The zinc finger protein NRIF interacts with the neurotrophin receptor p75^{NTR} and participates in programmed cell death

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NRIF (neurotrophin receptor interacting factor) is a ubiquitously expressed zinc finger protein of the Krüppel family which interacts with the neurotrophin receptor p75^{NTR}. The interaction was first detected in yeast and then biochemically confirmed using recombinant GST–NRIF fusions and p75^{NTR} expressed by eukaryotic cells. Transgenic mice carrying a deletion in the exon encoding the p75^{NTR}-binding domain of NRIF display a phenotype which is strongly dependent upon genetic background. While at the F₂ generation there is only limited (20%) embryonic lethality, in a congenic BL6 strain *nrif*^{−/−} mice cannot survive beyond E12, but are viable and healthy to adulthood in the Sv129 background. The involvement of NRIF in p75^{NTR}/NGF-mediated developmental cell death was examined in the mouse embryonic neural retina. Disruption of the *nrif* gene leads to a reduction in cell death which is quantitatively indistinguishable from that observed in p75^{NTR}^{−/−} and *ngf*^{−/−} mice. These results indicate that NRIF is an intracellular p75^{NTR}-binding protein transducing cell death signals during development.

Keywords: apoptosis/embryonic lethality/genetic background/p75^{NTR}/zinc finger protein

Introduction

The neurotrophins, including nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 and neurotrophin-4/5 have a well-established and essential role as agents preventing programmed cell death during the development of the mammalian nervous system (Snider, 1994). This function is mediated through the binding of neurotrophins to a family of tyrosine kinases, the trk receptors. In addition, there is a considerable body of evidence to suggest that the neurotrophins have a wider role in regulating neuronal function, including the modulation of synaptic plasticity (McAllister *et al.*, 1999), the regulation of dendritic arborization (Ruit *et al.*, 1990;

McAllister *et al.*, 1996) or the control of growth cone guidance *in vitro* (Zheng *et al.*, 1994; Gallo *et al.*, 1997). Recently, and somewhat surprisingly, the neurotrophins have also been shown to promote programmed cell death during normal development. In contrast to the survival activity mediated via the trk receptors, neurotrophin-induced apoptosis is a function mediated by a neurotrophin receptor unrelated to the trks, the p75^{NTR} receptor (Dechant and Barde, 1997). A role for p75^{NTR} in cell death has been documented (for a review, see Casaccia-Bonofil *et al.*, 1999) using cultured cells (Rabizadeh *et al.*, 1993; Casaccia-Bonofil *et al.*, 1996; Gu *et al.*, 1999; Soilu-Hänninen *et al.*, 1999), including neurons (Barrett and Bartlett, 1994; Davey and Davies, 1998). *In vivo*, p75^{NTR}-mediated cell death has been observed in the developing avian and murine retinae (Frade *et al.*, 1996; Frade and Barde, 1999) and in sympathetic neurons (Bamji *et al.*, 1998). However, the actual binding partners of p75^{NTR} involved in pro-apoptotic signalling are not well characterized.

Initially, p75^{NTR} was thought merely to modulate neurotrophin binding to the trks and the subsequent signal transduction through well-established tyrosine kinase receptor pathways (Segal and Greenberg, 1996). Indeed, substantial evidence supports such a co-receptor role for p75^{NTR}, including an increase in binding affinity (Hempstead *et al.*, 1991; Battleman *et al.*, 1993) and selectivity (Benedetti *et al.*, 1993) of a given trk for its cognate neurotrophin. Also, p75^{NTR} associates with all three trk receptors and can be co-immunoprecipitated with them (Bibel *et al.*, 1999). In addition, trk signalling is enhanced in the presence of p75^{NTR} (Davies *et al.*, 1993; Lee *et al.*, 1994; Verdi *et al.*, 1994). However, the widespread expression of p75^{NTR}, including in a variety of cells not expressing catalytic forms of trks, suggested that p75^{NTR} also has an independent function. The first indication that p75^{NTR} could initiate a signal transduction pathway independently of the trks was the observation that neurotrophin binding to p75^{NTR} stimulated the hydrolysis of sphingomyelin to produce the intracellular mediator ceramide (Dobrowsky *et al.*, 1995), although the function of this lipid second messenger is still not fully understood. Further, NGF was reported to activate the transcription factor NF-κB in neuroblastoma cells (Korner *et al.*, 1994) as well as in primary cultures of sensory (Wood, 1995) and sympathetic neurons (Maggirwar *et al.*, 1998), and in both Schwann cells (Carter *et al.*, 1996; Khursigara *et al.*, 1999) and oligodendrocytes (Ladiwala *et al.*, 1998; Yoon *et al.*, 1998). This activation of NF-κB was shown to occur through binding to the p75^{NTR} receptor (Carter *et al.*, 1996; Khursigara *et al.*, 1999). Neurotrophin binding to p75^{NTR} has also been observed to stimulate the stress-activated kinase, c-jun kinase, which has been implicated in mediating p75^{NTR}-induced apoptosis

Fig. 1. (A) Isolation of NRIF as a p75^{NTR} interactor from a yeast two-hybrid screen. Screening of a human fetal library for p75^{NTR}-interacting proteins resulted in the isolation of several candidate cDNAs. Yeast clones capable of growing under selective conditions (His⁻ Trp⁻ Leu⁻ on galactose plates) were tested further against other baits (bicoid, GST) and empty plasmid to confirm the specificity of the interaction. Only yeast expressing both p75^{ICD} and NRIF grew under selective conditions. (B) Mapping of the interacting regions. Deletion constructs expressing the indicated fragments of p75^{ICD} (left) and mouse NRIF (right) were co-transfected in yeast and their ability to support growth on selective media was compared with yeast co-expressing p75^{ICD} and NRIF (residues 521–832). Whereas the three p75^{ICD} fragments supported growth at comparable rates (left), the NRIF region N-terminal to the zinc fingers was clearly the most important for the interaction (right).

(Casaccia-Bonnet et al., 1996; Bamji et al., 1998; Yoon et al., 1998).

The p75^{NTR} receptor belongs to a large family of transmembrane proteins including the tumour necrosis factor (TNF) receptors R1 and R2, Fas, CD40, CD30, OX40, lymphotoxin- β receptor, CD27 and DR3, 4 and 5 (for a review, see Casaccia-Bonnet et al., 1998). Cysteine repeats in the extracellular domains originally formed the basis for defining members of this family. In addition, the cytoplasmic domains of several of these receptors have features in common, suggesting their involvement in protein–protein interactions. At the structural level, the cytoplasmic domain of both FAS/Apo-1/CD95 and of p75^{NTR} shows a similar motif consisting of a closely packed bundle of six short α -helices (Huang et al., 1996; Liepinsh et al., 1997). Several of these protein–protein interaction domains are now known to initiate signalling cascades leading to cell death or to the activation of the transcription factor NF- κ B. For receptors such as TNFR1 and R2, as well as Fas, CD40 or CD30, a number of cytoplasmic proteins have been identified which associate with these domains and appear to function as signal transducers (for a review, see Nagata, 1997). One such adaptor protein, TRAF6, initially identified on the basis of its interactions with CD40 and interleukin-1 (IL-1) (see Arch et al., 1998), has been implicated as mediating p75^{NTR} activation of NF- κ B (Khursigara et al., 1999).

We report here the identification of a novel protein which interacts with the intracellular domain of p75^{NTR} which we term NRIF, for Neurotrophin Receptor Interacting Factor. NRIF appears to play a role in p75^{NTR}-

mediated apoptosis since mice with the *nrif* gene deleted exhibit reduced cell death in early retinal cells, similar to p75^{-/-} and the *ngf*^{-/-} mice (Frade and Barde, 1999).

Results

Isolation of NRIF as an intracellular interactor of p75^{NTR} in a yeast two-hybrid screen

To identify proteins interacting with p75^{NTR}, the intracellular domain (ICD) of the rat p75^{NTR} receptor (residues 244–396) was fused to the C-terminus of the lexA DNA-binding protein in a yeast expression vector (Gyuris et al., 1993). This bait plasmid was co-transfected into a leucine-deficient yeast strain containing the lexA-binding domain upstream of the *LEU2* gene, along with a human fetal brain library fused to the B42 transcription activator under the control of a GAL1-inducible promoter in pJG4-5. We isolated 17 clones which demonstrated selective growth on galactose.

Upon sequence analysis, one of the clones, designated pJG-NRIF, was found to encode 1.3 kb of a novel sequence fused in the reading frame of B42 and having homology with a number of zinc finger proteins. This clone was also able to activate transcription of *lacZ* from the reporter plasmid pSH18-34 (which contains lexA-binding domains upstream of *lacZ*) when co-transfected with p75^{NTR}ICD, as evidenced by β -galactosidase staining (data not shown). To assess further the specificity of the interaction with p75^{NTR}, several ‘false baits’ were tested, including plasmids coding for the *Drosophila* protein bicoid, GST and the empty bait plasmid. Only co-transfection of

Fig. 2. Amino acid sequence of the predicted NRIF protein. Predicted amino acid sequence as translated from the *nrif* 6.3 cDNA clone (see text). Large arrows indicate the two potential translation initiation sites. The positions of the two N-terminal KRAB boxes, KRAB A and KRAB B, the pseudo-finger and the five zinc finger domains, are indicated. A group of regularly spaced leucine residues clustered at the N-terminal part of the protein, downstream of the KRAB domains, indicated in bold (L), could function as a nucleocytoplasmic signal (Nigg, 1997). The underlined sequence (residues 329–374) shares relatively high homology with several other KRAB-containing zinc finger proteins. The small arrow at position 530 marks the N-terminal end of the partial, human *nrif* cDNA isolated from the yeast two-hybrid screen. A potential nuclear localization signal is underlined. The sequence contains consensus sites for amidation, cAMP phosphorylation, CK2 phosphorylation, PK-C phosphorylation, myristoylation and Asn glycosylation. Sequence data have been submitted to the DDBJ/EMBL/GenBank database under accession No. AJ242914.

pJG-NRIF with p75^{NTR}ICD enabled the yeast to survive (Figure 1).

Cloning and sequence analysis of mouse NRIF

To obtain a full-length clone of mouse NRIF, a post-natal day 1 mouse cDNA library was screened with a 0.9 kb fragment from the human clone which contained the putative zinc finger domain. A 3.289 kb clone (clone 6.3) was isolated which contained a 2.645 kb uninterrupted, open reading frame with two consecutive, 5' Kozak consensus sequences (Kozak, 1995), two Krüppel-related boxes (KRAB) at the N-terminus (Bellefroid *et al.*, 1991; Rosati *et al.*, 1991; Constantinou-Deltas *et al.*, 1992), five zinc finger motifs followed by a potential nuclear localization signal (NLS) at the C-terminus and a 644 bp 3'-untranslated region (Figure 2). Primer extension analysis demonstrated that cDNA clone 6.3 corresponds to an NRIF full-length transcript (data not shown). Depending on whether the first or the second translation initiation codon is used, the predicted NRIF protein consists of 832 or 828 residues and has a mol. wt of 94 080.0 or 93 617.53 Da and a pI of 9.65 or 9.77, respectively.

Mouse NRIF clone 6.3 (Figure 2) is 56% identical overall to the partial human NRIF clone at the amino acid

Fig. 3. Tissue distribution of the *nrif* mRNA. Northern blot analysis (A) and poly(A)⁺ RNA dot blots (B) were used to characterize the RNA banding pattern and accumulation levels of the *nrif* mRNA. (A) The *nrif* cDNA probe detects a double band of ~3.7 kb (arrow) which is most abundant in adult testis, as well as a minor, larger transcript (asterisk). (B) The accumulation levels of the *nrif* mRNA were quantified with a dot blot (Clontech) where the loading was normalized to mRNA expression levels of eight housekeeping genes. The *nrif* mRNA is expressed ubiquitously and at relatively low levels in all tissues examined, except in testis. Sk. Musc., skeletal muscle; Sm. Musc., smooth muscle; Sub. gl., submaxillary gland; E7, E11, E15 and E17, whole embryos at the corresponding embryonic (E) stages. Note that the hybridization signal at E7 is 2-fold higher than at E11 and 3.7-fold higher than at E15. The signal detected in the negative control 'E.coli DNA' is likely to be due to the probe being prepared from an *E.coli* host.

level. Like human NRIF, it contains five predicted zinc fingers and a pseudo-finger which are 83% identical between human and mouse. The N-terminal half of the protein is much more divergent, sharing only 27% sequence identity. A search in the DNA and protein sequence databases while preparing this manuscript revealed that both the human and the mouse NRIF sequences have been identified independently. Human NRIF corresponds to the partial Zfp2 (DDBJ/EMBL/GenBank accession No. U71598) sequence submitted in 1996 by Petroni *et al.* (unpublished), who identified it as an up-regulated mRNA in an erythroleukaemia cell line. Similarly, a fragment of the mouse NRIF sequence is also present in the expressed sequence tag (EST) database as a 628 bp, Sugano mouse kidney mkia *Mus musculus* cDNA clone (EM_NEW:AI317700, submitted 18.12.98,

Fig. 4. Biochemical interaction between p75^{NTR} and NRIF. To confirm the interaction observed in yeast, recombinant GST–NRIF fusion proteins (diagrammed in **A**) were expressed in *E. coli* and immobilized on beads. (**B**) Whole extracts from 293 cells expressing HA-tagged p75 (left panel) and HA-tagged p75^{ICD} (right panel) were incubated with NRIF-loaded beads in a ‘pull-down’ assay. Co-precipitated p75^{NTR}, visualized with an anti-HA antibody, was detected only when beads were loaded with GST–NRIF (lanes 1 and 2), but not when the beads contained only GST (lane 3). Negative controls included beads loaded with non-NRIF-expressing bacterial extracts (control bacteria), and cell extracts from mock transfections (control cells). Lanes 5 (left panel) and 6 (right panel) correspond to whole-cell lysates from cells expressing HA-p75 or HA-p75^{ICD}, respectively. The unspecific band detected at ~26 kDa in the right panel is most likely due to an excess of GST protein as seen in Coomassie-stained gels (not shown).

WashU-HHMI Mouse EST Project; 99.4% identity in a 170 bp overlap with mouse NRIF).

The chromosomal locations of the mouse and the human NRIF genes have been determined. Mouse NRIF was mapped using a BSS DNA panel and two oligonucleotide primers which detected a single strand conformation polymorphism (SSCP) (Beier *et al.*, 1992; Beier, 1993) between the two parental strains (C57BL/6J × SPRET/Ei and SPRET/Ei) in the last intron of the *nrif* gene (see Figure 6A). The locus has been given the Zfp110 symbol and maps to chromosome 7, co-segregating with a large cluster of other loci at the most proximal position (see <http://www.jax.org/resources/documents/cmdata/bkmap/maps/7MapPage.html>). Given the presence of a zinc finger cluster on this chromosome which is conserved in human chromosome 19 (Stubbs *et al.*, 1996; Shannon and Stubbs, 1998), mapping of the human NRIF gene to the telomeric region of chromosome 19 further confirms that mouse NRIF (corresponding to cDNA clone 6.3) and the human NRIF sequence (originally isolated from the yeast two-hybrid screen) are indeed orthologues.

Mapping the interaction domains

The intracellular domain of p75^{NTR} can be divided into two sub-domains: the juxta-membrane region (residues

244–290) is 95% conserved between chick (Heuer *et al.*, 1990) and human (Johnson *et al.*, 1986; Huang *et al.*, 1996), suggesting some important, conserved function, and the C-terminus (residues 300–399) has homology to other proteins of this receptor family (Feinstein *et al.*, 1995). This latter region can be further subdivided into two segments showing a low degree of homology with the *Drosophila* death protein *Reaper* (residues 300–350 and 366–399). To determine which regions are important for the interaction between NRIF and p75^{NTR}, deletion mutants were tested in the yeast system (Figure 1B). The juxta-membrane sequence was sufficient for interaction with human NRIF and supported growth of the yeast on Leu– plates to a similar extent as the full-length p75^{ICD}. Interestingly, the so-called death domain (Liepinsh *et al.*, 1997) in the remaining C-terminus also interacted with NRIF, suggesting that these two regions may regulate the interaction co-operatively or that NRIF may bind as a dimer to both domains. The first *Reaper*-like region could not be tested alone for interaction because it auto-activated *LEU2* transcription in the absence of NRIF.

To determine which regions of mouse NRIF are responsible for association with p75^{NTR}, deletion constructs of the mouse clone were prepared from an initial construct containing only the last third of the cDNA

Fig. 5. Subcellular localization of NRIF in transfected 293 cells. **(A)** 293 cells were transiently transfected with EGFP (a–c) or EGFP–NRIF (d–f). Surviving cells (since EGFP–NRIF expression kills many cells that are removed as floaters) were fixed and stained with DAPI to visualize nuclei (b and e). In contrast to EGFP (c), EGFP–NRIF shows mainly nuclear localization, sometimes with a punctuate appearance (f). **(B)** 293 cells were transiently co-transfected with EGFP–NRIF and p75^{NTR}, fixed, stained with DAPI (h) and immunostained for p75 (k). Note the difference in efficiency of p75 expression (k) compared with EGFP–NRIF-expressing cells (i). While most cells still show nuclear localization of EGFP–NRIF, co-expression of the p75^{NTR} receptor causes three times as many cells to also express EGFP–NRIF in the cytoplasm (arrowhead), compared with EGFP–NRIF alone.

(region corresponding to the human NRIF fragment originally isolated from the yeast screen). Three mouse NRIF deletion constructs were generated (Figure 1B, right), deleting the pseudo-finger and the zinc fingers, only the zinc finger domain, or the N-terminal region. No significant interaction was observed between p75^{NTR} and the zinc fingers, as would be predicted if this region is a DNA- (or RNA-) binding domain. In contrast, the yeast co-transfected with the NRIF construct lacking the pseudo-finger grew best, suggesting that this remaining region is responsible for the association of NRIF with p75^{NTR}.

NRIF is expressed ubiquitously in mouse tissues

Northern blots probed with the mouse cDNA clone indicated a major double band of ~3.7 kb for the mRNA encoding NRIF, as well as a minor transcript of higher molecular weight, which most likely corresponds to a highly related transcript encoded by a homologous gene (Figure 3A; see Discussion). The NRIF mRNA appears to be ubiquitously expressed albeit at relatively low levels, except in testis (Figure 3A and B). Intact sciatic nerve shows no or very low transcript levels, as indicated by RT–PCR analysis, but NRIF is strongly expressed in dissociated Schwann cells (data not shown). RT–PCR analysis performed in mouse embryonic tissues prepared at E15 indicated that NRIF is also ubiquitously expressed during development, and at higher levels than in the adult. The NRIF mRNA is also expressed in all cell lines analysed (data not shown).

Biochemical interaction of NRIF and p75^{NTR} in vitro

The physical interaction between NRIF and p75^{NTR} was verified biochemically by co-precipitation of the p75 protein expressed in transfected 293 cells with recombinant mouse NRIF protein immobilized on Sepharose beads. Two different portions of the mouse NRIF cDNA were fused to GST: GST–NRIFΔN contains only the last third of the NRIF protein, and GST–NRIFΔZnf expresses full-length NRIF without its zinc fingers (Figure 4A). The levels of recombinant NRIF protein produced and their binding to the Sepharose beads vary depending on the portion of the protein expressed: GST–NRIFΔZnf is induced and bound to the beads at levels comparable to those of the GST moiety alone, while GST–NRIFΔN, containing the five zinc fingers, is poorly expressed, difficult to solubilize under a variety of conditions, and binds inefficiently to the beads. To test the interaction of these two proteins with p75^{NTR}, 293 cells were transfected with rat p75^{NTR} cDNA clones (Figure 4A) expressing either haemagglutinin (HA)-tagged p75^{NTR}ICD (HA-p75ICD) or HA-tagged full p75^{NTR} (HA-p75). As detected by immunoblot, GST–NRIF fusions immobilized on Sepharose beads were capable of binding both the intracellular domain of p75 (HA-p75ICD; Figure 4B right) and the full-length receptor (HA-p75; Figure 4B left) from transfected cells even under high salt conditions, whereas GST alone could not. This confirms the biochemical interaction of NRIF with p75^{NTR}. Curiously, GST–NRIFΔZnf pulls down a slightly different form of p75^{NTR} (Figure 4B, left, lane 2).

Fig. 6. Genomic targeting of the mouse *nrif* gene. (A) Deletion of the *nrif* exon encoding the p75^{NTR}-binding region. Shown are the mouse *nrif* cDNA clone 6.3 (predicted amino acid sequence shown in Figure 2), mouse genomic clone 4 isolated from a 129Sv genomic library, and the targeting construct, where the region encoding the p75-interacting portion was replaced by a pGKneo cassette in the antisense orientation. Following homologous recombination in ES cells, the targeted allele can be detected by Southern analysis of genomic *EcoRV* digests probed with the depicted 5' external probe. (B) Genotype analysis. Each PCR (performed on genomic DNA from tail biopsies) contains three primers capable of amplifying the wild-type 521 bp allele (+/+ lanes) and the 572 bp targeted allele (-/- lanes). Heterozygous mice amplify both bands (+/- lanes). Annealing positions for the three primers are indicated by arrows in (A). (C) RT-PCR analysis of *nrif*^{+/+} and *nrif*^{-/-} littermates. The absence of transcript from the *nrif* targeted region was confirmed by RT-PCR from RNA prepared from the listed tissues/organs. Wild-type samples contain the *nrif* transcript (upper panel, right), whereas mutant littermates (left) do not express any detectable levels of the targeted region, even after 40 PCR cycles. Equal cDNA levels were used in all PCRs as shown by comparable GAPDH amplification products (lower panel). DRG, dorsal root ganglia; Sp. cord, spinal cord; ScN, sciatic nerve; Sk. muscle, skeletal muscle.

Subcellular localization of the NRIF protein

In order to visualize the subcellular distribution of the NRIF protein in eukaryotic cells, we constructed an enhanced green fluorescent protein (EGFP)-NRIF protein fusion and monitored its expression by fluorescence microscopy. Already at this level, it became clear that overexpression of NRIF can kill transfected cells. Induction of apoptosis was quantified using an enzyme-linked immunosorbent assay (ELISA) method (see Frade and Barde, 1999). Between 20 and 28 h after transfection, NRIF-transfected cells accumulate 86% more free nucleosomes in their cytoplasm than do mock-transfected cells.

Despite the very low rate of successful transfection and expression, the few viable cells that express EGFP-NRIF show primarily nuclear localization of the protein (Figure 5A). Fluorescence levels are variable, ranging from diffuse to strong, with a granulate pattern which does not co-localize with nucleoli (Figure 5A, panel f, and data not shown). Co-transfection of p75^{NTR} substantially changes this exclusively nuclear localization: in some co-transfected cells, EGFP-NRIF can be seen both in the nucleus and in the cytoplasm (Figure 5B, panels i and k), or even only in the periphery, co-localizing with p75^{NTR}. This indicates that the physical interaction between NRIF and p75^{NTR} can also take place in living cells. Whether the mostly nuclear localization of NRIF is

an artefact of overexpression in transfected cells due to the cytomegalovirus (CMV) promoter is a possibility that cannot be excluded, but expression of NRIF under the control of weaker promoters does not yield detectable levels of expression.

Genomic targeting of the mouse *nrif* gene

In order to evaluate the functional significance of the interaction between NRIF and p75^{NTR}, we generated a mouse line with a targeted deletion in the *nrif* gene. The targeting strategy was designed so that the resulting mice would lack the NRIF fragment demonstrated to bind p75^{NTR} (as mapped in the yeast assay, see Figure 1B). A 6.5 kb *Bam*HI genomic clone (clone 4) isolated from a 129Sv library was identified and selected for genomic targeting of *nrif*. Subcloning of this phage fragment into a pBluescript vector and sequencing revealed the presence of two intronic regions interrupting the spacer region of NRIF (Figure 6A) and confirmed the identity of this clone as the genomic counterpart of the NRIF 6.3 cDNA described above. The 1.2 kb genomic fragment that encodes the p75^{NTR}-binding domain and four of the five zinc fingers were replaced by a pGK-neo selection cassette, and the resulting targeting construct (Figure 6A) was electroporated into R1 cells. Correct homologous recombination events were identified by Southern analysis

Genotypic ratio	+/+	+/-	-/-
Mixed (129Sv/BL6) background: F ₂ , postnatal	124	239	91
BL6 background, postnatal (7 backcross generations)	32	49	0
BL6 background, E11	11	21	17 (*4 readsorbed embryos)

Fig. 7. The *nrif* mutation is viable in mixed (BL6/129Sv) and 129Sv backgrounds, but embryonically lethal in congenic BL6 strains. Table: genotypic ratios obtained for the *nrif* mutation at the F₂ generation (mixed 129Sv/BL6 background) and at the N₇ F₁ generation (seven BL6 backcross generations followed by one intercross), after birth ('postnatal') and at E11. While at the F₂ generation there is only a 20% reduction in the proportion of *nrif*^{-/-} mice, in a BL6 background the *nrif* mutation is embryonically lethal, and no *nrif*^{-/-} mice survive beyond E12. Photo: severe growth retardation observed at E12 in *nrif*^{-/-} embryos in the BL6 background. The photograph shows six members of one litter analysed at E12. Note the difference between the three embryos of the upper row (*nrif*^{-/-}) as compared with their three wild-type littermates shown below. At E12, *nrif*^{-/-} embryos are severely growth retarded or in different stages of reabsorption. By E15, all *nrif*^{-/-} embryos presumably have been resorbed.

using an external 5' probe (Figure 6A). We identified 13 potential candidates amongst 650 isolated G418-resistant clones and, after confirming correct integration with an internal probe, four clones were further selected for injection into BL6 blastocysts. Chimeric mice generated from two of these ES clones (2/4 and 4/39) transmitted the mutation through their germline, and the progeny resulting from the two lines showed the same phenotypic traits at the following generations. A PCR-based assay was developed to show that only the genomic copy corresponding to the 6.3 cDNA was targeted and to screen for the mutant allele (Figure 6B). Complete absence of the targeted region was demonstrated by the lack of RT-PCR product in *nrif*^{-/-} tissues (Figure 6C).

The effect of the *nrif* mutation is strongly dependent on the genomic background

At the F₂ generation, *nrif*^{-/-} mice appear to be viable and fertile, with no obvious gross anatomical defect. Homozygous mutant males appear to be more docile and show an age-dependent, large reduction (up to 70% in young animals) in testis weight, although histological sections through testes show no obvious morphological alterations (data not shown). Genotypic segregation at the F₂ generation slightly deviates from Mendelian: ~20% of the *nrif*^{-/-} progeny die before or shortly after birth (Figure 7).

Fig. 8. Reduction of apoptosis in the retina of *nrif*^{-/-} embryos. Quantification of apoptotic levels in E15.5 retina was done from *nrif*^{+/+} and *nrif*^{-/-} littermates. (A) RT-PCR analysis performed on *nrif*^{+/+} (*n* = 4) and *nrif*^{-/-} (*n* = 7) retinæ demonstrating the presence of p75^{NTR} and NGF in *nrif*^{-/-} embryos (B: blank control). (B) Levels of soluble nucleosomes in *nrif*^{-/-} embryos were normalized to the amount of protein and then to the mean obtained for *+/+* embryos. In *nrif*^{-/-}, there is a 50% decrease in the levels of cell death, compared with 48% observed in *p75*^{-/-} and 56% in *ngf*^{-/-} embryos (Frade and Barde, 1999).

In order to generate congenic mouse strains harbouring the *nrif* mutation, F₁ heterozygous males were mated to BL6 females and founder F₀ chimeric males were mated to 129Sv females. In each case, this initial mating was followed by nine consecutive backcrosses of heterozygous males to BL6 or to 129Sv females, respectively. Surprisingly, to date, interbreeding of heterozygous animals at the N₆(BL6) backcross generation could never produce viable *nrif*^{-/-} progeny (Figure 7), whereas the 129Sv lineage produces *nrif*^{-/-} pups even after 10 generations of consecutive backcrosses. Embryonic death in the BL6 background seems to occur around E12, since no living *nrif*^{-/-} embryos were found at E13, E14 or E15, whereas several litters examined at E11 contained living homozygous mutants in Mendelian proportions (Figure 7). With wide variability, E12 *nrif*^{-/-} embryos showed very

different degrees of growth retardation in comparison with their wild-type littermates (Figure 7). Such a lethal phenotype could be rescued easily by interbreeding of heterozygous mice in the two congenic strains [*nrif*^{+/-}-N₁₀(BL6)×*nrif*^{+/-}-N₁₀(Sv129)], indicating that only the BL6 context cannot support the *nrif* mutation in homozygosis.

NRIF plays a role in developmental cell death in the mouse retina

In order to evaluate the possible involvement of NRIF in the pro-apoptotic pathway of p75^{NTR} *in vivo*, we quantified cell death levels in the retina of wild-type and *nrif*^{-/-} E15.5 F₂ littermates. Nine litters with both ^{+/+} and ^{-/-} embryos were analysed, and the amounts of free nucleosomes in retinal extracts quantified as described before (Frade and Barde, 1999). Despite variability, as previously observed with both the *ngf* and the p75^{NTR} mutations (see Frade and Barde, 1999), we observed a significant reduction in the levels of cell death in the *nrif*^{-/-} group when compared with their wild-type littermates (Figure 8). This reduction is quantitatively indistinguishable from what has been observed in p75^{-/-} and *ngf*^{-/-} mutants (Frade and Barde, 1999).

Discussion

Accumulating evidence suggests that p75^{NTR} can initiate programmed cell death in a number of specific cell types (Casaccia-Bonnel et al., 1998). However, unlike the well-characterized signal transduction pathways used by the trk receptors (Kaplan and Miller, 1997), the signalling molecules recruited by p75^{NTR} in its pro-apoptotic function are still unknown. To date, only TRAF6 has been reported as a p75^{NTR}-interacting molecule mediating p75^{NTR} effects, and it seems to be involved in the activation of NF-κB (Khursigara et al., 1999). To gain more insight into the underlying mechanisms of p75^{NTR} signalling, we used the yeast two-hybrid system and identified NRIF. This novel protein associates with the cytoplasmic domain of p75^{NTR}. Together with TRAF6, NRIF represents the first adaptor molecule found to interact directly with p75^{NTR} to transduce signals elicited by neurotrophin binding at the cell membrane.

The mouse *nrif* gene encodes a 94 kDa zinc finger protein of the Krüppel family. Although originally isolated on the basis of its interaction with the intracellular domain of p75^{NTR} in yeast cells (Figure 1), our pull-down assays demonstrated that full-length p75^{NTR} from mammalian cells also interacts with NRIF (Figure 4B, left). Further, the ICD of p75^{NTR} alone was precipitated by NRIF (Figure 4B, right), suggesting that the extracellular portion of the receptor is not required for the interaction. As mapped in yeast cells, NRIF and p75^{NTR} appear to interact between the region immediately N-terminal to the zinc fingers of NRIF, and two discrete portions of the p75^{NTR} ICD (Figure 1): the highly conserved juxta-membrane sequence of p75^{NTR}, shown to be the TRAF6-binding domain (Khursigara et al., 1999), and the death domain (Liepinsh et al., 1997). Deletion of either region of p75^{NTR} ICD did not prevent interaction with NRIF (Figure 1), indicating that NRIF can bind to both regions, possibly as a dimer. In yeast cells, the zinc fingers alone do not participate in the

binding of NRIF to p75^{NTR} (Figure 1), indicating that they are potentially free and capable of binding DNA or RNA.

The primary structure of NRIF (Figure 2), including its five zinc fingers, two N-terminal KRAB domains and a putative NLS, suggest that it may translocate to the nucleus and regulate gene expression. Indeed, several zinc finger proteins containing KRAB domains have been shown to be transcriptional repressors (Witzgall et al., 1994). The subcellular distribution of NRIF supports such a role also for this interactor: when expressed in 293 cells, EGFP-NRIF is primarily nuclear, but upon co-expression of p75^{NTR} both proteins can be seen co-localizing outside the nucleus (Figure 5B). This raises the possibility that the p75^{NTR}-NRIF signalling system may function in a manner similar to other receptor-transcription factor associations, such as Notch and Su(H) (Weinmaster, 1997) or the transforming growth factor-β (TGF-β) receptors and SMADs (Heldin et al., 1997). Upon activation of these receptors, their associated factors are released and translocate to the nucleus where they mediate their respective functions.

The *nrif* mRNA is expressed ubiquitously, but at higher and constant levels in the embryo, while in the adult mouse accumulation levels of the *nrif* mRNA vary among tissues and organs (Figure 3). The presence of a human *nrif* homologous and syntenic gene in the human genome further supports an evolutionarily conserved, important function. Furthermore, both the mouse and the human genome contain a second, highly related *nrif* gene which most likely arose as a result of a gene duplication. Zinc finger gene clusters in mouse chromosome 7 and human chromosome 19 are currently being fully sequenced (Lawrence Livermore Laboratories), so it will be interesting to see whether more members of the *nrif* family are described in the future.

In support of an essential role for NRIF, all cell lines examined express the *nrif* mRNA, as determined by RT-PCR studies (data not shown). However, the cellular function of NRIF has proven difficult to assess due to the very low levels of detectable expression upon transfection of the cDNA in a variety of cell lines, e.g. COS7, BHK, CV1, NIH-3T3 and HN10. Even 293 cells, known and widely used for their high efficiency of transfection, only rarely express transfected *nrif*, indicating that there may be a very narrow range of accumulation of the NRIF protein still allowing cell viability and/or proliferation. Overexpression of the NRIF protein seems to be toxic for transfected cells, and the proportion of successfully transfected cells that can express tagged *nrif* cDNAs is very low. It is possible that only cells in a particular stage of their cell cycle allow overexpression of NRIF.

To evaluate more directly the physiological significance of the NRIF-p75^{NTR} interaction *in vivo*, we generated *nrif*^{-/-} mice by homologous recombination. Based on our mapping studies in yeast (Figure 1) and with recombinant NRIF protein (Figure 4), we replaced the p75^{NTR}-binding region of NRIF with a pGKneo selection cassette (Figure 6). The resulting *nrif*^{-/-} mice express a truncated NRIF transcript lacking the p75^{NTR}-binding region and four of its five zinc fingers. These mice show a reduction in cell death in embryonic retina (Figure 8) which is virtually identical in its extent to what is observed in the p75 and *ngf* mutants (Frade and Barde,

1999), pointing to these three genes being operative in the same pathway. Our results are compatible with the hypothesis that during the period of naturally occurring cell death in the developing retina, NRIF actively participates in the apoptotic process mediated by p75^{NTR}. Several zinc finger proteins of the C2H2 type have already been shown to participate in programmed cell death. For example, TIEG, a TGF- β -inducible gene in pancreatic epithelial cells, has been shown to induce apoptosis when overexpressed (Cook *et al.*, 1998). Similarly, *Zac1* is a tumour suppressor gene expressed only in brain and pituitary gland and, like *p53*, it can regulate apoptosis and cell cycle arrest (Spengler *et al.*, 1997). Krox-20, originally identified as a serum response immediate early gene (IEG) (Chavrier *et al.*, 1988), activates transcription from a specific DNA recognition element (Swirnow and Milbrandt, 1995), controls segmentation during hindbrain development (Schneider-Maunoury *et al.*, 1993) but also regulates susceptibility to apoptosis in developing Schwann cells (Zorick *et al.*, 1999). Like Krox-20 and Nurr77 (Watson and Milbrandt, 1990), *nrif* is also induced in cultured cells by NGF; yet a direct interaction of a KRAB-containing zinc finger protein with a neurotrophin receptor has so far only been shown for NRIF. Presumably, binding of NGF to p75^{NTR} induces a conformational change in the receptor which then releases NRIF. NRIF, in turn, could translocate to the cell nucleus where its zinc fingers would bind specific DNA sequences, while its KRAB domains could silence basal and inducible transcription. Future work will attempt to identify downstream targets of the NRIF protein, the p75^{NTR} gene itself being a candidate. Indeed, p75^{NTR} mRNA levels are up-regulated in *nrif*^{-/-} mice (E.Casademunt, unpublished data, and Figure 8).

Unexpectedly, the phenotype of the *nrif* mutation appears to be masked in the mixed BL6/Sv129 background. Although at the F₂ generation (BL6:Sv129 50:50 hybrids) the mutation causes only limited lethality, after seven backcross generations to the BL6 strain *nrif*^{-/-} mice are no longer viable beyond E12 (Figure 7). The fact that the lethal phenotype can be rescued by only one generation of mating to *nrif*^{+/-} mice of Sv129 background strongly indicates that the BL6 genome cannot support the homozygous mutation. This situation is in principle analogous, but exactly opposite in the details to that described for the *Pax-2* (Schwarz *et al.*, 1997), *Egfr* (Threadgill *et al.*, 1995) and *Tgfb1* mutations (Bonyadi *et al.*, 1997), all of which have lethal consequences in a congenic Sv129 background. Our results are a further illustration of the need to evaluate the effect of targeted mutations in different congenic backgrounds (see e.g. Banbury Conference on Genetic Background in Mice, 1997; Frankel, 1998).

In conclusion, NRIF is the first zinc finger protein described to interact with the p75^{NTR} receptor and to play an active role in developmental programmed cell death. NRIF may therefore represent a new class of receptor-associated transcription factors important for regulating cell viability.

Materials and methods

Interaction trap

Plasmid vectors and yeast strain EGY48 required for the interaction trap were all generously provided by R.Brent (Gyuris *et al.*, 1993). The bait

p75ICD construct was generated by PCR amplification from a full-length rat p75^{NTR} cDNA clone (provided by E.Shooter) using the following primers: 5'-ATC GAA TTC TTC AAG AGG TGG AAC AG and 5'-GAG GGA TCC GTG AGT TCA CAC TGG GG, and in-frame cloning into the *EcoRI*-*Bam*HI sites of the yeast bait *lexA* fusion pEG202 vector. The interaction trap was performed as previously described (Gyuris *et al.*, 1993), using a human, 22-week-old, fetal, frontal cortex cDNA library cloned into the pJG4-5 plasmid (provided by D.Kraicnc). Out of 1.3×10^6 colonies plated, 200 grew under selective conditions and 17 of them showed galactose-dependent growth (conditions for induction of the library). Yeast DNA was isolated and retested for specific interaction with the p75^{NTR}ICD bait and with additional baits, e.g. bicoid and GST. Deletion constructs of rat p75^{NTR} were generated by PCR amplification of the cDNA encoding residues 244–290 and 295–396, and subcloned into pEG202. Deletion constructs of mouse *nrif* were PCR amplified from the cDNA encoding residues 521–661, 521–689 and 690–832, and subcloned into pJG4-5.

Cloning of the mouse *nrif* cDNA and genomic clones

Approximately 7.5×10^5 p.f.u. of a P1 mouse brain cDNA library in λ ZAPII (Stratagene) were plated and transferred to nylon membranes (Hybond N, Amersham). The filters were screened with a 0.9 kb, *EcoRI*-*XhoI* human *nrif* cDNA probe including the zinc finger domain, radiolabelled by random priming (Amersham). Hybridization was carried out with 5 $\times$ SSC, 5 $\times$ Denhardt's, 50 mM Tris-HCl pH 7.4, 1% SDS, 40% formamide and 100 μ g/ml salmon sperm DNA at 42°C overnight. The filters were washed at 65°C in 1 $\times$ SSC, 0.1% SDS. Positive clones were *in vivo* excised using Exassist phage and SOLR recipient cells (Stratagene), and recovered as pBluescript SK plasmids. Sequence analyses were done with the MacMolly or the Wisconsin Genetics Computer Group software.

Approximately 2×10^6 genomic clones from a mouse (129/Sv) genomic library made in the λ FIXII vector (Stratagene) and plated in LE392 host cells were screened using two radioactively labelled, overlapping fragments of the mouse NRIF 6.3 cDNA (*NcoI*-*EcoRI*, 1582 bp and *NcoI*-*Bam*HI, 653 bp). Hybridization and washing were done according to Church and Gilbert (1984). Six out of 14 independent clones which hybridized with both probes appeared to be independent isolates of the same gene, and all further work was with genomic clone 4 (Figure 6A).

Northern and RT-PCR analysis

Total RNA was extracted following a modified RNAzol B method (P.Chomczynski, Wak-Chemie Medical GmbH) from mouse embryonic (E15.5) and adult (1–2 months old) tissues. For Northern analysis, 20 μ g of total RNA was glyoxylated, electrophoresed on 1.2% agarose gels in 10 mM NaH₂PO₄/Na₂HPO₄ pH 6.5 and vacuum-transferred to Hybond N membranes. *nrif* mRNA levels were quantified using an RNA Master Blot (Clontech) following the manufacturer's instructions and a Molecular Dynamics phosphorimager. Northern blots were probed with a 1.1 kb *SpeI*-*EcoRV* fragment of the *nrif* cDNA which excludes the zinc finger region. RT-PCRs were done from 500 ng of total RNA essentially as described (Friedel *et al.*, 1997), using the following PCR primers: 5'-GTG ATG CTA GAG ACC TTG GG; 5'-CAC CAT GGC ATC GCT ACT GTC C. Because of the low levels of expression of the *nrif* mRNA, the PCR requires 5-fold higher levels of starting cDNA than those required to amplify the GAPDH transcript. Cycle parameters were 94°C 4 min; 94°C 30 s, 58°C 30 s, 72°C 40 s, 35 cycles.

Recombinant NRIF protein expression

Production of recombinant NRIF proteins in *E.coli* JM109 was induced for 4 h at room temperature by 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) with 0.1 mM ZnCl₂. Bacterial pellets were resuspended in lysis buffer [50 mM HEPES pH 7.9, 1% Triton X-100, 5 mM dithiothreitol (DTT), 0.5 M NaCl, 0.1 mM ZnCl₂, 1 mM phenylmethylsulfonyl fluoride (PMSF), 5 μ g/ml aprotinin, 5 μ g/ml leupeptin] and lysed by sonification. Soluble protein obtained by centrifugation was bound to glutathione-Sepharose 4B beads (Pharmacia Biotech) for 4 h at 4°C. Control beads were prepared by using the same host bacteria lacking any plasmid. Bound beads were washed extensively with 1 $\times$ phosphate-buffered saline (PBS), 0.3 M NaCl, 0.1 mM ZnCl₂. Protein binding to the beads was assessed by SDS-PAGE analysis of a small aliquot eluted with Laemmli sample buffer.

Cell culture and transfection

293 cells (Graham *et al.*, 1977) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal

calf serum (Sigma) and 1% penicillin/streptomycin (Gibco-BRL) at 37°C and 5% CO₂. Transient transfection was by the calcium phosphate precipitation method according to Chen and Okayama (1987), seeding 2.5×10^6 cells onto 10 cm plates the day preceding transfection. Transfection efficiency was estimated by parallel transfection with a pCMV-GFP vector (pEGFP-N1, Clontech).

'Pull-down assays'/Western blot

Two days after transfection, cells were washed with ice-cold PBS and lysed in 1 ml of lysis buffer/plate [25 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 0.8 % NP-40, 4 mM Pefabloc (Boehringer Mannheim), 5 µg/ml aprotinin, 5 µg/ml leupeptin] followed by centrifugation at 15 000 g, 4°C for 20 min. The supernatant (300 µl) was mixed with binding buffer (900 µl; 25 mM HEPES pH 7.5, 150 mM KCl, 5 mM MgCl₂, 0.13 mM ZnCl₂, 5 mM DTT, 4 mM Pefabloc, 5 µg/ml aprotinin, 5 µg/ml leupeptin), and incubated overnight with a 50 µl bed volume of protein-loaded glutathione–Sepharose 4B beads (see above). After washing the beads once with washing buffer 1 (WB1: 40 mM HEPES pH 7.5, 300 mM NaCl, 5 mM MgCl₂, 0.1 mM ZnCl₂, 1 mM DTT) and twice with WB2 (150 mM NaCl, otherwise like WB1), the proteins were eluted by boiling with 50 µl of 2× Laemmli buffer for 8 min, separated on a 10 or 12% SDS–polyacrylamide gel and transferred to PVDF membranes (Immobilon-P, Millipore). Protein blots were blocked with 5% non-fat milk powder in TBST (25 mM Tris pH 7.5, 150 mM NaCl, 0.1% Tween-20), incubated overnight at 4°C with 0.5 µg/ml anti-HA monoclonal antibody (Boehringer Mannheim) in blocking buffer, washed five times with TBST, incubated with goat anti-mouse IgG-POD (Pierce, 1:10 000 in blocking buffer) for 1 h at room temperature, washed again and finally developed using the ECL detection system (Amersham).

Construction of p75^{NTR} and NRIF expression plasmids

The intracellular domain of p75^{NTR} (starting with Lys251) used for pull-down experiments was amplified by PCR from rat p75^{NTR} cloned into pcDNA3 (Invitrogen) with the following primers: 5'-GTC AAG CTT GCC ACC ATG TAT CCT TAT GAC GTG CCT GAC TAT GCC GGG GGA AAG AGG TGG AAC AGC TG (which introduces a Kozak consensus sequence followed by a HA tag and a glycine linker), and 5'-GTC GCG GCC GCT CAC ACT GGG GAT GTG GCA G (primer 'p75end'), and then subcloned into *Hind*III–*Not*I-digested pRc/CMV (Invitrogen), creating HA-p75ICD. Full-length -p75 was amplified by PCR from the same template with primers 5'-GTC GCT AGC TTA TCC TTA TGA CGT GCC TGA CTA TGC CGG GGG AAA GGA GAC ATG TTC CAC AGC and 'p75end' and subcloned into pRc/CMV AC7 (Bibel *et al.*, 1999).

The full-length NRIF coding sequence (residues 2–832) was amplified by PCR with primers 5'-GAGGACAGTATGGCTTCC and 5'-CTAC-AATTTGGGCTGCTTC, and subcloned into pEGFP-C1, fusing EGFP at the N-terminus of NRIF to produce pEGFP-NRIF, or into pFLAG-CMV-2 creating N-terminally flagged NRIF (pFLAG-NRIF). The GST–NRIFΔZnf construct was made by subcloning the NRIF cDNA sequence excluding the zinc fingers from pFLAG-NRIF into pGEX-2T (Pharmacia). GST–NRIFΔN was generated by PCR amplification from cDNA clone 6.3 (Figure 6A) and subcloning into pGEX-4T-1 (Pharmacia).

Immunocytochemistry

Transiently transfected 293 cells grown on 13 mm glass coverslips (coated with poly-L-lysine, Sigma, 0.01%) were fixed with 2–4% paraformaldehyde in PBS for 15 min at room temperature, permeabilized with 0.2% Triton X-100/PBS, blocked in 10% bovine serum albumin, 10% normal goat serum (NGS)/PBS for at least 1 h at room temperature, and incubated overnight at 4°C with a polyclonal antibody against the intracellular domain of p75^{NTR} in PBS/5% NGS. After three 10 min washes with PBS, cells were incubated for 1 h at room temperature with LRSC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and 4',6-diamidino-2-phenylindole (DAPI) at 0.5 µg/ml in PBS/5% NGS. Following five 10 min washes with PBS, cells were mounted in 'Immuno Fluore Mounting Medium' (ICN Biochemicals) and analysed using fluorescence microscopy.

Nr1f gene targeting. Breeding scheme

To target the *nr1f* gene, the genomic region corresponding to the region between residues 520 and 777 (Figures 2 and 6A) was replaced by the neo cassette from the pGKneopA vector (from Soriano *et al.*, 1991), resulting in a targeting construct where the 5' arm was 3.2 kb, the 3' arm was 2.3 kb and the transcription orientation of the neo cassette was

opposite to that of the *nr1f* gene. R1 ES cells (Nagy *et al.*, 1993) were electroporated, selected with G418 and screened by Southern blot with an external 5' probe (Figure 6A) which recognizes a 6.5 kb wild-type band and a 9 kb mutant band in a genomic *Eco*RV digest.

BL6 mice were of the C57BL/6NJClrl (Charles River Laboratories) strain; Sv129 mice of the 129SvPas substrain. Chimeric mice were generated by transfer of BL6 blastocysts injected with targeted ES cells into pseudopregnant females. After generation of F₂ viable homozygous offspring, an intensive programme of backcrossing to BL6 and 129Sv was started by mating F₁ heterozygous females to BL6 males and founding chimeric mice to 129Sv females. In each case, heterozygous females were mated to BL6 and 129Sv males, respectively, for 10 consecutive generations in order to obtain congenic strains of each background. Mice were genotyped by Southern and PCR analysis from tail or yolk sac DNA extracted as described before (Laird *et al.*, 1991; Hogan *et al.*, 1994). PCR primers for *nr1f* genotyping were as follows: 5'-TCC ATG ACA CGA GGT TTA CG, 5'-CAG AAG TGC TAA GTT CCT CG and 5'-GAT TCG CAG CGC ATC GCC TTC, and cycle parameters: 94°C 4 min; 94°C 30 s, 57°C 30 s, 72°C 1 min, 35 cycles. The two first primers amplify the 521 bp wild-type band, whereas the first and third primer amplify the 572 bp targeted band.

Quantification of cell death in E15.5 retina

Heterozygous F₁ mice were mated until a vaginal plug (E0.5) was observed. Fifteen days later (E15.5), embryonic retinas were prepared and processed as described in Frade and Barde (1999), with duplicate measurements from each retina. The results are based on the analysis of nine litters (78 embryos). Six litters generated by homozygous +/+ and –/– parents were also analysed to evaluate the degree of variability between littermates of the same litter.

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