Figure 3 Photomicrographs of ice crystals grown in the presence of THP or AFP. The *c*-axis is horizontal in the plane of the page in all cases. **a,** Dilute haemolymph. **b,** Recombinant THP. **c,** Type-I fish AFP. **d,** Type-III fish AFP.

were unusual in that their surfaces were curved, whereas crystals generated by fish AFP types I and III are hexagonal bipyramids with flat, well-defined facets (Fig. 3 c,d), reflecting, in the case of type-I AFP, affinity for a specific pyramidal plane[10].

The maximum observed thermal hysteresis value (5.5 °C) is four times that obtained with fish AFPs[6]. At low concentrations the activity is up to 100 times that of fish AFPs, consistent with our observations that THP is a minor constituent of haemolymph. Contamination with as little as 0.1–1% THP could produce activities comparable to those obtained with fish AFPs, suggesting that past THP preparations[2–5] may have been impure.

A possible explanation for the hyperactivity of THP is that the protein has multiple ice-binding sites and that these sites either recognize several different features of ice, or a repeat expressed in several directions on the ice lattice. These properties could increase the frequency of THP binding to ice and might account for the curved ice crystal surfaces. The fourfold increase in maximum thermal hysteresis values might result from closer spacing of THP on the ice surface[11]. The exceptional activity of *Tenebrio* THP makes it an attractive reagent for applications requiring freeze resistance or the control of ice growth and morphology.

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Role of CED-4 in the activation of CED-3

Genetic analyses of the nematode *Caenorhabditis elegans* have identified three core components of the cell-death apparatus[1]. CED-3 and CED-4 promote, whereas CED-9 inhibits cell death. Recent studies indicate that CED-4 might interact independently with CED-3 and CED-9, forming the crux of a multicomponent death complex[2]. But except for its role as an adaptor molecule, little is known about CED-4 function. A clue came with the observation that mutation of the phosphate-binding loop (P-loop) of CED-4 disrupts its ability to induce chromatin condensation in yeast[3]. Further, a P-loop mutant of CED-4 (CED-4^{K165R}) fails to process CED-3 *in vivo*, both in insect[4] and mammalian cells (unpublished). We now confirm that CED-4 induces CED-3 activation and subsequent apoptosis, and that the process requires binding of ATP.

To test whether CED-4 could bind ATP, we used ATP analogues that label the nucleotide-binding sites on proteins. One such analogue is 5′-fluorosulphonyl-benzoyladenosine (FSBA)[5,6]. The natural nucleotide ATP (with Mg^{2+}), effectively inhibited FSBA incorporation whereas CTP (with Mg^{2+}) did not (Fig. 1a), showing that FSBA binds specifically to CED-4. The photoaffinity ATP analogue 8-azidoadenosine-5′-triphosphate[α-^{32}P] (8N$_3$-ATP) has also been used to identify ATP-binding proteins[7,8]. As predicted, CED-4 bound 8N$_3$-ATP and this photoaffinity labelling was attenuated with ATP and $MgCl_2$ (Fig. 1b). Unlike wild-type CED-4, a form of CED-4 (residues 171–549) in which the P-loop motif was deleted (CED-4ΔPL), failed to bind the azido analogue (Fig. 1c).

We next assessed the function of the CED-4 P-loop, and tested whether CED-4 activates CED-3 *in vitro*. We prepared

extracts from 293T cells transfected with CED-4 or the P-loop mutant CED-4^{K165R}. We saw proteolytic processing into active subunits when extracts containing CED-4 were incubated with *in vitro* translated ^{35}S-labelled CED-3, but not when incubated with the CED-3 active-site mutant, CED-3mt (Fig. 2a). Extracts generated from the vector or cells transfected with CED-4^{K165R} also failed to activate CED-3 *in vitro* (Fig. 2a). When CED-9 or CED-3mt were coexpressed with CED-4, CED-4 failed to proteolytically activate CED-3 (Fig. 2a).

To rule out the possibility that this phenomenon was due to a possible apoptotic nature of the CED-4-containing extract, we depleted CED-4 from the extract using Myc and histidine affinity tags. When we incubated CED-4-depleted extracts with CED-3, we found no CED-3 processing (Fig. 2a).

To confirm that CED-4 was responsible for CED-3 activation, we purified Myc/His affinity tagged CED-4 from the 293T extract by Ni^{2+} chromatography[9]. Purified CED-4, but not CED-4^{K165R}, stimulated CED-3 processing *in vitro*, and again the active-site mutant CED-3mt was not processed by CED-4 (Fig. 2b), implicating CED-4 as a catalyst for CED-3 self-processing.

In addition, a peptide inhibitor of caspases, DEVD aldehyde, abrogated CED-4-mediated auto-processing of CED-3. When the prodomain of CED-3 was truncated (CED-3(99–502)), CED-4 was unable to catalyse CED-3 processing (Fig. 2b). The prodomain of CED-3 interacts with CED-4 (ref. 10), and this CED-3 prodomain interaction with CED-4 seems to be required for CED-4-mediated activation of CED-3 processing. We also show that the ATP analogue FSBA blocks CED-4-mediated activation of CED-3 (Fig. 2c), confirming the functional importance of ATP binding in CED-4 activity as a catalyst for CED-3 self-processing.

We analysed the *C. briggsae* CED-4 sequence, and searched the non-redundant database, to find the highest similarity (after *C. elegans* CED-4) with the sequence
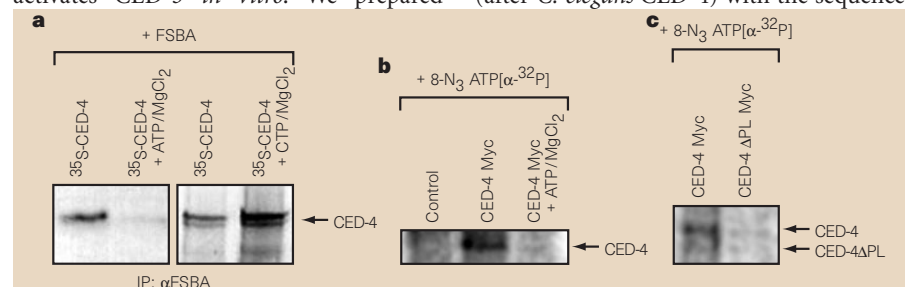

Figure 1 CED-4 binds ATP analogues. **a,** CED-4 binds FSBA, an inhibitor of P-loop-containing ATPases. ^{35}S-labelled CED-4 was incubated with 1 mM FSBA in DMSO in the presence or absence of excess ATP or CTP (10 mM) and $MgCl_2$ (10 mM), then immunoprecipitated with rabbit anti-FSBA (Boehringer), followed by SDS–PAGE. **b,** CED-4 binds an azido derivative of ATP. Lysates from 293T cells expressing Myc–CED-4 were incubated with 10 μM (final concentration) 8N$_3$-ATP in the presence or absence of excess ATP (1 mM) and $MgCl_2$ (1 mM). For azido-affinity labelling, samples were irradiated[7], and then immunoprecipitated with anti-Myc antibodies (Boehringer) followed by SDS–PAGE and phosphorimager analysis. Control represents lysates from vector-transfected cells. **c,** A P-loop deletion mutant of CED-4 fails to bind an azido derivative of ATP. Samples processed as in **b**. Expression of CED-4 and CED-4ΔPL was confirmed by immunoblotting.
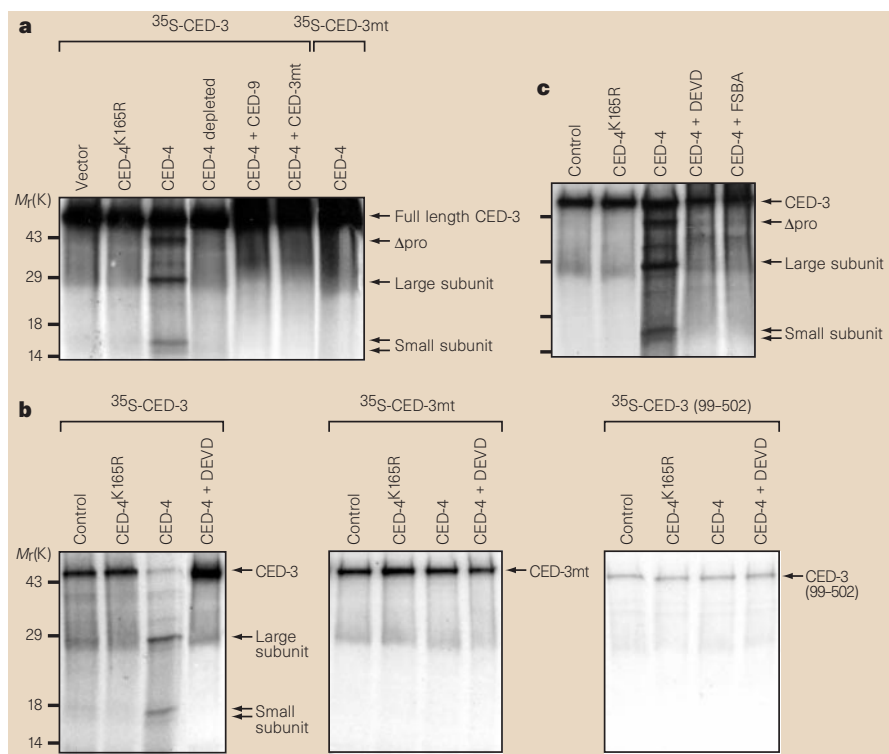
Figure 2 CED-4 facilitates CED-3 auto-activation *in vitro*. **a,** Cell extracts expressing CED-4 process *in vitro* translated, ³⁵S-labelled CED-3 but not CED-3mt. Myc/His–CED4 was depleted from an extract derived from CED-4-transfected 293T cells by incubation with Ni²⁺ beads and further depleted using anti-Myc antibodies and protein G. ³⁵S-labelled CED-3 was made by *in vitro* transcription/translation using the TNT rabbit reticulo-cyte lysate system (Promega; 30 min, 30 ℃). Prolonged incubation of the *in vitro* translate triggered auto-pro-cessing of CED-3 (ref. 13) and so was avoided. Reactions used 5 μl ³⁵S-labelled CED-3 and 20 μl lysate containing the indicated proteins, incubated for 1 h at 30 ℃. **b,** Purified CED-4 activates *in vitro* translated CED-3. CED-4 and CED-4^K165R were purified from transfected 293T cells[9]. Reactions used ~10–50 ng purified protein and *in vitro* translated ³⁵S-labelled CED-3, CED-3mt or CED-3(99–502) incubated for 1 h at 30 ℃[10]. DEVD-aldehyde was at 200 nM. **c,** CED-4 activation of CED-3 is blocked by FSBA (1 mM). Methods as in **b**.

of the flax rust-resistance protein L6 (Gen-Bank accession number 862905), a putative P-loop-containing ATPase[11]. The alignment of the *C. briggsae* CED-4 and L6 sequences consisted of 322 amino-acids with 23% identical and 43% similar residues. When the predicted ATPase domain of L6 was used to search the non-redundant database with the PSI-BLAST (position-specific iter-ative BLAST) program, which enhances BLAST search with profile analysis[12], the similarity to *C. elegans* CED-4 was detected at a statistically significant level ($P < 10^{-5}$). Analysis of a multiple alignment of the CED-4 sequences with those of plant resis-tance-response gene products homologous to L6 (GenBank accession numbers 862905, 1842251, 1086263, 699495, 1931650 and 1513144) showed a consistent pattern of conservation which was particularly promi-nent in the P-loop, the putative Mg²⁺-bind-ing site, and two additional, downstream motifs (unpublished). CED-4 and plant-resistance gene products may contain a homologous and probably functionally analogous ATPase domain.

In summary, CED-4 is central to the reg-ulation of the molecular framework of cell death. It has an intrinsic enzymatic activity which facilitates CED-3 auto-activation and subsequent apoptosis. CED-3 activation by CED-4 requires the activity of the putative CED-4 ATPase domain. The presence of a conserved ATPase domain in CED-4 and the plant resistance-response proteins indi-cates that there may be similarities between the cell-death machinery and mechanisms in animals and plants.

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Vaccination onto bare skin

We report here that applying genetic vectors onto the skin in a relatively non-invasive manner can elicit an immune response against the protein encoded by the vector. This procedure requires no special skill or equipment and so may reduce medical costs and offer a unique method for vaccination.

To elicit a specific immune response, we anaesthetized mice and removed hair and cornified epithelium over a restricted area of abdominal skin using a depilatory (for example Nair)[1]. We pipetted roughly 10⁸ plaque-forming units (PFU) of adenovirus vector in a volume of 10–50 μl onto the pre-shaved and Nair-treated skin. We allowed the adenovirus vector to incubate with the naked skin until the animal recovered from anaes-thesia, which took between 30 min and 1 h.

After application of AdCMV-hcea, an adenovirus vector encoding the human car-cinoembryonic antigen (CEA) gene, onto the skin of mice (strain C57BL/6), we mon-itored the production of antibodies against the human CEA protein by assaying sera from tail-bleeds. The test sera reacted in western blots with purified human CEA protein, but not with bovine serum albu-min (Fig. 1a). Of 24 vaccinated mice, 23 (96%) produced antibodies against the human CEA protein after a month, indicat-ing that specific antibodies were produced against exogenous proteins encoded by ade-novirus vectors as a result of the treatment. Antibodies against adenoviral proteins were also found in some of the treated animals.

To test whether this technique might be generally applicable, we applied AdCMV-hgmcsf, an adenovirus vector encoding the human granulocyte macrophage colony stimulating factor (hGM-CSF), in the same way. The test sera reacted with purified human GM-CSF protein (Fig. 1b) in 6 of 14 mice showing that 43% produced antibodies against the human GM-CSF protein after application of AdCMV-hgmcsf. Pre-immune sera collected before treatment, sera from untreated animals, and sera from animals treated with AdCMV-luc[2] all failed to react with the human CEA and GM-CSF proteins.