conserved four-amino-acid sequences called RHG (Reaper, Hid, and Grim) motif or IBM (IAP-Binding Motif) [3,10]. Unlike mammalian counterparts, however, RHG normally reside in the cytoplasm and their activities are regulated either at transcriptional levels or by phosphorylation [11]. The presence of two *Drosophila* Bcl-2 family proteins that localize to mitochondria [12,13] indicates that mitochondrial cell death pathway may also exist in flies [14]. Here, in order to investigate the role of mitochondria in *Drosophila* cell death pathway, we cloned and characterized DmHtrA2, a *Drosophila* homolog of HtrA2/Omi. Our data suggest that DmHtrA2 promotes cell death through a cleavage of *Drosophila* IAP1 (DIAP1) in the vicinity of mitochondria, which may represent a prototype of mitochondrial cell death pathway in evolution.

Materials and methods

Stress resistance. For UV-resistance, third-instar larvae were irradiated with 5 mJ/cm^2 UV and allowed to develop at 25 °C. Paraquat resistance was tested essentially as described [15]. Adult flies (age 10–20 days) were starved for 6 h and transferred to vials containing two 2 cm × 2 cm filter squares wetted with 20 mM paraquat (Sigma) in 5% sucrose solution. Survival was scored at 18 or 24 h after the transfer. The ingestion rate was determined by dye intake by adding 10 mg/ml bromophenol blue instead of paraquat. Fly lysate was analyzed by monitoring the absorbance at 595 nm at 18 h.

Recombinant proteins and cleavage assay. The C-terminally His_6-tagged recombinant human HtrA2 protein (HsHtrA2ΔN133-His_6) was described previously [6]. The N-terminally GST-tagged recombinant DmHtrA2 protein (GST-DmHtrA2ΔN92) was produced in the *Escherichia coli*. The protein was purified by affinity chromatography using Glutathione Sepharose™ 4B (Amersham Bioscience). N-terminally FLAG-tagged DIAP1 or Hop was translated *in vitro* in the presence of [^{35}S]-methionine using a TNT T7 Quick Coupled Transcription/Translation System (Promega). [^{35}S]-labeled proteins were incubated with recombinant HtrA2 proteins (100 nM) in Tris buffer containing 50 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 1 mM DTT for 1 h at 37 °C. The reaction mixtures were subjected to SDS–PAGE and visualized by autoradiography.

Cell death assay. Cell death assay was performed as described previously [12]. In brief, S2 cells were transfected with pUAST-derived expression constructs with a driver plasmid pWAGAL4 (actin promoter-GAL4), together with a reporter plasmid pCaspeR-hs-lacZ that encodes β-galactosidase under the control of the *hsp70* promoter. Twenty-four hours after transfection, the cells were heat-shocked at 37 °C and cultured for another 24 h. The cells were lysed at 48 h and assayed for β-galactosidase activity in a reaction mixture containing *o*-nitrophenyl-β-D-galactopyranoside.

For information regarding cloning, expression constructs, antibodies, cell culture, subcellular fractionation, Western blotting, and fly stocks, see Supplementary information.

Results and discussion

We cloned the DmHtrA2 cDNA (DDBJ/EMBL/GenBank, Accession No. AB112473) from the total RNA of wild-type fly embryo. DmHtrA2 encoded a protein of 422 amino acids with an N-terminal transmembrane (TM) domain, a central trypsin-like serine protease domain, and a C-terminal PDZ domain, as well as an IBM-like (ASKM) sequence that locates adjacent to the TM (Supplemental Fig. 1). RT-PCR analysis revealed that DmHtrA2 mRNA was expressed at all stages of *Drosophila* development (Supplemental Fig. 1).

To investigate the role of DmHtrA2 in cell death, we induced cell death in *Drosophila* S2 cells by UV-irradiation. Four hours after irradiation, the cells began to exhibit apoptotic morphological changes (Fig. 1A and B). We found that the protein level of DmHtrA2 was significantly up-regulated in the irradiated cells in a time-dependent

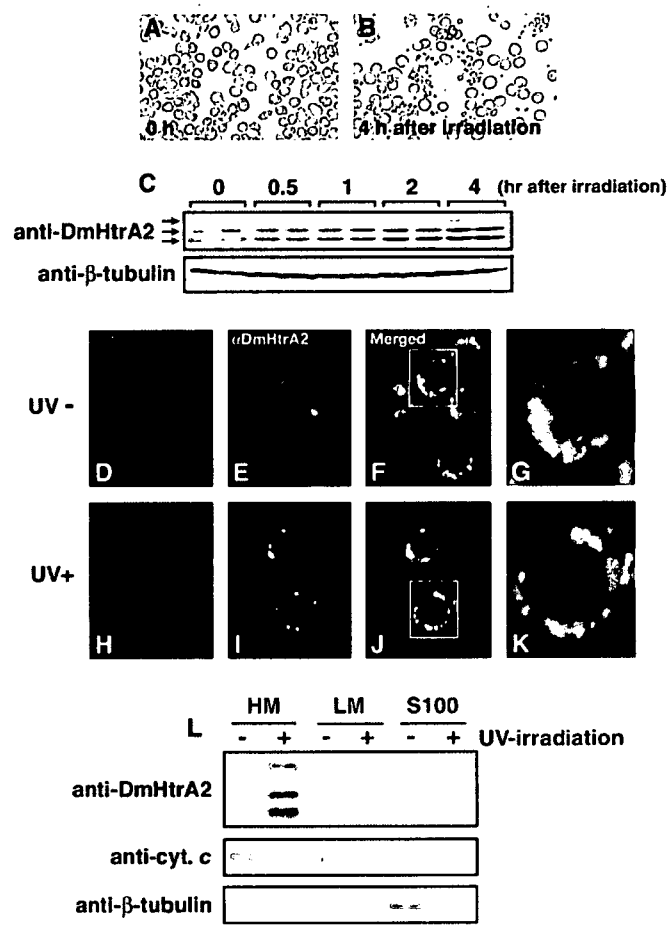

Fig. 1. Apoptotic stimulus up-regulates DmHtrA2 and induces its translocation from mitochondria to extramitochondrial compartment. (A,B) S2 cells were irradiated with UV (200 mJ/cm^2) and cultured for another 4 h. (C) Endogenous DmHtrA2 levels were assessed by Western blotting using an anti-DmHtrA2 antibody before and after UV-irradiation. An anti-β-tubulin antibody was used for a loading control. The anti-DmHtrA2 antibody recognized three bands; the highest band corresponded to the size of full-length form, and the lowest band corresponded to the size of putative mitochondrial mature (ΔN) form (C, upper panel). The experiments were performed using duplicate dishes and were repeated three times. (D–K) Confocal images of S2 cells co-stained with Mitotracker (magenta) and an anti-DmHtrA2 antibody (green). Mitochondria and the endogenous DmHtrA2 protein were visualized before (D–G) or 4 h after (H–K) UV-irradiation (200 mJ/cm^2). G and K show magnified images from F and J, respectively. (L) S2 cells were fractionated before (−) or 4 h after (+) UV-irradiation (200 mJ/cm^2), and the cell lysate was subjected to Western blot analysis using anti-DmHtrA2, anti-cytochrome *c*, and anti-β-tubulin antibodies. For DmHtrA2 protein, the total amount of protein from each fraction was adjusted to 2.5 μg. For cytochrome *c* and β-tubulin, the mitochondrial and cytosolic markers, respectively, an equal volume (10 μl) from each fraction was used for Western analysis.

manner (Fig. 1C). This up-regulation was observed even at 2 h after irradiation, preceding the morphological changes (Fig. 1C). Immunostaining of DmHtrA2 revealed that it exclusively localized to mitochondria under the normal condition (Fig. 1D–G). Four hours after UV-irradiation, the anti-DmHtrA2 staining still showed a punctate pattern with a higher signal intensity, but it no longer merged with the Mitotracker-labeled mitochondria (Fig. 1H–K). This suggests that DmHtrA2 is translocated to extramitochondrial conpartment in response to UV-irradiation. We further analyzed the subcellular localization of DmHtrA2 by fractionating S2 cell lysates before and after irradiation. DmHtrA2 protein was detected in the heavy membrane (HM) fraction, as was cyt *c*, but not in either the light membrane (LM) or cytosolic (S100) fraction (Fig. 1L). We found that UV-irradiation did not alter this distribution (Fig. 1L), suggesting that DmHtrA2 is released to the extramitochondrial compartment but stays in the vicinity of the mitochondria. Since no difference was observed in the mitochondrial membrane potential between control and UV-irradiated S2 cells (Fig. 1D and H; [16]), it is unlikely that the complementary staining of anti-DmHtrA2 and Mitotracker in irradiated cells was due to a loss of membrane potential in a subset of mitochondria.

To examine the physiological role of DmHtrA2, we generated DmHtrA2 knock-down flies using an RNAi construct of DmHtrA2 and a ubiquitous driver da-GAL4. In the knock-down larvae and adults, DmHtrA2 protein level was greatly reduced (Fig. 2A). The knock-down flies were viable and fertile with no detectable morphological abnormalities. We found that the DmHtrA2 knock-down larvae were more resistant to UV-induced lethality compared to control larvae (Fig. 2B). Furthermore, the DmHtrA2 knock-down adult flies were more resistant to dietary paraquat, a superoxide stress agent (Fig. 2C). The ingestion rate of the knock-down flies was not affected (data not shown). These observations suggest that DmHtrA2 is involved in stress-induced toxicity *in vivo*.

Mammalian HtrA2/Omi induces cell death by cleaving and thereby inactivating IAPs through its serine protease activity [4–7]. We therefore assumed that the stress-induced toxicity mediated by DmHtrA2 is through a cleavage of DIAP1. We performed an *in vitro* cleavage assay using recombinant DmHtrA2 and DIAP1 proteins, and found that both *Drosophila* and human HtrA2 proteins specifically cleaved DIAP1 (Fig. 3A). These HtrA2 proteins did not cleave a control protein FLAG-tagged Hop (Hsp70/Hsp90-organizing protein) (Fig. 3A). Thus, the specific serine protease activity of HtrA2/Omi is conserved in *Drosophila*.

Finally, we examined whether DmHtrA2 can kill the cell. Overexpression of DmHtrA2 in S2 cells significantly reduced their viability as potent as other *Drosophila* killer proteins such as Reaper (Fig. 3B and data not shown). This cell death could not be blocked by caspase inhibitors such as DIAP1, p35, or p49, similar to the one caused by human HtrA2/Omi [4,6]. We further examined the toxicity of DmHtrA2 *in vivo*. Overexpression of DmHtrA2 in developing *Drosophila* eye resulted in "no eye" phenotype (Fig. 3C), suggestive of an extensive cell death during development. This phenotype was also resistant to caspase inhibitors (Fig. 3C). Together, these data suggest that DmHtrA2 potently induces cell death through a cleavage of DIAP1.

Our observations suggest that stress stimuli such as UV-irradiation cause translocation of DmHtrA2 from mito-

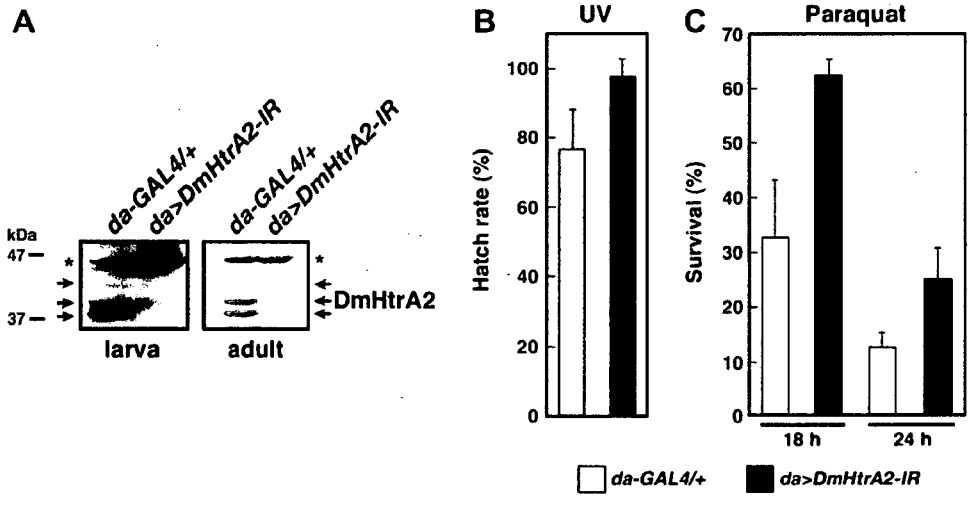

Fig. 2. DmHtrA2 mediates stress stimuli *in vivo*. (A) An RNAi construct (inverted-repeat; IR) for DmHtrA2 (UAS-DmHtrA2-IR) was driven by ubiquitous expression driver da-GAL4 in third-instar larvae or adult flies. Eight animals were homogenized with 96 μl of conventional SDS loading buffer and subjected to SDS–PAGE (4 μl/lane) followed by an anti-DmHtrA2 blotting. The non-specific bands detected by the anti-DmHtrA2 antibody (asterisks) show equal loadings of the protein. (B) Control (da-GAL4) or knock-down (da-GAL4, UAS-DmHtrA2-IR) third-instar larvae were irradiated with UV (5 mJ/cm^2), and the resistance was assessed by the number of adult flies that hatched. (C) Control or knock-down adult flies were starved and subjected to dietary paraquat. Survival was scored at 18 or 24 h after the beginning of ingestion.

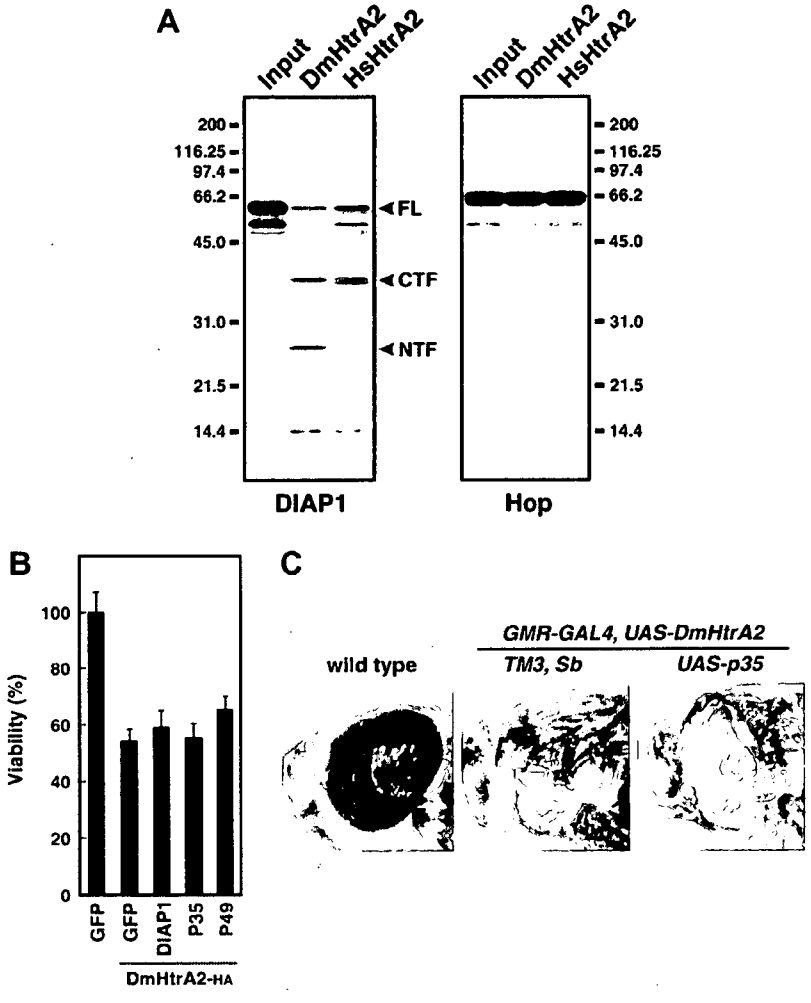

Fig. 3. DmHtrA2 is a potent cell death inducer that cleaves DIAP1. (A) [^{35}S]-labeled DIAP1 or Hop was incubated with *Drosophila* or human HtrA2 recombinant protein, subjected to SDS–PAGE, and visualized by autoradiography. The bands of C-terminal fragment (CTF) and N-terminal fragment (NTF) were predicted by Western blotting of S2 cell lysate co-expressing DmHtrA2 and FLAG-tagged DIAP1 (data not shown). (B) S2 cells were transfected with expression constructs for the indicated proteins and subjected to Cell Death assay. (C) DmHtrA2 was overexpressed in developing *Drosophila* eye using the GMR-GAL4 driver. The eyes at late pupal stage of wild-type, GMR-GAL4/UAS-DmHtrA2$^{2\ 6}$; *TM3, Sb*/+, and GMR-GAL4/UAS-DmHtrA2$^{2\ 6}$; UAS-p35/+ are shown.

chondria to extramitochondrial compartment, which in turn promotes cell death through inactivation of DIAP1. Indeed, a significant proportion of the *Drosophila* caspase DRONC, a fly counterpart of caspase-9, has been shown to localize near mitochondria [17]. Intriguingly, overexpression of DmHtrA2 caused caspase-independent cell death both *in vitro* and *in vivo*. This is consistent with the previous report that down-regulation of DIAP1 triggers a caspase activity-independent cell death pathway that is mediated by DRONC [18]. Our findings suggest that the mitochondrial regulation of cell death machinery could be conserved in *Drosophila*, and that the diverse roles of mitochondria in mammalian systems may have been coopted through the evolution of cell death mechanisms.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.03.079.

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