

Hrk/DP5 contributes to the apoptosis of select neuronal populations but is dispensable for haematopoietic cell apoptosis

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Summary

The pro-apoptotic BH3-only members of the Bcl2 family, crucial initiators of cell death, are activated by a diverse array of developmental cues or experimentally applied stress stimuli. We have investigated, through gene targeting in mice, the biological roles for the BH3-only family member HRK (also known as DP5) in apoptosis regulation. *Hrk* gene expression was found to be restricted to cells and tissues of the central and peripheral nervous systems. Sensory neurons from mice lacking *Hrk* were less sensitive to apoptosis induced by nerve growth factor (NGF) withdrawal, consistent with the induction of *Hrk* following NGF deprivation. By contrast, cerebellar granule neurons that upregulate *Hrk* upon transfer to low-K⁺ medium underwent apoptosis normally under these conditions in

the absence of *Hrk*. Furthermore, loss of *Hrk* was not sufficient to rescue the neuronal degeneration in *lurcher* mutant mice. Despite previous reports, no evidence was found for *Hrk* expression or induction in growth-factor-dependent haematopoietic cell lines following withdrawal of their requisite cytokine, and haematopoietic progenitors lacking HRK died normally in response to cytokine deprivation. These results demonstrate that HRK contributes to apoptosis signalling elicited by trophic factor withdrawal in certain neuronal populations but is dispensable for apoptosis of haematopoietic cells.

Key words: Apoptosis, Neurons, Hrk/DP5, Bim, Bcl2

Introduction

The development and survival of metazoan organisms is critically dependent on the removal of redundant, damaged or otherwise harmful cells by apoptosis (Jacobson et al., 1997; Strasser et al., 2000). Apoptosis culminates in the destruction of vital cellular components by caspases – proteases present in healthy cells as inactive zymogens. Caspase activation is primarily governed by the Bcl2 family of proteins, which consists of both death preventing and death promoting members (Cory et al., 2003; Strasser et al., 2000). Its pro-survival members share up to four conserved regions with Bcl2, termed Bcl2 homology (BH) regions 1 to 4 (BH1 to BH4). The pro-apoptotic Bax/Bak-like proteins [Bax, Bak and Bok (also known as Mtd)] contain the BH1, BH2 and BH3 regions and are structurally similar to Bcl2. Members of a more distant pro-apoptotic Bcl2 subfamily are related to Bcl2 only by the presence of the short (9–16 aa) pro-apoptotic BH3 domain and hence are termed ‘BH3-only’ proteins [BAD, BID, BCL2L11 (also known and hereafter referred to as Bim, or Bod), BIK (also known as Nbk), BMF, HRK (also known as DP5), NOXA and BBC3 (also known and hereafter referred to as PUMA)] (Huang and Strasser, 2000). BH3-only proteins are essential initiators of developmentally programmed cell death but their activity is dependent on the Bax/Bak-like proteins (Huang and Strasser, 2000; Willis and Adams, 2005).

Here, we have investigated the biological roles of the pro-apoptotic BH3-only Bcl2 family member *Hrk* (also known as DP5) in apoptosis regulation by generating mice deficient for *Hrk* by gene targeting. *Hrk* was first identified in a screen for genes induced in the phaeochromocytoma 12 (PC12) cell line, undergoing apoptosis in response to nerve growth factor (NGF) withdrawal (Imaizumi et al., 1997). NGF withdrawal also induces *Hrk* expression in primary sympathetic neurons (Harris and Johnson, Jr, 2001; Imaizumi et al., 1997). This trophic factor is crucial for survival of certain neuronal cell populations and to guide innervation of target organs during development (Huang and Reichardt, 2001; Patel et al., 2000). In mice, loss of NGF or its receptor TrkA results in massive death of neurons in the sensory and sympathetic ganglia around embryonic day (E)13.5 (Crowley et al., 1994; Patel et al., 2000; Smeyne et al., 1994; White et al., 1996). Trophic-factor-deprivation-induced neuronal death can be prevented by the absence of Bax (Deckwerth et al., 1996), demonstrating that NGF-mediated neuronal survival is regulated by the Bcl2 protein family. In addition to *Hrk*, the BH3-only protein Bim is also induced in sympathetic neurons following NGF withdrawal (Harris and Johnson, Jr, 2001; Putcha et al., 2001; Whitfield et al., 2001), but although sympathetic and sensory neurons from Bim-deficient mice were abnormally resistant to NGF deprivation in vitro, the residual apoptosis implicates

additional BH3-only proteins in their death (Putcha et al., 2001; Whitfield et al., 2001). Hrk has been proposed as a likely candidate because it is regulated by NGF and kills sympathetic neurons when transiently overexpressed (Harris and Johnson, Jr, 2001; Imaizumi et al., 1997; Putcha et al., 2001).

In cultures of cerebellar granule neurons (CGNs), high concentrations of K⁺ mimic the pro-survival effects of electrical stimulation from afferent synapses, which is believed to contribute to neuronal selection during development (D'Mello et al., 1993; Gallo et al., 1987; Nardi et al., 1997). The CGN death that follows K⁺ deprivation can be prevented by loss of Bax (Miller et al., 1997). Expression of the gene encoding Bim was also induced in CGNs following K⁺ withdrawal, and Bim-deficient CGNs were less sensitive than wild-type cells to K⁺ deprivation (Harris and Johnson, Jr, 2001; Putcha et al., 2001). However, death of Bim-deficient CGNs was delayed rather than prevented, indicating that other BH3-only proteins may cooperate with Bim to kill these cells (Putcha et al., 2001). HRK is again a candidate, as it is induced in CGN upon K⁺ deprivation and its forced expression kills CGNs in a Bax-dependent manner (Harris and Johnson, Jr, 2001).

In addition to the nervous system, *Hrk* expression has been reported in haematopoietic tissues (Inohara et al., 1997), and two studies have indicated that HRK is induced in primary haematopoietic cells and immortalised cell lines upon deprivation of their requisite growth factors (Sanz et al., 2000; Sanz et al., 2001). The death of growth-factor-starved haematopoietic cells can be inhibited by overexpression of Bcl2 (Strasser et al., 1991; Vaux et al., 1988) and in many of these cell types also by loss of Bim (Bouillet et al., 1999; Villunger et al., 2003b) or PUMA (Jeffers et al., 2003; Villunger et al., 2003a). Whereas loss of Bim or PUMA greatly protected lymphocytes from apoptosis induced by growth factor deprivation, it was less effective in preventing the death of haematopoietic progenitors compared with Bcl2 overexpression (Bouillet et al., 1999; Villunger et al., 2003a; Villunger et al., 2003b). Hence, HRK might contribute to the apoptosis of cytokine-deprived haematopoietic progenitors.

In a recent study, Imaizumi et al. found that HRK was required for NGF withdrawal-induced apoptosis of sympathetic neurons and axotomy-induced motoneuron death (Imaizumi et al., 2004); however, a detailed investigation of the haematopoietic system was not conducted. We have therefore sought to extend these observations, by both investigating the pro-apoptotic role of HRK in the haematopoietic system and expanding the analysis of the role of HRK in the nervous system to additional cell types and an in-vivo model of neurodegeneration, the *lurcher* mouse.

Results

Generation of mice lacking HRK

The *Hrk* gene was disrupted by replacing the single coding exon with a reporter gene that encodes nuclear-localised *E. coli* β -galactosidase and a *loxP*-flanked neomycin resistance cassette (Fig. 1A). Two independent C57BL/6 ES cell clones harbouring the correctly integrated targeting construct were identified. From each, an independent line of mice having heterozygous deletion of the *Hrk* locus was established and bred to homozygosity. Targeting was confirmed by Southern blot DNA analysis using 5' and 3' external genomic probes

(Fig. 1B and data not shown). One of these mouse lines was then bred to C57BL/6 Cre-deleter females (Schwenk et al., 1995), and deletion of the neomycin (*neo*) cassette confirmed by PCR. No *Hrk* mRNA was detected in the brains of homozygous mutant mice by either northern blotting or RT-PCR (Fig. 1C and data not shown), confirming correct targeting of the *Hrk* gene.

Hrk^{-/-} homozygotes were born at the expected Mendelian frequency from heterozygote intercross matings (*Hrk*^{+/+} 45, *Hrk*^{+/-} 100, *Hrk*^{-/-} 60). Their appearance, fertility and behaviour were indistinguishable from control littermates up to 2 years of age. Both *Hrk*^{-/-} males and females had normal body weights and weight of major organs (liver, lung, heart, thymus, spleen and kidney). Histological analysis of these and other organs revealed no obvious pathology or abnormality (data not shown). HRK is therefore not required for normal development, health or reproduction.

Haematopoietic progenitors lacking HRK respond normally to cytokine withdrawal

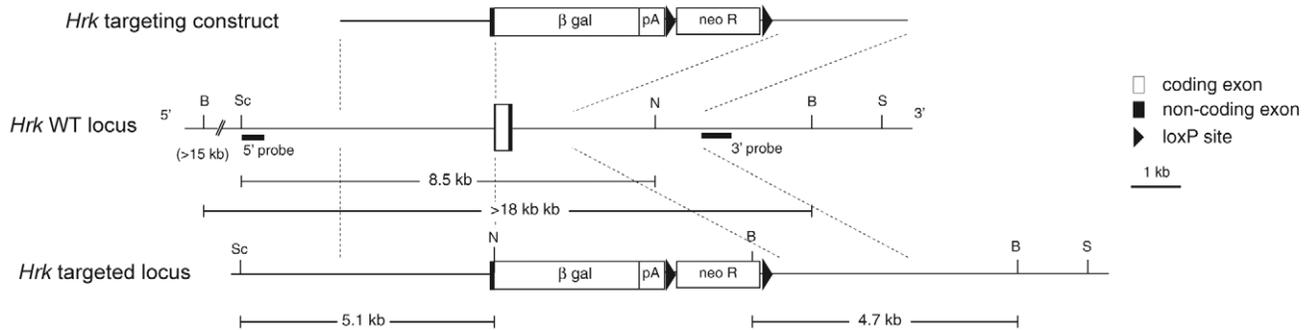
HRK was reportedly expressed in haematopoietic organs and induced following growth factor withdrawal in haematopoietic cell lines and normal haematopoietic progenitors (Inohara et al., 1997; Sanz et al., 2000; Sanz et al., 2001). We therefore investigated the requirement for *Hrk* in the apoptosis of haematopoietic progenitor cells in response to growth factor withdrawal. Foetal liver haematopoietic progenitors from *Hrk*^{-/-} or control (wt) embryos were plated in semi-solid agar to which interleukin 3 (IL3), stem cell factor (SCF) and erythropoietin (EPO) were added at the time of plating (0 hours) or after 24, 48 or 72 hours. The colony-forming potential of cytokine-starved *Hrk*^{-/-} haematopoietic progenitors was indistinguishable from that of cells from their wild-type littermates (Fig. 2A). Thus, HRK is not essential for apoptosis of haematopoietic progenitors following growth factor withdrawal.

HRK is not expressed in haematopoietic cells or induced in response to cytokine withdrawal

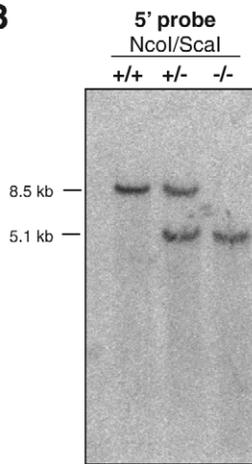
As HRK loss had no effect on haematopoietic progenitor apoptosis in response to cytokine withdrawal, we assessed whether the *Hrk* gene was actually regulated by cytokine withdrawal in haematopoietic cells as reported (Sanz et al., 2000; Sanz et al., 2001). In fact, although expression of *Hrk* mRNA was abundant in the brain, it proved to be below the level of detection in the IL3-dependent haematopoietic cell lines FDC-P1, BaF/3 and FL5.12 cells, and their derivatives stably transfected with a Bcl2 expression vector. Moreover, *Hrk* gene expression was not detectably induced in any of these cell lines following IL3 withdrawal (Fig. 2B). In contrast, expression of the gene encoding Bim increased upon IL3 withdrawal in these cells – as previously reported (Dijkers et al., 2000) – demonstrating that the cells were stressed and had upregulated death-promoting genes (Fig. 2B).

Further examination by northern blotting failed to detect evidence of *Hrk* gene expression in haematopoietic organs, such as spleen or bone marrow, or a panel of cell lines of haematopoietic origin (Fig. 2C and data not shown). Moreover, we failed to detect *Hrk* mRNA in FACS-sorted haematopoietic cell subsets, including B and T lymphocytes (at various stages of development and maturation), granulocytes, macrophages or

A



B



C

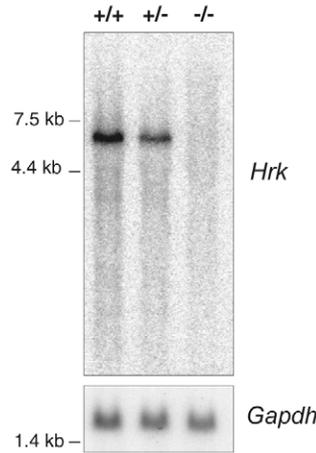


Fig. 1. Targeting of the *Hrk* locus. (A) Schematic diagram (to scale) of the *Hrk*-targeting construct, the wild-type (WT) *Hrk* locus and the targeted locus. Dashed lines demarcate regions of the WT locus used in the targeting construct. Location of 5' and 3' external probes for genomic Southern blot analysis and expected fragment sizes from indicated restriction digests are shown. B, *Bgl*II; Sc, *Sca*I; N, *Nco*I. (B) Confirmation by Southern blotting using 5' genomic probes of the loss of both wild-type *Hrk* alleles in *Hrk*^{-/-} mice. (C) Northern blot analysis of polyA⁺ mRNA extracted from brains of neonatal wild-type (+/+), *Hrk*^{+/-} (+/-) and *Hrk*^{-/-} (-/-) littermates. *Gapdh* RNA was loaded as control.

erythroid progenitors, by highly sensitive RT-PCR analysis (data not shown). These results do not support the published reports that *Hrk* is expressed in cells and tissues of the haematopoietic system and is upregulated in response to cytokine withdrawal (Inohara et al., 1997; Sanz et al., 2000; Sanz et al., 2001).

Hrk is expressed in the central and peripheral nervous systems

Hrk gene expression in the nervous system was examined in greater detail in embryos at embryonic day (E) 12.5, E13.5, E14.5, newborn brain and adult brain by radioactive in situ hybridisation using an *Hrk*-specific probe. *Hrk* mRNA expression was only detected in the nervous system. At E12.5, *Hrk* gene expression was very low (data not shown). At E13.5, *Hrk* mRNA was strongly expressed in dorsal root ganglia (DRG) and the ventral neural tube and at moderate levels in the cranial ganglia (Fig. 3B,E). At E14.5, a prominent *Hrk* mRNA signal was present in the ventral grey horn (VH) of the spinal cord, in

the area of the somatic motor neurons (Fig. 3G). Moderate levels were observed in the lateral grey horn (LH) in the area of the V0 ventral interneurons (long arrow, Fig. 3G) and low levels in the dorsal grey horn (DH) and the dorsal root ganglia (asterisk, Fig. 3G). In the newborn brain, *Hrk* was expressed at moderate levels in many areas and at slightly higher levels in the cortical plate, the olfactory tubercle, the piriform cortex, the hippocampus, the ventromedial nuclei of the hypothalamus, the cortical amygdaloid nuclei, the dorsal lateral geniculate nuclei of the thalamus and the facial motor nuclei of the pons (data not shown). In the adult brain, moderate levels were detected in the hippocampus. Thus, the *Hrk* gene exhibited a dynamic pattern of expression in of number of areas containing post-mitotic differentiating neurons.

Loss of *Hrk* affords moderate protection to sensory neurons against NGF withdrawal

Since *Hrk* expression is induced in response to NGF deprivation (Harris and Johnson, Jr, 2001; Imaizumi et al.,

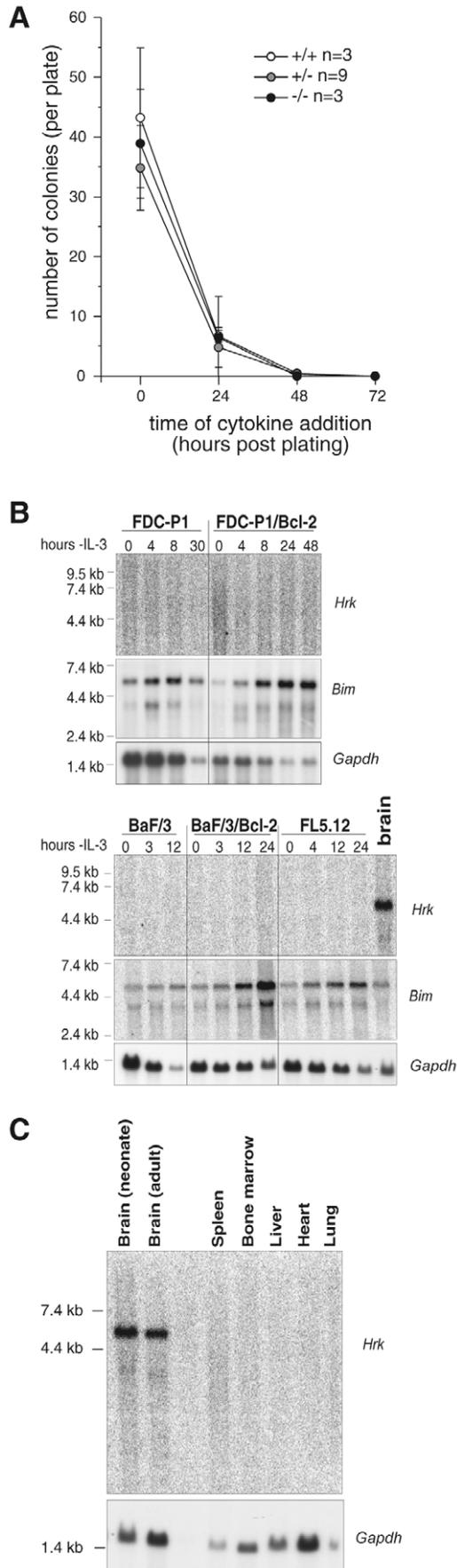


Fig. 2. HRK is not required for cytokine withdrawal-induced death of haematopoietic progenitors. (A) Foetal liver cells from E13.5 wt, *Hrk*^{+/-} and *Hrk*^{-/-} embryos were plated in soft agar and colonies scored 7 days after the addition of cytokines. Cytokines were added to the cultures at the time of plating (0 hours) or after 24, 48 or 72 hours. Data are the mean \pm s.d. from three or nine mice of each genotype. (B) Northern blot analysis of *Hrk* gene expression on polyA⁺ mRNA extracted from IL3-dependent haematopoietic cell lines and their derivatives over-expressing Bcl2. Each cell line was deprived of IL3 for the indicated times. PolyA⁺ mRNA from neonatal brain was used as a positive control. Northern blotting for the gene encoding Bim was included as a control to show that each cell line was stimulated to upregulate BH3-only gene expression. *Gapdh* RNA was loaded as control. (C) Northern blot analysis of *Hrk* gene expression on polyA⁺ mRNA extracted from the indicated tissues of C57BL/6 mice. *Gapdh* RNA was detected as a loading control.

2004; Imaizumi et al., 1997) and appears in DRG neurons at a time when they are critically dependent on NGF for survival, we tested the sensitivity of *Hrk*-deficient DRG neurons to NGF withdrawal. Indeed, DRG sensory neurons lacking *Hrk* were less sensitive to apoptosis induced by the withdrawal of NGF, as they displayed a small but highly reproducible increase in viability over cells from their control (wt) littermates (Fig. 4A). Results from heterozygotes (*Hrk*^{+/-}) and wt animals were pooled since we found no evidence for an intermediate heterozygote phenotype in this assay.

Unlike in sensory neurons, HRK loss did not reduce the sensitivity of sympathetic neurons from the superior cervical ganglia to NGF withdrawal (Fig. 4B), although this treatment did cause increased expression of *Hrk* mRNA in these cells (Fig. 4C). By contrast, loss of Bim partially protected sympathetic neurons against NGF deprivation (Fig. 4B), as previously reported (Putcha et al., 2001; Whitfield et al., 2001). This protection was protracted, as viable *Bcl2l1*^{-/-} (hereafter referred to as *Bim*^{-/-}) neurons were still observed 18 days after NGF withdrawal (Fig. 4B). We also performed MTT assays (to measure cellular proliferation) on sympathetic neurons of all three genotypes at 24, 36 and 48 hours post NGF deprivation. *Bim*-deficient but not HRK-deficient neurons were more resistant to apoptosis at all time points, whereas neurons (of all genotypes) maintained in NGF were still >95% viable after 72 hours (data not shown). These results show that HRK contributes to sensory neuron apoptosis in response to NGF withdrawal but is less important in sympathetic neurons.

HRK is not required for K⁺ withdrawal induced apoptosis of cerebellar granule neurons

We next investigated whether loss of HRK can prevent CGNs from undergoing apoptosis in vitro following K⁺ deprivation. CGNs from *Hrk*^{-/-} or wt littermates were cultured in medium containing high levels of K⁺ (25 mM) until they became dependent on K⁺ for survival (7 days), after which they were transferred to medium containing physiologic concentrations of K⁺ (5 mM) to promote apoptosis. Upon K⁺ deprivation, CGNs from *Hrk*^{-/-} mice underwent apoptosis as rapidly and as efficiently as cells from their wt littermates (Fig. 5A), although this treatment greatly increased the levels of *Hrk* mRNA expression in these cells (Fig. 5B). Thus, HRK loss alone is not sufficient to prevent apoptosis of CGNs after K⁺ withdrawal.

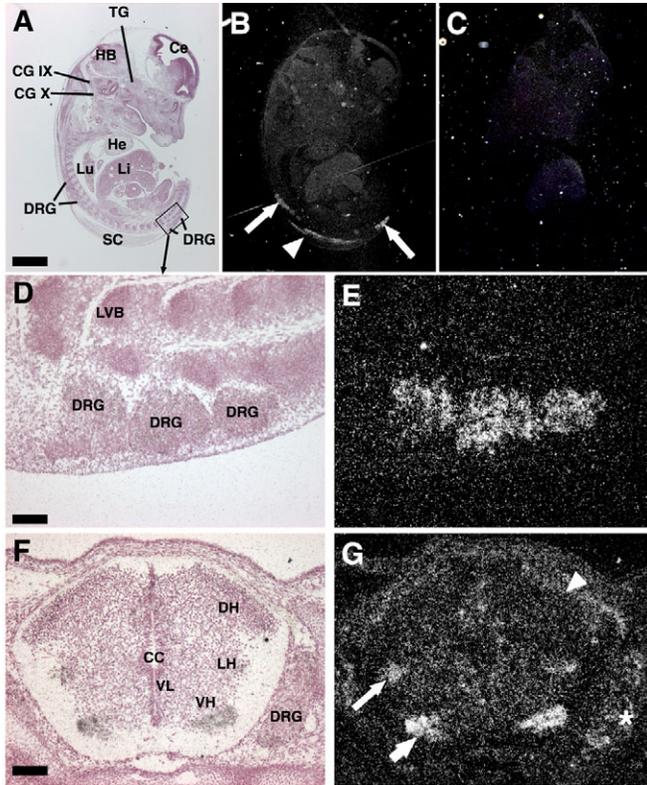


Fig. 3. Expression of the *Hrk* gene in the developing nervous system. (A-G) Para-sagittal paraffin sections of E13.5 (A-E) and transverse sections of E14.5 (F,G) wild-type embryos were hybridised with an *Hrk* anti-sense cRNA probe (A,B,D-E) or a sense control probe (C). The bright-field images in A,D,F identify the regions of the dark-field images (B,E,G), in which silver grains appear above areas of *Hrk* mRNA expression. D and E depict insert shown in A at a higher magnification. The *Hrk* gene shows highly restricted expression. Expression of *Hrk* was highest in dorsal root ganglia (DRG) at E13.5 (arrow in B; E), the spinal cord at E13.5 (arrowhead in B) and the ventral grey horn of the spinal cord (VH) at E14.5 (short arrow in G) in the area of the motor neurons. DRG at E14.5 (asterisk in G) exhibit a moderate *Hrk* mRNA signal, as do cells in the lateral grey horn (LH) ventral of the sulcus limitans (long arrow in G) in the area of V0 interneurons. Weak expression of *Hrk* mRNA is present in other areas of the spinal cord, e.g. the dorsal horn (arrowhead in G). Ce, cerebral cortex; CC, central canal; CG IX, inferior ganglion of glossopharyngeal nerve (IX); CG X, inferior ganglion of vagal nerve (X); DH dorsal grey horn; DRG, dorsal root ganglia; HB, hindbrain; He, heart; LH lateral grey horn; Li, liver; Lu, lung; LVB, cartilage primordium of a lumbar vertebral body; SC, spinal cord; TG, trigeminal ganglion; VL, ventricular layer. Bars, 1.2 mm (A-C) and 93 μ m (D-G).

HRK is dispensable for the neurodegeneration in *lurcher* heterozygote mice

We next investigated whether HRK loss could prevent neuronal death in a neurodegenerative disease model in vivo. The *lurcher* mutation is a semi-dominant gain-of-function mutation in the ionotropic glutamate receptor delta 2 (*Grid2*) gene that causes ataxia due to extensive loss of cerebellar neurons (Wetts and Herrup, 1982; Zuo et al., 1997). In *lurcher* mice, Purkinje neurons undergo a specialised form of cell death, associated

with autophagy (Yue et al., 2002) that is delayed but not prevented by Bcl2 overexpression or loss of Bax (Doughty et al., 2000; Selimi et al., 2000; Zanjani et al., 1998). Granule neuron death in *lurcher* mice, however, is overcome by Bax loss, implicating BH3-only proteins (Doughty et al., 2000; Selimi et al., 2000). We found that the *lurcher* heterozygotes displayed ataxia regardless of their *Hrk* genotype (*Hrk*^{-/-}*Grid2*^{+/-}, *Hrk*^{+/-}*Grid2*^{+/-} or *Hrk*^{+/+}*Grid2*^{+/-}). Furthermore, histological examination of the cerebellum showed that neuron loss in heterozygous *lurcher* animals still occurred in the absence of HRK (Fig. 6). Collectively, these results demonstrate that HRK is dispensable for neuronal loss in *lurcher* heterozygotes.

Discussion

We have used gene targeting in mice to investigate the biological roles of the pro-apoptotic BH3-only Bcl2 family member HRK in apoptosis regulation and further characterised its expression pattern in mice. We have extended the previous findings of Imaizumi et al. by investigating the pro-apoptotic role of HRK in sensory neurons, cerebellar granule neurons, and an in vivo model of neurodegenerative disease as well as in the haematopoietic system (Imaizumi et al., 2004).

We found prominent expression of the *Hrk* gene in the nervous system, consistent with some previous reports (Imaizumi et al., 2004; Imaizumi et al., 1997; Kanazawa et al., 1998). *Hrk* gene expression became detectable at E13.5 as a strong signal in the DRG and ventral neural tube. Expression in the DRG and spinal cord motor neurons was also evident at E14.5. *Hrk* gene expression at this age correlates with developmentally programmed cell death in both the DRG and motor neurons of the ventral spinal cord. The deaths in both these populations are known to be regulated by the Bcl2 protein family, because this cell loss is abolished by the absence of Bax (White et al., 1998; White et al., 1996). The survival of DRG neurons at this age is dependent on the target-derived trophic factor NGF, and indeed we found that HRK contributes to the death of DRG neurons deprived of NGF (see below). Likewise, HRK has been implicated in the death of motor neurons deprived of target-derived survival factors by axotomy (Imaizumi et al., 2004). Thus, HRK may also participate in the death of motor neurons that fail to make contact with their targets, thereby contributing to physiologic neuronal cell death during development.

In the brain, expression of the *Hrk* gene was widespread in newborn animals but became largely restricted to the hippocampus in adults. This restricted pattern in adults is of interest because the hippocampus is a site of normal postnatal neurogenesis, and the pro-apoptotic activity of HRK may be required in this region to balance neuronal production. Therefore, HRK might contribute to the control of cellular homeostasis in the postnatal nervous system in addition to the developing nervous system.

Given that *Hrk* was originally identified as a gene inducible in neurons following NGF withdrawal and is expressed in dorsal root ganglia at a time when NGF-dependent DRG neurons undergo physiological cell death, we assessed the sensitivity of HRK-deficient DRG neurons to NGF deprivation. Consistent with the regulation of HRK by NGF, HRK-deficient DRG neurons were abnormally resistant to NGF withdrawal-induced apoptosis. Since HRK loss did not

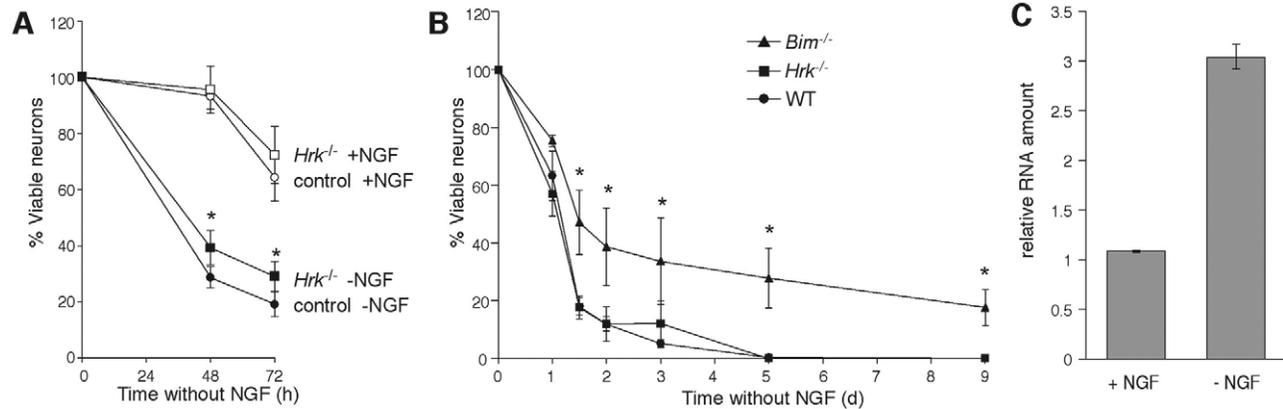


Fig. 4. HRK contributes to NGF-deprivation-induced apoptosis in DRG neurons. (A) DRG neurons were isolated from control (wt and *Hrk*^{+/-}, *n*=4) or *Hrk*^{-/-} (*n*=5) littermates, cultured for 24 hours in NGF, then transferred to medium with or without NGF (supplemented with anti-NGF antibodies) for 72 hours. Cell survival was assessed by counting the number of viable neurons in an assigned field before NGF withdrawal (0 hours), then counting the same field again at 48 and 72 hours after NGF withdrawal. Percent survival was determined relative to the viable neuron number at 0 hours. Asterisks indicate statistically significant survival difference between control and *Hrk*^{-/-} neurons in the absence of NGF ($P < 0.05$, Student's *t*-test). (B) SCG neurons were isolated from WT, *Hrk*^{-/-} or *Bim*^{-/-} mice and cultured with NGF for 5 to 6 days, then washed and cultured in medium with or without NGF (supplemented with anti-NGF antibodies) for up to 48 hours. Cell survival was assessed from the number of viable neurons remaining in each well at each time point relative to the number present in the same well before NGF withdrawal and were expressed in percent. Data represent the mean \pm s.d. Asterisks indicate statistically significant difference between survival of wild-type (WT) and *Bim*^{-/-} neurons in the absence of NGF ($P < 0.05$, Student's *t*-test). (C) Q-PCR analysis of *Hrk* mRNA expression in wt SCG neurons cultured with or without NGF for 16 hours. Expression of neurofilament 3 was used for normalisation. Data are the mean \pm s.d. of nine cultures of neurons from three mice (triplicates).

abolish DRG neuron apoptosis, other BH3-only proteins may also contribute. Indeed, Bim is induced in response to NGF withdrawal (Harris and Johnson, Jr, 2001; Putcha et al., 2001; Whitfield et al., 2001), and loss of Bim reduced the sensitivity of both sensory and sympathetic neurons to NGF withdrawal (Putcha et al., 2001; Whitfield et al., 2001). It would therefore be of interest to test the resistance of neurons lacking both HRK and Bim to NGF deprivation.

Interestingly, although we found that HRK loss alone partially protected sensory neurons from NGF-withdrawal-induced apoptosis, it did not appear to do so in sympathetic neurons. This finding suggests that the activity of an individual BH3-only protein depends not only on the type of stimulus but also the cell type. Similarly, Bim loss conveys greater protection to glucocorticoid treatment in B than T lymphocytes (Bouillet et al., 1999; Erlacher et al., 2005) (L.C. and A.S., unpublished observations). Such cell-context dependence may, for example, reflect the relative levels of expression and activity of the BH3-only proteins and their pro-survival targets. Imaizumi et al. also tested the resistance of HRK-deficient sympathetic neurons to NGF withdrawal and found a small but significant protective effect (Imaizumi et al., 2004). That effect, however, was subtle and required a large number of animals to reach statistical significance. Differences in genetic background (inbred C57BL/6 for our *Hrk*^{-/-} mice versus mixed C57BL/6x129SV for the previously reported *Hrk*^{-/-} mice) might account for the differences in results. Although we might have observed such an effect if more animals had been studied, our data and those reported by Imaizumi et al. (Imaizumi et al., 2004) collectively indicate that the role of HRK in this form of neuronal cell death must be minor relative to other apoptosis initiators, such as Bim. This is consistent with the observation that Bim is a more potent BH3-only protein than HRK, because

Bim binds avidly to all pro-survival Bcl2 family members, whereas HRK binds to only a subset (Chen et al., 2005; Kuwana et al., 2005). The role of HRK in sympathetic neuron death may become more apparent in mice lacking both HRK and Bim, because HRK loss might enhance the phenotype of the Bim-deficient neurons. Our preliminary observations, however, suggest that the combined loss of HRK and Bim does not protect sympathetic neurons as potently as loss of Bax from NGF withdrawal-induced apoptosis. Hence, yet another BH3-only family member may well contribute. An obvious candidate is PUMA, because it is induced in sympathetic neurons following NGF deprivation (Besirli et al., 2005) and is required to a similar extent as Bim for their apoptosis (Wytenbach and Tolkovsky, 2006).

Although CGNs undergo rapid apoptosis when shifted to low-K⁺ medium and HRK expression is induced (Harris and Johnson, Jr, 2001), HRK-deficient CGNs exhibited normal sensitivity to K⁺ deprivation. Bim is also regulated by K⁺ deprivation in CGNs and, in contrast to HRK, its loss does delay their death (Putcha et al., 2001; Whitfield et al., 2001). It remains possible that concurrent loss of HRK further enhances the protective effect of Bim loss in CGNs but other BH3-only proteins, such as PUMA, which is also induced in CGNs in response to K⁺ deprivation (Besirli et al., 2005), may also play a crucial role.

To investigate whether loss of HRK rescues neuronal apoptosis in a neurodegenerative setting in vivo, we investigated its effect on the *lurcher* phenotype. Overall, our observations suggest that HRK is dispensable for both granule cell and Purkinje neuron death in *lurcher* mice. Similarly, loss of Bim was found to be insufficient to rescue Purkinje or granule neuron apoptosis in *lurcher* mice (Bouillet et al., 2003). Removal of both HRK and Bim and perhaps yet other

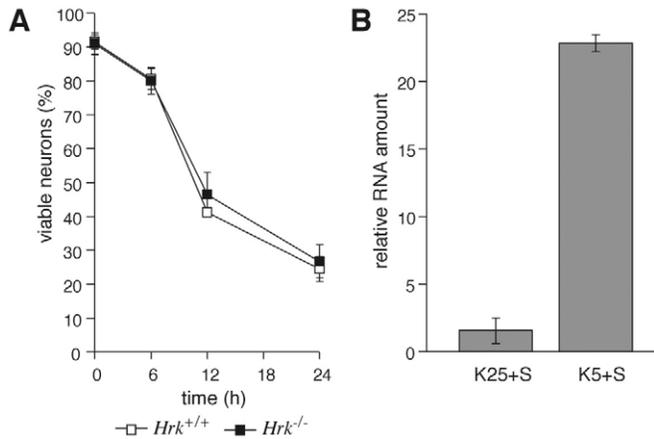


Fig. 5. Loss of HRK does not protect cerebellar granule neurons from K⁺ deprivation. (A) Cerebellar granule neurons were isolated from wild-type (*Hrk*^{+/+}, *n*=3) and *Hrk*^{-/-} (*n*=3) littermates, cultivated in high-K⁺ medium for 7 days, then transferred to low-K⁺ medium to induce apoptosis. Viability was determined 0, 6, 12 and 24 hours after transfer to low K⁺ using the Live/dead assay (Molecular Probes) to assess the number of viable and dead neurons in five independent fields at each time point for each animal and expressed in percent. Data are the mean \pm s.d. (B) Q-PCR analysis of *Hrk* mRNA expression in wt cerebellar granule neurons cultured for 4 hours in the presence of high (K25+S) or low levels (K5+S) of K⁺. Expression of neurofilament 3 was used for normalisation. Data are the mean \pm s.d. from nine cultures of neurons from three mice (triplicates).

BH3-only members may therefore be needed to prevent the abnormal apoptosis of *lurcher* granule neurons.

Following observations that HRK expression can be induced in haematopoietic progenitors in response to growth factor withdrawal (Sanz et al., 2000; Sanz et al., 2001), we tested the sensitivity of HRK-deficient haematopoietic progenitors to cytokine deprivation but found that loss of HRK afforded no protection. Since Bim is induced in haematopoietic cells in response to cytokine withdrawal (Dijkers et al., 2000) and both Bim and PUMA are crucial for their death (Bouillet et al., 1999; Jeffers et al., 2003; Villunger et al., 2003a), it can be argued that HRK is made redundant by the activities of Bim and PUMA. However, we do not think that HRK plays a role in haematopoietic cells, because we found no evidence that it is expressed in any haematopoietic cell type (primary or immortalised) or, indeed, that it is induced in response to cytokine withdrawal. In conclusion, we believe that HRK contributes to programmed cell death and stress-induced apoptosis in the nervous but not the haematopoietic system.

Materials and Methods

Generation of *Hrk*^{-/-} mice

The murine *Hrk* locus was defined from two clones (458-f11 and 476-j2) isolated from an RPCI-23 female C57BL/6J mouse bacterial artificial chromosome (BAC) library (Roswell Park Cancer Inst, NY). The location of exon 2 (non-coding) was determined to lie between 12.5 kb and 24 kb downstream of the sole coding exon. The targeting construct for *Hrk* consists of 3.1 kb of 5' and 2.6 kb of 3' homologous *Hrk* locus sequence surrounding the *Escherichia coli lacZ* reporter gene fused in-frame with the *Hrk* initiator ATG and a *loxP*-flanked neomycin (neo)-resistance cassette driven by the PGK promoter, replacing the *Hrk* coding exon. The targeting construct was electroporated into C57BL/6 (Bruce4) embryonic stem (ES) cells.

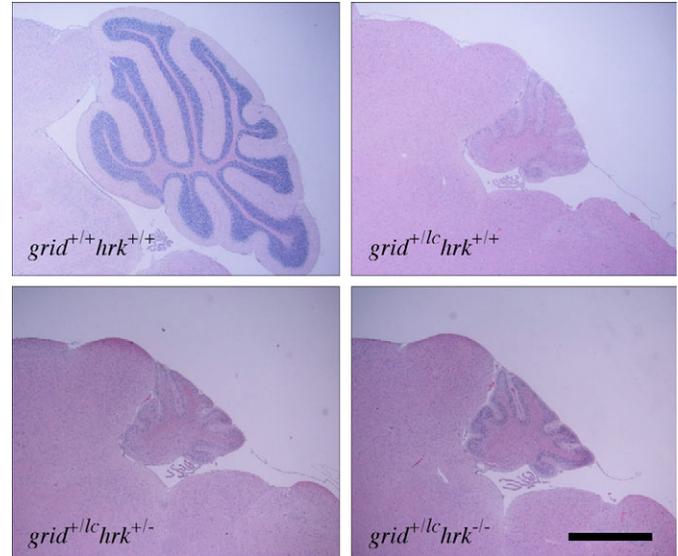


Fig. 6. Loss of HRK does not rescue cerebellar Purkinje cells or granule neurons in *lurcher* mutant mice. Mid-sagittal cerebellar sections stained with H&E showing that the *Hrk* genotype (*Hrk*^{+/+}, *Hrk*^{+/-} or *Hrk*^{-/-}) does not affect the size of the cerebellum in *lurcher* (lc) heterozygous (*grid*^{+/-}) animals. Bar, 1 mm.

Targeting was confirmed by genomic Southern blotting using external 5' and 3' genomic probes and a *neo*-specific probe to confirm single construct integration. Two correctly targeted ES cell lines were selected for blastocyst injection and the mouse lines of each (denoted line 354 and line 355) were maintained on an inbred C57BL/6 background. The neomycin resistance cassette was deleted from one line (mouse 354) by crossing to female C57BL/6 Cre-deleter mice (Schwenk et al., 1995), to generate the del354 line; *neo*-cassette deletion was confirmed by PCR. All analysis reported was performed using del354 mice and verified where possible with line-355 mice. Targeted *Hrk* mice of the del354 line were genotyped by PCR using a four-oligonucleotide strategy. The wild type allele-specific oligonucleotides were 3' (anti-sense) 5'-GCGACCCCGACCGACACTTCTTG-3' in combination with 5' (sense) 5'-CAGCTTGGCCTCCTATCACCTTCC-3'. Targeted allele-specific oligonucleotides were 5' (sense) 5'-CCGGTCCGCTACCATTACCAGT-TGG-3' and 3' (anti-sense) 5'-CCTCTACAGATGTGATATGGCTGATTATGATC-3'. This reaction, designed to co-amplify a wild type allele-specific product of 209 bp and a targeted allele-specific product of 113 bp, was performed using 25 cycles and an annealing temperature of 60°C.

The generation of the *Bcl2l1*^{-/-} (referred to as *Bim*^{-/-}) mice has been described previously (Bouillet et al., 1999). These mice, originally on a mixed C57BL/6x129SV background, had been backcrossed with C57BL/6 mice for more than ten generations. All experiments with animals were conducted according to the guidelines of the Melbourne Research Directorate Animals Ethics Committee.

Northern blotting, Southern blotting, RT-PCR, Q-PCR and in situ hybridisation

Northern blotting, Southern blotting and RT-PCR were performed as described (Coults et al., 2004). Probing for *Hrk* by northern blotting was performed with a cDNA template that was amplified using the *Hrk*-specific oligonucleotides (sense) 5'-GGTCCAGCTACAGAGAGACCACC-3' and (anti-sense) 5'-CTCTCCCT-GCCACCCTAGACATTACG-3'.

Real-time (RT)-PCR was performed using the 7900HT Fast Real-time PCR System (Applied Biosystems) according to the manufacturer's instructions. The expression assays used are: Mm01962376_s1 for *Hrk* and Mm00456201_m1 for the normalisation control neurofilament3. Each cDNA sample was analysed in triplicate with both the HRK and the Nef3 assays and only samples with standard deviation less than 0.25 were considered.

For in situ hybridisation, a 423 bp probe coding for bases 364-786 of the mouse *Hrk* cDNA (accession number NM007545) was cloned into pBluescript KS+ and sequenced. To produce an antisense riboprobe, this plasmid was linearised with *Bam*HI and transcribed with T3 RNA polymerase incorporating [³³P]-labeled UTP (Amersham). In situ hybridisation was performed essentially as described previously (Thomas et al., 2000). Briefly, paraffin-embedded sections were de-waxed, re-hydrated through graded concentrations of ethanol, incubated for 10 minutes at room temperature in 10 mg/ml proteinase K, fixed in 4%

paraformaldehyde for 10 minutes, then dehydrated through graded concentrations of ethanol. Sections were air-dried and a hybridisation solution, containing 2×10^7 cpm/ml of the cRNA probe, was placed over the sections. Slides were incubated overnight at 56°C and then washed at 60°C in 50% formamide 0.3 M NaCl, 0.03 M Na_3 citrate. Slides were then washed in 0.3 M NaCl, 0.03 M Na_3 citrate and dehydrated through graded concentrations of ethanol. Sections were dipped in liquid emulsion (Kodak) and exposed to autoradiography for 2 weeks at 4°C before being counterstained with haematoxylin.

Dorsal root ganglia neuron culture assays

Dorsal root ganglion neurons were prepared essentially as described (Murphy et al., 1991). DRG were isolated from post-natal day 1 *Hrk*^{-/-} or control (wt) pups and each animal was processed separately. Ganglia were incubated with 0.2 mg/ml trypsin and 10 $\mu\text{g}/\text{ml}$ DNaseI for 30 minutes at 37°C with occasional gentle agitation. Ganglia were then gently triturated sequentially through 20G, 22G and 23G needles. Cells were cultured at $1.6 \times 10^3/\text{cm}^2$ in Greiner dishes (Cellstar), pre-coated with 0.5 mg/ml poly-ornithine (Sigma) and 20 $\mu\text{g}/\text{ml}$ laminin (Invitrogen) in growth medium (1:1 mix of Dulbecco's modified Eagle's and RPMI media with Monomed supplement (CSL, Melbourne) containing 1% FCS and 50 ng/ml recombinant human NGF β (Peprotech) at 37°C , 5% CO_2 in a humidified incubator. Twenty-four hours after plating, cells were washed and medium was replaced with growth medium containing NGF or 500 ng/ml anti-NGF monoclonal antibody (Chemicon). The viable neurons within a randomly chosen segment of each well were counted before NGF withdrawal and at the indicated times after withdrawal. Cell viability is presented as a percentage of the total number of neurons viable in that segment at time 0. Neuron viability was assessed on the basis of morphology and scored blind relative to genotype.

Superior cervical ganglia neuron culture assays

Sympathetic neurons were isolated from the superior cervical ganglia (SCG) of P0-P1 mice and cultured as described previously (Whitfield et al., 2004). Briefly, 3500-4500 neurons were plated on 13-mm glass coverslips coated with poly-L-lysine (Sigma) and laminin (Invitrogen) and cultured in medium supplemented with 50 ng/ml 2.5S NGF (Cedarlane), 20 μM fluorodeoxyuridine and 20 μM uridine. After 5-6 days, 100-200 neurons over two to four randomly selected fields of each coverslip were counted and then NGF was withdrawn as described (Whitfield et al., 2004). The same selected fields were scored again at different time points after NGF withdrawal to assess the proportion of attached (healthy) neurons compared with those at time 0. Cell viability is expressed as the percentage of neurons attached at a given time versus that at time 0.

Cerebellar granule neuron culture assays

CGNs were isolated from postnatal day 7 (P7) pups as described (Miller and Johnson, Jr, 1996). Briefly, cerebella were cleaned of meninges, diced and incubated in 0.3 mg/ml trypsin at 37°C for 15 minutes. Cerebellar fragments were triturated and the resulting cell suspension filtered through a Nitex 3-20/14 mesh filter (Sefar). Neurons were plated in Greiner dishes pre-coated with poly-L-lysine (Sigma). Cells were cultured in K25+S (basal medium Eagle (Gibco BRL) containing 10% heat inactivated FCS (dialysed against balanced salt solution), 2 mM L-glutamine and 25 mM KCl) at 35°C , 5% CO_2 in a humidified incubator. 10 μM AraC (Sigma) was added 36-48 hours post plating. Medium was changed after 4 days and K^+ withdrawn from cultures after 7 days by replacing medium with K5+S (like K25+S medium but with 5 mM KCl). Neuron viability was assessed using the Live/Dead assay (Molecular Probes). Viability was determined from photomicrographs of five representative fields from each well and scored blind relative to treatment and genotype.

Haematopoietic cell colony forming assays

Foetal livers were isolated from E13.5 wild-type and *Hrk*^{-/-} embryos from the same litter and dissociated by gentle pipetting. Cells were plated in 35-mm Petri dishes at 2.5×10^4 cells per plate in 1 ml of 0.3% bacto-agar (Difco) prepared in modified DME with 10% iron-fortified bovine calf serum (Hyclone). Cytokines were added either at the time of plating, or 24, 48 and 72 hours post plating: mouse SCF at 100 ng/ml (a kind gift from Helene Martin, WEHI), mouse interleukin 3 (IL3) at 2500 U/ml (Peprotech) and human EPO at 2U/ml (Janssen Cilag). Cells were cultured at 37°C , 10% CO_2 in a humidified incubator and the resulting colonies counted 7 days after addition of cytokines.

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