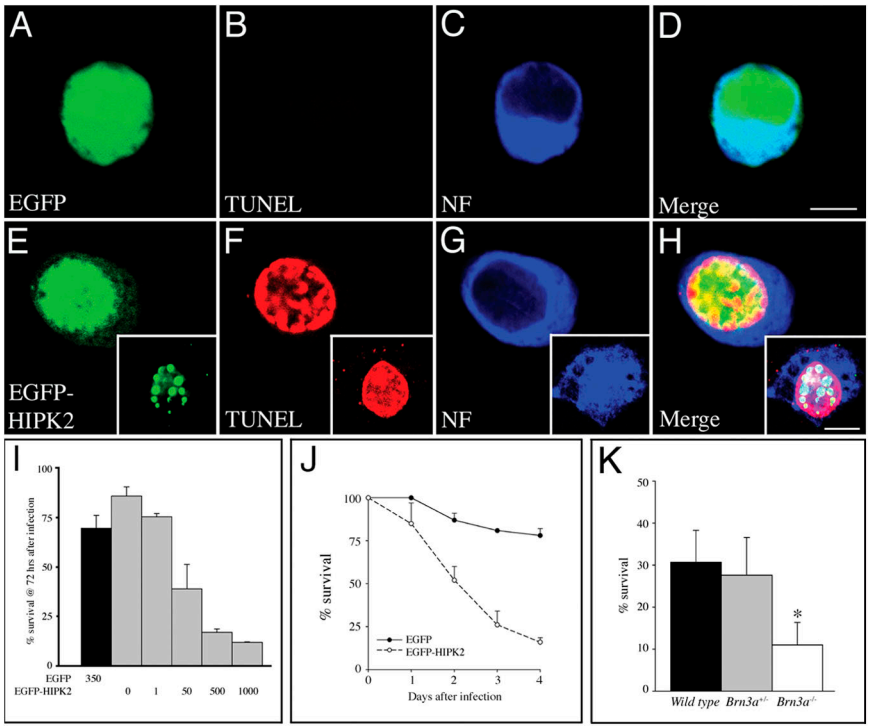

Figure 3. **HIPK2 induces neuronal apoptosis.** (A–H) Although EGFP has no effect on the survival of cultured trigeminal neurons (A–D), EGFP-HIPK2 induces apoptosis (highlighted by TUNEL stain) (E–H). The distribution of EGFP-HIPK2 varies in neurons, with some showing diffuse localization in the nucleus and others in structures similar to nuclear bodies (E, inset). (I and J) HIPK2-induced apoptosis is dose dependent. More than 50% of neurons die at 72 after the addition of 50 MOI of EGFP-HIPK2 HSV, whereas neurons infected by EGFP at 350 MOI have only slight reduction in survival (I). Neurons expressing EGFP-HIPK2 undergo apoptosis over a period of 3–4 d. By the fourth day, >70% of neurons expressing EGFP-HIPK2 show morphological features consistent with cell death, whereas >80% of neurons expressing EGFP continue to survive (J). Dosage of HSV used in J is 100 MOI for both EGFP and EGFP-HIPK2. All data represent mean ± SEM ($n = 3$). (K) Compared with *wild type* or *Brn3a*$^{+/-}$, overexpression of HIPK2 induces significantly more apoptosis in *Brn3a*$^{-/-}$ trigeminal neurons (*wild type*, $n = 4$, *Brn3a*$^{+/-}$, $n = 10$, and *Brn3a*$^{-/-}$, $n = 8$, *t* test, * indicates $P < 0.05$).

pro-apoptotic function of HIPK2 was solely dependent on its interaction with Brn3a, we examined the effect of HIPK2 expression in sensory neurons of *Brn3a*$^{-/-}$ mutants. One would predict that if HIPK2 function depends exclusively on its ability to suppress Brn3a, HIPK2-induced apoptosis should be attenuated in *Brn3a*$^{-/-}$ neurons. Alternatively, if HIPK2 continues to induce apoptosis in these neurons, it is likely that HIPK2 may also activate additional Brn3a-independent cell death mechanisms. Our results indicated that, similar to the data in J, ~30% of wild-type and *Brn3a*$^{+/-}$ neurons survived at 72 h after infection with HSV-HIPK2 (Fig. 3 K). However, significantly fewer (~10%) neurons from *Brn3a*$^{-/-}$ mutants survived under the same treatment (Fig. 3 K). These results support the notion that HIPK2 and Brn3a antagonize each other and the interaction between these two regulates a delicate balance of gene expression critical for neuronal survival. They also suggest that overexpression of HIPK2 in the absence of Brn3a can lead to significantly more apoptosis, probably due to activation of Brn3a-independent cell death pathway(s) (D'Orazi et al., 2002; Hofmann et al., 2002; Zhang et al., 2003).

Expression of HIPK2 during sensory neurogenesis

To begin to understand the in vivo function of HIPK2, especially in the context of sensory neuron survival during development, we investigated the expression pattern of HIPK2. In situ hybridization using an RNA probe derived from the 39 UTR indicated that HIPK2 mRNA was present in the developing sensory ganglia, including the trigeminal, vestibulocochlear, nodose-petrosal, and dorsal root ganglia (Fig. 4 A). HIPK2 mRNA was also detected in mid-brain/hindbrain regions and in nonneural tissues, such as liver, heart, and kidney (Fig. 4 A and not depicted). Using an antibody that recognized the COOH terminus of HIPK2, HIPK2 was detected in the developing trigeminal and dorsal root ganglia at E12.5 (Fig. 4, C, F, and G). In contrast to the more restricted expression of Brn3a, HIPK2 protein was also detected in the developing spinal cord (Fig. 4, B and C). Interestingly, whereas Brn3a was confined to the nuclei, the distribution of HIPK2 appeared to be more heterogeneous. At E12.5, some neurons showed dense nuclear staining, whereas the staining in others was more diffuse (Fig. 4, F and G, arrows). The presence of HIPK2 protein in the neuronal cytoplasm appeared to be more distinct at postnatal stages, suggesting that the subcellular localization of HIPK2 may change at different stages of neuronal development (not depicted).

Targeted deletion of *HIPK2* gene

The suppressive effects of HIPK2 in vitro and the coexpression of Brn3a and HIPK2 in sensory ganglia suggest that both may be important components in the transcriptional mechanism that controls sensory neuron survival and cell death by regulating a common set of prosurvival genes. To test this hypothesis, we deleted *HIPK2* in embryonic stem (ES) cells. The mouse *HIPK2* locus encompasses ~150 kb and contains 16 exons (unpublished data). We targeted exon 3, which encodes the first ATG and the entire HIPK2 kinase domain (from amino acids 192 to 520). The majority of exon 3 was replaced with the reporter gene *LacZ*, followed by a polyadenylation signal sequence and the selection marker *neomycin* gene. Four positive ES clones were identified by Southern blot analysis using specific 5′ and 3′ probes, three were injected into blastocysts (Fig. 5, A and B). Germline transmission of the targeted allele was confirmed by Southern blot analyses (Fig. 5 C). Northern blot analysis using a probe that hybridized with a sequence in the exons 3 and 4 confirmed that HIPK2 mRNA was undetectable in mouse embryonic fibroblasts (MEFs) derived from homozygous *HIPK2* (*HIPK2*$^{-/-}$) mutants

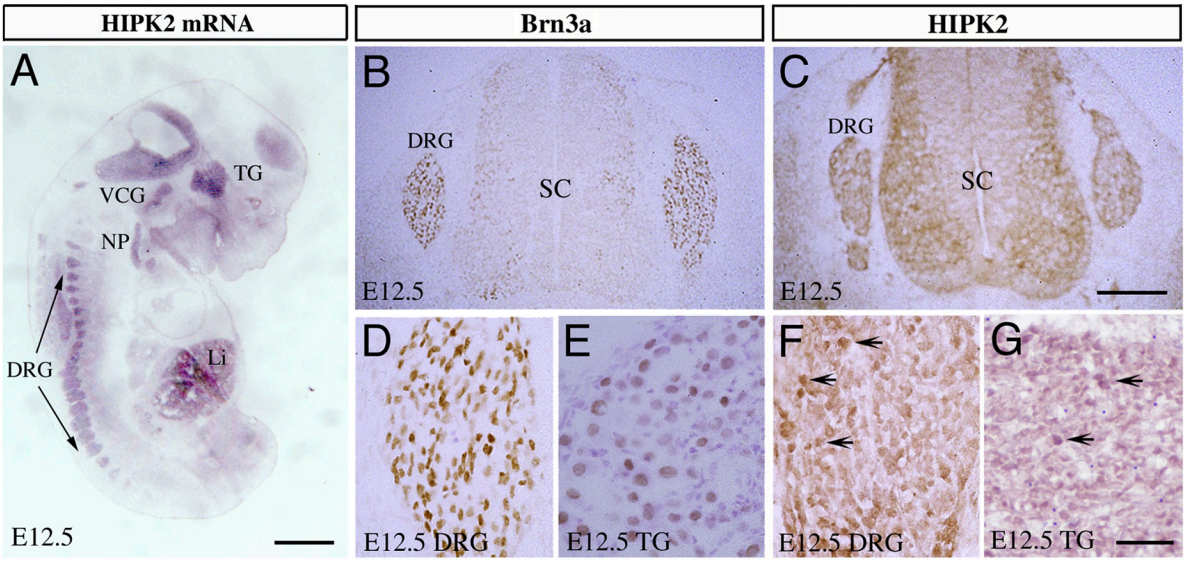

Figure 4. **Expression of HIPK2 in the developing sensory nervous system.** (A) HIPK2 mRNA is present in the sensory ganglia, including trigeminal ganglion (TG), vestibulocochlear ganglion (VCG), nodose-petrosal ganglion (NP), and the dorsal root ganglia (DRG) at E12.5. Li, liver. (B–G) Antibodies detect Brn3a and HIPK2 proteins in the DRG (B–D, and F) and TG (E and G) at E12.5. The staining intensity of HIPK2 appears to vary among neurons at E12.5 with a few showing more intense HIPK2 staining (F and G, arrows). Bars: (A) 500 μm; (B and C) 200 μm; (D–G) 50 μm.

(Fig. 5 D). The absence of HIPK2 transcripts in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants was further confirmed by RT-PCR using primers that detected sequences in exon 3 and in exons 15–16 of HIPK2 (Fig. 5 E). The insertion of LacZ in–frame with the coding sequence of HIPK2 (HIPK2^{LacZ}) provided an excellent lineage tracer for cells expressing HIPK2. Consistent with the in situ hybridization and immunostaining results (Fig. 4), significant HIPK2^{LacZ} staining intensity was detected in the developing sensory ganglia, including the trigeminal and dorsal root ganglia, diencephalon, and spinal cord at E13.5 (Fig. 5, F and G).

Loss of HIPK2 results in up-regulation of Brn3a and Brn3a target genes

The coexpression of Brn3a and HIPK2 in sensory ganglion and the suppressive effects of HIPK2 on Brn3a-mediated gene expression suggest that loss of HIPK2 could lead to increases in

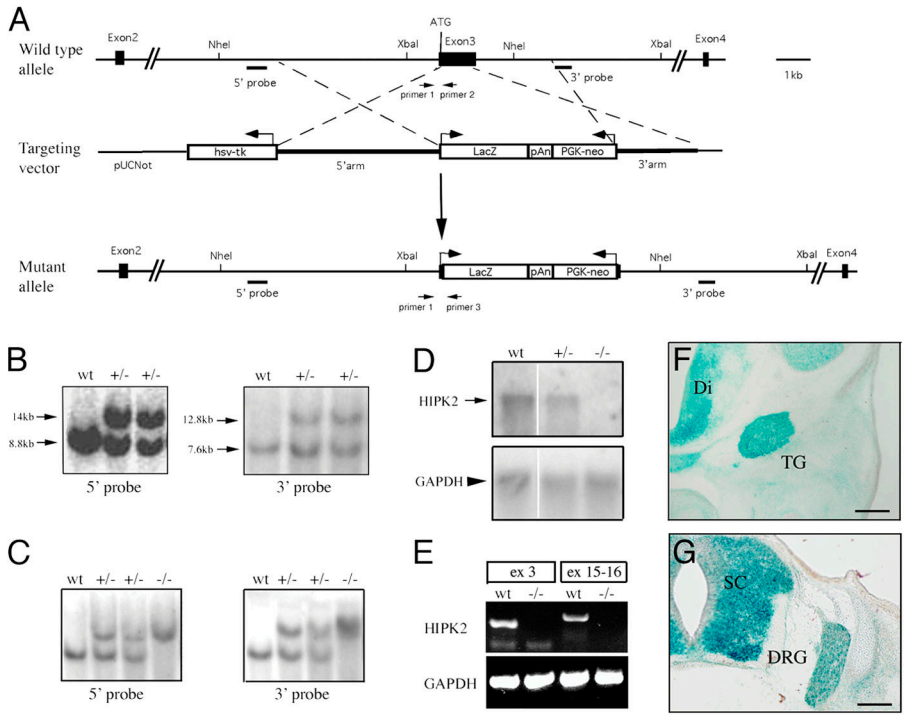

Figure 5. **Targeted deletion of HIPK2 and generation of *HIPK2* null mice.** (A) Homologous recombination with the targeting construct deletes exon 3 (1st coding exon), and simultaneously inserts *LacZ* and a selection marker *neomycin* (PGK-*neo*) gene in-frame with the first ATG of HIPK2. Arrows indicate the orientation of PGK-*neo*, *thymidine kinase* (hsv-tk) and *LacZ* genes. Primers 1–3 are used for genotyping. (B) Genomic DNA from G418-resistant ES colonies was screened by Southern blots using 5′ and 3′ probes indicated in A. (C) Southern blot genotyping of wild type (wt), *HIPK2*$^{+/-}$ (+/−), and *HIPK2*$^{-/-}$ (−/−) progeny using the same probes. (D) Total RNA from MEFs derived from wild type, *HIPK2*$^{+/-}$, and *HIPK2*$^{-/-}$ mutants was probed with a cDNA fragment containing 5′ UTR and first coding exon of HIPK2. Level of HIPK2 expression is reduced in *HIPK2*$^{+/-}$ mutants and is completely absent in *HIPK2*$^{-/-}$ mutants. (E) mRNA from trigeminal ganglia was amplified by RT-PCR using primers for the first coding exon (exon 3) of HIPK2 and the last two exons (exon 15–16). Consistent with results from the Northern blot, HIPK2 expression is not detected in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants. (F and G) The presence of LacZ in HIPK2 locus provides a convenient tool to detect HIPK2 expression in the developing trigeminal ganglion (F) and dorsal root ganglion (G) at E13.5. Bars: (F) 250 μm; (G) 100 μm.

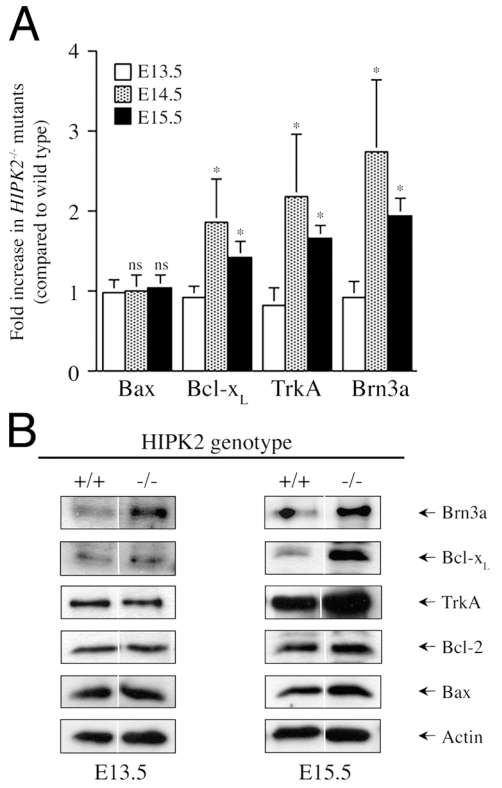

Figure 6. **Stage-specific up-regulation of Brn3a, TrkA, and Bcl-x_L in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants.** (A) The mRNA levels of Brn3a, TrkA, Bcl-x_L, and Bax in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants and wild-type littermates are determined using qRT-PCR assays. No alteration in the mRNA level is detected at E13.5. In contrast, significant increases in the mRNA levels of Brn3a, TrkA, and Bcl-x_L are present at E14.5 and E15.5. The level of Bax mRNA level remains unchanged from E13.5 to E15.5. The numbers of samples examined for each age group are: E13.5, $n = 3$; E14.5, $n = 3$; and E15.5, $n = 5$. All data represent mean ± SEM. ns indicates no significant difference and * indicates $P < 0.01$ (*t* test). (B) Western blot and quantitative analyses of protein expression in trigeminal ganglion of *HIPK2*$^{-/-}$ mutants and wild-type littermates. At E13.5, only Brn3a protein shows a modest increase in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants, whereas the protein levels of TrkA, Bcl-x_L, Bcl-2, and Bax remain unchanged. In contrast, the protein levels of Brn3a, TrkA, and Bcl-x_L increase by 2.6-, 1.6-, and 1.7-fold in *HIPK2*$^{-/-}$ mutant trigeminal ganglion at E15.5, whereas the levels of Bax and Bcl-2 show no change.

the expression of Brn3a and its downstream targets. To test this hypothesis, we used qRT-PCR assays to determine the mRNA levels of Brn3a, TrkA, Bcl-x_L, and Bax in trigeminal ganglion from individual wild-type and *HIPK2*$^{-/-}$ embryos collected at E13.5, 14.5, and 15.5. Loss of HIPK2 resulted in a significant increase in Brn3a, TrkA, and Bcl-x_L mRNA at E14.5 and E15.5, with no significant change at E13.5. Specifically, the level of Brn3a mRNA increased by 2.7-fold at E14.5 and two-fold at E15.5 (Fig. 6 A; $P < 0.01$, *t* test). The levels of TrkA and Bcl-x_L mRNA also showed significant, albeit smaller increases at E14.5 and E15.5 (Fig. 6 A). In contrast, the level of Bax mRNA remained unchanged from E13.5 to E15.5. Although Brn3a mRNA showed no significant increase at E13.5, Brn3a protein was modestly increased in some *HIPK2*$^{-/-}$ mutants at E13.5 (~1.2-fold), suggesting that HIPK2 may also regulate Brn3a protein stability at this stage (Fig. 6 B). At E15.5, the protein levels of Brn3a, TrkA, and Bcl-x_L proteins showed consistent increases by 2.6-, 1.6-, and 1.7-fold, respectively (Fig. 6 B). In contrast, levels of Bcl-2 and Bax proteins were not altered at any time point examined. Together, increases in the mRNA and proteins of Brn3a, TrkA, and Bcl-x_L in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants suggest that HIPK2 negatively regulates Brn3a-mediated gene expression in vivo.

Reduced apoptosis and increased neuron number in trigeminal ganglion of *HIPK2*$^{-/-}$ mutants

Previous data have indicated that loss of Brn3a, TrkA, or Bcl-x_L results in prominent cell death and dramatic loss of neurons in developing sensory ganglia (Motoyama et al., 1995; Huang et al., 1999a,b). Hence, the observed increase in the expression of Brn3a, TrkA, and Bcl-x_L in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants should create a condition rendering *HIPK2*$^{-/-}$ sensory neurons more resistant to apoptosis during development (Ensor et al., 2001). To test this possibility, we determined the number of neurons undergoing apoptosis and the total number of neurons in the trigeminal ganglion of wild-type and *HIPK2*$^{-/-}$ mutants at different developmental stages. In the wild-type trigeminal ganglion, apoptotic profiles were detected as early as E10.5 and reached a maximum between E11.5 to E13.5, followed by a progressive decline at E15.5 and P0 (Fig. 7, A, C, and E; Huang et al., 1999b). Most of the apoptotic profiles stained positive for neurofilament, indicating that they are indeed neurons (Fig. 7 A, arrowheads). Whereas the number of apoptotic neurons was similar between wild type and *HIPK2*$^{-/-}$ at E10.5 and E11.5, there was significantly reduced apoptosis in the mutant ganglion at E13.5, E15.5, and P0 (Fig. 7, B, C, and E; 50–60% reduction, $P < 0.01$; *t* test). In agreement with this, there were significantly more neurons in the *HIPK2*$^{-/-}$ ganglion at E13.5 and P0 ($0.01 < P < 0.025$ at E13.5 and $P < 0.01$ at P0, $n = 4$, *t* test). Thus, these data indicate that loss of HIPK2 leads to up-regulation of Brn3a and its target genes and reduces apoptotic cell death during the development of sensory ganglia.

Discussion

Maintenance of neuronal survival depends on a balanced expression of prosurvival and proapoptotic genes. Results from this work demonstrate that transcriptional corepressor HIPK2 negatively regulates Brn3a-mediated neuronal survival in the developing sensory ganglia. The mechanism by which HIPK2 regulates Brn3a is mediated through protein–protein interaction, which suppresses the Brn3a-dependent transcription of *brn3a*, *trkA*, and *bcl-x_L* loci. As a consequence, overexpression of HIPK2 mimics the phenotype of the *Brn3a*$^{-/-}$ mutants, resulting in apoptotic cell death in cultured sensory neurons. Conversely, loss of HIPK2 leads to a stage-specific up-regulation of Brn3a and Brn3a target genes, reduced apoptosis, and increased neuron number in the trigeminal ganglion in vivo. Together, these data support the model that interaction between Brn3a and HIPK2 regulates gene expression, apoptosis, and neuronal survival during trigeminal ganglion development (Fig. 8 A). Due to their important roles in regu-

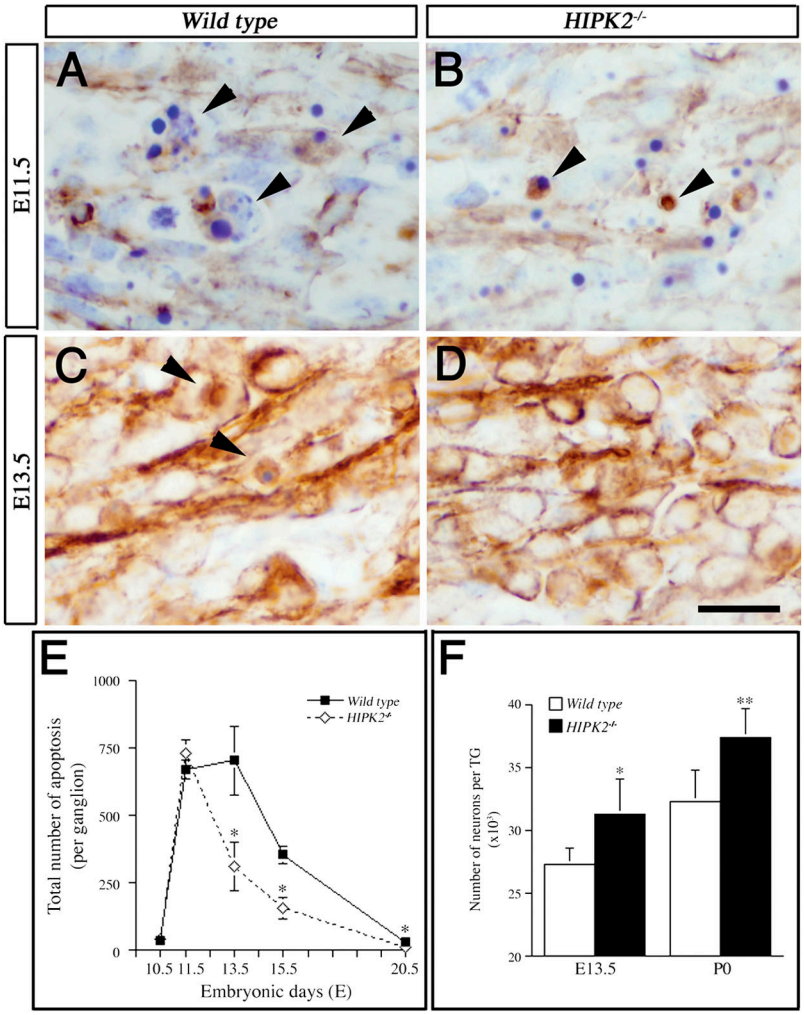

Figure 7. **Reduced apoptosis and increased neuron numbers in the developing trigeminal ganglion of *HIPK2*$^{-/-}$ mutants.** (A–D) At E11.5, the number of neurons undergoing apoptosis is similar in the trigeminal ganglion of wild-type (A) and *HIPK2*$^{-/-}$ mutants (B; arrowheads highlight apoptotic profiles that are neurofilament positive). In contrast, neuronal apoptosis in the trigeminal ganglion is significantly reduced in *HIPK2*$^{-/-}$ mutants at E13.5 (C and D). Bar, 20 μm. (E) Quantification of neuronal apoptosis in the trigeminal ganglion of wild-type and *HIPK2*$^{-/-}$ mutants from E10.5 to postnatal day 0 (P0). Although no difference is detected at E10.5 and E11.5, trigeminal ganglion in *HIPK2*$^{-/-}$ mutant show much reduced apoptosis at E13.5, E15.5 and P0 ($P < 0.01$, *t* test). (F) Number of neurons, as assessed by neurofilament-positive cells, is increased in *HIPK2*$^{-/-}$ mutants at E13.5 ($0.01 < P < 0.025$) and at P0 ($P < 0.01$, *t* test). Data represent mean ± SEM ($n = 4$).

lating the expression of TrkA and Bcl-x_L, loss of Brn3a or HIPK2 leads, respectively, to either increased or reduced programmed cell death (Fig. 8 B).

It is well-established that a cascade of transcriptional factors controls the specification of cell fate and determination of cell survival and differentiation during the development of sensory ganglia (Anderson, 1999). For instance, expression of the basic helix-loop-helix factors Ngn1 and Ngn2 promotes neuronal fate in cranial and spinal sensory ganglia (Ma et al., 1998, 1999). Indeed, ectopic expression of Ngn1 results in ectopic Brn3a and TrkA expression in mesenchymal cells (Greenwood et al., 1999). In contrast, characterization of *Brn3a*$^{-/-}$ mutants indicates that Brn3a is essential for survival and differentiation of sensory neurons, but not neuronal cell fate specification (Huang et al., 1999b, 2001; McEvilly et al., 1996; Xiang et al., 1996; Eng et al., 2001). In the trigeminal ganglion of *Brn3a*$^{-/-}$ mutants, >70% of the neurons undergo apoptotic cell death between E15.5 and E17.5 (Fig. 8 B). Mechanisms of apoptotic cell death in *Brn3a*$^{-/-}$ mutant sensory ganglia involve disruption of neurotrophin signaling as a result of a progressive down-regulation of TrkA (Fig. 2; Huang et al., 1999b; Ma et al., 2003). In addition, the current work also demonstrates that Brn3a is required for the maintenance of Bcl-x_L in vivo with a time course similar to that of TrkA (Fig. 2).

Recent analyses of cis-regulatory elements in *trkA*, *brn3a*, and *bcl-x_L* extend these in vivo findings and provide convincing data for a direct involvement of Brn3a in transcriptional control of these target genes (Trieu et al., 1999; Ma et al., 2000, 2003; Smith et al., 2001). Mutations in Brn3a-binding sites abolish the ability of the *trkA* enhancer to drive transgene expression in sensory neurons (Ma et al., 2003). Similar to the *trkA* enhancer, multiple Brn3a binding sites have also been identified in the *brn3a* enhancer (Trieu et al., 1999). Brn3a activates luciferase constructs containing these binding sites, consistent with the notion that Brn3a positively regulates its own expression through a feed forward mechanism (Fig. 2 and Fig. 8 A; Trieu et al., 1999). Interestingly, all the Brn3a binding sites are located several kilobases upstream from the transcriptional initiation site, suggesting that a long-range control through the recruitment of multiple transcriptional cofactors may regulate Brn3a expression. Indeed, analyses of Brn3a autoregulation in transgenic mice expressing *LacZ* under the control of *brn3a* enhancer indicate that, rather than activating, Brn3a suppresses its own expression at the early stages of sensory ganglion development (Trieu et al., 2003). These observations have led to the hypotheses that either Brn3a suppresses its own expression or a yet unidentified transcriptional corepressor may inhibit Brn3a functions in a stage-dependent manner

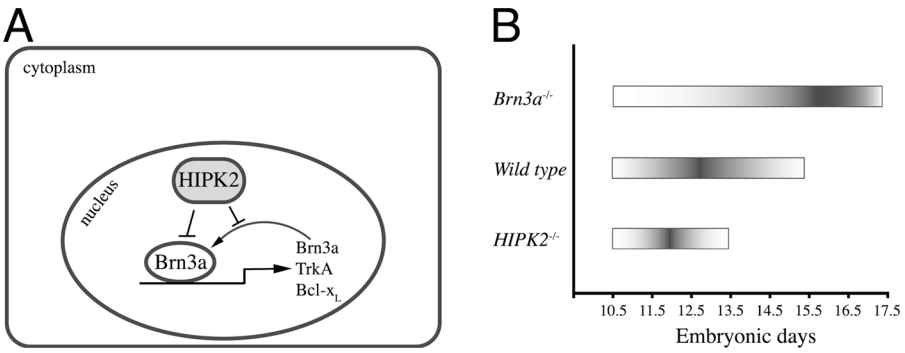

Figure 8. **Proposed model for Brn3a and HIPK2 in transcriptional control of gene expression in sensory neurons.** (A) HIPK2 is a transcriptional corepressor for Brn3a-mediated gene expression. HIPK2 interacts with Brn3a and suppresses Brn3a auto-regulatory activity and Brn3a downstream targets, including TrkA and Bcl-x_L. (B) In control (wild type) trigeminal ganglion, programmed cell death can be detected from E10.5 to E15.5, and reaches its maximum at E11.5 and E13.5. Due to the opposing effects of Brn3a and HIPK2 in regulating the expression of TrkA and Bcl-x_L, loss of Brn3a leads to increase in neuronal apoptosis and expansion of the period of programmed cell death in trigeminal ganglion at E15.5 and E17.5. In contrast, loss of HIPK2 leads to reduced apoptosis at E13.5 and E15.5.

(Trieu et al., 2003). Data from this current work favor the latter hypothesis and indicate that HIPK2 is a candidate corepressor that suppresses Brn3a auto-regulation and Brn3a target genes. Consistent with this model, removal of HIPK2 results in up-regulation of Brn3a, TrkA, and Bcl-x_L, a reduction in apoptosis during the period of programmed cell death, and a slight increase in neuron numbers during the development of trigeminal ganglion (Figs. 6 and 7).

A diversity of molecular mechanisms contributes to the execution of cell death through the canonical and noncanonical pathways (Yuan et al., 2003). The extensive cell death in the sensory ganglia of *trkA*$^{-/-}$ and *bcl-x*$_L^{-/-}$ mutants coincides with the period of programmed cell death in these ganglia and underscores the important roles of these two molecules in the canonical cell death pathway (Motoyama et al., 1995; Huang et al., 1999a; Yuan et al., 2003). The fact that Brn3a regulates the expression of TrkA and Bcl-x_L indicates that Brn3a-mediated transcriptional regulation is an integral regulatory component of the cell death process. Loss of Brn3a leads to down-regulation of TrkA and Bcl-x_L, which precedes and most definitively contributes to the dramatic increase in cell death observed in the trigeminal ganglia of *Brn3a*$^{-/-}$ mutants (Huang et al., 1999b; Ma et al., 2003). The reduced apoptosis observed in the trigeminal ganglion of *HIPK2*$^{-/-}$ mutants provides supporting evidence that the interaction between Brn3a and HIPK2 regulates a delicate balance of gene expression that is critical for cell death. Interestingly, however, loss of HIPK2 appears to have no effect on apoptosis in the trigeminal ganglion at E10.5 or E11.5. Rather, the reduction in neuronal apoptosis in *HIPK2*$^{-/-}$ mutants is most significant at E13.5, E15.5, and P0, with a slight increase in neuron number at the same stages (Fig. 7, E and F; Fig. 8 B). One possible explanation for the lack of a detectable effect at the early stage of programmed cell death in *HIPK2*$^{-/-}$ mutant is that the efficiency of HIPK2-mediated suppression of Brn3a may require the formation of a larger and more effective corepressor complex. Indeed, recent data have indicated that HIPK2 can interact with additional corepressors, including Groucho, histone deacetylase (HDAC) and CtBP (Choi et al., 1999; Zhang et al., 2003). Thus, if a larger HIPK2-based corepressor complex does exist in vivo, removal of more than one component of this complex may be required to produce a more significant change in Brn3a-mediated gene expression.

Given the role of HIPK2 as a transcriptional repressor, how might the activity of HIPK2 be regulated during sensory neuron development? Recent evidence indicates that transcriptional corepressors of class II HDACs show a progressive switch from nucleus to the cytoplasm during muscle cell differentiation, whereas muscle specific transcription factor MEF remains constantly in the nucleus (McKinsey et al., 2000). Nuclear export of HDACs is regulated by Ca^{2+} signals at the cell surface and by the subsequent activation of calcium-calmodulin kinases (McKinsey et al., 2000). Due to the interaction between HIPK2 and HDAC (Choi et al., 1999), it is tempting to speculate that a similar mechanism may regulate HIPK2 functions during sensory neuron development. Future more in-depth analyses of HIPK2 protein distribution should help test this hypothesis. Alternatively, it is possible that other signaling pathways, such as those involving neurotrophins, TGFβ and Wnts, may also regulate the subcellular localization of HIPK2 or its interaction with other proteins (Harada et al., 2003; Kanei-Ishii et al., 2004).

In addition to its ability to interact with Brn3a, HIPK2 has also been shown to interact with several other factors that are important for cell survival and apoptosis, most notably, p53 and CtBP (D'Orazi et al., 2002; Hofmann et al., 2002; Zhang et al., 2003). Indeed, overexpression of HIPK2 in *Brn3a*$^{-/-}$ sensory neurons or sympathetic neurons, which do not depend on Brn3a for survival (Ensor et al., 2001), continues to cause apoptotic cell death (Fig. 3 K; unpublished data). These results support the notion that HIPK2 can activate additional Brn3a-independent cell death pathways and that such mechanisms might become more prominent in the absence of Brn3a (Fig. 3 K). Unlike Brn3a, TrkA, Bcl-x_L, or HIPK2, however, the level of CtBP expression is extremely low in the sensory ganglia, making it a less likely contributor to sensory neuron survival (Hildebrand and Soriano, 2002; unpublished data). In contrast, the reported antagonistic roles of Brn3a and p53 in transcriptional regulation of Bcl-x_L raises the interesting possibility that, in addition to suppressing Brn3a, HIPK2, and p53 may cooperatively suppress Bcl-x_L expression (Sugars et al., 2001). Alternatively, HIPK2 could potentially up-regulate p53-dependent activation of Bax (Budram-Mahadeo et al., 2002). Our data indicate that the level of Bax is essentially unchanged in either *Brn3a*$^{-/-}$ or *HIPK2*$^{-/-}$ mutants (Figs. 2 and 7) and we did not detect any synergistic effect of HIPK2 and p53 on Bax or Bcl-x_L luciferase activity

(unpublished data). Future investigations using genome-wide screens for the expression of p53-dependent genes in *HIPK2*$^{-/-}$ mutants should provide more information regarding common target genes that are regulated by both HIPK2 and p53.

Materials and methods

Yeast two-hybrid screen, coimmunoprecipitation, and Western blot analyses
To identify Brn3a cofactors, we screened a yeast two-hybrid cDNA library prepared from E10.5-11.5 mouse embryos (Hollenberg et al., 1995). Of the 10^7 transformants, 67 were His^+ and $LacZ^+$. After ruling out false-positive clones, nine clones showed specific interactions with full-length Brn3a. Among these, five interacted with the POU domain of Brn3a, but not the non-POU domain. Sequencing data showed that this clone encoded partial sequence (amino acids 782–897) of HIPK2 (Kim et al., 1998). To further define which region in Brn3a interacted with HIPK2, we prepared yeast constructs that contained only the POU-specific domain (POU_S) or POU homeodomain (POU_{HD}) and determined their ability to interact with HIPK2 (amino acids 752–897) in liquid X-gal assays. Full-length HIPK2 cDNA was provided by Y. Kim (National Institutes of Health, Bethesda, MD).

For coimmunoprecipitation, COS cells were transfected with EGFP-HIPK2 and Brn3a cDNA using LipofectAMINE (Invitrogen). Cell lysates were collected 48 h after transfection in RIPA buffer. Supernatant was incubated for 1–2 h at 4°C with Brn3a antibody and with protein A/G agarose beads (Santa Cruz Biotechnology, Inc.) overnight at 4°C. Beads were washed in buffers containing 1% NP-40 (wash 1), 1% NP-40/0.5% sodium deoxycholate (wash 2), and 1% NP-40/0.5% sodium deoxycholate/0.1% SDS (wash 3). EGFP-HIPK2 was detected with anti-GFP (BD Biosciences). Quantification of the protein levels in Western blots (Fig. 6 B) was performed using the NIH Image program (developed at the National Institutes of Health and available at http://rsb.info.nih.gov/nih-image/). A central lane that contained proteins from *HIPK2*$^{+/-}$ trigeminal ganglia was spliced out in Fig. 6 B according to the guidelines by *JCB* (Rossner and Yamada, 2004).

Luciferase assays and EMSA
The luciferase reporter constructs for Brn3a and Bcl-x_L (provided by E. Turner, University of California, San Diego, San Diego, CA; and G. Packham, Imperial College, London, UK) have been characterized previously (Budhram-Mahadeo et al., 1999; Trieu et al., 1999; Sugars et al., 2001; Thornborrow et al., 2002). The TrkA luciferase construct was prepared by placing two tandem repeats of 5′-end Brn3a binding site in trkA promoter into pGL3-basic (Promega; Ma et al., 2003). To determine the effects of Brn3a and HIPK2 on the activity of these promoters, 293 cells (1.5×10^5) were transfected with Brn3a and EGFP-HIPK2 cDNA at designated concentrations. The total amount of DNA added was normalized using pcDNA3.1. Measurement of luciferase activity was done using the dual luciferase system using TD 20/20 luminometer (Turner Designs). The effects of HIPK2 in Brn3a binding to the consensus DNA sequence (b3s1) were determined using in vitro translated full-length Brn3a and HIPK2 protein (Coupled TNT System; Promega; Liu et al., 2000).

In situ hybridization and immunohistochemistry
Two relatives of HIPK2, HIPK1, and HIPK3, were reported to share high DNA sequence homology with HIPK2 (Kim et al., 1998). To circumvent the problem of cross reactivity, a 600-bp probe from the 3′ UTR of HIPK2 was isolated. Sequence homology of this probe to similar regions in HIPK1 and HIPK3 is <40%. Northern blot analyses using this probe showed a very different expression pattern from that of HIPK and HIPK3, and sense probes were used as negative controls and showed no detectable signals. A pAb recognizing NF150 was used (CHEMICON International, Inc.). Brn3a antibody was previously characterized (Huang et al., 1999a, 2001) and HIPK2 antibody was generated in rabbits using a maltose binding protein fused with COOH terminus of HIPK2 (MBP-HIPK2C) and purified using GST-HIPK2C affinity column.

Sensory neuron cultures
Trigeminal ganglia from P0-P2 mice were trypsinized and triturated in 1 ml of F-14 culturing medium with a fire-polished siliconized Pasteur pipette. The dissociated cell suspension was diluted and plated in F-14 culturing medium in tissue culture dishes or slide chambers. NGF (10 ng/ml) or glia-derived neurotrophic factor (50 ng/ml, for cultures of *Brn3a*$^{-/-}$ neurons; Huang et al., 1999b) was added to the medium at the time of plating. For real-time PCR experiments, cells were plated at a density of ~2,500 cells per dish, whereas for cell counting and immunohistochemical analysis, ~300 cells per dish were plated. After plating, cells were incubated at 37°C for 3 d before infection with HSV. The preparation of recombinant HSV carrying EGFP-HIPK2 or Brn3a was generated using the standardized methods and the titers of HSV were determined using PC12 cells (Coopersmith and Neve, 1999).

Assessment of cell survival and apoptosis
The number of living neurons in culture was determined by counting the neurons within a marked grid in the center of the culture dishes before HSV infection and at various times after infection. Neurons were observed by phase-contrast microscopy (model TE2000U; Nikon). Living neurons were identified as having a smooth, spherical, bright cell body and multiple neurites, whereas dead cells had a shrunken irregular cytoplasm and either fragmented or complete loss of neurites. Apoptosis was assessed using TUNEL with the in situ cell death detection kit, TMR red (Roche Molecular Biochemicals). Cells were analyzed using a three laser confocal microscope (model TCS SP; Leica) and were considered to be apoptotic by the presence of condensed, TUNEL positive nuclear material accompanied by shrinking of the cytoplasm and loss of axons. Laser intensity (measured as the PMT levels) for each fluorophor was kept within the linear range. Images were captured using confocal software (Leica) and imported into Photoshop 7.0. Countings of apoptotic neurons and total neuron number in the trigeminal ganglion were performed as described previously (Huang et al., 1999a,b).

qRT-PCR assays
A qRT-PCR technique was used to measure the levels of TrkA, Bcl-x_L, Brn3a, and Bax mRNAs. Total RNAs were extracted using an RNeasy Kit (QIAGEN). Reverse transcription reactions were performed using random hexamer primers and the SuperScript first-strand synthesis system (Invitrogen). Quantification of cDNA was done on ABI Prism 7900HT detection system (PE Applied Biosystems). The levels of GAPDH were also quantified allowing TrkA, Bcl-x_L, Brn3a, and Bax expression to be calculated relative to GAPDH mRNA.

Gene targeting of *HIPK2* and generation of *HIPK2* null mice
Murine *HIPK2* genomic DNA was isolated from a mouse 129/SvJ λ library (Stratagene) using a cDNA probe containing the first coding exon (exon 3) of *HIPK2*. Recombination arms were generated by PCR and subcloned into the targeting vector pPNT, which contained *neomycin* (PGK-neo) and *thymidine kinase* (hsv-tk) genes (Tybulewicz et al., 1991). The reporter gene *LacZ*, followed by a poly(A) sequence, was inserted in frame with the first ATG of HIPK2. The targeting construct was electroporated into 129SvJ ES cells and selected with both G418 and gancyclovir. Genomic DNA from G418-resistant ES colonies was digested with Nhe1 and Xba1 and screened with 5′ and 3′ probes. 5′ probe detected a 8.8-kb Nhe1 fragment in wild-type allele and a 14-kb fragment in mutant allele, whereas 3′ probe detected a 7.6-kb Xba1 fragment in wild-type allele and a 12.8-kb fragment in mutant allele. Three independent ES lines were injected into C57Bl/6J blastocysts. Chimeras were mated to C57Bl/6J females to generate F1 heterozygotes. All mice were maintained in 129 and B6 mixed background. Animal care was performed according to the Institute of Animal Care and Use Committee guidelines. Genotypes were confirmed by Southern blot and PCR analysis. Northern blots using mRNA from MEFs and a probe containing exons 3 and 4 was performed to confirm the absence of HIPK2 expression. RT-PCR was also performed on mRNA from MEFs and trigeminal ganglia by using primers for the first coding exon (exon 3) of HIPK2 and the last two exons (exon 15 and 16).

Online supplemental material
The sequences of primers and probes used in qRT-PCR and genotyping of HIPK2 mutants and the experimental details are available in Table S1 (primers and probes for qRT-PCR for Brn3a and its target genes), Table S2 (primers for HIPK2 mutant mice genotyping), and Table S3 (primers for RT-PCR to detect HIPK2 cDNA). Online material is available at http://www.jcb.org/cgi/content/full/jcb.200406131/DC1.

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