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Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis

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Background: Inhibitor of apoptosis proteins (IAPs) suppress apoptotic cell death in several model systems and are highly conserved between insects and mammals. All IAPs contain at least one copy of the ~70 amino-acid baculovirus IAP repeat (BIR), and this domain is essential for the anti-apoptotic activity of the IAPs. Both the marked structural diversity of IAPs and the identification of BIR-containing proteins (BIRPs) in yeast, however, have led to the suggestion that BIRPs might play roles in other, as yet unidentified, cellular processes besides apoptosis. Survivin, a human BIRP, is upregulated 40-fold at G2–M phase and binds to mitotic spindles, although its role at the spindle is still unclear.

Results: We have identified and characterised two *Caenorhabditis elegans* BIRPs, BIR-1 and BIR-2; these proteins are the only BIRPs in *C. elegans*. The *bir-1* gene is highly expressed during embryogenesis with detectable expression throughout other stages of development; *bir-2* expression is detectable only in adults and embryos. Overexpression of *bir-1* was unable to inhibit developmentally occurring cell death in *C. elegans* and inhibition of *bir-1* expression did not increase cell death. Instead, embryos lacking *bir-1* were unable to complete cytokinesis and they became multinucleate. This cytokinesis defect could be partially suppressed by transgenic expression of survivin, the mammalian BIRP most structurally related to BIR-1, suggesting a conserved role for BIRPs in the regulation of cytokinesis.

Conclusions: BIR-1, a *C. elegans* BIRP, is probably not involved in the general regulation of apoptosis but is required for embryonic cytokinesis. We suggest that BIRPs may regulate cytoskeletal changes in diverse biological processes including cytokinesis and apoptosis.

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Background

The basal machinery of apoptosis is highly conserved in metazoa, comprising at least three functional components: caspases, a family of cysteine proteases whose activity is required for apoptosis in all metazoans thus far examined [1]; caspase activators, such as *Caenorhabditis elegans* CED-4 and mammalian Apaf-1; and the Bcl-2 family of apoptotic regulators [2]. In addition to these, the anti-apoptotic inhibitor of apoptosis protein (IAP) family is highly conserved between insects and mammals and may thus represent a fourth component of the basal conserved apoptotic machinery (reviewed in [3]).

IAPs were initially identified as baculovirus proteins that prevent host cell apoptosis following infection [4,5] and a number of structurally related cellular homologues have since been identified [6–10]. These classical IAPs have either two or three copies of a ~70 amino-acid domain — the baculovirus IAP repeat (BIR) — at the amino terminus and a C_3HC_4 RING finger domain at the carboxyl terminus [5]. BIRs contain an invariant C_2HC motif, suggesting a role in metal ion coordination [4], but these regions contain no other known motifs [3].

Cellular IAPs have been shown to suppress apoptosis in a number of model systems. In *Drosophila*, loss-of-function mutations in the IAP Diap-1, encoded by the *thread* locus, enhance killing by the pro-apoptotic gene *reaper* [8]. Ectopic expression of Diap-1 suppresses killing by all three of the *Drosophila* apoptotic triggers — *reaper*, *hid* and *grim* — both *in vivo* [11] and in insect cells in culture [12,13]. In mammals, overexpression of IAPs [6,7,9,14] and addition of certain IAPs to cell lysates [15,16] have been shown to suppress apoptosis induced by a variety of stimuli, including treatment of cells with drugs and addition of either active caspases or the caspase activator cytochrome c to cell lysates.

The suppression of apoptosis in both mammalian and insect cells by IAPs requires the BIRs of these proteins. Recently, BIRs have been suggested to suppress apoptosis by direct and selective inhibition of specific caspases

[15–18]. But, although the X-linked IAP Xiap, the human IAPs c-Iap1 and c-Iap2, and the neural inhibitory protein (NAIP) all display a similar anti-apoptotic activity in mammalian cells, their ability to inhibit caspases varies by over three orders of magnitude. There is therefore a substantial discrepancy between the ability of an IAP to inhibit apoptosis and its ability to inhibit caspases, suggesting that BIRs may regulate apoptosis via other mechanisms in addition to the direct inhibition of caspases.

BIRs have more recently been identified in structurally diverse proteins including NAIP, a gene whose mutation may contribute to spinal muscular atrophy [19], survivin [20], which contains a single BIR, and BRUCE, a giant ubiquitin-conjugating enzyme that contains a BIR [21]. The structural diversity of BIR-containing proteins (BIRPs) suggests either that BIRs link many diverse pathways to the regulation of apoptosis or that BIRs are involved in a wide range of biological processes in addition to apoptosis. The identification of BIRPs in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* [3] — organisms that have no identified apoptotic programme (reviewed in [22]) — would tend to support the latter view.

Here, we report our characterisation of two BIRPs in *C. elegans*, one of which is required for the completion of cytokinesis. We demonstrate that this is a conserved function of BIRPs and suggest that BIRPs can regulate cytoskeletal rearrangements involved in multiple biological processes, including both cytokinesis and apoptosis.

Results

The *C. elegans* genome encodes two BIR-containing proteins

The *C. elegans* genome sequence consortium has identified two *C. elegans* genes encoding proteins with homology to the BIRs of IAPs (T27F2.3 and C50B8.2) [23] which we call *bir-1* and *bir-2*. No other proteins containing significant homology to BIRs were found in *C. elegans* and there are unlikely to be more BIRPs in this organism as the sequencing of the genome is essentially complete. We cloned the *bir-1* and *bir-2* cDNAs (see Materials and methods); in both cases the sequence of the predicted open-reading frames (ORFs) was identical to that predicted by the *C. elegans* genome sequence consortium (Figure 1a). The BIR-1 and BIR-2 proteins contain one and two BIRs, respectively, and have no other domains of known homology (Figure 1b). The BIRs of these proteins are most closely related to those of survivin and to the *S. pombe* and *S. cerevisiae* BIRs (Figure 1c). BIR-1 is also structurally similar to survivin: both are approximately 150 amino acids in length and contain a single amino-terminal BIR.

The *bir-1* and *bir-2* genes each have a single transcript (Figure 2). Although *bir-1* is highly expressed in embryos, with a stable lower level of expression throughout all other developmental stages, *bir-2* expression is very low and detectable only in adults and embryos. The transcript sizes were the same as those predicted by the sizes of the cloned cDNAs, confirming that full-length cDNAs had been isolated for both genes. Both the structural similarity between BIR-1 and survivin proteins and the greater and more extensive expression of *bir-1* compared with *bir-2* led us to concentrate on the characterisation of *bir-1*.

Overexpression of *bir-1* does not affect cell death *in vivo*

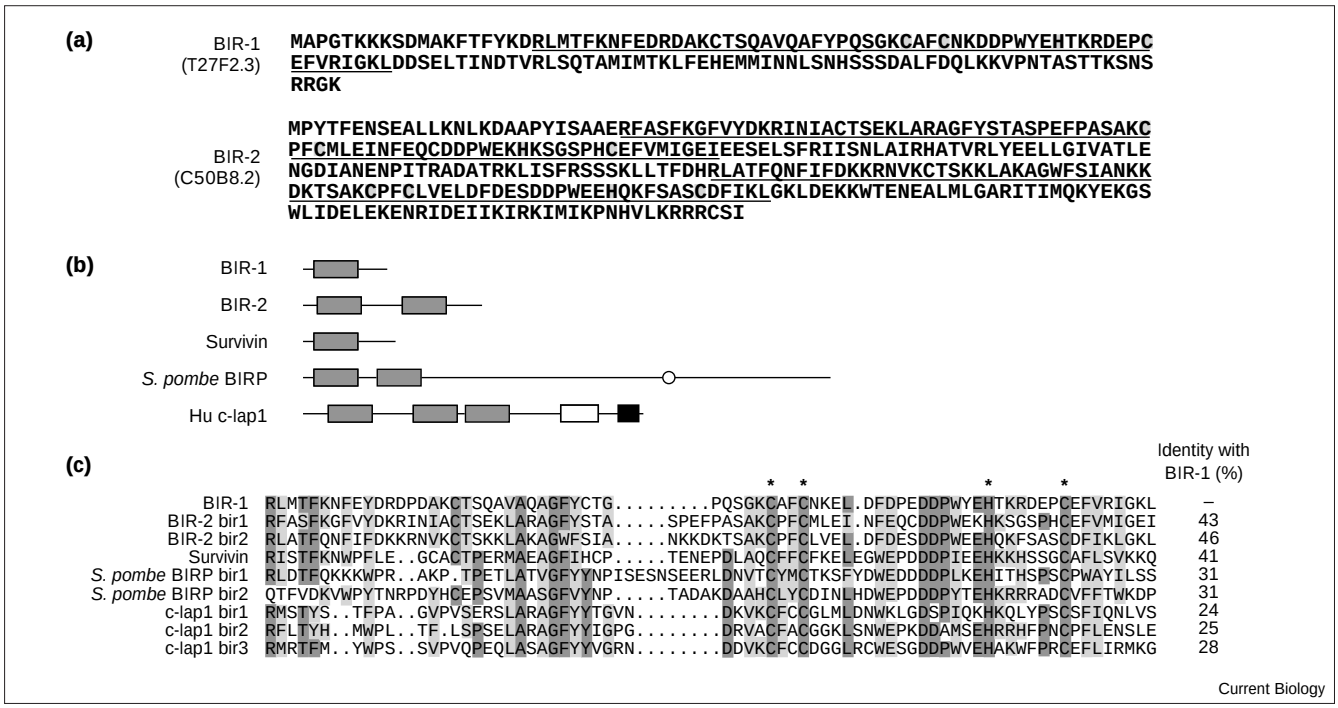
Overexpression of BIRPs has been shown to suppress cell death in a wide variety of contexts. BIRs are required for the anti-apoptotic activity of BIRPs and have been shown to inhibit caspases directly *in vitro*; inhibition of caspase activity has therefore been suggested to be the mechanism by which BIRPs exert their anti-apoptotic effects. The activity of the *C. elegans* caspase CED-3 is required for cell death in this nematode [24]. Inhibition of CED-3 activity *in vivo*, either by genetic mutations in *ced-3* itself [24] or by overexpression of the caspase inhibitor p35 [25], results in the suppression of developmentally occurring cell deaths.

To investigate whether, like p35, BIR-1 could suppress cell death *in vivo*, we generated transgenic worms in which sequences encoding BIR-1 amino-terminally tagged with a haemagglutinin (HA) epitope (HA–BIR-1) were placed under control of a heatshock-inducible promoter (transgenic lines hsHA–BIR-1 a, b, e and f); these lines also express green fluorescent protein (GFP) under the control of the same heatshock promoters to allow this protein to serve as a marker for heatshock-inducible expression. During normal *C. elegans* development, 14 specific cells in the anterior pharynx undergo apoptosis. Perturbation of the cell death machinery (for example, through mutations in *ced-3* [26], *ced-4* [26], *ced-9* [27] or *egl-1* [28]) alters the number of deaths and even very weak effects on cell death (such as 2% additional survival) can be observed.

We induced HA–BIR-1 expression by subjecting HA–BIR-1-expressing *C. elegans* lines to heatshock at a stage before the onset of developmental cell deaths and counted the number of surviving cells in the anterior pharynx 48 hours later. To ensure that the experimental conditions would permit us to observe a suppression of cell death, a parallel experiment was carried out with *ced-9*, the nematode *bcl-2* homologue that suppresses cell death in *C. elegans* [27,29]. Although induced overexpression of *ced-9* markedly suppressed cell death in the pharynx, no significant effect was seen following induction of HA–BIR-1 expression (Figure 3).

To ensure that the HA epitope had not affected any putative BIR-1 anti-apoptotic activity, we repeated analogous experiments with transgenic lines expressing either carboxy-terminally Myc-tagged BIR-1 or untagged BIR-1 under control of the same heatshock promoters; none of

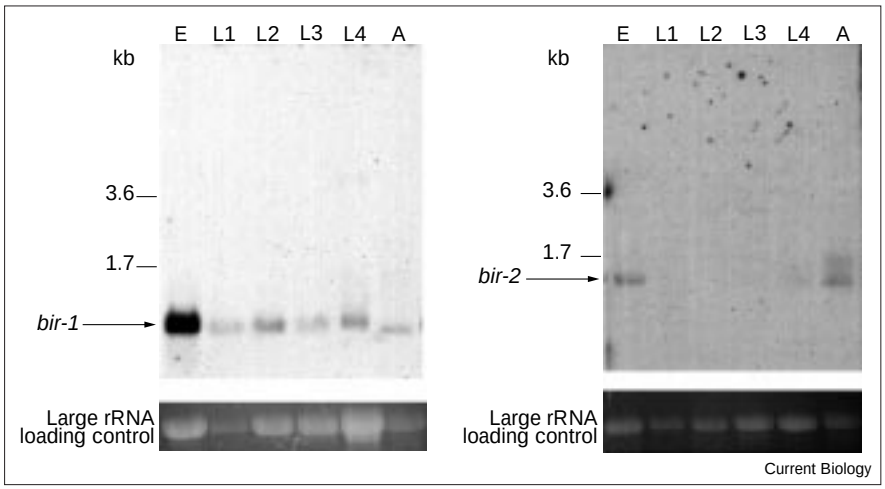
Figure 1



Predicted sequences of BIR-1 and BIR-2 and sequence analysis. (a) Primary predicted amino-acid sequences of BIR-1 and BIR-2. The single BIR of BIR-1 and the two BIRs of BIR-2 are underlined; conserved C_2HC residues are shaded in grey. (b) Schematic depiction of the domain structure of BIR-1, BIR-2, survivin, *S. pombe* BIRP and human c-lap1. BIRs are shown as grey shaded boxes, the RING finger of c-lap1 as a black box and the caspase-recruiting domain (CARD) of c-lap1 as a white box. The P loop (a predicted ATP-binding motif) of *S. pombe* BIRP is shown as an open circle. (c) Alignment of BIRs of BIR-1, BIR-2, survivin, *S. pombe* BIRP and human c-lap1. BIRs were aligned using PileUp (gap creation penalty = 12; gap extension penalty = 4). Residues identical in five or more sequences are shaded in dark grey; residues conserved in five or more sequences are shaded in light grey. The conserved C_2HC residues are marked with asterisks.

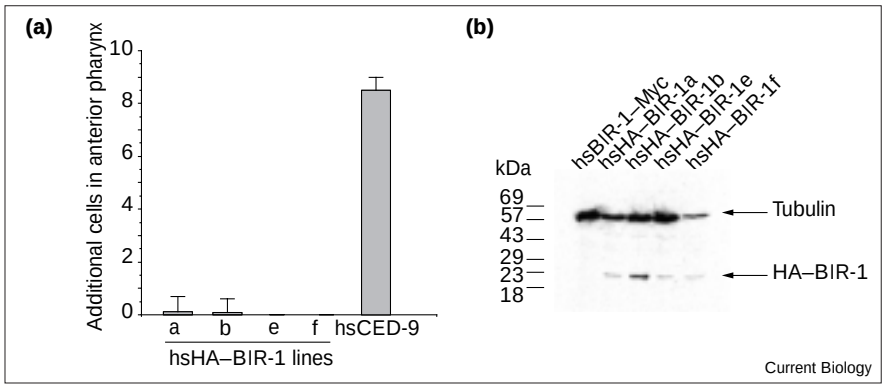
these lines demonstrated any ability of BIR-1 to suppress apoptosis following heatshock (data not shown). To ensure that the heatshock promoters could drive expression in the cells destined to die, we examined GFP expression in all transgenic lines tested following heatshock. In all lines tested, GFP expression was high and almost ubiquitous

Figure 2



Expression of *bir-1* and *bir-2* during *C. elegans* development. Northern blots containing 10 μg total RNA purified from embryos (E), larval stages 1–4 (L1–L4) or adult worms (A) were probed with ^{32}P-labelled DNA probes corresponding to the ORFs of either *bir-1* or *bir-2*. The positions of size markers are indicated on the left of each blot and represent the positions of rRNAs. Ethidium-bromide-stained gels with large rRNA levels as loading controls are shown below the blots.

Figure 3



Expression of HA–BIR-1 does not suppress cell death in the pharynx of *C. elegans*. (a) Lines expressing either heatshock-inducible amino-terminally HA-tagged BIR-1 (hsHA–BIR-1) or CED-9 (hsCED-9) were subjected to heatshock before the onset of developmental cell death. Embryos were allowed to hatch and the number of additional cells in the pharynges of transgenic L3–L4 larvae was quantitated 48 h later. Four independent heatshock HA–BIR-1 transgenic lines were analysed (a,b,e,f). In each case, data shown are the mean number of additional cells in the anterior pharynx ± standard deviation. As a comparison, strong loss-of-function *ced-3* worms (*n717*) have an average of 12.5 ± 0.4 extra cells [56]. (b) HA–BIR-1 is efficiently expressed in transgenic lines following heatshock. The transgenic lines described in (a) were subjected to heatshock and lysates made 90 min later. Blots were probed with rabbit polyclonal anti-HA antibodies or murine monoclonal anti-tubulin antibodies (to examine tubulin levels as a loading control). The hsBIR-1–Myc lysate is included as a negative control for the anti-HA antibody probing.

(data not shown), mirroring the pattern of heatshock-promoter-induced expression previously described [30].

Our data indicate that overexpression of *bir-1* has no effect on cell death in the pharynx. We conclude that if *bir-1* overexpression can affect the execution of cell death in *C. elegans*, this effect is negligible in comparison to that of *ced-9* or p35. We performed analogous overexpression experiments with *bir-2* and also failed to detect any effect on cell death (unpublished observations).

Inhibition of *bir-1* does not affect cell death in the hermaphrodite germ line

The adult hermaphrodite germ line of *C. elegans* has a continuously proliferating population of cells that give rise to oocytes [31]. A low level of cell death occurs in wild-type germ lines, increasing with the age of the adult animal [32]. Mutations that suppress somatic cell death (such as loss-of-function mutations in either *ced-3* or *ced-4*) also suppress this germ-cell death, suggesting that germ-cell death uses the same cell death machinery as all somatic apoptotic cell deaths. Loss-of-function mutations of *ced-9* greatly increase the number of germ-line deaths [32]. If, like *ced-9*, *bir-1* has a general anti-apoptotic function, then inhibition of *bir-1* in the germ line might be expected to result in an increase in the number of germ-cell deaths.

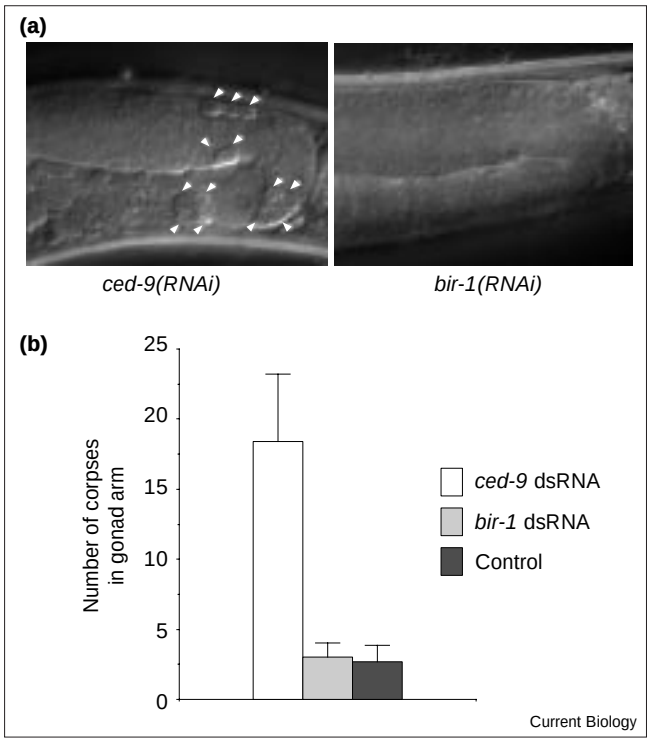
We used RNA-mediated interference (RNAi) to inactivate either *bir-1* or *ced-9* in the germ line. RNAi is a technique whereby the injection of double-stranded RNA (dsRNA) corresponding to sequences in the gene of interest results in the potent and specific inhibition of gene activity [33]. Animals were injected in a single gonad arm with dsRNA corresponding to either *bir-1* or *ced-9* ORFs or with water alone and the germ line analysed 28 hours later. Whereas *ced-9(RNAi)* treatment greatly increased the number of germ-cell deaths observed, injection of *bir-1* dsRNA had no observable effect on cell death in the germ line (Figure 4). Similarly, injection of *bir-2* dsRNA failed to affect germ-cell death (unpublished observations). We conclude that, unlike *ced-9*, *bir-1* has no major role in the suppression of apoptosis in the germ line.

Inhibition of *bir-1* causes a defect in cytokinesis

To determine whether *bir-1* is required for any other aspect of *C. elegans* development, we again used RNAi to inactivate *bir-1* and then analysed the phenotype of injected worms and their progeny. We found that ablation of *bir-1* resulted in 100% embryonic lethality. The embryos were multinucleate (Figure 5a) and over 75% of embryos failed to form more than two cells. The observed defects appeared to result from an inability of *bir-1(RNAi)* embryos to complete cytokinesis (Figure 5b). Whereas cytokinesis initiated normally, and the cleavage furrow progressed to a great extent, cytokinesis failed to complete and the cleavage furrow subsequently regressed. During early divisions in a *bir-1(RNAi)* embryo, both the position and timing of cytokinesis was almost identical to that seen in wild-type embryos, suggesting that polarity was unaffected by *bir-1* inactivation (Figure 5b). Following the regression of the cleavage furrow, the nuclei entered a new cell cycle, resulting in highly multinucleate zygotes.

The *bir-1(RNAi)* embryos were also defective in the extrusion of the polar body, a process which is essentially a highly asymmetric cytokinesis [34]. During normal germline development, oocytes are arrested at prophase of meiosis I until fertilisation. Following fertilisation, meiosis I and II are completed and additional chromosomes are extruded by cytokinesis to form the polar body. The polar

Figure 4



Inhibition of *bir-1* by RNAi does not affect cell death in the adult hermaphrodite germ line. (a) Young adult hermaphrodite worms were injected with dsRNA corresponding to either *bir-1* or *ced-9* ORFs and observed 28 h later. Cell corpses are marked with arrowheads. Anterior is to the left. (b) The numbers of corpses observed in the germ lines of young adult hermaphrodite worms were counted 28 h after injection with dsRNA corresponding to either *bir-1* or *ced-9* ORFs or with water as a control. Values are means ± standard deviation for at least 10 animals.

body was clearly observed in all wild-type embryos (marked in Figure 5b) and was absent in *bir-1(RNAi)* embryos.

To ensure that the cytokinesis defect that we observed was genuinely an effect of *bir-1* inhibition and not a non-specific effect of injection of *bir-1* dsRNA, we carried out several controls. First, we showed that two dsRNA species corresponding to non-overlapping regions of the *bir-1* cDNA (nucleotides 25–310 and 311–605, respectively) gave an identical phenotype to that obtained following injection of dsRNA corresponding to the *bir-1* ORF (data not shown), demonstrating that the phenotype was indeed specific to *bir-1*. Second, to confirm that *bir-1* was indeed being silenced, we showed that injection of dsRNA corresponding to the *bir-1* ORF silenced a transgene expressing a GFP–BIR-1 fusion protein under the control of a 3 kb region of the *bir-1* promoter, but had no effect on a transgene expressing GFP alone under the control of the same promoter region (data not shown). Finally, injection of over a dozen other dsRNA species (including *bir-2*, which failed to give any detectable phenotype) did not result in a similar defect (unpublished observations; Mona Spector, personal communication). These controls confirmed that *bir-1* RNAi treatment silences only transcripts containing *bir-1* sequences (and does not affect the *bir-1* promoter activity) and that the phenotype observed was genuinely due to *bir-1* inactivation.

Survivin expression partially suppresses the cytokinesis defect caused by *bir-1* inhibition

The mammalian BIRP with the highest degree of structural and sequence similarity to BIR-1 is survivin [20]. To determine whether survivin expression could rescue the cytokinesis defect that arose following *bir-1* ablation, we carried out the following experiment. GFP or an amino-terminally FLAG-tagged survivin ORF were cloned downstream of approximately 3 kb of the *bir-1* 5′ region into a vector to give constructs p*bir-1*GFP and p*bir-1*Svn, respectively. In this way, survivin could be expressed transgenically *in vivo* under the control of *bir-1* 5′ regulatory elements, and GFP could be used as a co-injection marker to identify embryos with a transgenically active *bir-1* promoter.

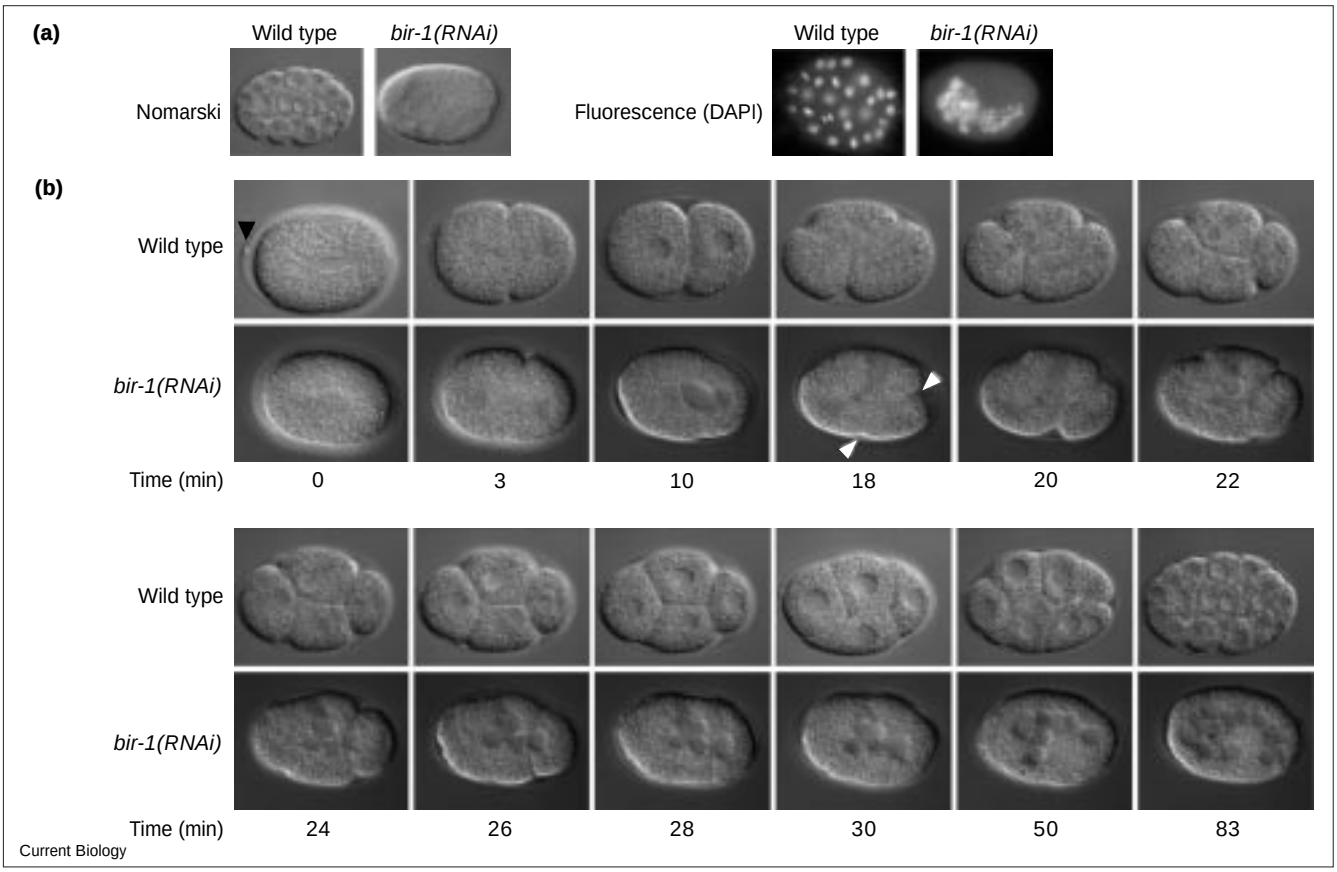
Very young adult wild-type hermaphrodite worms were injected with *bir-1* dsRNA to inhibit *bir-1* expression as described above. Worms displaying a 100% penetrant cytokinesis defect in both gonad arms 15 hours later (as assessed by the presence of multinucleate embryos *in utero*) were re-injected either with an equimolar mixture of p*bir-1*GFP and p*bir-1*Svn or with p*bir-1*GFP alone. At 36 hours after the injection of p*bir-1*GFP alone or of p*bir-1*GFP and p*bir-1*Svn, embryos that had been laid were collected from the plate and examined.

To determine the extent of cytokinesis (and hence of rescue), we counted the number of cells present in individual embryos. We also assessed GFP fluorescence to determine which embryos were expressing the injected transgenes (survivin and GFP under control of the *bir-1* promoter). We found that, whereas most embryos expressing GFP alone failed to cellularise, most embryos expressing survivin showed a substantial degree of cellularisation (Figure 6). These data suggest that expression of survivin under the control of the *bir-1* promoter can significantly suppress the cytokinesis defect resulting from *bir-1* ablation. In no case was the observed partial restoration of cellularisation following survivin expression sufficient to prevent embryonic lethality, however, suggesting that survivin can only partially substitute for BIR-1 in *bir-1(RNAi)* embryos.

Discussion

In summary, we have identified two *C. elegans* genes encoding proteins containing regions that have homology to the BIRs of IAPs. One of these, *bir-1*, is unable to suppress cell death following ectopic expression *in vivo* and inhibition of *bir-1* does not increase cell death in the adult germ line.

Figure 5



Inhibition of *bir-1* by RNAi causes a defect in cytokinesis resulting in a lack of cellularisation and polyploidy. (a) Comparison of morphology and DNA localisation in *bir-1*-ablated embryos. Embryos were removed from dissected *bir-1(RNAi)* adult worms and either observed by Nomarski optics or fixed and stained with DAPI to visualise DNA and observed by fluorescence microscopy. Embryos are ~50 μm in length. (b) Time course of development of wild-type and *bir-1*-ablated embryos. Embryos were removed from dissected wild-type adult worms or *bir-1(RNAi)* adult worms and observed using Nomarski optics at 20°C. Time is given in min following dissection of the adult. Initiation of an individual cytokinesis is marked with white arrowheads. The polar body of a wild-type embryo is marked with a black arrowhead. Embryos are ~50 μm in length. Anterior is to the left.

Inactivation of *bir-1* causes a defect in embryonic cytokinesis, however, and this can be partially suppressed by transgenic expression of survivin, the most closely related mammalian homologue. We conclude that BIR-1 is not a general regulator of cell death in *C. elegans* and that BIRPs have a conserved role in the regulation of cytokinesis.

Expression of *bir-1* and *bir-2* has no effect on cell death

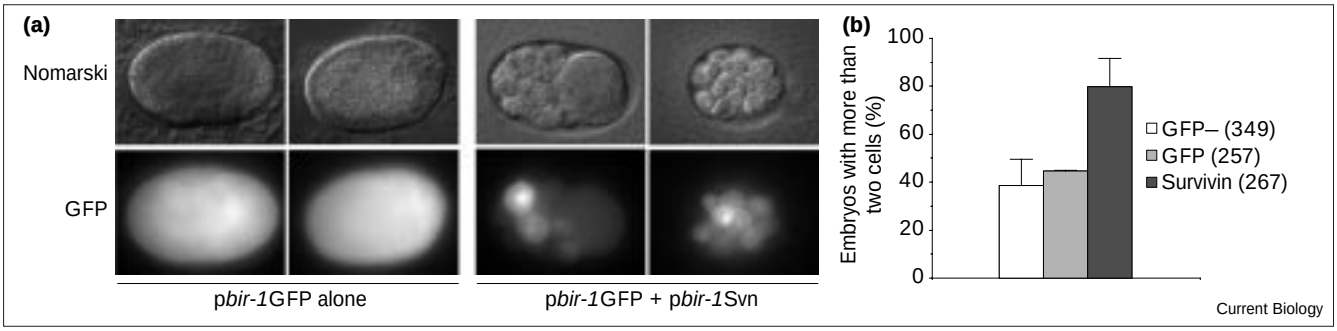
We find that overexpression of *bir-1* has no significant effect on developmentally occurring cell death in the nematode pharynx and that ablation of *bir-1* has no effect on the rate of cell death in the hermaphrodite germ line. We conclude that BIR-1 is unlikely to act as a general cell death inhibitor in *C. elegans*.

This finding clearly does not exclude the possibility that BIRPs regulate cell death in other organisms: the complete suppression of *reaper*-induced cell death in insect cells, both *in vivo* [8,11] and in tissue culture [12], by Diap-1 is compelling, and Xiap is a potent inhibitor of certain mammalian caspases [15,16]. The identification of BIRPs in both fission and budding yeast [3], however, combined with our demonstration that in *C. elegans* BIRPs do not suppress cell death, argue that any anti-apoptotic activity of BIRs evolved later and that BIRs have a more ancient, conserved function.

bir-1 is required for cytokinesis

Inhibition of *bir-1* by RNAi causes a defect in the late stages of cytokinesis. The phenotype of *bir-1(RNAi)* embryos is morphologically similar to that following inhibition of either the *cyk-1* [35] or the *CeMKLP1 C.elegans* genes [36]. The *cyk-1* gene encodes a protein with formin homology (FH) domains that is required for cytokinesis; FH-domain-containing proteins have an essential role in

Figure 6



Expression of human survivin under the *bir-1* promoter can partially rescue the cytokinesis defect arising from inhibition of *bir-1*. (**a**) Morphology of *bir-1(RNAi)* embryos following expression of FLAG-tagged survivin under control of the *bir-1* promoter. Adult wild-type worms in which *bir-1* expression had been inhibited by RNAi treatment were injected either with p*bir-1*GFP alone or with both p*bir-1*GFP and p*bir-1*Svn (p*bir-1*GFP + p*bir-1*Svn) into a single gonad. At 36 h after injection, embryos that had been laid were examined by both Nomarski and fluorescence microscopy (GFP). (**b**) Expression of FLAG-tagged survivin under the control of the *bir-1* promoter partially rescues the cytokinesis defect in *bir-1*-ablated embryos. Adult worms displaying a 100% *bir-1(RNAi)* phenotype were injected either with p*bir-1*GFP alone (GFP) or with both p*bir-1*GFP and p*bir-1*Svn (survivin). At 36 h after injection, embryos that had been laid were examined and both the number of cells and the GFP fluorescence in each embryo determined; embryos not expressing detectable GFP are designated GFP–. Results represent the mean ± standard deviation from three independent experiments. Total number of embryos counted is shown in parenthesis for each sample.

cytokinesis in both yeast and *Drosophila* [37]. *CeMKLP1* encodes a microtubule-associated kinesin that is required for cleavage furrow advancement during cytokinesis. The morphological similarity between *cyk-1(RNAi)*, *CeMKLP1(RNAi)* and *bir-1(RNAi)* embryos also argues that the result of *bir-1* inhibition is principally a defect in cytokinesis.

Cytokinesis uses a complex machinery comprising at least actin and myosin (reviewed in [38]), the small GTPase RhoA [39], RhoA effectors such as citron homologues [40] and IQGAPs [41–44], kinesins [36,45,46], and FH-domain-containing proteins [35,47]. Although there is no coherent molecular model of cytokinesis that integrates the function of such diverse players, one thing is clear: the requirement that we report here for a BIRP in cytokinesis is unexpected and, thus far, mechanistically inexplicable. Analysis of BIR-1 function using the powerful genetics available in *C. elegans* should provide insight into how BIRPs like BIR-1 are involved in cytokinesis, which may in turn shed light on cytokinesis in general.

Expression of survivin can partially rescue the cytokinesis defect caused by ablation of *bir-1*

The finding that transgenic expression of survivin can partially suppress the embryonic cytokinesis defect that arises following ablation of *bir-1* suggests that an involvement in cytokinesis may be conserved between these two BIRPs. Structurally, survivin is the BIRP most similar to BIR-1 and the BIR of survivin is very similar to that of BIR-1 (41.4% identity). We have not determined whether the BIR of survivin is required for survivin's ability to rescue the *bir-1(RNAi)* cytokinesis defect; however, as the only region with significant homology between BIR-1 and survivin is in the BIRs, we favour this hypothesis. Survivin is upregulated ~40-fold at G2–M phase in human cells and associates with the mitotic spindle via an interaction with polymerised tubulin. This BIRP is therefore both expressed at the right time and localised to the correct place to have a role in cytokinesis.

The structural similarity between BIR-1 and survivin might suggest that only small BIRPs containing a single BIR are involved in cytokinesis. There are hints that structurally different BIRPs may also play a similar role in *Drosophila*, however. The *thread* locus encodes Diap-1, a *Drosophila* BIRP that has a carboxy-terminal RING finger [8] and is structurally more similar to the mammalian and viral IAPs than to survivin and BIR-1. Diap-1 was identified in a genetic screen for mutations that enhance cell death caused by ectopic expression of the pro-apoptotic *Drosophila* gene *reaper* [8]. Flies heterozygous for *thread* loss-of-function alleles have enhanced levels of *reaper*-mediated killing, and ectopic expression of Diap-1 suppresses *reaper*-induced killing [8,11,12]. Despite homozygous mutations in the *thread* locus in *Drosophila* being embryonic lethal, however, the lethality does not appear to be due to an increase in cell death [8] (as might be predicted if the sole function of Diap-1 is the suppression of cell death), but instead is due to a defect in cellularisation [48]. Whether Diap-1, like BIR-1, is required for cytokinesis in *Drosophila* is not known, but the similarity in terminal phenotype is intriguing.

A model for BIRs as regulators of cytoskeleton organisation

The findings that BIR-1 is required for a late event in cytokinesis, that survivin is functionally homologous to

BIR-1, and that *Drosophila* embryos with homozygous loss-of-function mutations in the *thread* locus are defective in cellularisation [48], all suggest that BIRs have conserved functions in worms, flies and mammals. Cytokinesis involves major changes in the cytoskeleton, resulting in profound focal alterations at the plasma membrane and, ultimately, in the cleavage of a cell into two daughter cells (for a review, see [38]). One of the main morphological features of apoptosis is extensive membrane 'blebbing' and the release of membrane-enclosed apoptotic bodies [49]. 'Pinching-off' of apoptotic bodies from a dying cell — a caspase-independent process [50,51] — is reminiscent of the cleavage of a cell into two daughter cells during cytokinesis. On this basis, we suggest that BIRs mediate interactions with the cytoskeleton and play a role in various biological processes requiring focal alterations at the plasma membrane; these may include cytokinesis and the formation and release of apoptotic bodies. The identification of proteins that interact with BIRs should provide further insight into their function.

Materials and methods

Maintenance of C. elegans *strains*

All strains described were maintained as hermaphrodites at 20°C, grown on NGMl standard plates and fed with the OP50 bacterial strain as described [52]. Worms were observed either under a dissecting microscope (Leica MZ8) or using a Zeiss Axioplan for Nomarski optics. All injections were performed using standard protocols.

Identification and cloning of bir-1 *and* bir-2

The *bir-1* and *bir-2* genes were identified by searching the non-redundant GenBank database with human c-*iap1* as a probe with Advanced Blast. ORFs were cloned by RT–PCR and sequenced.

RNA preparation and northern blot analysis

For RNA purification, wild-type N2 *C. elegans* were grown in liquid culture as described [31]. RNA was purified using a standard guanidinium-isothiocyanate-based RNA isolation procedure. RNA (10 μg) from each sample was run on a 0.8% agarose gel containing 1.85% formamide, 1 μg/ml ethidium bromide. The gel was washed for 45 min in 20 × SSC, blotted overnight onto Hybond-N^+ (Amersham) and the RNA UV-crosslinked to the membrane using a Stratalinker (Stratagene). DNA probes corresponding to either the *bir-1* or *bir-2* ORFs were ^{32}P-labelled and used to probe the blots for 18 h at 65°C in Church buffer. Blots were washed twice in 0.2 × SSC, 0.1% SDS at 65°C and exposed for 36 h to Kodak XAR-5 film at –70°C.

Generation of transgenic worms and expression of genes under control of heatshock promoters

Sequences encoding either amino-terminally HA-tagged BIR-1 (HA–BIR-1), carboxy-terminally Myc-tagged BIR-1 (BIR-1–Myc) or untagged BIR-1 were generated by PCR and cloned into vectors pPD49.78 and pPD49.83, placing them under control of heatshock promoters [30,53]. To generate transgenic lines, N2 adult worms were injected in both gonads with 20 ng/μl of each of the DNAs of interest along with 80 ng/μl pRF4 which contains a dominant *rol-6* allele [54]. Transgenes were maintained in these lines as extrachromosomal arrays [55]. To assay for the effect of heatshock-induced expression of *bir-1* or *ced-9* (*ced-9* strain described in [29]), 30 young rolling transgenic adult worms were placed on plates and allowed to lay embryos for 45 min. The plates were placed at 30°C in a waterbath for 45 min, removed to 20°C for 90 min and adult worms removed. Embryos on the plates subjected to heatshock were allowed to hatch and the pharynges of the resultant L3–L4 transgenic worms were scored 48 h later. Lysates were prepared from worms 90 min after heatshock, run on a 12.5% SDS–polyacrylamide gel and blotted onto PVDF membrane (Amersham). Blots were probed with anti-HA antibodies (rabbit polyclonal; Upstate Biotech) and anti-tubulin antibodies (mouse monoclonal DM1A; Sigma) and antibodies detected by enhanced chemiluminescence (Amersham).

RNA synthesis and RNA-mediated inhibition

RNA was synthesised using the Ribomax kit (Promega) and processed according to manufacturer's protocols. Sense and antisense strands were mixed prior to injection to give a final RNA concentration of 1 mg/ml and then annealed. Young adult N2 worms were injected in both gonads with dsRNA and observed at least 15 h later. Individual embryos were obtained by dissecting worms in a concave slide and mouthpipetting released embryos onto a 4% agar pad in M9 buffer and the embryos observed using a Zeiss Axioplan microscope.

DAPI staining of embryos

Embryos were mouthpipetted onto a slide coated with poly-L-lysine and glycerine in 4 μl 4% formaldehyde in PBS. A coverslip was placed over the embryos and the slide flash-frozen in liquid nitrogen. The coverslip was rapidly removed and the slide immersed in acetone at 0°C. Slides were washed twice in PBS, fixed in 4% formaldehyde in PBS (5 min, room temperature), washed a further two times in PBS and 10 μl DAPI solution (10 μg/ml in PBS) was overlaid onto the slide.

Rescue of cytokinesis defect caused by bir-1 *inhibition by transgenic expression of human survivin*

A region of genomic DNA spanning ~3 kb immediately 5′ to the start ATG of *bir-1* was amplified by PCR using the primers AAACTGCAGGGTTCTCGCGGAGCTTGAGCTTGAGCC and AAAGGTACCTTTCTGAAATCAATGAAAAACAACTAGG (oligos from Operon). We expected that this region would contain not only the *bir-1* promoter, but most of the 5′ sequences that direct endogenous *bir-1* expression. This ~3 kb region was cloned into the *Pst*I–*Kpn*I sites of vector pPD117.01 to give the vector p*bir-1*GFP. An ORF encoding human survivin with an amino-terminal FLAG epitope tag was generated by PCR and cloned into p*bir-1*GFP to generate vector p*bir-1*Svn. Very young adult wild-type N2 hermaphrodite worms were injected with *bir-1* dsRNA to inhibit *bir-1*. Worms were assessed for the presence of multinucleate embryos *in utero* 15 h after injection, and worms containing only mutant embryos in the uterus were reinjected in a single gonad with either p*bir-1*GFP alone or with p*bir-1*GFP and p*bir-1*Svn. Embryos were removed from the plate 36 h after injection of the pbir constructs and examined with both Nomarski and fluorescence microscopy. The experiment was repeated completely three times and results are the mean of the three experiments; the error is the standard deviation.

Supplementary material

A figure showing the constructs p*bir-1*GFP and p*bir-1*Svn is published with this paper on the internet.

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References

1. Thornberry NA, Lazebnik Y: Caspases: enemies within. *Science* 1998, 281:312-316.
2. Adams JM, Cory S: The Bcl-2 protein family: arbiters of cell survival. *Science* 1998, 281:1322-1326.
3. Uren AG, Coulson EJ, Vaux DL: Conservation of baculovirus inhibitor of apoptosis repeat proteins (BIRPs) in viruses, nematodes, vertebrates and yeasts. *Trends Biochem Sci* 1998, 23:159-162.

4. Crook NE, Clem RJ, Miller LK: An apoptosis-inhibiting baculovirus gene with a zinc finger-like motif. *J Virol* 1993, 67:2168-2174.
5. Birnbaum MJ, Clem RJ, Miller LK: An apoptosis-inhibiting gene from a nuclear polyhedrosis virus encoding a polypeptide with Cys/His sequence motifs. *J Virol* 1994, 68:2521-2528.
6. Duckett CS, Nava VE, Gedrich RW, Clem RJ, Van Dongen JL, Gilfillan MC, *et al*.: A conserved family of cellular genes related to the baculovirus iap gene and encoding apoptosis inhibitors. *EMBO J* 1996, 15:2685-2694.
7. Uren AG, Pakusch M, Hawkins CJ, Puls KL, Vaux DL: Cloning and expression of apoptosis inhibitory protein homologs that function to inhibit apoptosis and/or bind tumor necrosis factor receptor-associated factors. *Proc Natl Acad Sci USA* 1996, 93:4974-4978.
8. Hay BA, Wassarman DA, Rubin GM: ***Drosophila*** homologs of baculovirus inhibitor of apoptosis proteins function to block cell death. *Cell* 1995, 83:1253-1262.
9. Liston P, Roy N, Tamai K, Lefebvre C, Baird S, Cherton-Horvat G, *et al*.: Suppression of apoptosis in mammalian cells by NAIP and a related family of IAP genes. *Nature* 1996, 379:349-353.
10. Rothe M, Pan MG, Henzel WJ, Ayres TM, Goeddel DV: The TNFR2-TRAF signaling complex contains two novel proteins related to baculoviral inhibitor of apoptosis proteins. *Cell* 1995, 83:1243-1252.
11. Hay BA, Maile R, Rubin GM: P element insertion-dependent gene activation in the ***Drosophila*** eye. *Proc Natl Acad Sci USA* 1997, 94:5195-5200.
12. Vucic D, Kaiser WJ, Harvey AJ, Miller LK: Inhibition of reaper-induced apoptosis by interaction with inhibitor of apoptosis proteins (IAPs). *Proc Natl Acad Sci USA* 1997, 94:10183-10188.
13. Vucic D, Kaiser WJ, Miller LK: Inhibitor of apoptosis proteins physically interact with and block apoptosis induced by ***Drosophila*** proteins hid and GRIM. *Mol Cell Biol* 1998, 18:3300-3309.
14. Hawkins CJ, Uren AJ, Hacker G, Medcalf RL, Vaux DL: Inhibition of interleukin 1 beta-converting enzyme-mediated apoptosis of mammalian cells by baculovirus IAP. *Proc Natl Acad Sci USA* 1996, 93:13786-13790.
15. Deveraux QL, Takahashi R, Salvesen GS, Reed JC: X-linked IAP is a direct inhibitor of cell-death proteases. *Nature* 1997, 388:300-303.
16. Deveraux QL, Roy N, Stennicke HR, Van Arsdale T, Zhou Q, Srinivasula SM, *et al*.: IAPs block apoptotic events induced by caspase-8 and cytochrome c by direct inhibition of distinct caspases. *EMBO J* 1998, 17:2215-2223.
17. Seshagiri S, Miller LK: Baculovirus inhibitors of apoptosis (IAPs) block activation of Sf-caspase-1. *Proc Natl Acad Sci USA* 1997, 94:13606-13611.
18. Roy N, Deveraux QL, Takahashi R, Salvesen GS, Reed JC: The c-IAP-1 and c-IAP-2 proteins are direct inhibitors of specific caspases. *EMBO J* 1997, 16:6914-6925.
19. Roy N, Mahadevan MS, McLean M, Shutler G, Yaraghi Z, Farahani R, *et al*.: The gene for neuronal apoptosis inhibitory protein is partially deleted in individuals with spinal muscular atrophy. *Cell* 1995, 80:167-178.
20. Ambrosini G, Adida C, Altieri DC: A novel anti-apoptosis gene, ***survivin***, expressed in cancer and lymphoma. *Nat Med* 1997, 3:917-921.
21. Hauser HP, Bardroff M, Pyrowolakis G, Jentsch S: A giant ubiquitin-conjugating enzyme related to IAP apoptosis inhibitors. *J Cell Biol* 1998, 141:1415-1422.
22. Fraser A, James C: Fermenting debate: do yeast undergo apoptosis? *Trends Cell Biol* 1998, 8:219-221.
23. The *C. elegans* Sequencing Consortium: Genome sequence of the nematode ***C. elegans***: a platform for investigating biology. *Science* 1998, 282:2012-2018.
24. Ellis HM, Horvitz HR: Genetic control of programmed cell death in the nematode ***C. elegans.*** *Cell* 1986, 44:817-829.
25. Xue D, Horvitz HR: Inhibition of the ***Caenorhabditis elegans*** cell-death protease CED-3 by a CED-3 cleavage site in baculovirus p35 protein. *Nature* 1995, 377:248-251.
26. Yuan JY, Horvitz HR: The ***Caenorhabditis elegans*** genes ***ced***-3 and ***ced***-4 act cell autonomously to cause programmed cell death. *Dev Biol* 1990, 138:33-41.
27. Hengartner MO, Ellis RE, Horvitz HR: ***Caenorhabditis elegans*** gene ced-9 protects cells from programmed cell death. *Nature* 1992, 356:494-499.
28. Conradt B, Horvitz HR: The ***C. elegans*** protein EGL-1 is required for programmed cell death and interacts with the Bcl-2-like protein CED-9. *Cell* 1998, 93:519-529.
29. Hengartner M, Horvitz H: C. ***elegans*** cell survival gene ***ced***-9 encodes a functional homolog of the mammalian proto-oncogene ***bcl***-2. *Cell* 1994, 76:665-676.
30. Stringham EG, Dixon DK, Jones D, Candido EP: Temporal and spatial expression patterns of the small heat shock (hsp16) genes in transgenic ***Caenorhabditis elegans***. *Mol Biol Cell* 1992, 3:221-233.
31. Riddle DL, Blumenthal T, Meyer BJ, Preiss JR: *C. elegans II*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press; 1997.
32. Gumienny TL, Lambie E, Hartweig E, Horvitz HR, Hengartner MO: Genetic control of programmed cell death in the ***Caenorhabditis elegans*** hermaphrodite germline. *Development* 1999, 126:1011-1022.
33. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC: Potent and specific genetic interference by double-stranded RNA in ***Caenorhabditis elegans***. *Nature* 1998, 391:806-811.
34. Albertson DG, Thomson JN: Segregation of holocentric chromosomes at meiosis in the nematode, ***Caenorhabditis elegans***. *Chromosome Res* 1993, 1:15-26.
35. Swan KA, Severson AF, Carter JC, Martin PR, Schnabel H, Schnabel R, Bowerman B: cyk-1: a ***C. elegans*** FH gene required for a late step in embryonic cytokinesis. *J Cell Sci* 1998, 111:2017-2027.
36. Powers J, Bossinger O, Rose D, Strome S, Saxton W: A nematode kinesin required for cleavage furrow advancement. *Curr Biol* 1998, 8:1133-1136.
37. Frazier JA, Field CM: Actin cytoskeleton: are FH proteins local organizers? *Curr Biol* 1997, 7:R414-R417.
38. Glotzer M: The mechanism and control of cytokinesis. *Curr Opin Cell Biol* 1997, 9:815-823.
39. Kishi K, Sasaki T, Kuroda S, Itoh T, Takai Y: Regulation of cytoplasmic division of ***Xenopus*** embryo by rho p21 and its inhibitory GDP/GTP exchange protein (rho GDI). *J Cell Biol* 1993, 120:1187-1195.
40. Madaule P, Eda M, Watanabe N, Fujisawa K, Matsuoka T, Bito H, *et al*.: Role of citron kinase as a target of the small GTPase Rho in cytokinesis. *Nature* 1998, 394:491-494.
41. Adachi H, Takahashi Y, Hasebe T, Shirouzu M, Yokoyama S, Sutoh K: ***Dictyostelium*** IQGAP-related protein specifically involved in the completion of cytokinesis. *J Cell Biol* 1997, 137:891-898.
42. Eng K, Naqvi NI, Wong KC, Balasubramanian MK: Rng2p, a protein required for cytokinesis in fission yeast, is a component of the actomyosin ring and the spindle pole body. *Curr Biol* 1998, 8:611-621.
43. Epp JA, Chant J: An IQGAP-related protein controls actin-ring formation and cytokinesis in yeast. *Curr Biol* 1997, 7:921-929.
44. Osman MA, Cerione RA: Iqg1p, a yeast homologue of the mammalian IQGAPs, mediates cdc42p effects on the actin cytoskeleton. *J Cell Biol* 1998, 142:443-455.
45. Adams RR, Tavares AA, Salzberg A, Bellen HJ, Glover DM: pavarotti encodes a kinesin-like protein required to organize the central spindle and contractile ring for cytokinesis. *Genes Dev* 1998, 12:1483-1494.
46. Raich WB, Moran AN, Rothman JH, Hardin J: Cytokinesis and midzone microtubule organization in ***Caenorhabditis elegans*** require the kinesin-like protein ZEN-4. *Mol Biol Cell* 1998, 9:2037-2049.
47. Castrillon DH, Wasserman SA: Diaphanous is required for cytokinesis in ***Drosophila*** and shares domains of similarity with the products of the limb deformity gene. *Development* 1994, 120:3367-3377.
48. Moore LA, Broihier HT,. Van Doren M, Lunsford LB, Lehmann R: Identification of genes controlling germ cell migration and embryonic gonad formation in ***Drosophila***. *Development* 1998, 125:667-678.
49. Kerr J, Wyllie A, Currie A: Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer* 1972, 26:239-257.
50. McCarthy NJ, Whyte MK, Gilbert CS, Evan GI: Inhibition of Ced-3/ICE-related proteases does not prevent cell death induced by oncogenes, DNA damage, or the Bcl-2 homologue Bak. *J Cell Biol* 1997, 136:215-227.
51. Xiang J, Chao DT, Korsmeyer SJ: BAX-induced cell death may not require interleukin 1 beta-converting enzyme-like proteases. *Proc Natl Acad Sci USA* 1996, 93:14559-14563.
52. Brenner S: The genetics of ***Caenorhabditis elegans***. *Genetics* 1974, 77:71-94.

53. Jones D, Russnak RH, Kay RJ, Candido EP: Structure, expression, and evolution of a heat shock gene locus in ***Caenorhabditis elegans*** that is flanked by repetitive elements. *J Biol Chem* 1986, 261:12006-12015.
54. Mello CC, Kramer JM, Stinchcomb D, Ambros V: Efficient gene transfer in ***C.elegans***: extrachromosomal maintenance and integration of transforming sequences. *EMBO J* 1991, 10:3959-3970.
55. Mello C, Fire A: DNA transformation. *Methods Cell Biol* 1995, 48:451-482.
56. Hengartner MO, Horvitz HR: Activation of ***C. elegans*** cell death protein CED-9 by an amino-acid substitution in a domain conserved in Bcl-2. *Nature* 1994, 369:318-320.

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Supplementary material

Caenorhabditis elegans inhibitor of apoptosis protein (IAP) homologue BIR-1 plays a conserved role in cytokinesis

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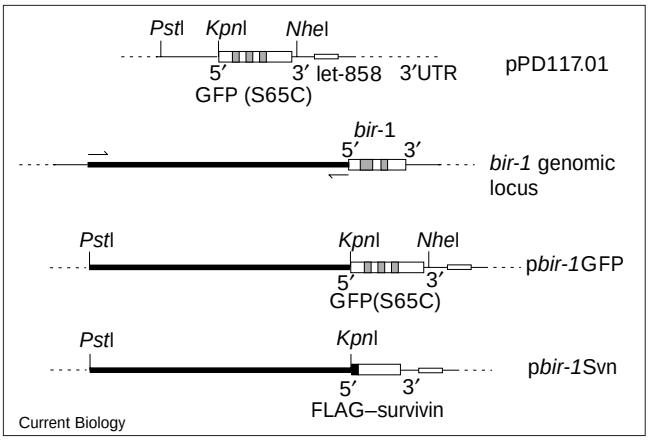
Figure S1



Schematic depiction of constructs p*bir-1*GFP and p*bir-1*Svn. A ~3 kb region of genomic DNA immediately 5′ to the start ATG of *bir-1* was cloned into vector pPD117.01, placing it directly upstream of a S65C variant of GFP that contains three synthetic introns to enhance expression, to generate construct p*bir-1*GFP. The GFP-encoding sequences of p*bir-1*GFP were excised and replaced by an ORF encoding an amino-terminally FLAG-epitope-tagged version of survivin to make construct p*bir-1*Svn. Exons or coding sequences are depicted by open boxes, introns are depicted as grey shaded boxes and the amino-terminal FLAG epitope tag as a black box. The portion of the 3′ UTR of the *let-858* gene that provides the polyadenylation site and the 3′ UTR of the message from p*bir-1*GFP and p*bir-1*Svn is shown as a thin open box. The ~3 kb region of the *bir-1* promoter is shown as a thin black box.