Intracellular FACS staining

Cells were stained for surface CD19 (Pharmingen), fixed by resuspension in 4% paraformaldehyde, permeabilized by the addition of 0.1% saponin and subsequently incubated with the anti-SLP-65 antibody B-211. A fluorescein isothiocyanate-labelled goat anti-mouse IgG2a was used as a secondary antibody. FACS analysis was performed with a FACSCalibur (Becton Dickinson).

Received 30 January; accepted 28 March 2003; doi:10.1038/nature01608.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank E. Bergsträsser (Zürich), U. zur Stadt (Hamburg), J. Harbott (Giessen) and O. G. Ottmann (Frankfurt) for pre-B ALL samples; W.-D. Ludwig (Berlin) and J. Harbott (Giessen) for immunophenotyping and molecular genetic studies of the patients from Freiburg and Hanover; P. Nielsen for reading the manuscript and H. Mossmann for help in the mouse experiments; and C. Eschbach and U. Stauffer for technical support. Financial support for these experiments was provided by the Deutsche Forschungs Gemeinschaft and the Leibniz programme to M.R.

Competing interests statement The authors declare that they have no competing financial interests.

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Humanin peptide suppresses apoptosis by interfering with Bax activation

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Bax (Bcl2-associated X protein) is an apoptosis-inducing protein that participates in cell death during normal development and in various diseases[1]. Bax resides in an inactive state in the cytosol of many cells. In response to death stimuli, Bax protein undergoes conformational changes that expose membrane-targeting domains, resulting in its translocation to mitochondrial membranes, where Bax inserts and causes release of cytochrome *c* and other apoptogenic proteins[2]. It is unknown what controls conversion of Bax from the inactive to active conformation. Here we show that Bax interacts with humanin (HN), an anti-apoptotic peptide of 24 amino acids encoded in mammalian genomes[3,4]. HN prevents the translocation of Bax from cytosol to mitochondria. Conversely, reducing HN expression by small interfering RNAs sensitizes cells to Bax and increases Bax translocation to membranes. HN peptides also block Bax association with isolated mitochondria, and suppress cytochrome *c* release *in vitro*. Notably, the mitochondrial genome contains an identical open reading frame, and the mitochondrial version of HN can also bind and suppress Bax. We speculate therefore that HN arose from mitochondria and transferred to the nuclear genome, providing a mechanism for protecting these organelles from Bax.

To identify proteins that might bind selectively to inactive Bax, we performed a yeast two-hybrid screen of a human testis complementary DNA library using as a bait human Bax(S184K)—a mutant form of Bax locked into an inactive conformation[5]. One cDNA clone confirmed to interact specifically with Bax(S184K) contained an in-frame cDNA encoding HN.

HN was originally identified as an anti-apoptotic peptide encoded in a cDNA that rescued neuronal cells from apoptosis induced by presenilin mutants associated with familial Alzheimer's disease and by amyloid-β protein[3]. Mutagenesis studies showed that the cysteine at position 8 of the HN peptide is critical for its anti-apoptotic function[3,4]. We therefore contrasted the ability of wild-type and Cys8Pro (C8P) mutant HN to interact with Bax in co-immunoprecipitation assays, expressing HN peptides as fusions with green fluorescent protein (GFP) in human cells. GFP–HN co-immunoprecipitated Myc-tagged Bax from HEK293T cells, but not GFP or GFP–HN(C8P) (Fig. 1a). Immunoblot analysis confirmed production of all proteins. GFP–HN also co-immunoprecipitated endogenous Bax from certain cell lines (not shown).

We surveyed multiple Bcl2-family proteins for interactions with HN by co-immunoprecipitation experiments (Fig. 1a). GFP–HN did not co-immunoprecipitate with other Bcl2-family proteins that are predicted to share structural similarity with Bax, including Bcl2, Bcl-xL, Bak, Bcl-B, Mcl1 and Bok[6].

Expression of endogenous HN has been demonstrated in the testis and colon of mice[7]. Using a rabbit polyclonal antibody raised against HN peptide (gift of I. Nishimoto) for co-immunoprecipitations, we determined that endogenous HN peptide interacts with endogenous Bax in mouse testis. Endogenous HN was also determined to be a predominantly cytosolic protein (see Supplementary Information).

To characterize further the binding of HN to Bax, we generated an *in vitro* binding assay using the fluorescence polarization technique.

Full-length Bax protein was produced in bacteria and purified[8], and was then incubated with rhodamine-conjugated synthetic purified HN peptide. As shown in Fig. 1c, Bax bound rhodamine–HN in a concentration-dependent and saturable manner, with an estimated dissociation constant (K_d) of 2 nM. In contrast, various control peptides of similar length, such as rhodamine–CD40 (residue P250 to G266), did not display interactions with Bax in fluorescence polarization assays.

We next performed various cell-based experiments to explore the functional significance of HN-binding to Bax (Fig. 2; see also Supplementary Information). First, we tested the effects of HN in yeast (*Saccharomyces cerevisiae*), where ectopic expression of Bax induces cell death through a mechanism similar to mammalian cells (reviewed in ref. 9). We expressed Bax using a conditional promoter, where Bax is produced when yeast are plated on galactose- but not glucose-containing medium[10]. Co-expression of wild-type HN peptide, expressed as a fusion protein with TAD, rescued yeast from Bax-induced lethality, whereas HN(C8P) mutant did not (Fig. 2a). Immunoblot analysis demonstrated that both the HN and HN(C8P) fusion proteins were produced at comparable levels in yeast, excluding differences in expression as a trivial explanation for the findings (not shown).

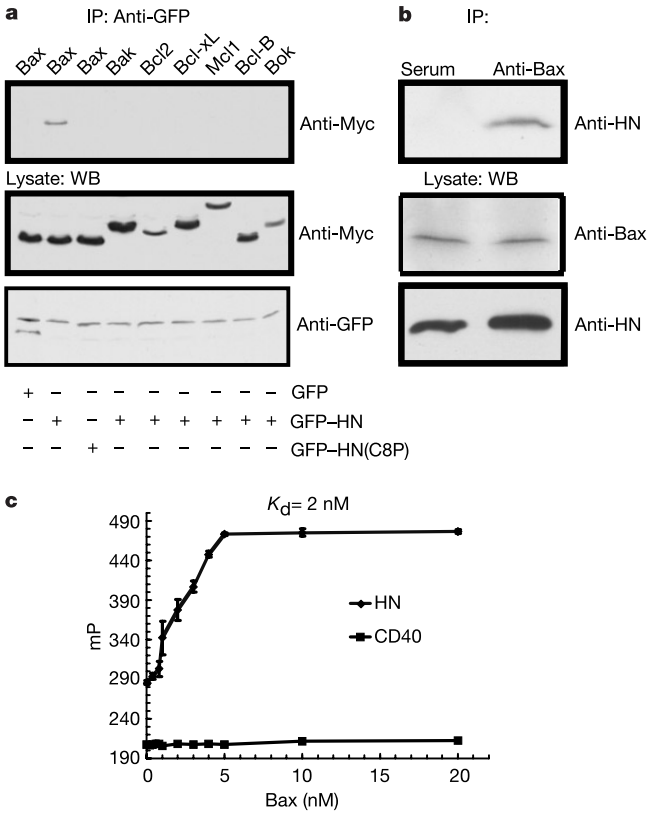

Figure 1 HN interacts with Bax. **a**, HEK293T cells were transfected with pcDNA3-Myc plasmids encoding Bax or other Bcl2-family proteins with GFP, GFP–HN or GFP–HN(C8P). Cell lysates were immunoprecipitated with polyclonal anti-GFP antibody. The immunoprecipitates (IP) or the lysates were blotted with anti-Myc or anti-GFP antibodies, respectively. The slower migrating form of GFP seen in lane 1 represents an alternative form of GFP produced from the wild-type (control) p-EGFP-C1 plasmid (http://www.clontech.com). **b**, Testes of three-week-old mice were homogenized in lysis buffer (0.1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, plus protease inhibitor cocktail). The lysates were immunoprecipitated with control rabbit serum or polyclonal anti-mouse Bax antibody. The immunoprecipitates or the lysates were subjected to electrophoresis in Tris/Tricine gels (16%T, 3%C), electroblotted to PVDF membrane, and immunoblotted with polyclonal anti-HN antibody or monoclonal anti-Bax antibody 6A7 (Trevigen). **c**, The affinity (K_d) of Bax–HN interaction was measured by fluorescence polarization assay, using various concentrations of purified recombinant Bax and 40 nM rhodamine-conjugated HN peptide. Control peptide from CD40 is also shown (mean ± s.d.; $n = 3$).

Next, we examined the effects of HN overexpression in mammalian cells using several cell death stimuli known to induce apoptosis partly through Bax-dependent mechanisms[11]. When Flag-tagged HN or Flag-tagged control (27-amino-acid) peptides were expressed in CSM14.1 cells (immortalized rat hippocampal neurons)[12], apoptosis induced by kinase inhibitor (staurosporine (STS)), serum deprivation and ultraviolet irradiation was selectively suppressed by HN–Flag (Fig. 2b, c). Conversely, apoptosis induced by a Bax-independent death stimulus, tumour necrosis factor (TNF), was not suppressed (Fig. 2c). Although HN reduced by half apoptosis induced by 0.2 μM STS (52 ± 4% compared with 28 ± 3%; $P = 0.03$), HN-mediated protection was overcome at higher doses of STS (1 μM) (Fig. 2b), consistent with invoking Bax-independent (HN-insensitive) mechanisms of apoptosis at higher doses[11].

As an alternative to over-expression studies, synthetic small interfering RNA (siRNA)[13] was used to knock down expression of endogenous HN in SF268 cells, a glioblastoma cell line empirically determined to contain high levels of endogenous HN. Transfection into SF268 cells of HN siRNA, but not control siRNAs, reduced endogenous levels of HN, as determined by immunoblotting, correlating with increased sensitivity to apoptosis induced by STS and serum-deprivation (mitochondrial-dependent) but not by TNF-related apoptosis-inducing ligand (TRAIL; mitochondria-independent) (Fig. 2d). The specificity of the siRNA-mediated effect was further confirmed by various control transfections, which did not nonspecifically sensitize cells to apoptosis (Supplementary Information). Again, whereas HN siRNA doubled the percentage of cell death in cultures exposed to low doses of STS (53 ± 4% compared with 27 ± 3%), this sensitization was overcome by high doses of STS (Fig. 2d), consistent with the studies of HN overexpression discussed above.

As HN immunoprecipitates with Bax but not Bak, we next contrasted the effects of HN on apoptosis induced by these two Bcl2-family proteins; performing transient transfection experiments using CSM14.1 neurons or prostate cancer PC-3 cells. HN suppressed by half the percentage of apoptosis induced by Bax, whereas Bak-induced apoptosis was unaffected (Fig. 2e).

For the next experiment investigating the functional significance of HN-binding to Bax, we contrasted the effects of HN in Bax-expressing and Bax-deficient human cells, reasoning that if the mechanism by which HN suppresses apoptosis involves Bax binding, then HN should not protect Bax-deficient cells. For these experiments, we used HCT116 colon cancer cells, which contain one intact and one mutant *BAX* allele, and a mutant of HCT116 (gift of B. Vogelstein) in which the remaining allele was disrupted by homologous recombination, producing Bax-deficient cells[14]. In contrast to their differences in Bax expression, these cell lines both express comparable amounts of Bid (Fig. 2f) and several other Bcl2-family proteins (not shown). In HCT116 parental cells, HN–Flag significantly reduced the percentage of cells undergoing STS-induced apoptosis, suppressing apoptosis by approximately half when modest doses of STS were used (62 ± 5% compared with 32 ± 3%; $P = 0.002$) (Fig. 2f). In contrast, HN–Flag failed to suppress apoptosis in Bax-deficient HCT116 cells. On the basis of previous studies using cells from gene knockout mice that have shown that either Bax or Bak is sufficient for STS-induced apoptosis[11], we presume the reason why HN-mediated protection is overcome at high doses of STS in parental HCT116 cells is because HN does not interfere with Bak.

Finally, using a series of HN mutants, we observed a perfect correlation between Bax binding and suppression of apoptosis induced by overexpression of Bax (Supplementary Information).

To explore the mechanism by which HN suppresses apoptosis induced by Bax, we examined the effects of HN overexpression on

STS-induced translocation of GFP–Bax protein from cytosol to mitochondria in cells[2,5,15]. For these experiments, synthetic HN or HN(C8P) peptides were introduced into cells[16], and we monitored the translocation of GFP–Bax to mitochondria by microscopy[2,5,15], determining the percentage of cells in which cytosolic fluorescence was diffuse versus punctate. Treatment of GFP–Bax-expressing cells with STS induced mitochondrial translocation of GFP–Bax in most cells (Fig. 3a; see also Supplementary Information), whereas GFP–Bax translocation was suppressed by about half in cells transduced with HN but not HN(C8P) control peptide. These findings were also confirmed by subcellular fractionation, where cytosol and mitochondria-enriched heavy membrane fractions were

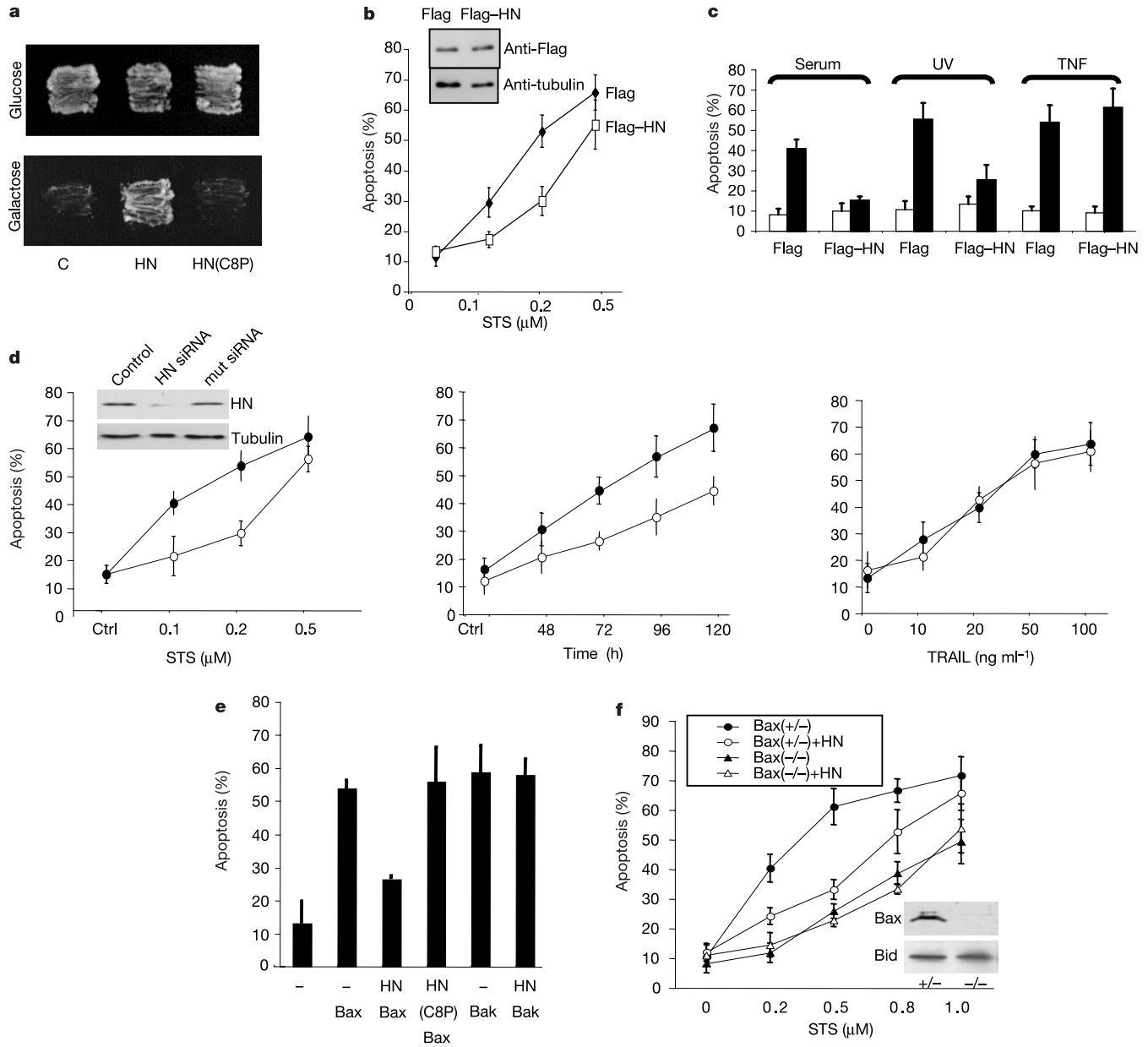

Figure 2 HN inhibits cell death induced by Bax. **a**, HN prevents Bax-induced cell death in yeast. Cells were co-transformed with YEp51-Bax (encoding Bax under control of a *GAL10* promoter) and plasmid control (C) or plasmids encoding HN or HN(C8P). Cells grown in glucose-containing medium were streaked onto either glucose or galactose plates to suppress or induce Bax expression, respectively. **b**, CSM14.1 neuronal cells were transfected with plasmids encoding Flag–HN or Flag–control peptide, together with GFP-encoding plasmid at a 4:1 ratio. Lysates prepared from replicate cultures of transfected cells were normalized for total protein content and analysed by immunoblotting using anti-Flag and anti-tubulin antibodies, confirming production of similar amounts of Flag–control and Flag–HN peptides (inset). **c**, CSM14.1 cells transfected as above were cultured either without further treatment (open bars) or subjected to apoptotic stimuli (filled bars), including serum deprivation for 3 days, 10 J m^{-2} ultraviolet irradiation, followed by 24 h culture, or 50 ng ml^{-1} TNF plus 1 μM 2-cyano-3,12-dioxooleana-1,9-dien-28-oic acid (CDDO) for 24 h. Apoptosis (%) was determined by DAPI staining. **d**, SF268 cells were transfected with HN siRNAs (filled circles) or scrambled control siRNAs (open circles). After 72 h, cell lysates were prepared and 100 μg was analysed by immunoblotting using anti-HN and anti-tubulin (as a loading control) antibodies (inset). (See Methods for full details.) **e**, HN protects against Bax- but not Bak-induced apoptosis. CSM14.1 cells were co-transfected with pcDNA3-HA-Bax or pcDNA3-HA-Bak together with plasmids encoding with GFP (−), GFP–HN or GFP–HN(C8P). Apoptosis was measured 48 h later among GFP$^+$ cells. **f**, HN inhibits STS-induced apoptosis in wild-type HCT116 cells, but not HCT116 *BAX*$^{-/-}$ cells. HCT116 cells heterozygous or homozygous for *BAX* gene inactivation were transfected with plasmids encoding Flag (filled symbols) or HN–Flag (open symbols), together with GFP-encoding plasmids at a 4:1 ratio. After 24 h, cells were cultured with or without 0.2–1.0 μM STS for 8 h, then apoptosis was determined for GFP$^+$ cells (mean ± s.d.; $n = 3$). Lysates from the cells were analysed by immunoblotting (inset; 20 μg) using anti-Bax and anti-Bid antibodies.

prepared from cells transduced with untagged HN or HN(C8P) peptides. We measured the relative amounts of endogenous Bax protein in these two fractions by immunoblotting (Fig. 3b).

The peptide transfer findings were extended to address the role of endogenous HN using siRNA methods. In SF268 cells, which contain high levels of endogenous HN peptide (unlike Cos7 cells), most of the Bax protein remained in the cytosol after treatment with low doses of STS (0.1 μM) (Fig. 3c). In contrast, when HN expression was knocked down by siRNA in SF268 cells, then STS-induced translocation of Bax to membranes was enhanced (Fig. 3c). We conclude therefore that HN suppresses translocation of Bax to mitochondria.

It has been suggested that HN might block apoptosis by acting outside cells, through HN-binding cell surface receptors, rather than inside cells[3,4]. To determine whether HN can act directly on Bax, we tested the effects of HN on isolated mitochondria. Addition of recombinant purified Bax protein to mitochondria *in vitro* induces cytochrome *c* release[17]. Pre-incubating Bax with HN peptide suppressed Bax association with mitochondria and reduced cytochrome *c* release (Fig. 3d). In contrast, the non-Bax-binding HN(C8P) mutant peptide did not interfere with the effects of Bax on isolated mitochondria. We conclude that HN can directly suppress the targeting of Bax to mitochondria. Furthermore, *in vitro* protein binding experiments suggested that HN stabilizes the conformation of Bax in which the carboxy-terminal hydrophobic α-helical transmembrane domain is docked onto the body of the Bax protein (Supplementary Information).

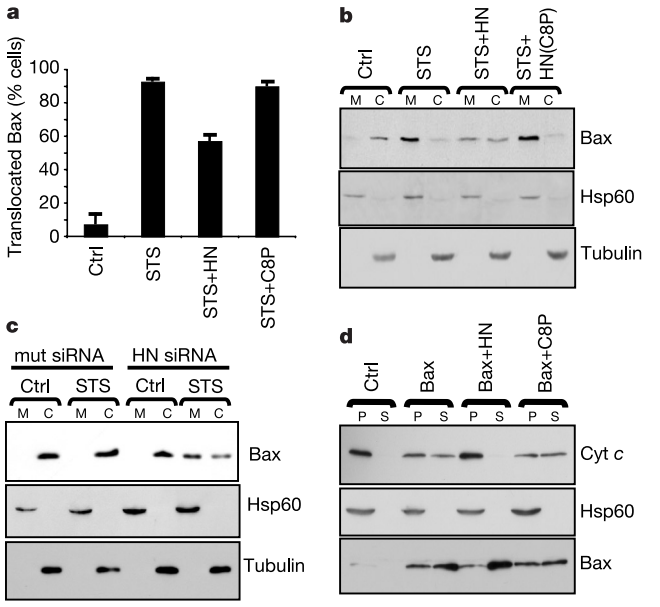

Figure 3 HN blocks Bax translocation to mitochondria. **a**, HN peptide prevents STS-induced Bax translocation *in vivo*. Cos7 cells were first transfected with GFP–Bax plasmids. After 24 h, untagged wild-type or HN(C8P) mutant peptides were introduced into cells using Chariot reagent, and 2 h later cells were treated with 1 μM STS. After 4 h, the percentage of cells with translocated Bax was determined (mean ± s.d.; $n = 3$) by confocal microscopy. **b**, Wild-type or HN(C8P) mutant peptides were introduced into Cos7 cells using Chariot reagent. Cytosolic (C) and mitochondria-containing (M) fractions were isolated by differential centrifugation[22] and analysed by immunoblotting using anti-Bax (top), Hsp60 (middle; mitochondrial marker), or tubulin (bottom; cytosol marker) antibodies. **c**, HN or mutant control double-stranded siRNAs were transfected into SF268 cells. After 48 h, cells were cultured without (control) or with 0.1 μM STS. After 24 h, cells were fractionated and analysed by immunoblotting as above. **d**, Isolated mitochondria were mixed with 400 ng Bax protein, with or without pre-incubating Bax with 100 μM HN or HN(C8P) peptides for 10 min. After 3 h at 30 °C, mitochondria were centrifuged and the resulting pellets (P) and supernatants (S) were analysed by immunoblotting using anti-cytochrome *c* (top), Hsp60 (middle) and Bax (bottom) antibodies.

During analysis of HN-encoding sequences in the human genome, we noticed an identical open reading frame (ORF) embedded in the 16S ribosomal RNA gene of the mammalian mitochondrial genome (Fig. 4a). Differences in codon usage by the endogenous protein translation machinery of mitochondria predict a slightly different HN peptide (Fig. 4b), if produced within these organelles[18]. We therefore contrasted the ability of the predicted nuclear-encoded and mitochondria-encoded HN peptides (termed HN(N) and HN(M), respectively) to bind Bax and to suppress apoptosis induced by overexpression of Bax. Using HN(N) and HN(M) fused to GFP, comparable amounts of Bax co-immunoprecipitated with both proteins (Fig. 4c). Bax-induced apoptosis was also suppressed to comparable extents by GFP–HN(N) and GFP–HN(M) (Fig. 4d). Thus, the nuclear and the mitochondrial translations of the HN ORF bind and suppress Bax.

It was proposed previously that the HN peptide is secreted from cells, acting on neighbouring cells[3]. We demonstrate here that Bax is the molecular target of HN relevant to its anti-apoptotic phenotype. Mutants of HN that fail to bind Bax also fail to suppress apoptosis. Moreover, HN peptide blocks the effects of Bax on isolated

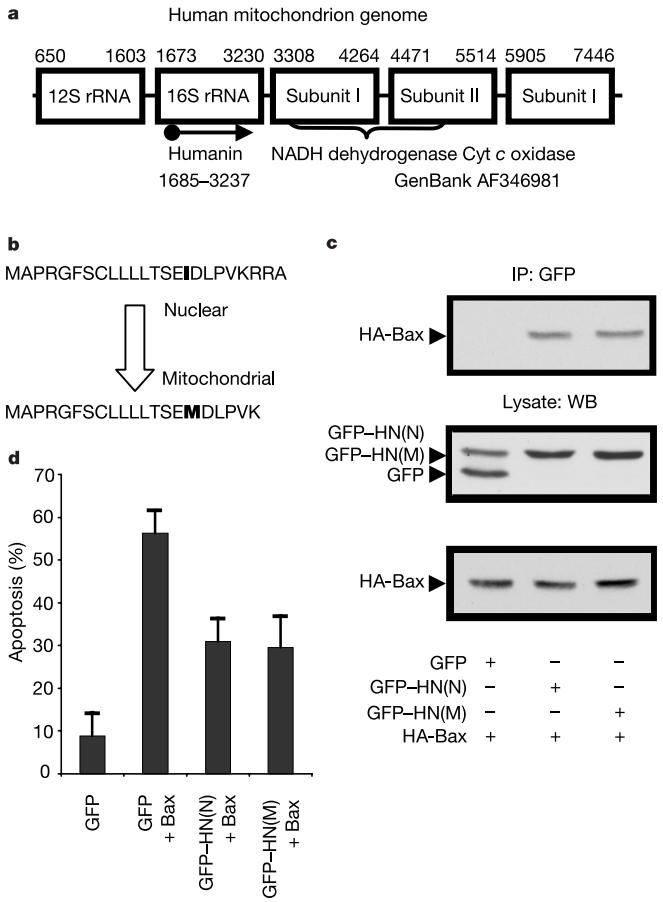

Figure 4 Comparison of nuclear (N)- and mitochondria (M)-encoded HN. **a**, An ORF identical in nucleotide sequence to the previously reported HN cDNA[3] is embedded in the 16S rRNA gene in the mitochondrial genome. **b**, The amino-acid sequence of the putative mitochondrial HN peptide was predicted according to mitochondrial codon usage. **c**, HEK293T cells were transfected with pcDNA3-HA-Bax together with plasmids encoding GFP, GFP–HN(N) or GFP–HN(M). Cell lysates were immunoprecipitated with polyclonal anti-GFP antibody. Immunoprecipitates (IP) or cell lysates were analysed by immunoblotting using anti-haemagglutinin (HA) or anti-GFP antibodies, respectively. **d**, CSM14.1 neuronal cells were co-transfected with pcDNA3-HA-Bax together with plasmids encoding GFP, GFP–HN(N) or GFP–HN(M). The percentage of apoptosis was determined 48 h later by DAPI staining (mean ± s.d.; $n = 3$).

mitochondria, excluding a need for a plasma membrane receptor (also the case in yeast), thus dissociating its mechanism of action from other mammalian proteins.

The HN peptide is encoded in several long messenger RNAs that have been detected *in vivo*, and the endogenous HN peptide, with a relative molecular mass of 3,000, has been detected in certain normal tissues[7]. Using a BLAST search, we found cDNAs identical or similar to HN in plants, nematodes, rats, mice and many other species (see Supplementary Information). Although we have no reason to suspect this ORF is expressed in mitochondria currently, the presence of an identical HN-encoding ORF embedded in the mitochondrial genome raises the possibility that replicas of this ORF were transferred during evolution from these organelles into mammalian genomes. Given that mitochondria are thought to have arisen from bacteria that established residence in the cytosol of eukaryotic cells[19], it is also intriguing that the Bax protein shares marked structural similarity with the pore-forming domains of bacterial colicin proteins, molecules which are secreted and used as weapons to kill competing bacteria (reviewed in ref. 20). Although the particular mechanisms differ, the ability of HN peptides to suppress Bax translocation to mitochondrial membranes is reminiscent of the immunity proteins (such as Cai and Cbi) of bacteria that bind and inhibit colicins[21]. HN, however, shares no sequence homology with these bacterial proteins.

Although additional mechanisms besides HN binding may be involved in the control of Bax translocation *in vivo*, determination of the structure of HN bound to Bax may suggest strategies for developing small-molecule drugs that inhibit Bax. In this regard, Bax has been implicated through gene ablation studies in mouse models in numerous diseases associated with pathological cell loss, including stroke, Parkinson's disease, and oocyte depletion during menopause, making it a promising target for new therapies. □

Methods

Yeast two-hybrid assays

Yeast two-hybrid screening of cDNA libraries was performed using hu-Bax(S184K) in pGilda. A human adult testis cDNA library (Invitrogen) was screened using standard yeast two-hybrid procedures (see Supplementary Information).

Cell culture, transfections, imaging and apoptosis assays

CSM14.1, HCT116, Cos7 and SF268 cells were cultured in DMEM high-glucose medium (Irvine Scientific) and PC-3 cells in RPMI 1640 medium, containing 10% fetal bovine serum (FBS). Transfection of cells was performed using SuperFect (Qiagen) or LipofectaminePLUS reagent (Invitrogen). CSM14.1 cells were cultured at 39 °C after transfection to inactive temperature-sensitive large T antigen, as described[12]. In Fig. 2b, CSM14.1 cells were cultured for 8 h with 0.1–0.5 μM STS, then fixed and stained with 4,6-diamidino-2-phenylindole (DAPI) to determine the percentage of apoptotic cells, evaluating ≥200 GFP^+ cells per sample (mean ± s.d.; $n = 3$). We performed confocal microscopy and apoptosis assays as described[15,22] (see also Supplementary Information). In Fig. 2d, the percentage of apoptosis was measured after the following treatments: (1) SF268 cells cultured with various concentrations of STS for 8 h, at 72 h after siRNA transfection (mean ± s.d.; $n = 3$) (left panel); (2) SF268 cells washed 4 h after transfection with HN siRNA or control dsRNA and then cultured for 2–5 days without serum (middle panel); (3) 3 days after transfection, SF268 cells were treated with 10–100 ng ml^{-1} TRAIL (Alexis) plus 25 μg ml^{-1} cycloheximide for 8 h (right panel).

Immunoblotting and immunoprecipitations

Immunoblotting was performed as described previously[22]. For co-immunoprecipitations, cells were cultured in 50 μM benzyloxycarbonyl-Val-Ala-Asp (O-methyl)-fluoromethyl ketone (zVAD-fmk) to prevent apoptosis. Cells were suspended in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 20 mM EDTA, 50 mM NaF, 0.5% NP-40, 0.1 mM Na_3VO_4, 20 μg ml^{-1} leupeptin, 20 μg ml^{-1} aprotinin, 1 mM dithiothreitol and 1 mM phenylmethyl sulphonyl fluoride (PMSF)). Lysates (200 μl diluted in 1 ml final volume of lysis buffer) were cleared by incubation with 15 μl protein G Sepharose 4B (Zymed) and then incubated with 15 μl polyclonal antibody and 15 μl protein G at 4 °C overnight. Beads were then washed four times with 1.5 ml lysis buffer before boiling in Laemmli sample buffer and performing SDS–polyacrylamide gel electrophoresis or immunoblotting.

Fluorescence polarization assays

Recombinant Bax protein was isolated from *Escherichia coli* BL21 harbouring pTYB1-Bax essentially as described[8]. For fluorescence polarization assays, various concentrations of Bax protein were incubated with 40 nM of rhodamine-conjugated synthetic purified HN peptide dissolved in water for 30 min in dark. Fluorescence polarization was measured using an Analyst AD Assay Detection System (LJL Biosystem).

Peptides

Rhodamine-conjugated HN peptide and unconjugated HN and HN(C8P) peptides were synthesized using fluorenylmethoxycarbonyl chemistry with DIC coupling[23] (see Supplementary Information).

Peptide transfections

HN or HN(C8P) peptides were transfected into GFP–Bax-transfected Cos7 cells using Chariot reagent (Active Motif)[16]. One microgram of peptide was mixed with 6 μl of Chariot reagent in 200 μl water and incubated for 30 min. Two hours before STS treatment, GFP–Bax-expressing cells in 6-well plates were washed with PBS and incubated with Chariot–peptide complex in serum-free medium at 37 °C for 1 h. Cells were incubated for an additional hour after 1 ml complete growth medium was added. Cells were then treated with STS to induce Bax translocation. Pilot experiments with fluorochrome-conjugated peptide indicated >80% cell transfection.

Preparation and transfection of siRNA

Two different double-stranded ribo-oligonucleotides with overhanging 3′ deoxy TT were prepared that target HN mRNAs (HN siRNA1: r(CCAGUGAAAUUGACCUGCC)d(TT); HN siRNA2: r(GGGUUCAGCUGUCUCUUAC)d(TT)), along with two scrambled controls (mut siRNA1: r(UUCGACCUAGGAUCACACG)d(TT); mut siRNA2: r(CGCAUCUGUUAUCGGUGUC)d(TT)). Double-stranded (ds)RNAs were dissolved in sterile 100 mM potassium acetate, 30 mM HEPES-KOH, 2 mM magnesium acetate, pH 7.4, to a final concentration of 20 μM. SF268 cells were cultured in 6-well plates in 2 ml DMEM medium containing 10% FBS. Cells at 40% confluency were transfected with a 1:1 mixture of both siRNAs or both control dsRNAs using 10 μl oligofectamine (Invitrogen) per 10 μl siRNA (final concentration 100 nM) in serum-free medium. Cells were rinsed with medium after 16 h incubation and cultured for a further 56 h before analysis. Control transfection was also performed using oligofectamine without nucleic acids (Supplementary Fig. 2 and data not shown).

Subcellular fractionation

A total of 10^7 cells were resuspended with five volumes of buffer A (20 mM HEPES-KOH, pH 7.5, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM Na_2-EDTA, 1 mM Na_2-EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethyl sulphonyl fluoride) containing 250 mM sucrose. Cells were homogenized with 25 strokes of a Teflon homogenizer, and centrifuged two times at 750*g* for 10 min at 4 °C. Supernatants were centrifuged at 10,000*g* for 20 min at 4 °C. The resulting mitochondria-containing pellets were washed twice with buffer A, then resuspended in buffer A containing 250 mM sucrose. The supernatants of the 10,000*g* spin were further centrifuged at 100,000*g* for 1 h at 4 °C to produce cytosol.

Cytochrome *c* release assays

Mitochondria were isolated from HCT116 cells by differential centrifugation as above. Purified recombinant human Bax protein[8] (400 ng for each sample) was pre-incubated with or without 100 μM synthetic HN peptide or mutant HN(C8P) peptide for 10 min at 4 °C. The untreated or peptide pre-treated Bax protein samples were then mixed with an equal amount of HCT116 mitochondria (in a volume of 40 μl) at 30 °C for 3 h. Samples were then centrifuged at 10,000*g* for 20 min to obtain pellet and supernatant fractions, measuring cytochrome *c* by immunoblotting.

Received 25 February; accepted 31 March 2003; doi:10.1038/nature01627.
Published online 4 May 2003.

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Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We wish to thank the NIH and the Department of Defense for its generous support. We also thank R. Cornell and A. Sawyer for manuscript preparation, and B. Vogelstein, D. Bredesen, R. Youle and I. Nishimoto for reagents.

Competing interests statement The authors declare that they have no competing financial interests.

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corrigenda

Fungus-growing ants use antibiotic-producing bacteria to control garden parasites

C. R. Currie, J. A. Scott, R. C. Summerbell & D. Malloch

Nature **398**, 701–704 (1999).

We reported in this Letter that, on the basis of its cell-wall chemistry, the bacterium associated with the fungus-growing ant *Acromyrmex octospinosus* is in the genus *Streptomyces* (Streptomycetaceae: Actinomycetes). It has been brought to our attention by *Nature* that R. Wirth, T. Wagner, C. Kost, I. Böttcher, W.-R. Arendholz and M. Redenbach (manuscript submitted) do not find evidence of a specialized relationship between bacteria in the genus *Streptomyces* and fungus-growing ants in the genus *Acromyrmex*. Our ongoing molecular phylogenetic analyses reveal that the specialized symbiotic bacterium associated with *Acromyrmex* is not a species of *Streptomyces*, but is instead in the actinomycetous family Pseudonocardiaceae (C.R.C. and M. Cafaro, manuscript in preparation). This genus-level misidentification does not affect our other conclusions. □

High brightness electron beam from a multi-walled carbon nanotube

Niels de Jonge, Yann Lamy, Koen Schoots & Tjerk H. Oosterkamp

Nature **420**, 393–395 (2002).

The small round spot visible in Fig. 3 does not represent the actual emission pattern, but is an artefact caused by a low-operation voltage of the micro-channel plate. This measurement error has no effect on the value of the reduced brightness as it was not determined from the measurement of the emission pattern. □

addendum

HIV-1 superinfection despite broad $CD8^+$ T-cell responses containing replication of the primary virus

Marcus Altfeld, Todd M. Allen, Xu G. Yu, Mary N. Johnston, Deepak Agrawal, Bette T. Korber, David C. Montefiori, David H. O'Connor, Ben T. Davis, Paul K. Lee, Erica L. Maier, Jason Harlow, Philip J. R. Goulder, Christian Brander, Eric S. Rosenberg & Bruce D. Walker

Nature **420**, 434–439 (2002).

The partial length HIV consensus sequences for virus A (day 18) and virus B (day 1,170) have been submitted to GenBank as accession numbers AY247251 and AY268493, respectively. □

erratum

Subsecond dopamine release promotes cocaine seeking

Paul E. M. Phillips, Garret D. Stuber, Michael L. A. V. Heien R. Mark Wightman & Regina M. Carelli

Nature **422**, 614–618 (2003).

In this Letter, the *x* axis of Fig. 4b should have ranged from −60 s to +60 s with 0 s at the grey triangle. □