

### 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: E293/402 (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 X buffer (Akt Kinase Assay Kit, Cell Signaling Technology), 100 μM ATP, 0.5 mM peptide (ENNEKQMRQLSMRT(253–493)).

For radioactivity incorporation assays, 250 μCi/ml ^32P-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 gel.

### 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 Previously unpublished constructs used in this study are: pCMX-N-CoR-RAKA-Flag, pCMX-N-CoR C-terminal (amino acids 1501–2300), pCMX-N-CoR(S401A)-Flag, pCMX-Gal4-VP16-Flag, pCMX-N-CoR(R13–R23)-Flag, and pCMX-N-CoR-(253-493)-Flag for glial fibrillary acidic protein.

### Chromatin immunoprecipitation

Chromatin immunoprecipitation was performed on normal neural stem cells largely as described. 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 IgGs or antibodies against N-CoR or CBF1 overnight at 4°C. The extracts were then incubated with protein A–sepharose beads (Sigma) for 1 h. After extensive washing of the beads, proteins were eluted and reversed by crosslinking for 6 h at 65°C. After DNA purification, PCR was performed on 29–35 cycles. Primers were: GAPB, 5’-GACTAAGGTGTTTCCTCCGGC-3’ (sense), 5’-CAAGGCTAGTCTGACCGAG-3’ (antisense); HESSID, 5’-CGTGTCCTCTCCGAGTTGAG-3’ (sense), 5’-GTACAGTGATGTCGCCGAG-3’ (antisense).

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

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Necrotic cell death underlies the pathology of numerous human neurodegenerative conditions1. 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 protein α-subunit gene gsa-1 evoke an analogous pattern of degenerative (necrotic-like) cell death in neurons that express the mutant proteins2,3,4. An increase in concentrations of cytoplasmic calcium in dying cells, elicited either by external calcium influx or by release of endoplasmic reticulum stores, is thought to comprise a major death-signalling event5,6. 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 AS3 and ASP-4. Our findings show that two

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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. Electron microscopy studies in humans. Neurodegeneration by introducing various death-inducing mutations into aspartyl-protease-deficient cad-1, daf-4 and unc-52 mutant strains. Cell death initiated by toxic deg-1(d) and deg-3(d) alleles and by overexpressing the hyperactivated Gaα(Q227L) variant (αQ(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 αQ(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

![Image](311x358 to 541x413)

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 peptatin 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(d) gene (right). Aspartyl protease deficiency in cad-1; mec-4(d) (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 < 0.0001, unpaired t-test). e, Vacuolated PVC interneurons at the L1 stage per 100 αQ(gf) animals in genetic backgrounds with reduced aspartyl protease activity and under conditions of starvation (n = 250, P < 0.0001, unpaired t-test).
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 αs(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 αs(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 cort-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).

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 αs(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(1j); mec-4(d) animals carrying each of the indicated asp genes on extrachromosomal arrays. Ex[asp-3] and Ex[asp-4] restore cell death in cad-1(1j); 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 αs(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 αs(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 αs(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 thapsigargin 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 dau-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 lysosomes16. Notably, ASP-3 and ASP-4 contain a conserved lysosome-targeting, 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 signals14. 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 to drive over-expression 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 vacuolization 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 over-expression 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 ([Ca2+]i) homeostasis has been implicated in necrotic cell death both in mammals and in C. elegans3,4. But the mechanism by which Ca2+ contributes to cell death remains unclear. Calpains are diverse intracellular papain-like cysteine proteases that require Ca2+ for activation20. In primate hippocampal neurons, degeneration after acute ischaemia is accompanied by an increase in [Ca2+]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 [Ca2+]i activates calpains, which in turn mediate rupture of lysosomes and leakage of killer cathepsins that dismantle the cell21.

To elucidate the role of calpain activity in C. elegans neurodegeneration, we treated mec-4(d), deg-1(d), deg-3(d) and αs(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 αs(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 trv-3, a previously characterized gene that is involved in C. elegans sex determination22. The TRA-3 protease is regulated by Ca2+, but it lacks a typical calmodulin-like Ca2+-binding domain23. In TRA-3, a C2-like domain and two Ca2+-binding sites in the protease core are probably Ca2+ 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 Ca2+-binding domain, whereas the other two lack either or both
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 \( \alpha_c(gf) \) 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-4(d), tra-3;deg-3(d) and tra-3;\( \alpha_c(gf) \) double mutants (Fig. 3e), further confirming the requirement for TRA-3 in neurodegeneration. Expression of mec-4, deg-3 and \( \alpha_c(gf) \) 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 Gia, (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\(^{2+} \)], induces necrotic cell death in \( C. \) \( \) elegans. 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 \( c r t-1(h229) \) null mutants, which blocks neurodegeneration induced by mec-4(d) and \( \alpha_c(gf) \), 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\(^{2+} \)], calpain and aspartyl proteases function downstream of [Ca\(^{2+} \)], 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 Table 1). 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 proteomes 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\(^{2+} \)], 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, 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.\(^ {8} \) The cultivation temperature was kept at 20 °C, unless noted otherwise. We used the following strains: wild-type N2 Bristol isolate, cad-1(1)I; cad-1(144)II, esp-1(n2083)II, unc-52(n250)II, daf-4(m63)III, tra-3(e1107); dpy-4(e1106)IV, deg-3(s662)VI, referred to in the text as deg-3(d); deg-3(s662)X, referred to in the text as deg-3(d); mec-4(e231)X, referred to in the text as mec-4(d); and nsdu5(gf-GFP[A227L], PGLR-1GFP, referred to in the text as \( \alpha_c(gf) \) (ref. 6). The following double mutants were examined for neurodegeneration: cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X, cad-1(1)I; mec-4(e231)X, cad-1(144)I; mec-4(e231)X.
Induction of somatic hypermutation in immunoglobulin genes is dependent on DNA polymerase iota

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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 mutations1,2. The Y family of DNA polymerases—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 template3,4. 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 line5, or can be induced, as in the BL2 cell line, after the engagement of several surface receptors—a stimulus that resembles the process in vivo6,7. In the BL2 cell line, mutations are induced in the G1 phase of the cell cycle, occur on one DNA strand of the rearranged V(H) gene, and eventually become fixed by replication in one of the daughter cells8. 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 vivo10,11. 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 activity12,13,14. 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 morphology. In the second experiment, mutations are induced in the G1 phase of the cell cycle, occur on one DNA strand of the rearranged V(H) gene, and eventually become fixed by replication in one of the daughter cells8. 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 vivo10,11. 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 activity12,13,14. 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 morphology. In the second experiment, mutations are induced in the G1 phase of the cell cycle, occur on one DNA strand of the rearranged V(H) gene, and eventually become fixed by replication in one of the daughter cells8. 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 vivo10,11. 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 activity12,13,14. 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 morphology. In the second experiment, mutations are induced in the G1 phase of the cell cycle, occur on one DNA strand of the rearranged V(H) gene, and eventually become fixed by replication in one of the daughter cells8. 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 vivo10,11.