with secondary antibodies from various sources (Molecular Probes, Chemicon, Vector Laboratories).

Pharmacological reagents

Treatment with pharmacological reagents occurred 30–60 min before growth factor or cytokine administration. The reagents were either present throughout the experiment or alternatively the medium was replaced after 6–12 h incubation. Reagents in this study were: LY294002 (10–50 μM; Calbiochem) and okadaic acid (1 nM to 1 μM; Calbiochem and Alomone Laboratories).

In vivo and *in vitro* kinase assays

The *in vivo* kinase assay was performed largely as recommended by the supplier (Akt Kinase Assay Kit, Cell Signaling Technology). *In vitro* kinase assays were performed using 1 × buffer (Akt Kinase Assay Kit, Cell Signaling Technology), 100 μM ATP, 0.5 mM peptide (ENNEKQMRQLSVIPPMMFDA), 300 U ml^{-1} Akt1 protein kinase. For radioactivity incorporation assay, 250 μCi ml^{-1} [γ-^{32}P]ATP were included in the reaction. The reactions were quenched by addition of SDS gel buffer, and were either spotted on nitrocellulose membranes for immunoblotting, or were analysed by PAGE using 18% acrylamide gels.

Transfections and constructs

Transfections were performed with lipofection reagents (Effectene, Qiagen) largely following the supplier's recommendations. Typically, 1–2 μg DNA was lipofected on a 60-mm dish for 6–12 h in the presence of FGF2, after which the cells received fresh medium with growth factors as indicated. Previously unpublished constructs used in this study are: pcDNA3-HA-N-CoR C-terminal (amino acids 1501–2300), pCMX-N-CoR(S401A)-Flag, pCMX-N-CoR-RAKA-Flag, pCMX-N-CoRΔ1501–1800-Flag, and pCMX-Gal4-SMRT(253–493).

Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed on neural stem cells largely as described[28]. Neural stem cells grown on 150-mm dishes were collected and crosslinked using 1% formalin for 10 min at room temperature, and the extracts were sonicated until the DNA fragments were 500–800 bp in size. Cell extracts were subsequently incubated with 5 μg IgG or antibodies against N-CoR or CBF1 overnight at 4 °C. The extracts were incubated with protein A-sepharose beads (Sigma) for 1 h. After extensive washing of the beads, proteins were eluated and reversed by crosslinking for 6 h at 65 °C. After DNA purification, PCR was performed at 29–33 cycles. Primers were: GFAP, 5′-GACTAAGCTGTTTCCTCGGC-3′ (sense), 5′-CAAGGTCACTGTACCCAGAG-3′ (antisense); HES5, 5′-CGTGTCTCTTCCTCCCATTG-3′ (sense), 5′-GATCCAGTGTGATCCGCAGG-3′ (antisense).

Received 13 June; accepted 23 September 2002; doi:10.1038/nature01156.
Published online 16 October 2002.

1. Temple, S. The development of neural stem cells. *Nature* **414,** 112–117 (2001).
2. Ghosh, A. & Greenberg, M. E. Distinct roles for bFGF and NT-3 in the regulation of cortical neurogenesis. *Neuron* **15,** 89–103 (1995).
3. Johe, K. K., Hazel, T. G., Muller, T., Dugich-Djordjevic, M. M. & McKay, R. D. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* **10,** 3129–3140 (1996).
4. Palmer, T. D., Markakis, E. A., Willhoite, A. R., Safar, F. & Gage, F. H. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* **19,** 8487–8497 (1999).
5. Temple, S. & Alvarez-Buylla, A. Stem cells in the adult mammalian central nervous system. *Curr. Opin. Neurobiol.* **9,** 135–141 (1999).
6. Hörlein, A. J. *et al.* Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. *Nature* **377,** 397–404 (1995).
7. Kao, H. Y. *et al.* A histone deacetylase corepressor complex regulates the Notch signal transduction pathway. *Genes Dev.* **12,** 2269–2277 (1998).
8. Frisén, J. & Lendahl, U. Oh no, Notch again! *Bioessays* **23,** 3–7 (2001).
9. Faux, C. H., Turnley, A. M., Epa, R., Cappai, R. & Bartlett, P. F. Interactions between fibroblast growth factors and Notch regulate neuronal differentiation. *J. Neurosci.* **21,** 5587–5596 (2001).
10. Hitoshi, S. *et al.* Notch pathway molecules are essential for the maintenance, but not the generation, of mammalian neural stem cells. *Genes Dev.* **16,** 846–858 (2002).
11. Jepsen, K. *et al.* Combinatorial roles of the nuclear receptor corepressor in transcription and development. *Cell* **102,** 753–763 (2000).
12. Raballo, R. *et al.* Basic fibroblast growth factor (Fgf2) is necessary for cell proliferation and neurogenesis in the developing cerebral cortex. *J. Neurosci.* **20,** 5012–5023 (2000).
13. Rao, M. S., Noble, M. & Mayer-Proschel, M. A tripotential glial precursor cell is present in the developing spinal cord. *Proc. Natl Acad. Sci. USA* **95,** 3996–4001 (1998).
14. Choi, B. H. Prenatal gliogenesis in the developing cerebrum of the mouse. *Glia* **1,** 308–316 (1988).
15. Yasui, Y. *et al.* Roles of Rho-associated kinase in cytokinesis; mutations in Rho-associated kinase phosphorylation sites impair cytokinetic segregation of glial filaments. *J. Cell Biol.* **143,** 1249–1258 (1998).
16. Perissi, V. *et al.* Molecular determinants of nuclear receptor-corepressor interaction. *Genes Dev.* **13,** 3198–3208 (1999).
17. Besnard, F. *et al.* Multiple interacting sites regulate astrocyte-specific transcription of the human gene for glial fibrillary acidic protein. *J. Biol. Chem.* **266,** 18877–18883 (1991).
18. Kaneko, R., Hagiwara, N., Leader, K. & Sueoka, N. Glial-specific cAMP response of the glial fibrillary acidic protein gene cell lines. *Proc. Natl Acad. Sci. USA* **91,** 4529–4533 (1994).
19. Kahn, M. A. *et al.* Ciliary neurotrophic factor activates JAK/Stat signal transduction cascade and induces transcriptional expression of glial fibrillary acidic protein in glial cells. *J. Neurochem.* **68,** 1413–1423 (1997).
20. Bellacosa, A., Testa, J. R., Staal, S. P. & Tsichlis, P. N. A retroviral oncogene, akt, encoding a serine-threonine kinase containing an SH2-like region. *Science* **254,** 274–277 (1991).
21. Morrison, S. J. Pten-uating neural growth. *Nature Med.* **8,** 16–18 (2002).
22. Rhee, Y., Gurel, F., Gafni, Y., Dingwall, C. & Citovsky, V. A genetic system for detection of protein nuclear import and export. *Nature Biotechnol.* **18,** 433–437 (2000).
23. Yaffe, M. B. *et al.* A motif-based profile scanning approach for genome-wide prediction of signaling pathways. *Nature Biotechnol.* **19,** 348–353 (2001).
24. Egloff, M. P. *et al.* Structural basis for the recognition of regulatory subunits by the catalytic subunit of protein phosphatase 1. *EMBO J.* **16,** 1876–1887 (1997).
25. Aggen, J. B., Nairn, A. C. & Chamberlin, R. Regulation of protein phosphatase-1. *Chem. Biol.* **7,** R13–R23 (2000).
26. Molne, M. *et al.* Early cortical precursors do not undergo LIF-mediated astrocytic differentiation. *J. Neurosci. Res.* **59,** 301–311 (2000).
27. Sauvageot, C. M. & Stiles, C. D. Molecular mechanisms controlling cortical gliogenesis. *Curr. Opin. Neurobiol.* **12,** 244–249 (2002).
28. Braunstein, M., Rose, A. B., Holmes, S. G., Allis, C. D. & Broach, J. R. Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.* **7,** 592–604 (1993).

Acknowledgements We are grateful to L. van Grunsven and R. McKay for their help in neural stem cell biology, and for providing advice throughout this study. Constructs and reagents were provided by D. Altomare, J. Testa, E. Lamar, C. Kintner, J. De Vellis, B. Andersen, T. Sugihara, D. Rose, and the Campagnoni Laboratory. We are also grateful to S. McMullen for microscopy assistance; C. Nelson and A. Krones for various cell culture assistance; H. Taylor for animal care; P. Myer for artwork; M. Fisher for assistance with the manuscript; V. Perissi for numerous reagents, discussions, and advice on ChIP; and members of the Rosenfeld laboratory for comments, discussions and various reagents, in particular L. Erkman, A. Gleiberman, V. Kumar, R. McEvilly and D. Solum. M.G.R. is an investigator with the Howard Hughes Medical Institute. O.H. was partially funded by the Swedish Brain Foundation. This work was funded by a grant from NIH.

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

Correspondence and requests for materials should be addressed to M.G.R. (e-mail: mrosenfeld@ucsd.edu).

Specific aspartyl and calpain proteases are required for neurodegeneration in *C. elegans*

Popi Syntichaki*, Keli Xu†‡, Monica Driscoll† & Nektarios Tavernarakis*

** Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology, Heraklion 71110, Crete, Greece*
† Department of Molecular Biology and Biochemistry, Rutgers, The State University of New Jersey, 604 Allison Road, Piscataway, New Jersey 08855, USA

Necrotic cell death underlies the pathology of numerous human neurodegenerative conditions[1]. In the nematode *Caenorhabditis elegans*, gain-of-function mutations in specific ion channel genes such as the degenerin genes *deg-1* and *mec-4*, the acetylcholine receptor channel subunit gene *deg-3* and the G_s protein α-subunit gene *gsa-1* evoke an analogous pattern of degenerative (necrotic-like) cell death in neurons that express the mutant proteins[2–6]. An increase in concentrations of cytoplasmic calcium in dying cells, elicited either by extracellular calcium influx or by release of endoplasmic reticulum stores, is thought to comprise a major death-signalling event[7,8]. But the biochemical mechanisms by which calcium triggers cellular demise remain largely unknown. Here we report that neuronal degeneration inflicted by various genetic lesions in *C. elegans* requires the activity of the calcium-regulated CLP-1 and TRA-3 calpain proteases and aspartyl proteases ASP-3 and ASP-4. Our findings show that two

‡ Present address: Program in Developmental Biology, The Hospital for Sick Children, 555 University Avenue, Toronto Ontario, M5G 1X8, Canada.

distinct classes of proteases are involved in necrotic cell death and suggest that perturbation of intracellular concentrations of calcium may initiate neuronal degeneration by deregulating proteolysis. Similar proteases may mediate necrotic cell death in humans.

Neuronal degeneration initiated by hyperactive MEC-4, an ion channel subunit that is normally required for mechanotransduction in the six touch receptor neurons of *C. elegans*, is reminiscent of excitotoxic cell death in mammals[9,10]. Electron microscopy studies of dying neurons in animals expressing a gain-of-function *mec-4* allele (*u231* or *d*; dominant) have shown extensive degradation of cellular contents during mid to late stages of cell death[9]. This ultrastructural feature suggests that proteolysis may be central to the biochemical mechanism underlying neuronal degeneration. The main executioner protease, caspase CED-3, which mediates programmed cell death (apoptosis) in *C. elegans*, and three additional CED-3-related proteases (CSP-1, CSP-2 and CSP-3) that are encoded in the nematode genome are not required for cell death induced by *mec-4(d)* (ref. 11; P.S. and N.T., unpublished data). This indicates that a distinct, non-apoptotic mechanism, which probably involves different proteases, functions in neurodegeneration in the nematode.

Aspartyl proteases are a class of catabolic hydrolases that, among others, includes lysosomal (cathepsin D) and non-lysosomal (cathepsin E) enzymes. We tested the requirement for aspartyl protease activity in neurodegeneration inflicted by hyperactive MEC-4 in touch receptor neurons in three ways. First, we used genetic backgrounds that have diminished levels of aspartyl protease activity. Nematode strains carrying mutations in three genes, *cad-1*, *daf-4* and *unc-52*, that encode otherwise unrelated proteins have been shown to maintain aspartyl protease activity that is 90% lower than in wild-type animals[12–14]. We found that neurodegeneration induced by *mec-4(d)* was suppressed in *cad-1;mec-4(d)*, *daf-4;mec-4(d)* or *unc-52;mec-4(d)* mutant strains (Fig. 1a and Supplementary Information).

Second, we treated *mec-4(d)* animals with pepstatin A, an inhibitor of aspartyl proteases. Treatment ameliorated necrosis of the six touch receptor neurons (Fig. 1a). Third, we subjected *mec-4(d)* animals to starvation conditions under which aspartyl protease activity has been shown to drop to 5–10% that of well-fed nematodes[12,15]. We observed a reduction in the number of dying neurons in the progeny of starved animals (Fig. 1a). Survival of touch receptor neurons was confirmed by the presence of cells expressing green fluorescent protein (GFP) from the *mec-4* promoter in adult animals.

Suppression of *mec-4(d)*-induced cell death in the genetic backgrounds and under the conditions examined was not a consequence of a reduction in the quantities of toxic MEC-4(d) protein. We used the *mec-4* promoter to drive expression of both *lacZ* and GFP reporter genes in *cad-1*, *daf-4* or *unc-52* mutant strains, as well as in animals treated with pepstatin A and starved animals. We did not detect any difference in reporter gene expression between these animals and well-fed wild-type animals (Fig. 1b, left, *cad-1(j1)* is shown as an example). Similarly, we assayed the relative stability of MEC-4 by using reporter genes with either *lacZ* or GFP fused at the carboxy terminus of the full-length MEC-4 protein. Quantities of protein were either not affected or even slightly increased by manipulations that reduced aspartyl protease activity (Fig. 1b, right, *cad-1(j1)* is shown).

We next determined whether aspartyl protease deficiency is generally protective against necrotic cell death. Gain-of-function (*d*) mutations in three otherwise unrelated genes, the degenerin *deg-1*, the α-7 nicotinic acetylcholine receptor Ca^{2+} channel subunit gene *deg-3*, and *gsa-1*, which encodes the $G\alpha_s$ subunit, trigger degeneration of specific sets of neurons expressing the toxic variants[2,4–6]. We tested the general requirement for aspartyl protease in neurodegeneration by introducing various death-inducing mutations into aspartyl-protease-deficient *cad-1*, *daf-4* and *unc-52* mutant strains. Cell death inflicted by toxic *deg-1(d)* and *deg-3(d)* alleles and by overexpressing the hyperactivated $G\alpha_s$(Q227L) variant (α_s*(gf)*) was suppressed in genetic backgrounds deficient in aspartyl proteases. Starvation, which results in diminished aspartyl protease activity, also ameliorated neurodegeneration (Fig. 1c–e). Neuron survival was confirmed in adult animals by scoring expression of GFP. Similar to expression of *mec-4*, expression of *deg-1*, *deg-3* and α_s*(gf)* was not reduced in protease-deficient genetic backgrounds or under starvation conditions. We conclude that aspartyl protease activity is required generally for neurodegeneration caused by deleterious mutations in

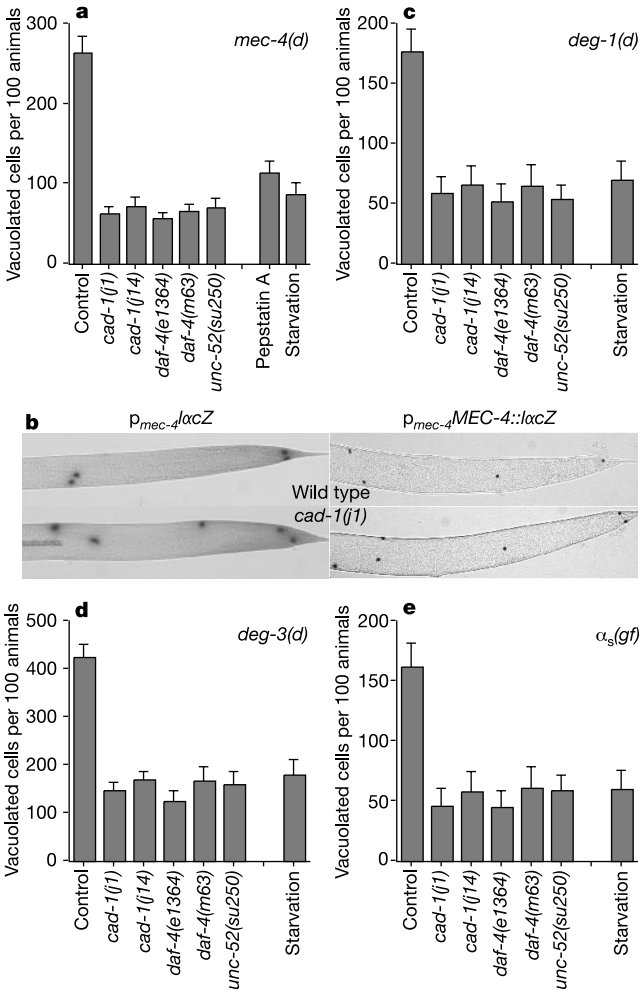

Figure 1 Aspartyl protease deficiency suppresses neurodegeneration in *C. elegans*. **a**, Number of vacuolated touch receptors at the L1 stage per 100 animals carrying the *mec-4(d)* allele in genetic backgrounds with reduced aspartyl protease activity, after treatment with pepstatin A and under conditions of starvation ($n = 250$, $P < 0.0001$, unpaired *t*-test). **b**, Expression of *lacZ* in touch receptor neurons driven solely by the *mec-4* promoter (left) or fused at the end of the full-length *mec-4* gene (right). Aspartyl protease deficiency in *cad-1(j1)* (bottom) does not affect *mec-4* expression or stability, as compared with the wild-type background (top). **c**, Vacuolated PVC interneurons at the L2 stage per 100 *deg-1(d)* animals in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation ($n = 250$, $P < 0.0001$, unpaired *t*-test). **d**, Vacuolated IL1 sensory neurons and PVC interneurons per 100 L1 *deg-3(d)* mutant larvae in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation ($n = 250$, $P < 0.001$, unpaired *t*-test). **e**, Vacuolated PVC interneurons at the L1 stage per 100 α_s*(gf)* animals in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation ($n = 250$, $P < 0.0001$, unpaired *t*-test).

many different *C. elegans* genes.

There are at least six expressed aspartyl protease genes (*asp-1* to *asp-6*) encoded in the nematode genome (ref. 16; see Supplementary Information for multiple sequence alignment and phylogenetic tree). To identify those that contribute to the protease activity required for neurodegeneration, we systematically knocked down the expression of each *asp* gene by RNA interference (RNAi) in *mec-4(d)*, *deg-3(d)* and $\alpha_s(gf)$ genetic backgrounds. As a positive control in these experiments, we knocked down *crt-1*, which encodes calreticulin, an endoplasmic reticulum (ER) chaperone required for neurodegeneration induced by *mec-4(d)* and $\alpha_s(gf)$, but not *deg-3(d)* (ref. 8). Although RNAi is relatively ineffective for genes expressed in mature *C. elegans* neurons (ref. 17; N.T. and P.S., unpublished data), we observed suppression of neurodegeneration triggered by *mec-4(d)* in *crt-1(RNAi)* animals (Fig. 2a, c). It seems likely that RNAi is more effective with genes that are required at early developmental stages in the nervous system (degeneration occurs soon after the touch receptor neurons are born during late embryogenesis and the first larval stage in *mec-4(d) C. elegans* mutants[9]).

Of the six aspartyl protease genes examined, *asp-3* and *asp-4* were specifically required for neurodegeneration (Fig. 2a–c). *asp-1* knockdown also detectably reduced neurodegeneration but to a much lower extent. In a reciprocal approach, we introduced each *asp* gene into *cad-1(j1);mec-4(d)* double mutant animals, in which

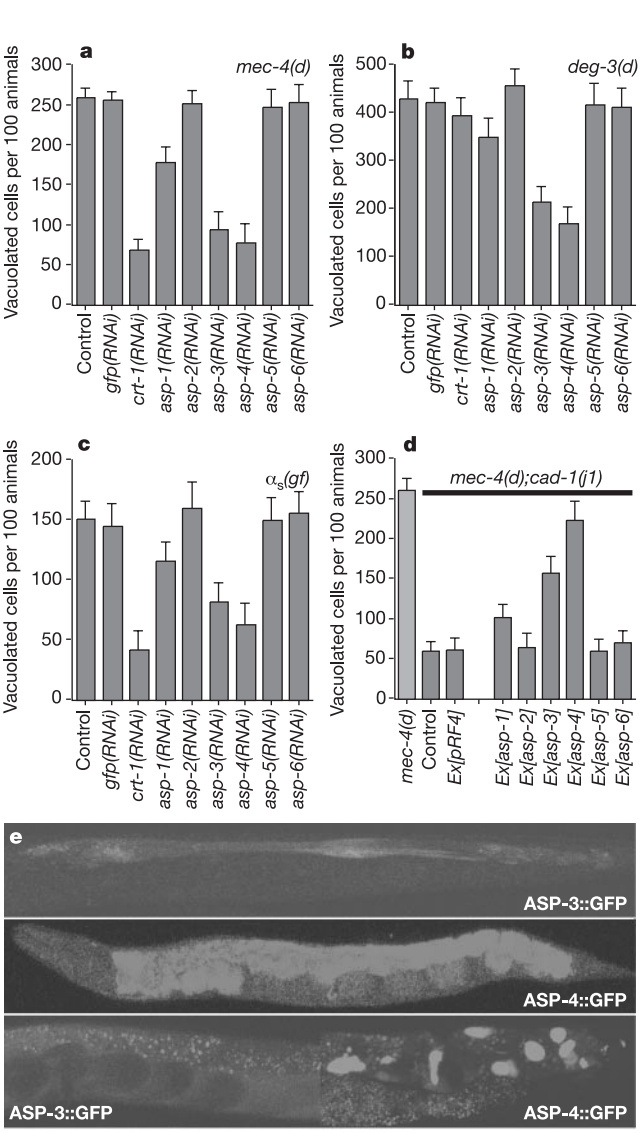

Figure 2 Specific aspartyl proteases are required for neurodegeneration in *C. elegans*. **a**–**c**, Effect of RNAi with the indicated *asp* genes in *mec-4(d)*, *deg-3(d)* and $\alpha_s(gf)$ mutants. RNAi with *crt-1* was used as a positive control; RNAi with *gfp* was used as a negative control. Interference with *asp-3* and *asp-4* results in significant suppression in all three genetic backgrounds ($n = 200$, $P < 0.0001$, unpaired *t*-test). Efficacy of RNAi was assessed as described in Methods. **d**, Degenerating touch receptors in transgenic *cad-1(j1);mec-4(d)* animals carrying each of the indicated *asp* genes on extracromosomal arrays. *Ex[asp-3]* and *Ex[asp-4]* restore cell death in *cad-1(j1);mec-4(d)* double mutants ($n = 150$, $P < 0.0001$, unpaired *t*-test). **e**, Top and middle, images of animals expressing ASP-3::GFP and ASP-4::GFP. Bottom, confocal images of the subcellular localization of ASP-3::GFP and ASP-4::GFP (see Supplementary Information for details).

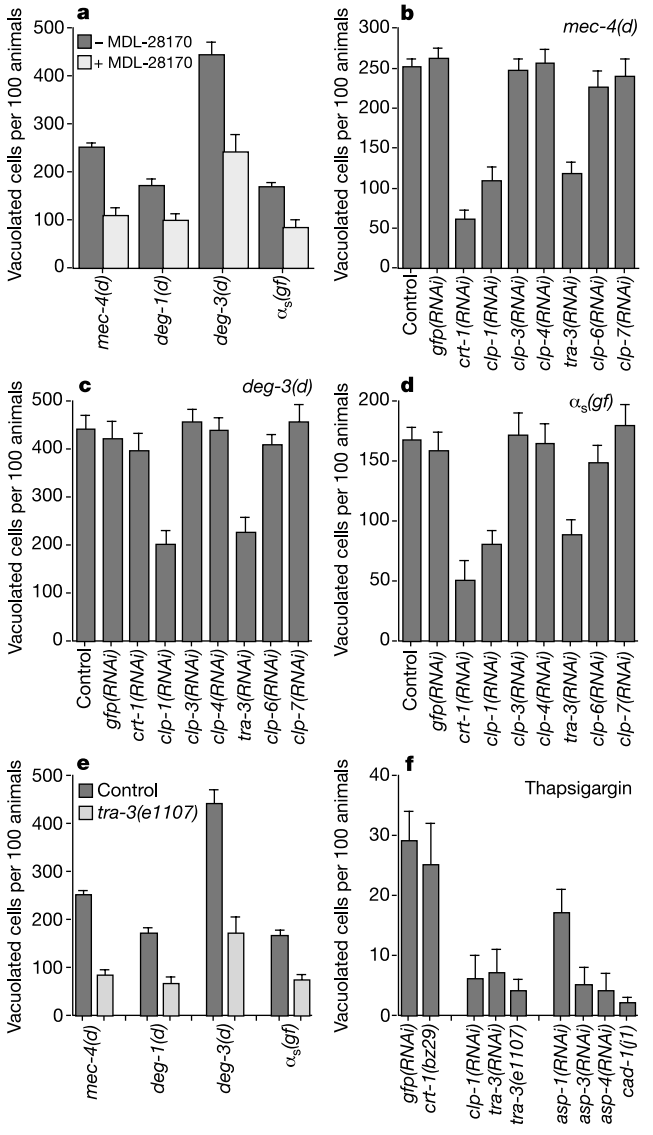

Figure 3 Specific calpain proteases are required for neurodegeneration in *C. elegans*. **a**, Degenerating neurons in *mec-4(d)*, *deg-1(d)*, *deg-3(d)* and $\alpha_s(gf)$ animals without or after treatment with calpain inhibitor MDL-28170 ($n = 150$, $P < 0.001$, unpaired *t*-test). **b**–**d**, Effect of RNAi with the indicated *clp* genes in *mec-4(d)*, *deg-3(d)* and $\alpha_s(gf)$ mutants. RNAi with *crt-1* was used as a positive control; RNAi with *gfp* was used as a negative control. Interference with *clp-1* and *tra-3* results in significant suppression in all three genetic backgrounds ($n = 200$, $P < 0.0001$, unpaired *t*-test). **e**, Neurodegeneration in single *mec-4(d)*, *deg-3(d)* and $\alpha_s(gf)$ mutants, and in double *tra-3(1107)* homozygotes originating from *tra-3(1107)* homozygous parents ($n = 100$, $P < 0.001$, unpaired *t*-test). **f**, Vacuolated cells per 100 L1 progeny of animals treated with thaspigargin and subjected to RNAi with the indicated genes ($n = 80$, $P < 0.001$, unpaired *t*-test).

neuronal degeneration caused by *mec-4(d)* is suppressed owing to the aspartyl protease deficiency of *cad-1(j1)*. We observed that degeneration was restored in animals carrying the *asp-3* and *asp-4* transgenes and to a much lower extent in animals carrying the *asp-1* transgene (Fig. 2d). Similarly, overexpression of *asp-3* and *asp-4* restored degeneration in *daf-4(e1364);mec-4(d)* and *unc-52(su250);mec-4(d)* double mutants (see Supplementary Information). Together, our results indicate that ASP-3 and ASP-4 aspartyl proteases are required for neurodegeneration inflicted by diverse genetic insults in *C. elegans*, but that ASP-1 contributes only marginally.

ASP-1 contains a conserved lysosome-targeting, *N*-glycosylation site (Asp 71) that is typical of cathepsin D lysosomal proteases that are predominantly localized to lysosomes[16]. Notably, ASP-3 and ASP-4 do not contain this *N*-glycosylation site but have another potential *N*-glycosylation site that is common in non-lysosomal cathepsin E proteases (ref. 16 and see Supplementary Information). We examined the expression and subcellular localization of ASP-3 and ASP-4 by fusing GFP at the C termini of both proteins. Strong expression was observed in intestinal cells and to a much lesser extent in other types of cell, including muscle cells, the hypodermis and neurons (Fig. 2e). Both fusion proteins were localized mainly in the cytoplasm, but were also found in lysosomes that appear as distinct autofluorescent puncta (Fig. 2f).

Overexpression of caspase CED-3, the protease that mediates execution of programmed cell death, is sufficient to induce apoptosis in the absence of upstream death initiator signals[18]. We examined whether, by analogy, increased expression of the aspartyl proteases ASP-1, ASP-3 and ASP-4 is sufficient to inflict degeneration of specific neurons in *C. elegans*. We used the *mec-4* promoter to overexpress *asp-1*, *asp-3* and *asp-4* in the touch receptor neurons, and the motor neuron-specific *unc-8* promoter[19] to drive overexpression in the ventral nerve cord motor neurons. A low percentage of transgenic animals expressing *asp-3* and *asp-4*, but not *asp-1*, in touch receptor neurons showed spontaneous vacuolation of these neurons during late embryogenesis and the early L1 larval stage and failed to respond normally to gentle touch as adults (for *asp-3*, 12.3 ± 0.8%; for *asp-4*, 13.1 ± 1.2%; $n = 250$, background is zero). Similarly, increased expression of *asp-3* and *asp-4* in motor neurons resulted in variably uncoordinated animals with vacuolated cells in the ventral nerve cord (for *asp-3*, 9.2 ± 0.5%; for *asp-4*, 11.6 ± 0.7%; $n = 250$, background is zero). Staining of cell nuclei with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) in affected adults revealed missing neurons, indicating that overexpression of aspartyl protease caused neuronal death rather than mere malfunction (data not shown).

What is the mechanism that relays signals generated by upstream death initiating events to executioner aspartyl proteases? Perturbation of cytosolic calcium ($[Ca^{2+}]_i$) homeostasis has been implicated in necrotic cell death both in mammals and in *C. elegans*[7,8]. But the mechanism by which Ca^{2+} contributes to cell death remains unclear. Calpains are diverse intracellular papain-like cysteine proteases that require Ca^{2+} for activation[20]. In primate hippocampal neurons, degeneration after acute ischaemia is accompanied by an increase in $[Ca^{2+}]_i$ and concomitant activation of calpain. In addition, activated calpain seems to be localized to disrupted lysosomal membranes (reviewed in ref. 21). These findings have culminated in formulation of the 'calpain–cathepsin' hypothesis, whereby an increase in $[Ca^{2+}]_i$ activates calpains, which in turn mediate rupture of lysosomes and leakage of killer cathepsins that dismantle the cell[1,21].

To elucidate the role of calpain activity in *C. elegans* neurodegeneration, we treated *mec-4(d)*, *deg-1(d)*, *deg-3(d)* and $\alpha_s(gf)$ mutant animals with Z-Val-Phe-CHO (MDL-28170), a potent calpain inhibitor. Treatment markedly reduced the number of degenerating neurons in all four mutants without reducing the expression of *mec-4*, *deg-1*, *deg-3* or $\alpha_s(gf)$ (Fig. 3a). This observation suggested that calpain proteases are involved in the cell death process.

The *C. elegans* genome encodes 17 genes with similarity to calpain, 7 of which show significant identity to mammalian calpains over their whole length (*clp-1* to *clp-7*, see WormBase (http://www.wormbase.org) and Supplementary Information for multiple sequence alignment and phylogenetic tree). *clp-5* corresponds to *tra-3*, a previously characterized gene that is involved in *C. elegans* sex determination[22]. The TRA-3 protease is regulated by Ca^{2+}, but it lacks a typical calmodulin-like Ca^{2+}-binding domain[23]. In TRA-3, a C2-like domain and two Ca^{2+}-binding sites in the protease core are probably Ca^{2+} sensors that activate this enzyme (see Supplementary Information). Examination of the other CLP sequences showed that CLP-1, CLP-2, CLP-6 and CLP-7 contain motifs that are typical of calpains, including a thiol (cysteine) protease active site and a Ca^{2+}-binding domain, whereas the other two lack either or both

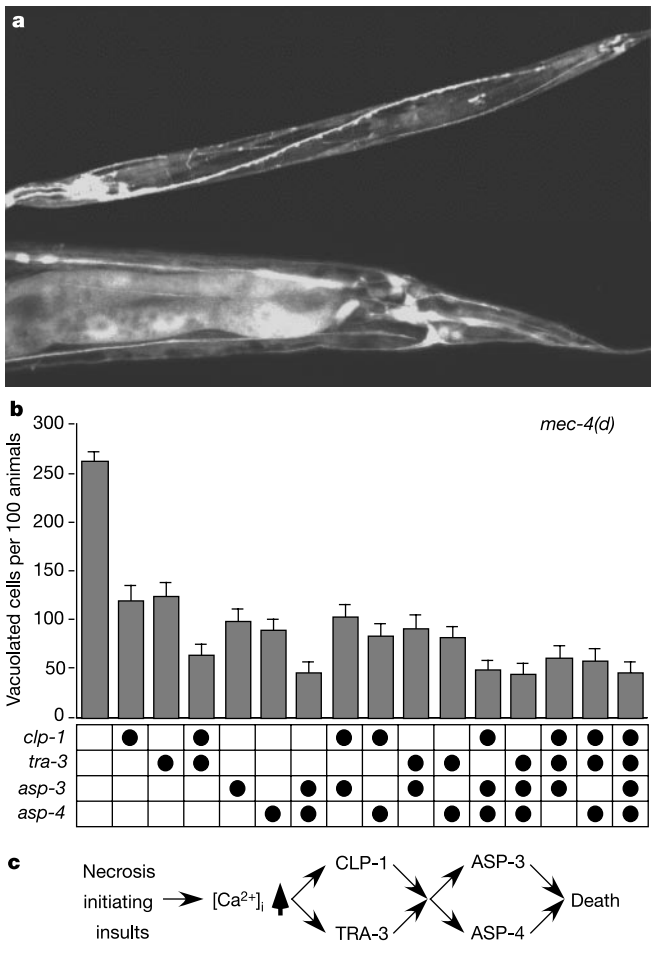

Figure 4 Calpains act sequentially with aspartyl proteases to facilitate cell death. **a**, Expression of *clp-1*. Top, image of animal expressing GFP from the *clp-1* promoter widely in the nervous system. Bottom, confocal image detailing expression in the intestine and neurons of the tail ganglion (see also Supplementary Information). **b**, Neurodegeneration assays in *mec-4(d)* animals subjected to RNAi with protease genes. *In vitro* synthesized dsRNAs, corresponding to calpain genes *clp-1* and *tra-3* and aspartyl proteases *asp-3* and *asp-4*, were injected either individually or in pools as indicated. Bars represent the average of three independent experiments ($n = 300$). Knockdown of both calpain or both aspartyl protease genes results in significantly more extended quenching of neurodegeneration than for any single gene ($P < 0.001$, unpaired *t*-test). **c**, Working model for a necrotic cell death pathway in *C. elegans*. Death triggering signals are relayed to a specific set of executioner aspartyl proteases through sensing of $[Ca^{2+}]_i$ perturbations by calpains.

(see Supplementary Information for multiple sequence alignment). We investigated the role of calpain proteases in neurodegeneration by RNAi-mediated knockdown of *clp-1*, *clp-3*, *clp-4*, *clp-6*, *clp-7* and *tra-3* in *mec-4(d)*, *deg-3(d)* and $\alpha_s(gf)$ mutant strains. Cell death was suppressed in all three strains when animals were subjected to RNAi with *clp-1* and *tra-3* but not *clp-3*, *clp-4*, *clp-6* or *clp-7* (Fig. 3b–d). We obtained similar results with *tra-3*;*mec-4(d)*, *tra-3*;*deg-1(d)*, *tra-3*;*deg-3(d)* and *tra-3*;$\alpha_s(gf)$ double mutants (Fig. 3e), further confirming the requirement for TRA-3 in neurodegeneration. Expression of *mec-4*, *deg-3* and $\alpha_s(gf)$ genes was not detectably reduced in these experiments.

Studies have suggested that release of Ca^{2+} from ER stores to the cytoplasm contributes to neurodegeneration initiated by hyperactive MEC-4 or $G\alpha_s$ (ref. 8). Forced release of Ca^{2+} from ER stores by treatment with thapsigargin, a compound that also inhibits the ER Ca^{2+} re-uptake pump SERCA and results in a net increase in $[Ca^{2+}]_i$, induces necrotic cell death in *C. elegans*[8]. We found that calpain activity is required for thapsigargin-induced cell death: treatment with thapsigargin was not effective in animals subjected to RNAi with *clp-1*, or in *tra-3* mutants (Fig. 3f). We also examined the requirement for aspartyl protease activity in thapsigargin-induced cell death. *cad-1* mutants and animals subjected to RNAi with *asp-3* or *asp-4* were resistant to the toxic effects of thapsigargin, but RNAi with *asp-1* slightly ameliorated cell death. Loss of calreticulin function in *crt-1(bz29)* null mutants, which blocks neurodegeneration induced by *mec-4(d)* and $\alpha_s(gf)$, but not *deg-3(d)*, did not suppress thapsigargin toxicity (Fig. 3f). These observations indicate that although CRT-1 is required for the build-up of noxious $[Ca^{2+}]_i$, calpain and aspartyl proteases function downstream of $[Ca^{2+}]_i$ signalling to facilitate death.

clp-1 is expressed strongly in many types of cell and tissue, including muscle cells and neurons (Fig. 4a and Supplementary Information). *tra-3* is also expressed in the nervous system of the animal (S. Sokol and P. Kuwabara, personal communication). We examined synergy between proteases of the same type, as well as between aspartyl proteases and calpains, in neurodegeneration. We observed that simultaneous RNAi with both *asp-3* and *asp-4* resulted in an enhanced suppression of neurodegeneration induced by *mec-4(d)*. Similarly, RNAi with both calpains further increased suppression. But we did not observe synergy between aspartyl proteases and calpains (Fig. 4b). Therefore, aspartyl and calpain proteases function in the same pathway that facilitates neurodegeneration in *C. elegans*. We did not achieve complete or near-complete blockage of neurodegeneration in these experiments. Incomplete suppression of cell death by aspartyl or calpain protease deficiency in our trials could be due to the limited capacity of RNAi to knockdown genes efficiently in the nematode nervous system, the contribution of other additional biochemical pathways and protease activities, or both. A comprehensive study of the remaining calpain proteases including CLP-2, which contains all of the catalytic residues that are typical of calpains (Supplementary Table 1), may illuminate this issue.

We propose that diverse death-initiating conditions converge, in part, to increase $[Ca^{2+}]_i$, which signals the activation of calpain proteases that subsequently engage executioner lysosomal and cytoplasmic aspartyl proteases, leading to cell destruction (Fig. 4c). Consistent with this model, neurodegeneration inflicted by cell-specific overexpression of *asp-3* and *asp-4* cannot be bypassed by a deficiency in either or both calpains (Supplementary Table 2). The identification of two specific classes of protease that are required for neurodegeneration in *C. elegans* may provide insight into similar pathologies in mammals. The lysosomal degradation system has been found to be upregulated in neurons of individuals affected with Alzheimer's disease[24], and cathepsin D expression is induced under conditions of excitotoxic cell death (reviewed in ref. 25). In addition, calpain inhibitors can be protective in certain cases of nerve or muscle degeneration after ischaemic episodes[26,27]. These findings suggest that, similar to apoptosis, necrotic cell death mechanisms are conserved from nematodes to humans, and they highlight specific executioner proteases as potential targets for therapeutic intervention in an effort to battle neurodegenerative disorders. □

Methods

Strains and genetics

We used standard procedures for *C. elegans* maintenance, crosses and other genetic manipulations[28]. The cultivation temperature was kept at 20 °C, unless noted otherwise. We used the following strains: wild-type N2 Bristol isolate, *cad-1(j1)II*, *cad-1(j14)II*, *csp-1(nr2018)II*, *unc-52(su250)II*, *daf-4(e1364)III*, *daf-4(m63)III*, *tra-3(e1107)/dpy-4(e1166)IV*, *deg-3(u662)V*, referred to in the text as *deg-3(d)*; *deg-1(u38)X*, referred to in the text as *deg-1(d)*; *mec-4(u231)X*, referred to in the text as *mec-4(d)*; and *nuIs5*[$p_{glr\text{-}1}G\alpha_s(Q227L)$ $p_{glr\text{-}1}$*GFP*], referred to in the text as $\alpha_s(gf)$ (ref. 6). The following double mutants were examined for neurodegeneration: *cad-1(j1)II;mec-4(u231)X*, *cad-1(j14)II;mec-4(u231)X*, *cad-1(j1)II;deg-3(u662)V*, *cad-1(j1)II;*$\alpha_s(gf)$, *csp-1(nr2018)II;mec-4(u231)X*, *csp-1(nr2018)II;deg-3(u662)V*, *csp-1(nr2018)II;*$\alpha_s(gf)$, *unc-52(su250)II;mec-4(u231)X*, *unc-52(su250)II;deg-1(u38)X*, *unc-52(su250)II;deg-3(u662)V*, *unc-52(su250)II;*$\alpha_s(gf)$, *daf-4(e1364)III;mec-4(u231)X*, *daf-4(m63)III;mec-4(u231)X*, *daf-4(e1364)III;deg-1(u38)X*, *daf-4(e1364)III;deg-3(u662)V*, *daf-4(e1364)III;*$\alpha_s(gf)$, *tra-3(e1107)IV;mec-4(u231)X*, *tra-3(e1107)IV;deg-3(u662)V*, *tra-3(e1107)IV;*$\alpha_s(gf)$, *tra-3(e1107)/+IV;mec-4(u231)X*, *tra-3(e1107)/+IV;deg-3(u662)X* and *tra-3(e1107)/+IV;*$\alpha_s(gf)$.

The *tra-3(e1107)/+* heterozygotes segregate *tra-3(e1107)* homozygotes, which, owing to maternal effects are phenotypically wild type and segregate abnormal sterile males or intersex[22]. We assayed cell death in *tra-3(e1107)* homozygotes originating from *tra-3(e1107)/+* heterozygotes and in abnormal progeny of *tra-3(e1107)* homozygotes, as well as in *tra-3(e1107)/+* heterozygotes. Neurodegeneration was suppressed only in the abnormal progeny of *tra-3(e1107)* homozygotes, indicating the maternal contribution of TRA-3 in cell death. *uvEx[asp-nrol-6(su1006)];cad-1(j1)II;mec-4(u231)* (referred to in the figure legend as *Ex[asp-n] cad-1(j1)II;mec-4(u231)*, where *n* is 1–6) were constructed by injecting PCR fragments encompassing each *asp* gene, together with plasmid pRF4 carrying the dominant transformation marker *rol-6(su1006)*, into the gonads of *cad-1(j1)II;mec-4(u231)* gravid hermaphrodites.

Plasmid constructs and RNA interference

We generated reporter constructs by fusing GFP at the C terminus of ASP-3 and ASP-4, and at the second exon of *clp-1*. The *mec-4* and *unc-8* promoters were fused upstream of *asp-1*, *asp-3* and *asp-4* coding sequences to achieve ectopic overexpression of aspartyl protease genes in touch receptor neurons and motor neurons, respectively. Overexpression of *clp-1* and *tra-3* using a similar strategy did not result in detectable neurodegeneration (data not shown). For RNAi experiments, we generated plasmid constructs for *in vitro* synthesis of double-stranded RNAs (dsRNAs) corresponding to various aspartyl and calpain proteases that were subsequently injected as described[29]. There is no sequence identity in the DNA between the protease genes tested, which eliminates incident dsRNA cross-interference in our experiments.

We carried out neurodegeneration assays (see below) in the L1 progeny of injected individuals. In a complementary approach, we also constructed plasmids for synthesis of dsRNAs in *Escherichia coli* bacteria that were fed to animals as described[30]. In all cases, injecting dsRNA resulted in the strongest interference effects in the nervous system. We assayed the effectiveness of RNAi with aspartyl protease gene expression by measuring aspartyl protease activity in total animal extracts[12] and by monitoring expression of full-length *asp*::GFP reporter genes. RNAi with *tra-3* phenocopied the *tra-3* loss-of-function phenotype, confirming effective interference.

Cell death assays

Animals were staged using the criteria of developmental timing and extent of gonadal development. Neurodegeneration was generally scored during the mid-L1 larval stage. We prepared L1 larvae by washing gravid adult populations of plates and allowing the remaining eggs to hatch for 4–5 h. For the strains expressing *mec-4(d)*, and ASP-1, ASP-3 and ASP-4 from the *mec-4* promoter, we scored neurodegeneration by the characteristic vacuolated appearance of the six touch receptor neurons using differential interference contrast (Nomarski) microscopy. For *deg-1(d)* and $\alpha_s(gf)$, we scored vacuolation of the two PVC interneurons in the tail, and for *deg-3(d)*, we scored vacuolation of IL1 sensory neurons in the head and PVC interneurons in the tail. For strains expressing ASP-1, ASP-3 and ASP-4 from the *unc-8* promoter, we assayed vacuolation of ventral nerve cord motor neurons. Cell death or survival was confirmed by the lack or presence of GFP expression in these neurons and DAPI staining of cell nuclei, in adult animals. Thapsigargin-induced cell death was assayed as described[8]. Animals were treated with 10 μg ml^{-1} thapsigargin. We injected protease inhibitors (5 μM pepstatin A, Sigma; 10 μM MDL-28170, Calbiochem) in the body cavity of gravid adults and assayed neurodegeneration in the progeny of injected individuals. We imposed starvation by moving well-fed young adults to plates devoid of bacteria and allowing them to lay eggs that were hatched in the absence of food. Neurodegeneration was assayed in the L1 progeny of starved animals. For *unc-52(su250)*, *daf-4(e1364)* and *daf-4(m63)* alleles, which are temperature-sensitive, we assayed neurodegeneration at 25 °C, where effects were maximal. Statistical analysis was carried out using the Microsoft OfficeXP Excel software package.

Received 3 July; accepted 21 August 2002; doi:10.1038/nature01108.

1. Syntichaki, P. & Tavernarakis, N. Death by necrosis: uncontrollable catastrophe, or is there order behind the chaos? *EMBO Rep.* **3,** 604–609 (2002).
2. Chalfie, M. & Wolinsky, E. The identification and suppression of inherited neurodegeneration in *Caenorhabditis elegans*. *Nature* **345,** 410–416 (1990).
3. Driscoll, M. & Chalfie, M. The *mec-4* gene is a member of a family of *Caenorhabditis elegans* genes that can mutate to induce neuronal degeneration. *Nature* **349,** 588–593 (1991).
4. Treinin, M. & Chalfie, M. A mutated acetylcholine receptor subunit causes neuronal degeneration in *C. elegans*. *Neuron* **14,** 871–877 (1995).
5. Korswagen, H. C., Park, J. H., Ohshima, Y. & Plasterk, R. H. An activating mutation in a *Caenorhabditis elegans* G_s protein induces neural degeneration. *Genes Dev.* **11,** 1493–1503 (1997).
6. Berger, A. J., Hart, A. C. & Kaplan, J. M. $G\alpha_s$-induced neurodegeneration in *Caenorhabditis elegans*. *J. Neurosci.* **18,** 2871–2880 (1998).
7. Sattler, R. & Tymianski, M. Molecular mechanisms of calcium-dependent excitotoxicity. *J. Mol. Med.* **78,** 3–13 (2000).
8. Xu, K., Tavernarakis, N. & Driscoll, M. Necrotic cell death in *C. elegans* requires the function of calreticulin and regulators of Ca^{2+} release from the endoplasmic reticulum. *Neuron* **31,** 957–971 (2001).
9. Hall, D. H. *et al.* Neuropathology of degenerative cell death in *Caenorhabditis elegans*. *J. Neurosci.* **17,** 1033–1045 (1997).
10. Lee, J. M., Zipfel, G. J. & Choi, D. W. The changing landscape of ischaemic brain injury mechanisms. *Nature* **399,** A7–A14 (1999).
11. Chung, S., Gumienny, T. L., Hengartner, M. O. & Driscoll, M. A common set of engulfment genes mediates removal of both apoptotic and necrotic cell corpses in *C. elegans*. *Nature Cell Biol.* **2,** 931–937 (2000).
12. Jacobson, L. A. *et al.* Identification of a putative structural gene for cathepsin D in *Caenorhabditis elegans*. *Genetics* **119,** 355–363 (1988).
13. Rogalski, T. M., Mullen, G. P., Bush, J. A., Gilchrist, E. J. & Moerman, D. G. UNC-52/perlecan isoform diversity and function in *Caenorhabditis elegans*. *Biochem. Soc. Trans.* **29,** 171–176 (2001).
14. Estevez, M. *et al.* The *daf-4* gene encodes a bone morphogenetic protein receptor controlling *C. elegans* dauer larva development. *Nature* **365,** 644–649 (1993).
15. Hawdon, J. M., Emmons, S. W. & Jacobson, L. A. Regulation of proteinase levels in the nematode *Caenorhabditis elegans*. Preferential depression by acute or chronic starvation. *Biochem. J.* **264,** 161–165 (1989).
16. Tcherepanova, I., Bhattacharyya, L., Rubin, C. S. & Freedman, J. H. Aspartic proteases from the nematode *Caenorhabditis elegans*. Structural organization and developmental and cell-specific expression of *asp-1*. *J. Biol. Chem.* **275,** 26359–26369 (2000).
17. Tavernarakis, N., Wang, S. L., Dorovkov, M., Ryazanov, A. & Driscoll, M. Heritable and inducible genetic interference by double-stranded RNA encoded by transgenes. *Nature Genet.* **24,** 180–183 (2000).
18. Miura, M., Zhu, H., Rotello, R., Hartwieg, E. A. & Yuan, J. Induction of apoptosis in fibroblasts by IL-1 beta-converting enzyme, a mammalian homolog of the *C. elegans* cell death gene *ced-3*. *Cell* **75,** 653–660 (1993).
19. Tavernarakis, N., Shreffler, W., Wang, S. & Driscoll, M. *unc-8*, a DEG/ENaC family member, encodes a subunit of a candidate mechanically gated channel that modulates *C. elegans* locomotion. *Neuron* **18,** 107–119 (1997).
20. Sorimachi, H. & Suzuki, K. The structure of calpain. *J. Biochem. (Tokyo)* **129,** 653–664 (2001).
21. Yamashima, T. Implication of cysteine proteases calpain, cathepsin and caspase in ischemic neuronal death of primates. *Prog. Neurobiol.* **62,** 273–295 (2000).
22. Barnes, T. M. & Hodgkin, J. The *tra-3* sex determination gene of *Caenorhabditis elegans* encodes a member of the calpain regulatory protease family. *EMBO J.* **15,** 4477–4484 (1996).
23. Sokol, S. B. & Kuwabara, P. E. Proteolysis in *Caenorhabditis elegans* sex determination: cleavage of TRA-2A by TRA-3. *Genes Dev.* **14,** 901–906 (2000).
24. Adamec, E., Mohan, P. S., Cataldo, A. M., Vonsattel, J. P. & Nixon, R. A. Up-regulation of the lysosomal system in experimental models of neuronal injury: implications for Alzheimer's disease. *Neuroscience* **100,** 663–675 (2000).
25. Ferri, K. F. & Kroemer, G. Organelle-specific initiation of cell death pathways. *Nature Cell Biol.* **3,** E255–E263 (2001).
26. Stracher, A. Calpain inhibitors as therapeutic agents in nerve and muscle degeneration. *Ann. NY Acad. Sci.* **884,** 52–59 (1999).
27. Huang, Y. & Wang, K. K. The calpain family and human disease. *Trends Mol. Med.* **7,** 355–362 (2001).
28. Brenner, S. The genetics of *Caenorhabditis elegans*. *Genetics* **77,** 71–94 (1974).
29. Fire, A. *et al.* Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391,** 806–811 (1998).
30. Kamath, R. S., Martinez-Campos, M., Zipperlen, P., Fraser, A. G. & Ahringer, J. Effectiveness of specific RNA-mediated interference through ingested double-stranded RNA in *Caenorhabditis elegans*. *Genome Biol.* **2,** research0002.1–0002.10 (2001).

Supplementary Information accompanies the paper on *Nature*'s website (**http://www.nature.com/nature**).

Acknowledgements We thank P. Kuwabara and S. Sokol for communicating results before publication; C. Samara for technical help; and colleagues for discussions and comments on the manuscript. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. Certain strains were from the NemaPharm Group of Axys Pharmaceuticals. We thank A. Fire for plasmid vectors and L. Jacobson for *cad-1* mutant strains. This work was funded in part by grants from NIH NINDS to M.D. and by grants from HFSPO and IMBB to N.T.

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

Correspondence and requests for materials should be addressed to N.T. (e-mail: tavernarakis@imbb.forth.gr).

Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota

Ahmad Faili*, Said Aoufouchi*, Eric Flatter*, Quentin Guéranger, Claude-Agnès Reynaud & Jean-Claude Weill

INSERM U373, Faculté de Médecine Necker-Enfants Malades, 156 rue de Vaugirard, 75730, Paris Cedex 15, France
** These authors contributed equally to this work*

Somatic hypermutation of immunoglobulin genes is a unique, targeted, adaptive process. While B cells are engaged in germinal centres in T-dependent responses, single base substitutions are introduced in the expressed V_H/V_L genes to allow the selection of mutants with a higher affinity for the immunizing antigen. Almost every possible DNA transaction has been proposed to explain this process, but each of these models includes an error-prone DNA synthesis step that introduces the mutations[1,2]. The Y family of DNA polymerases[3]—pol η, pol ι, pol κ and rev1—are specialized for copying DNA lesions and have high rates of error when copying a normal DNA template[4,5]. By performing gene inactivation in a Burkitt's lymphoma cell line inducible for hypermutation, we show here that somatic hypermutation is dependent on DNA polymerase iota.

Burkitt's lymphoma cell lines represent *in vitro* models of the hypermutation of immunoglobulin (Ig) genes. Mutation either can occur constitutively, as in the Ramos cell line[6], or can be induced, as in the BL2 cell line, after the engagement of several surface receptors—a stimulus that resembles the process *in vivo*[7,8]. In the BL2 cell line, mutations are induced in the G1 phase of the cell cycle, occur on one DNA strand of its rearranged V_H gene, and eventually become fixed by replication in one of the daughter cells[9]. The feasibility of gene targeting in the BL2 cell line has been shown by inactivation of the *activation-induced cytidine deaminase* (*AID*) gene, which totally abolishes hypermutation in BL2 (ref. 9), as it does *in vivo*[10,11].

We used the same gene inactivation procedure to generate BL2 clones deficient in polymerase iota (pol ι), one of the two human homologues of yeast RAD30, which shows short-gap filling, highly error-prone polymerase activity[12,13,14]. Figure 1a shows the organization of the human POLI exons and the constructs used to inactivate both alleles. Two pol-ι-null clones (54 and 267) were generated from a pol-ι heterozygous clone. The two clones had the same proliferation rate as the original BL2 cell line, with a similar mitotic index and cell-cycle profile, and showed no chromosomal aberrations or translocations in spreads of metaphase cells (data not shown). No pol ι expression was detected when cell extracts from the 54 and 267 ι-null clones were immunoblotted using rabbit polyclonal antibodies raised against human pol ι (Fig. 1b). Expression of pol ι was restored by transfecting the cells with pIRES vectors driving the expression of the complete human pol-ι complementary DNA under the control of the promoter for cytomegalovirus (CMV; Fig. 1b): within the variations inherent to quantification by western blot, expression was comparable to that observed in the normal BL2 cell line. In spite of several attempts, we could not obtain clones overexpressing pol ι, either in a normal or in a pol-ι-null background. We selected two restored clones for each targeted cell for further analysis.

The BL2 cell line can be induced for hypermutation of its rearranged V_H gene by two different procedures. In the first, crosslinking IgM in co-culture with a T-helper (T_H) clone or T_H cell line results in the occurrence of mutations after 3 d in culture[7,15]. We have adapted this somatic hypermutation (SHM) induction