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Flightless-I regulates proinflammatory caspases by selectively modulating intracellular localization and caspase activity

Juying Li,¹ Helen L. Yin,² and Junying Yuan¹

¹Department of Cell Biology, Harvard Medical School, Boston, MA 02115

²Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, TX 75390

Caspase-1 and caspase-11 are proinflammatory caspases that regulate cytokine production and leukocyte migration during pathogen infection. In an attempt to identify new intracellular regulators of caspase-11, we found that Flightless-I, a member of the gelsolin superfamily of actin-remodeling proteins, interacts and regulates both caspase-11 and caspase-1. Flightless-I targets caspase-11 to the Triton X-100-insoluble cytoskeleton fraction and the cell leading edge. In addition, Flightless-I inhibits caspase-1 activation and caspase-1-mediated

interleukine-1 β (IL-1 β) maturation. The physiological relevance of these findings is supported by the opposing effects of Flightless-I overexpression and knockdown on caspase-1 activity and IL-1 β maturation. Our results suggest that Flightless-I may be a bona fide caspase-1 inhibitor that acts through a mechanism similar to that of cytokine response modifier A, a potent caspase-1 inhibitor from the cowpox virus. Our study provides a new mechanism controlling the localization and activation of proinflammatory caspases.

Introduction

Caspases are a family of cysteine proteases regulating both apoptosis and inflammation (Cryns and Yuan, 1998). Caspases are divided into two subfamilies based on their major functions in vivo, proapoptotic subfamily, or proinflammatory subfamily (Martinon and Tschoop, 2004). The proapoptotic subfamily of caspases (caspase-3, -6, -7, -8, -9, and -10) mediates a cascade of proteolytic cleavage events that culminate in apoptotic cell death. However, human caspase-1, -4, and -5 and mouse caspase-11 and -12 constitute another group referred to as the caspase-1 subfamily of proinflammatory caspases. Importantly, the activation of caspase-1 results in the cleavage and activation of the proinflammatory cytokines interleukine-1 β (IL-1 β) and IL-18 (Thornberry et al., 1992; Ghayur et al., 1997; Gu et al., 1997). Although caspase-11 does not process pro-IL-1 β directly, coexpression of caspase-11 with caspase-1 promotes the activity of caspase-1 in processing pro-IL-1 β (Wang et al., 1996). Both caspase-1-deficient mice (Kuida et al., 1995; Li et al., 1995)

and caspase-11-deficient mice (Wang et al., 1998) develop normally but have a defect in the maturation of IL-1 β and are resistant to the lethal effect of endotoxins, which indicates the importance of the proper regulation of proinflammatory caspases in host defense. Several viral proteins, e.g., cytokine response modifier A (CrmA) and p35, acting as pseudosubstrates, are potent caspase-1 inhibitors produced by the viruses to dampen IL-1 β secretion and apoptosis during host defense response (Clem et al., 1991; Ray et al., 1992). In addition, the serpin PI9 has been identified as a human homologue of CrmA (Sprecher et al., 1995). The reactive center of PI9 shows 54% identity with CrmA and contains a Glu residue at the P1 position rather than the Asp found in CrmA (Annand et al., 1999). It has been shown that PI9 inhibits active caspase-1 in vitro (Annand et al., 1999) and in human vascular smooth muscle cells (Young et al., 2000). PI9 also inhibits apical proapoptotic caspases and death receptor-mediated apoptosis (Kummer et al., 2007). However, the expression of PI9 in monocytes/macrophages is relatively low and is further diminished after lipopolysaccharide (LPS) stimulation or differentiation (Young et al., 2000).

Flightless-I protein, originally identified from a *Drosophila melanogaster* mutant unable to fly (Campbell et al., 1993), contains a C-terminal gelsolin-like domain (GLD) and belongs to the gelsolin superfamily of actin-remodeling proteins (Silacci et al., 2004).

Correspondence to J. Yuan: jyuan@hms.harvard.edu

Abbreviations used in this paper: Aip1, actin-interacting protein 1; CARD, caspase recruitment domain; CrmA, cytokine response modifier A; GLD, gelsolin-like domain; HSS, high-speed supernatant; IL-1 β , interleukine-1 β ; LPS, lipopolysaccharide; LRR, leucine-rich repeat; LRRFIP2, LRR in Flightless-I-interacting protein 2; LSP, low-speed pellet; shRNA, short hairpin RNA; TLR, toll-like receptor; wt, wild type.

The online version of this paper contains supplemental material.

Although there is evidence that Flightless-I regulates the actin cytoskeleton, its GLD is far more divergent from that in many other gelsolin family members, which suggests that the GLD of Flightless-I may have other unique regulatory functions. In addition, Flightless-I has an N-terminal leucine-rich repeat (LRR) protein-protein interaction domain that has many identified binding partners, including FLAP1 and LRR in Flightless-I-interacting proteins (LRRFIPs; Liu and Yin, 1998; Fong and de Couet, 1999). The unique important role of Flightless-I is supported by the finding that Flightless-I-deficient mice are embryonic lethal (Campbell et al., 2002), whereas mice deficient in several other gelsolin family proteins (e.g., gelsolin, villin, and capG) are not (Witke et al., 1995, 2001; Pinson et al., 1998). The human Flightless-I gene maps within the microdeletion critical region of Smith-Magenis Syndrome, which is associated with a spectrum of developmental and behavioral abnormalities (Chen et al., 1995).

Through a Flag tag-based affinity purification approach, we identified Flightless-I as a new caspase-11-binding protein. Here, we show that Flightless-I regulates two major proinflammatory caspases, caspase-11 and caspase-1, in multiple ways. Flightless-I regulates the subcellular distribution of caspase-11 by promoting its localization at the cell leading edge. Furthermore, Flightless-I inhibits caspase-1 activation in a manner similar to CrmA.

Results

Identification of Flightless-I as a caspase-11-binding protein

We have previously found that caspase-11 promotes actin depolymerization and leukocyte migration through interaction with Aip1 (actin-interacting protein 1; Li et al., 2007). In the previous study (Li et al., 2007), we established two stable cell lines of mouse macrophage-like J774 cells expressing an N-terminal Flag-tagged enzymatic activity site C254G mutant form of caspase-11 (J774-C11-C254G) or a control vector (J774-pBabe). Total lysates of LPS-treated J774-C11-C254G and J774-pBabe cells were affinity purified using an anti-Flag (M2) agarose column, and the proteins eluted by the Flag peptide were analyzed by SDS-PAGE. In addition to Aip1 (66 kD), we recovered a second protein in the affinity-purified proteins from J774-C11-C254G cells with molecular mass of ~50 kD (Fig. 1 A, left). Mass spectrometry analysis revealed that it is mouse LRRFIP2.

We first attempted to reproduce the interaction of caspase-11 and LRRFIP2 using coimmunoprecipitation after cotransfection of expression vectors encoding caspase-11 and LRRFIP2 but failed to detect any interaction (Fig. 1 B). LRRFIP2 was originally identified from a yeast two-hybrid screen using the LRR region of Flightless-I as the bait (Fong and de Couet, 1999). We examined the possibility that Flightless-I might be required for LRRFIP2 to interact with caspase-11. We cotransfected Flag-tagged caspase-11, HA-tagged LRRFIP2, and nontagged Flightless-I expression constructs into 293T cells. The cell lysates were immunoprecipitated with anti-Flag agarose beads and the immunity-purified protein complexes were analyzed by

Western blotting using anti-HA and anti-Flightless-I antibodies. Interestingly, Flightless-I interacted with caspase-11 regardless of LRRFIP2 coexpression (Fig. 1 B) and LRRFIP2 coimmunoprecipitated with caspase-11 only when Flightless-I was coexpressed. This result suggests that the interaction between caspase-11 and LRRFIP2 might be mediated through Flightless-I. We probed a Western blot of the protein elute from the original affinity purification experiment with anti-Flightless-I antibody and confirmed the presence of significantly higher amount of Flightless-I in the J774-C11-C254G immunocomplex than in the control (Fig. 1 A, right).

Flightless-I (144 kD) contains an N-terminal LRR domain and a C-terminal GLD (Fig. 1 C); the latter is responsible for actin binding (Liu and Yin, 1998). Caspase-11 contains an N-terminal CARD (caspase recruitment domain), which is followed by the cysteine protease catalytic domain (the p30 domain; Fig. 1 C). To determine the binding domains between Flightless-I and caspase-11, we generated a series of expression constructs for the full-length and truncated mutants of Flightless-I and caspase-11. Caspase-11 coimmunoprecipitated with the full-length Flightless-I as well as with the LRR or GLD of Flightless-I (Fig. 1 D). Flightless-I interacted with the full-length and p30 domain of caspase-11 much more strongly than with the CARD domain of caspase-11 (Fig. 1 E). This mode of interaction between Flightless-I and caspase-11 is different from the interaction between Aip1 and caspase-11, which is mediated through the CARD domain but not the p30 domain of caspase-11 (Li et al., 2007).

Overexpression of Flightless-I changes the subcellular distribution of caspase-11

Because Flightless-I is an actin-binding protein, we fractionated Triton X-100 cell extracts into different pools by differential centrifugation (Fig. 2 A; Yamamoto et al., 2001). Low-speed centrifugation sediments highly cross-linked actin filaments (low-speed pellet [LSP]). Actin filaments that are not cross-linked into thick bundles can be sedimented into a high-speed pellet by centrifugation at a higher speed. Actin monomers and short oligomers remain in the high-speed supernatant (HSS). Flightless-I was preferentially present in the Triton X-100-insoluble fraction, mostly in the LSP when it was expressed in 293T cells, which suggests that Flightless-I might prefer to bind cross-linked actin filaments. The preference of Flightless-I for the LSP was not affected by coexpression of the full-length or the truncated forms of caspase-11 (Fig. 2 B). Unlike Flightless-I, Aip1, also an actin-binding protein, was preferentially present in the Triton X-100-soluble HSS fraction (Fig. 2 C). This is consistent with the relatively low affinity of Aip1 for F-actin (Ono, 2003).

Caspase-11 was predominantly associated with the Triton X-100-soluble HSS fraction. However, a small fraction of both the full-length and the p30 domain but not the CARD domain of caspase-11 could be detected in the Triton X-100-insoluble fraction (Fig. 2 D). Coexpression of Flightless-I increased the amount of the full-length and p30 domain of caspase-11 in the Triton X-100-insoluble LSP fraction, which is consistent with their ability to bind to Flightless-I. In contrast, the CARD domain, which is much weaker in its ability to interact with Flightless-I

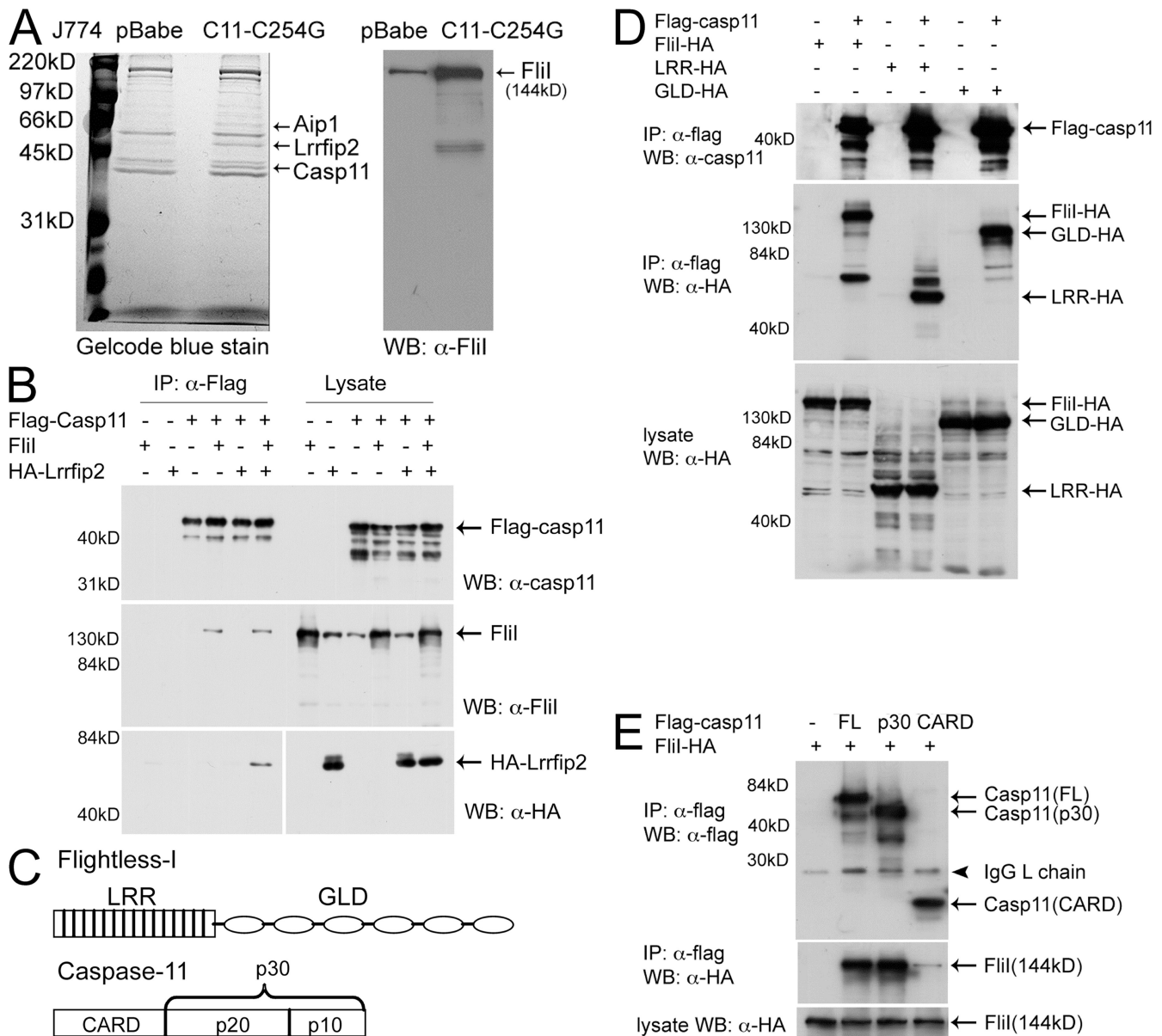


Figure 1. Identification of Flightless-I as a caspase-11-binding protein. (A) Affinity purification of caspase-11-interacting proteins. The cell lysates of J774 cells stably expressing an N-terminal Flag-tagged caspase-11 active site mutant (C11-C254G) or vector alone (pBabe) were affinity purified through an anti-Flag M2 agarose column and the proteins bound to the column were separated by 12% SDS-PAGE and stained by Gelcode blue dye. A 50-kD protein specific for J774 C11-C254G cells (arrow) was identified by mass spectrometry to be LRRFIP2 (left). A Western blot analysis of affinity-purified proteins using an anti-Flightless-I antibody confirmed the presence of Flightless-I in the immunopurified protein complex from J774 C11-C254G cells (right). Flii, Flightless-I. (B) LRRFIP2 interacts with caspase-11 through Flightless-I. The cell lysate of 293T cells expressing Flag-tagged caspase-11, HA-tagged LRRFIP2, and nontagged Flightless-I was immunoprecipitated with anti-FLAG M2 beads. Western blots of the immunocomplexes were probed with anti-HA, anti-Flightless-I, or anti-caspase-11 antibodies. (C) A diagram of Flightless-I and caspase-11. (D) Both the LRR domain and the GLD domain of Flightless-I interact with caspase-11. The cell lysate of 293T cells expressing Flag-tagged caspase-11 and HA-tagged full-length or truncated mutants of Flightless-I was immunoprecipitated with anti-Flag M2 beads. Western blots of the immunocomplexes and the lysate input were probed with anti-HA or anti-caspase-11 antibodies. (E) The p30 domain of caspase-11 interacts with Flightless-I. The lysate of 293T cells expressing Flag-tagged full-length or truncated mutants of caspase-11 and HA-tagged Flightless-I was immunoprecipitated with anti-Flag M2 beads. Western blots of the immunocomplexes and the lysate input were probed with anti-HA or anti-Flag antibodies.

as compared with that of the full-length or p30 domain of caspase-11 (Fig. 1 E), remained in the HSS fraction regardless of Flightless-I expression (Fig. 2 D). Finally, the distribution of caspase-11 did not change significantly when Aip1 was co-expressed (Fig. 2 D). Collectively, these results suggest that the increased partition of the full-length or p30 domain of caspase-11 into the Triton X-100-insoluble LSP fraction is dependent on Flightless-I binding.

To further characterize the subcellular localization of caspase-11 and Flightless-I by immunofluorescence, we expressed EGFP-tagged caspase-11 (caspase-11-EGFP) in HeLa and COS cells. Caspase-11 was predominantly localized in the cytosol (Fig. 2 E). Flightless-I has been shown to colocalize with actin arcs, membrane ruffles, and the leading edge of migrating fibroblasts after serum stimulation (Davy et al., 2001). When we expressed an HA-tagged Flightless-I expression vector in

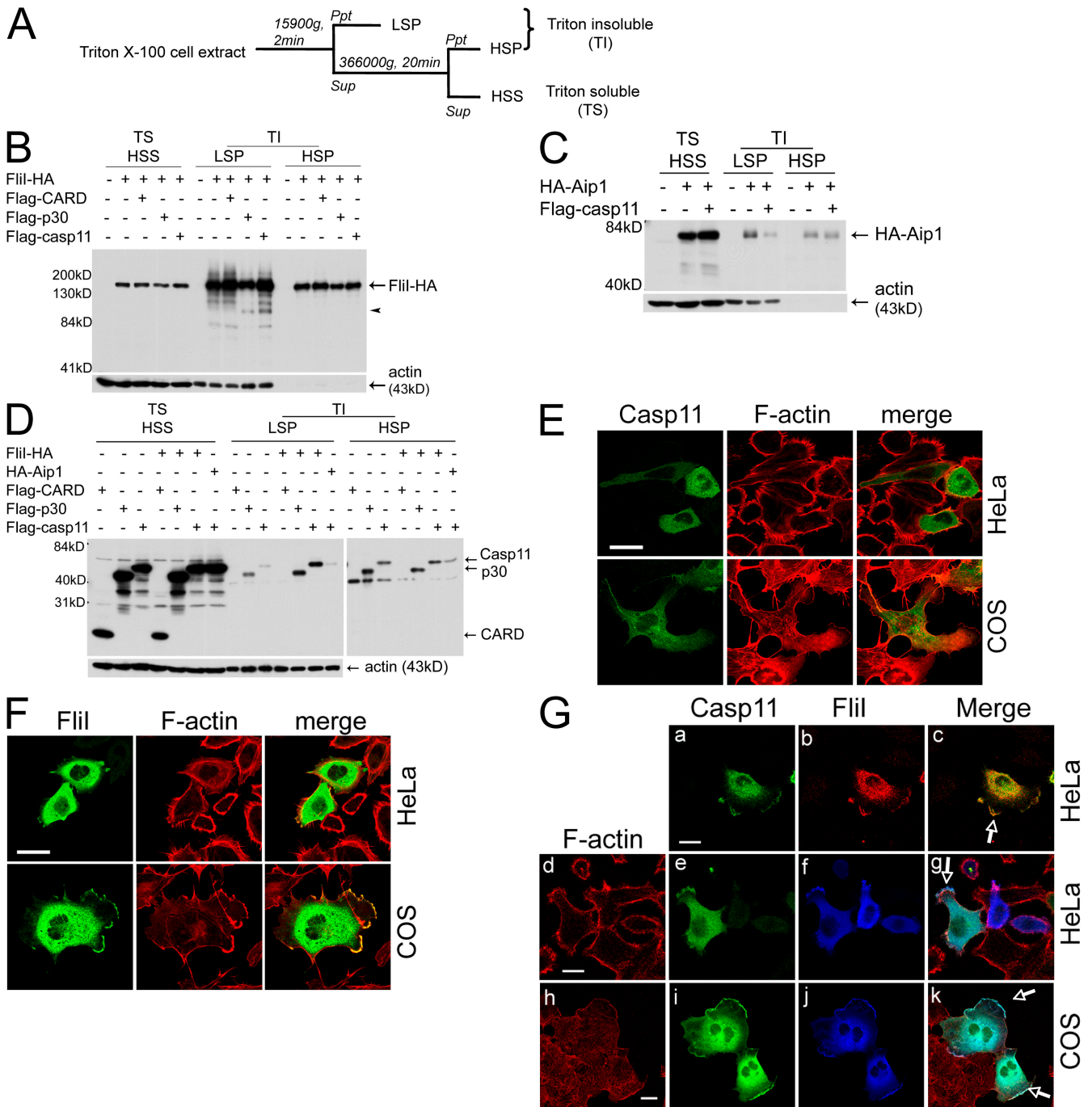


Figure 2. Overexpression of Flightless-I changes the subcellular distribution of caspase-11. (A) Fractionation of Triton X-100 cell extract by differential centrifugation. HSP, high-speed pellet; Ppt, pellet; Sup, supernatant. (B and C) Distribution of Flightless-I and Aip1 among Triton X-100-soluble (TS) and insoluble (TI) fractions. The cell lysates of 293T cells expressing HA-tagged Flightless-I (B) or Aip1 (C) and Flag-tagged caspase-11 were fractionated as in A. Equal fractions of each pool were electrophoresed on SDS-PAGE and subjected to Western blotting with anti-HA or anti-actin antibodies. Flii, Flightless-I. Arrowhead indicates the cleavage product of Flightless-I. (D) Overexpression of Flightless-I but not Aip1 increased the distribution of caspase-11 in the Triton X-100-insoluble LSP fraction. The cell lysate of 293T cells expressing indicated proteins was fractionated as in A. Western blots of the proteins in each pool were probed with anti-Flag or anti-actin antibodies. (E) Subcellular localization of caspase-11 in HeLa and COS cells. C-terminal EGFP-tagged caspase-11 was overexpressed in HeLa and COS cells (green). F-actin was stained with rhodamine-conjugated phalloidin (red). (F) Subcellular localization of Flightless-I in HeLa and COS cells. HA-tagged Flightless-I was overexpressed in HeLa and COS cells and stained with a rat anti-HA primary antibody and an anti-rat-IgG-Alexa 488 secondary antibody (green). F-actin (red) was stained as in E. (G) Colocalization of Flightless-I and caspase-11 at the cell leading edge. EGFP-tagged caspase-11 (a, e, and i) and HA-tagged Flightless-I (b, f, and j) were coexpressed in HeLa and COS cells. Flightless-I was stained with mouse anti-HA primary antibody and anti-mouse-IgG-Alexa 594 (b, red) or Alexa 647 (f and j, blue) secondary antibody. F-actin was stained with rhodamine-conjugated phalloidin (d and h, red). Arrows indicate the leading edge. Bars: (E and F) 30 μ m; (G) 15 μ m.

HeLa and COS cells, we could detect Flightless-I in the cytosol as well as at the cell leading edge by immunofluorescence using an anti-HA antibody (Fig. 2 F). In cells coexpressing caspase-11–EGFP and HA-tagged Flightless-I, there was an increased localization of caspase-11 at the cell leading edge, where caspase-11 colocalized with Flightless-I (Fig. 2 G, a–c, arrow) as well as the F-actin-rich region (Fig. 2 G, d–k, arrows). These results suggest that by interacting with caspase-11, Flightless-I might be able to bring caspase-11 to the F-actin-rich region at the cell leading edge.

Flightless-I inhibits caspase-1 activation in vivo and in vitro

Caspase-11 belongs to the caspase-1 subfamily of proinflammatory caspases. The amino acid sequences of caspase-11 and caspase-1 at the catalytic domain (p30 domain) are 50% identical, whereas the sequences in the CARDS are more divergent (21% identical; Wang et al., 1996). Because Flightless-I interacts with caspase-11 through the highly conserved p30 domain, we went on to examine whether Flightless-I could interact with caspase-1 as well. As shown in Fig. 3 A, Flightless-I but not LRRFIP2 coimmunoprecipitated with Flag-tagged caspase-1 in 293T cells. Similar to that of caspase-11 (Fig. 1 D), caspase-1 could interact with both the LRR domain and the GLD domain of Flightless-I (Fig. 3 A). Endogenous caspase-1 and Flightless-I also interact in J774 cells (Fig. S1 A, available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>). The interaction of Flightless-I with proinflammatory caspases is specific because Flightless-I did not bind to caspase-9 (Fig. S1 B). Expression of caspase-11 did not interfere with the binding between Flightless-I and caspase-1 (Fig. S1 C).

To examine whether the expression of Flightless-I could affect caspase-1 activation, we coexpressed caspase-1 and Flightless-I in 293T cells. Overexpression of caspase-1 induced autocleavage, as evidenced by the appearance of p20 (Fig. 3 B, arrowheads). Interestingly, coexpression of Flightless-I significantly inhibited caspase-1 self-cleavage in a dose-dependent manner (Fig. 3, B and C; and Fig. S2 A, available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>). The expression of the LRR and GLD domains of Flightless-I also had an inhibitory effect, though to a less extent compared with full-length Flightless-I (Fig. 3, B and C). In contrast, the expression of X-linked inhibitor of apoptosis protein or cellular inhibitor of apoptosis 1, inhibitors of caspase-3 and caspase-9 (Deveraux and Reed, 1999), did not inhibit caspase-1 activation (Fig. 3 B).

Caspase-1 cleaves pro-IL-1 β to generate mature IL-1 β (Thornberry et al. 1992). To examine whether Flightless-I could inhibit IL-1 β maturation mediated by caspase-1, we coexpressed pro-IL-1 β , caspase-1, and Flightless-I in 293T cells. Mature IL-1 β secreted into the culture media was detected by ELISA (Fig. 3 D, right). Overexpression of caspase-1 induced self-activation as well as mature IL-1 β production (Fig. 3 D, lane 2), as has been reported (Wang et al., 1996). Caspase-11 is an activator of caspase-1 (Wang et al., 1996). Coexpression of caspase-11 enhanced caspase-1 activation and IL-1 β pro-

duction, and caspase-11 itself was cleaved into p30 (Fig. 3 D, lane 3). Coexpression of Flightless-I significantly inhibited caspase-1 activation and reduced IL-1 β production 84% (Fig. 3 D, lane 4). The addition of pan-caspase inhibitor zVAD-fmk together with Flightless-I coexpression further inhibited caspase-1 activation and IL-1 β production (Fig. 3 D, lane 6). These results suggest that Flightless-I might function as a caspase-1 inhibitor.

To determine whether Flightless-I could inhibit caspase-1–induced apoptosis, we transfected HeLa cells with either a Flag-tagged caspase-1 expression vector alone or caspase-1 together with HA-tagged Flightless-I constructs and immunostained transfected cells with anti-Flag and anti-HA antibodies. As shown in Fig. 3 E, 24 h after transfection, expression of caspase-1 induced apoptosis with typical blebbing morphology in $6.2 \pm 3.4\%$ of transfected cells that were still attached to the coverslips (Fig. 3 E, a, arrows). When Flightless-I and caspase-1 expression constructs were cotransfected, apoptotic cells were found to express only caspase-1 (Fig. 3 E, b, arrow). None of the cells that expressed both caspase-1 and Flightless-I were apoptotic in all the fields examined (Fig. 3 E, b and c). These results indicate that Flightless-I could inhibit caspase-1–induced apoptosis.

To determine whether Flightless-I could directly inhibit caspase-1 activity in vitro, we preincubated recombinant caspase-1 with either GST or GST-tagged Flightless-I and examined the caspase-1 activity in cleaving in vitro translated [35 S]Met-labeled substrate pro-IL-1 β (Fig. 4 A). GST and GST-tagged Flightless-I had no effect on pro-IL-1 β processing at 0.014 μ M. At a concentration above 1.4 μ M, compared with GST, GST-tagged Flightless-I showed an inhibitory effect on caspase-1–mediated mature IL-1 β production. To confirm this result, we also tested the activity of caspase-1 in cleaving colorimetric peptide substrate Ac-YVAD-pNA (Fig. 4 B). In this assay, GST-tagged Flightless-I showed a significant inhibitory effect on caspase-1 activity at 0.3 μ M, whereas GST had no effect at this concentration. CrmA is a more potent inhibitor of caspase-1 than Flightless-I (Fig. S2 B; Komiyama et al., 1994). In contrast, GST-tagged Flightless-I did not significantly inhibit caspase-11 activity compared with GST alone at the similar concentrations at which Flightless-I could inhibit caspase-1 activity (Fig. 4 C). Flightless-I did not inhibit caspase-11–induced cell death in HeLa cells (Table S1, available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>).

Knockdown of endogenous Flightless-I enhances caspase-1 activity

To examine whether endogenous Flightless-I functions as a caspase-1 inhibitor, we transiently knocked down endogenous Flightless-I in J774 cells using synthetic siRNA oligos. Three independent siRNA oligos targeting different coding regions of Flightless-I significantly reduced the endogenous protein levels of Flightless-I (Fig. 5 A). The levels of caspase-1 were not affected in these three Flightless-I transient knockdown cells (Figs. 5 B and S3 A, available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>). In response to LPS plus ATP stimulation, mature IL-1 β secretion was significantly increased

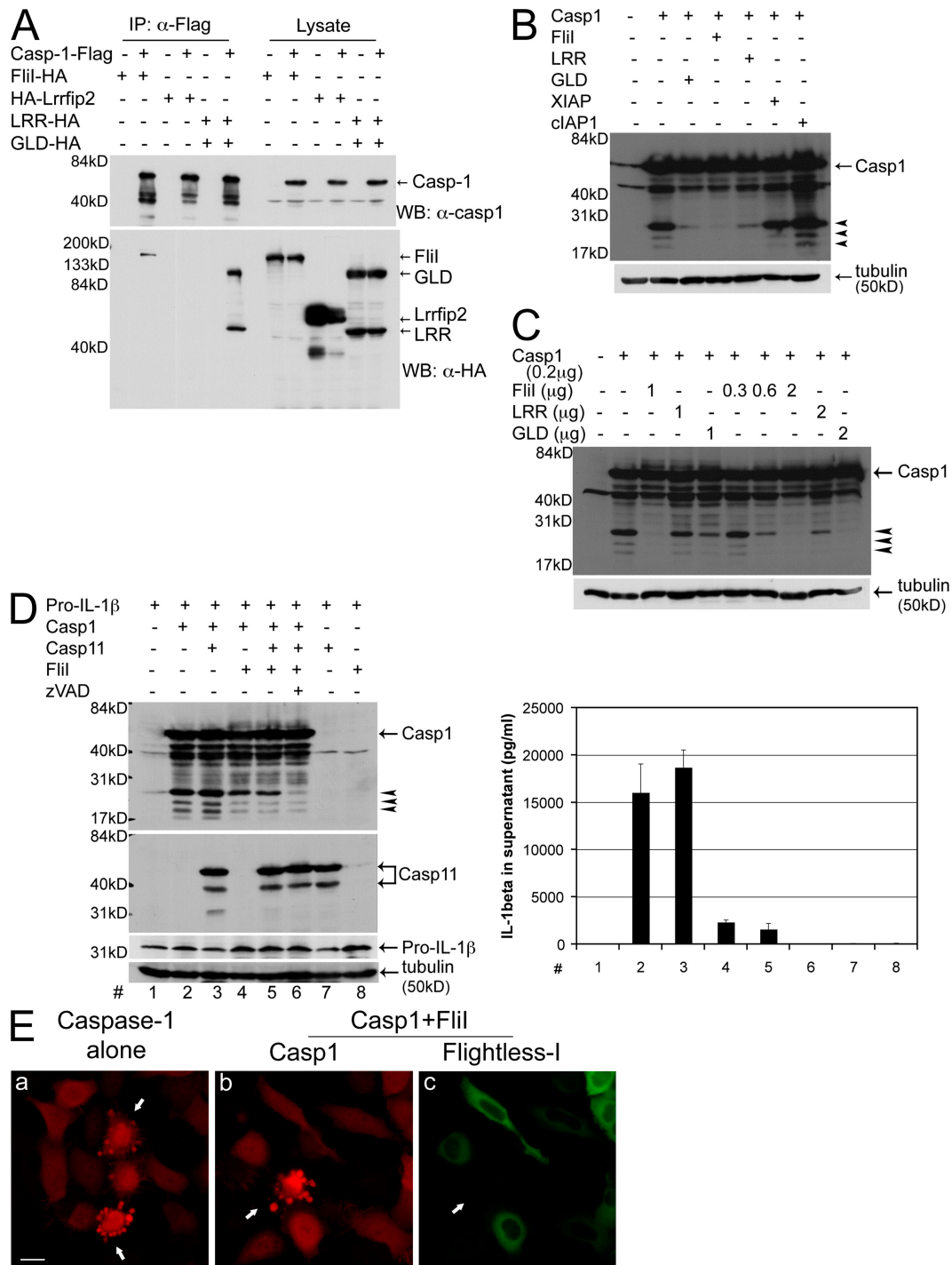


Figure 3. Flightless-I inhibits caspase-1 activation in vivo. (A) Caspase-1 interacts with Flightless-I. The cell lysates of 293T cells expressing indicated proteins were immunoprecipitated with anti-Flag M2 beads. Western blots of the immunocomplexes and the lysate input were probed with anti-caspase-1 or anti-HA antibodies. Flil, Flightless-I. (B) Overexpression of Flightless-I but not cellular inhibitor of apoptosis 1 or X-linked inhibitor of apoptosis protein inhibit the self-cleavage of caspase-1. Expression plasmids encoding caspase-1 (0.2 μ g) and other indicated proteins (1 μ g) were cotransfected into 293T cells. The Western blot of cell lysates was probed using an anti-caspase-1 antibody. Arrowheads indicate the cleavage products of caspase-1. (C) Flightless-I inhibits the self-cleavage of caspase-1 in a dose-dependent manner. Expression constructs of full-length or truncated mutants of Flightless-I at indicated amounts were cotransfected with the expression vector of caspase-1 (0.2 μ g) into 293T cells. The Western blot of cell lysates was probed against an anti-caspase-1 antibody. (D) Flightless-I inhibits caspase-1-dependent IL-1 β maturation in vivo. Expression vectors encoding caspase-1 (0.2 μ g) and other indicated proteins (1 μ g) were cotransfected into 293T cells. The Western blots of cell lysates were probed against anti-pro-IL-1 β , anti-caspase-1, anti-caspase-11, or anti-tubulin antibodies. The amount of mature IL-1 β secreted into the culture supernatants was measured using ELISA. Arrowheads indicate the cleavage products of caspase-1. Error bars represent SD of triplicates. (E) Flightless-I inhibits caspase-1-induced apoptosis. An expression vector encoding Flag-tagged caspase-1 was transfected into HeLa cells with or without the expression construct of HA-tagged Flightless-I. 24 h after transfections, cells were fixed. The distribution of caspase-1 was determined by immunostaining with mouse anti-Flag primary antibody and anti-mouse-IgG-Alexa 594 secondary antibody (red). The distribution of Flightless-I was determined by immunostaining with rat anti-HA primary antibody and anti-rat-IgG-Alexa 488 secondary antibody (green). Bar, 30 μ m.

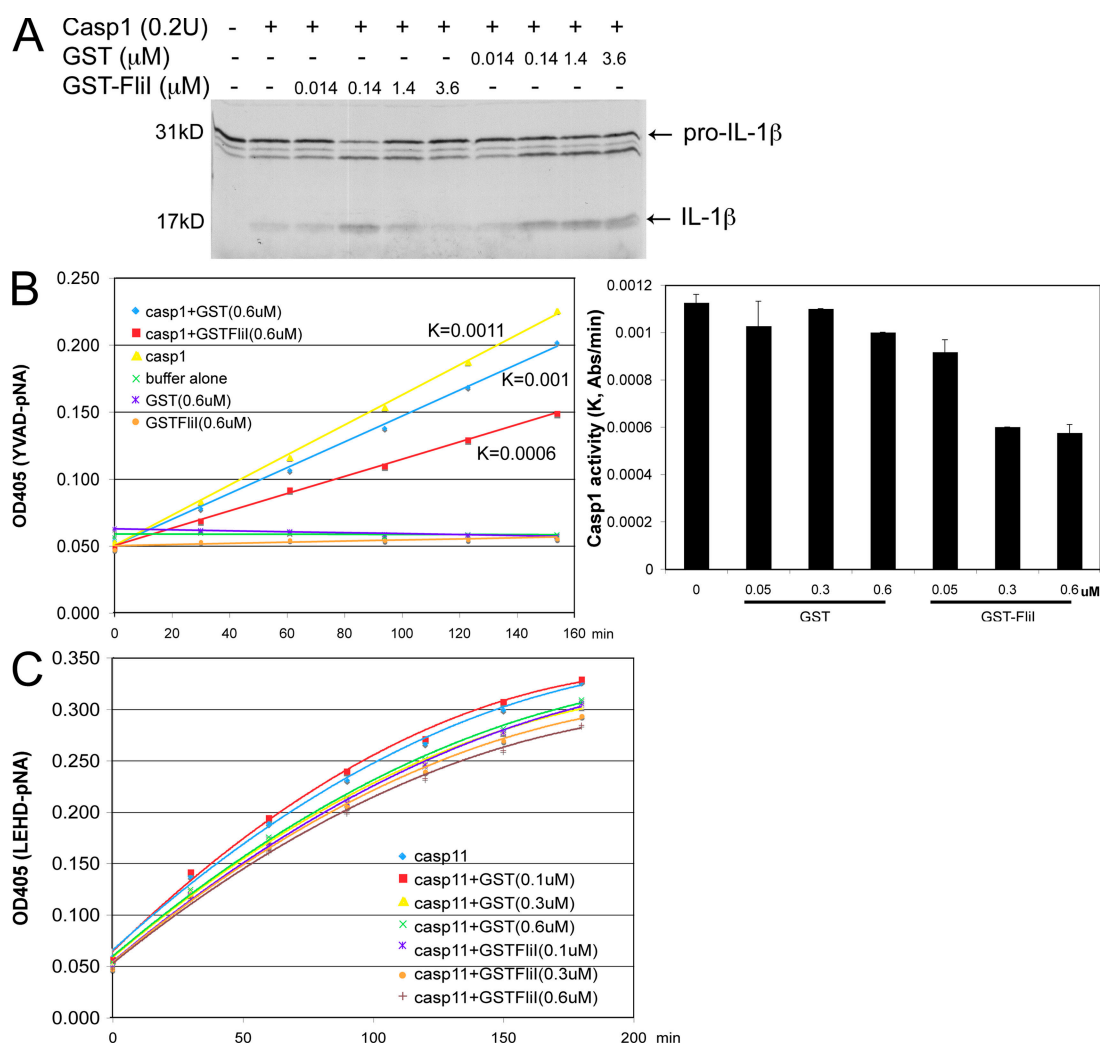


Figure 4. **Flightless-I inhibits caspase-1 activation in vitro.** (A) Flightless-I inhibits caspase-1-dependent pro-IL-1 β processing in vitro. Recombinant caspases-1 (0.2 U) was preincubated with GST or GST-tagged Flightless-I (GST-Flii) at 30°C for 1 h and then reacted with [³⁵S]Met-labeled in vitro translated pro-IL-1 β for another 1.5 h at 30°C in 20- μ l volume. The cleavage of pro-IL-1 β into IL-1 β was visualized by autoradiography. (B) Flightless-I inhibits caspase-1 activity in vitro. Recombinant caspase-1 (1 U) was preincubated with GST or GST-tagged Flightless-I (GST-Flii) at 37°C for 30 min and caspase-1 activity was measured using the colorimetric substrate Ac-YVAD-pNA at OD 405 nm in 100- μ l volume. Error bars represent SD of duplicates. Abs, absorbance at OD 405 nm; K (Abs/min), the rate of hydrolysis. (C) Flightless-I does not inhibit caspase-11 activity in vitro. The recombinant p30 domain of 10 μ g caspase-11 was preincubated with GST or GST-tagged Flightless-I (GST-Flii) at 37°C for 30 min and caspase-11 activity was measured using colorimetric substrate, Ac-LEHD-pNA at OD 405 nm in 100- μ l volume.

in Flightless-I knockdown cells compared with control cells (Fig. 5 C). This result suggests that the LPS-induced endogenous caspase-1 activation that is required for cleaving pro-IL-1 β into mature IL-1 β may be enhanced when the endogenous level of Flightless-I is reduced.

Flightless-I has been reported to negatively modulate the LPS-induced toll-like receptor (TLR) pathway by interfering with the TLR4 and MyD88 interaction to reduce nuclear factor- κ B (NF- κ B) activation (Wang et al., 2006). Because both pro-IL-1 β and caspase-11 are transcriptional targets of NF- κ B in response to LPS stimulation (Cogswell et al., 1994; Schauvliege et al., 2002), we considered the possibility that increased IL-1 β production in the Flightless-I knockdown cells might result from the increased pro-IL-1 β synthesis or increased caspase-1 activity caused by the higher level of upstream activator caspase-11 rather than directly relieving the inhibitory effect of

Flightless-I on caspase-1 when the endogenous Flightless-I level was reduced. Therefore, we further examined the protein levels of pro-IL-1 β and caspase-11 in Flightless-I transient knockdown cells. Consistent with the study of Wang et al. (2006), the protein level of pro-IL-1 β was higher in Flightless-I knockdown cells than in control cells after LPS stimulation (Figs. 5 D and S3 B). However, the caspase-11 protein level did not change significantly in Flightless-I knockdown cells compared with control cells (Fig. 5 D).

To directly address the issue of whether caspase-1 activity was affected when the endogenous Flightless-I level was reduced, we further characterized the Flightless-I knockdown cells. We generated short hairpin RNA (shRNA) of Flightless-I in a pSRP retroviral vector using the same targeting sequence as siRNA No. 1 and stably knocked down endogenous Flightless-I in J774 cells (Fig. 5 E). Similar to transient knockdown cells

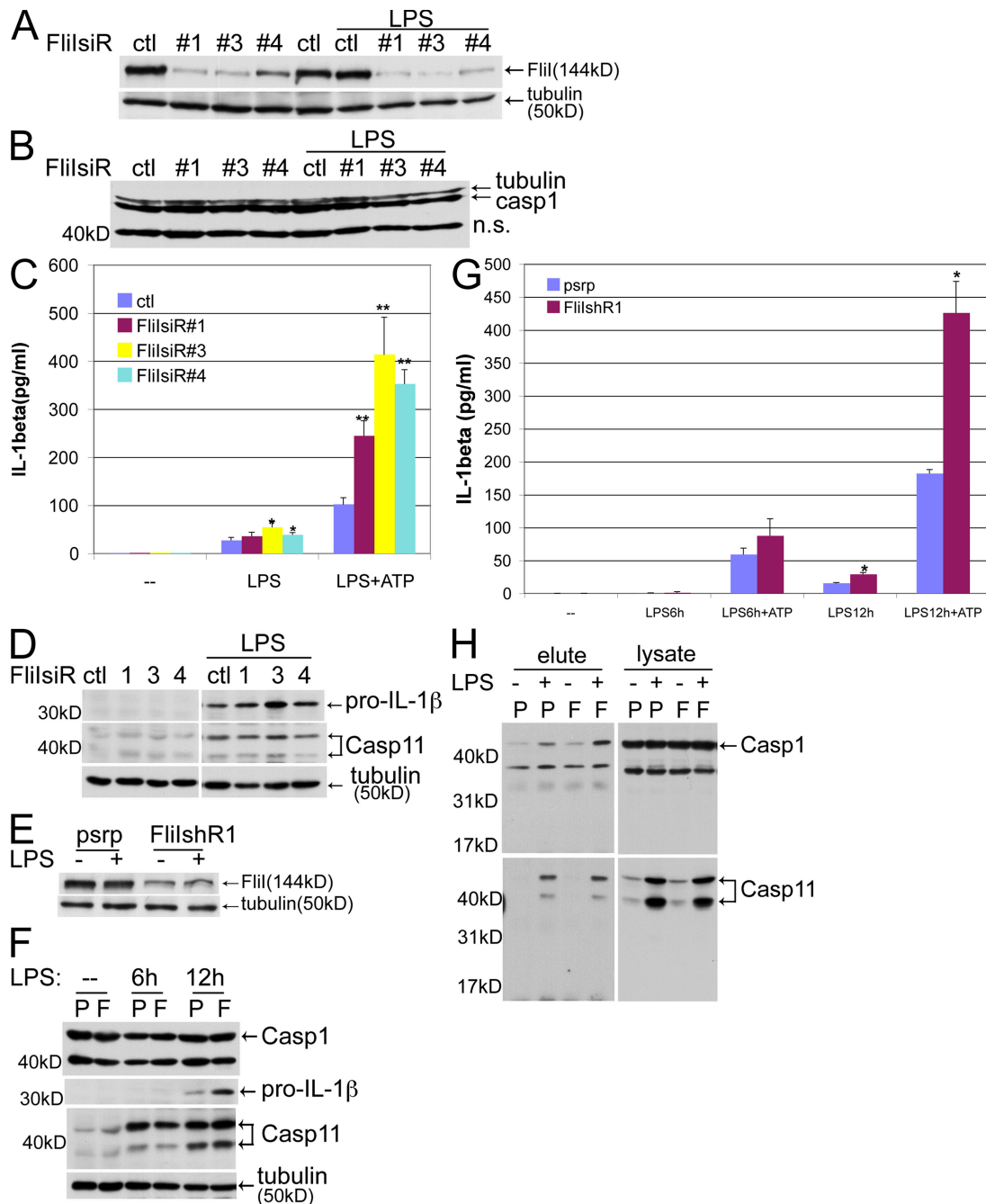


Figure 5. Knockdown of endogenous Flightless-I enhances caspase-1 activity. (A) Transient knockdown of endogenous Flightless-I in J774 cells using Thermo Fisher Scientific synthetic siRNA oligos. Nos. 1, 3, and 4 are three different siRNA oligos targeting Flightless-I. ctl, nontargeting control siRNA oligo. (B) The expression levels of caspase-1 in Flightless-I transient knockdown cells with or without 12 h of LPS (1 µg/ml) treatment. n.s., a nonspecific band recognizable by an anti-caspase-1 antibody. (C) IL-1β secretion was increased in Flightless-I transient knockdown cells in response to LPS plus ATP stimulation. 6×10^4 control and Flightless-I transient knockdown cells were incubated with 1 µg/ml LPS for 12 h then stimulated with 5 µM ATP for 30 min. The amount of secreted IL-1β in the culture supernatant was measured by ELISA. (D) The expression levels of pro-IL-1β and caspase-11 in Flightless-I transient knockdown cells with or without 12 h of LPS (1 µg/ml) treatment. (E) Stable knockdown of endogenous Flightless-I in J774 cells by shRNA1 in a retroviral pSRP vector. shRNA1 targets the same sequence on Flightless-I as siRNA1 in A. (F) The expression levels of caspase-1, pro-IL-1β, and caspase-11 in Flightless-I stable knockdown cells (FliIshR1) and control cells (pSRP) with or without 1 µg/ml LPS treatment for 6 or 12 h. F, FliIshR1; P, pSRP. (G) IL-1β secretion was increased in Flightless-I stable knockdown cells in response to LPS plus ATP stimulation. The experimental procedures were the same as in C. (H) Active caspase-1 and active caspase-11 levels in Flightless-I stable knockdown cells (FliIshR1) and control cells (pSRP). Cell lysates from untreated or 12-h LPS (1 µg/ml) treated cells were incubated with biotinylated YVAD-cmk at 37°C for 45 min and precipitated with avidin beads. Western blots of the protein elute and the lysate input were probed with anti-caspase-1 or anti-caspase-11 antibodies. F, FliIshR1; P, pSRP. Error bars represent SD of duplicates; *, $P < 0.05$; **, $P < 0.01$.

(Fig. 5 D), Flightless-I stable knockdown cells (FliIshR1) showed enhanced protein levels of pro-IL-1β but not caspase-11 compared with control cells (pSRP) after LPS stimulation (Fig. 5 F).

Mature IL-1β production in Flightless-I stable knockdown cells (FliIshR1) was increased to a similar extent as in the transient knockdown cells (FliIshR1; Fig. 5, G and C). We measured the

endogenous caspase-1-like proteolytic activity against the colorimetric peptide substrate Ac-YVAD-pNA in Flightless-I stable knockdown cells and control cells (Fig. S4 A, available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>). Under unstimulated conditions, we detected higher spontaneous caspase-1-like protease activity in Flightless-I stable knockdown cells than in the control cells. However, after 12 h of LPS stimulation, caspase-1-like protease activity was enhanced in both Flightless-I stable knockdown cells and control cells to a similar level (Fig. S4 A).

To specifically determine the effect of Flightless-I knockdown on caspase-1 activation, because YVAD-pNA is not an absolutely specific substrate for caspase-1, we labeled the active caspase-1-like proteases in the total cell lysate from unstimulated or LPS-treated cells with biotinylated YVAD-cmk. The labeled proteins were precipitated with avidin beads and the protein elute was analyzed by Western blotting with anti-caspase-1 and anti-caspase-11 antibodies (Fig. 5 H). LPS stimulation alone did not induce the cleavage of either caspase-11 or caspase-1. LPS treatment enhanced the labeling of both full-length active caspase-1 and caspase-11 by biotinylated YVAD-cmk. Interestingly, LPS treatment induced a higher level of active caspase-1 labeled by biotinylated YVAD peptide in Flightless-I stable knockdown cells than in the control cells. In contrast, the level of active caspase-11 was not altered by the changes in the level of Flightless-I expression (Fig. 5 H). Thus, endogenous Flightless-I specifically regulates the activation of caspase-1 but not caspase-11, which is consistent with the *in vitro* data that recombinant Flightless-I inhibits caspase-1 but not caspase-11 activity (Fig. 4, B and C). After LPS plus ATP stimulation, we did not observe the cleavages of either caspase-1 or pro-IL-1 β in the cell lysate (Fig. S3, C and D), however, there was increased cleavage of both caspase-1 and pro-IL-1 β in the culture media of Flightless-I knockdown cells compared with control cells (Fig. S3, E and F), which is consistent with the ELISA assay result in Fig. 5 G.

Flightless-I inhibits caspase-1 as a pseudosubstrate

Because overexpression of Flightless-I could increase the distribution of caspase-11 into the Triton X-100-insoluble LSP fraction (Fig. 2 D), we examined whether overexpression of Flightless-I had the same effect on caspase-1. Caspase-1 was present predominantly in the Triton X-100-soluble HSS fraction (Fig. 6 A). Expression of full-length Flightless-I, the LRR domain, or the GLD domain increased the distribution of caspase-1 into the Triton X-100-insoluble LSP fraction. Coexpression of Flightless-I with caspase-1 in COS cells also enriched the localization of caspase-1 at the cell leading edge (Fig. S4 B). When the full-length caspase-11 or the p30 domain of caspase-11 was overexpressed, we observed a C-terminal cleavage fragment of the C-terminal HA-tagged Flightless-I around 100 kD in the LSP fraction, recognized by an anti-HA antibody (Fig. 2 B, arrowhead). In the LSP fraction from the cells transfected with caspase-1 expression vector (Fig. 6 B), two additional Flightless-I C-terminal fragments were observed, one around 90 kD (Fig. 6 B, single asterisk) and the other around 60 kD (double asterisks), as well as the 100-kD fragment (arrowhead).

To determine whether caspase-1 could cleave Flightless-I directly *in vitro*, we used *in vitro* translated [³⁵S]Met-labeled Flightless-I as a substrate and examined the cleavage by autoradiograph. There were four prominent *in vitro* cleavage products of Flightless-I generated in the presence of caspase-1 (Fig. 6 C, lines). The cleavage of Flightless-I was inhibited in the presence of caspase-1 inhibitor YVAD-cmk (Fig. S4 C). The cleavage of Flightless-I by caspase-1, however, is less efficient than that of pro-IL-1 β (Fig. 6 C).

We searched for potential canonical caspase cleavage sites on Flightless-I using the ExPASy Proteomics Server (<http://ca.expasy.org/>) and found only one potential cleavage site (Asp526) for caspase-1 and no site for any other caspases. This potential caspase-1 recognition site (⁵²³YEADC⁵²⁷) is localized at the GLD domain of Flightless-I and evolutionarily conserved in the Flightless orthologues of *D. melanogaster*, human, mouse, and rat (Fig. 6 D). Interestingly, the sequence, YEADC (P4-P1'), is highly reminiscent of the pseudosubstrate recognition site (LVADC) of CrmA, a potent caspase-1 inhibitor identified from the cowpox virus (Ray et al., 1992). CrmA forms an inhibitory complex with caspase-1 in its intact form (Komiya et al., 1994). To determine whether caspase-1 cleaves after Asp526 of Flightless-I, we mutated Asp526 to Glu526 and cotransfected expression constructs of C-terminal HA-tagged Flightless-I (wild type [wt]) or Flightless-I (D526E) together with caspase-1 into 293T cells. The cleavage of Flightless-I was detected with an anti-HA antibody. The cleavage at Asp526 is expected to generate a C-terminal fragment of Flightless-I around 90 kD. Surprisingly, D526E mutation of Flightless-I appeared to have enhanced the caspase-1-dependent cleavage at the predicted Asp526 site as the level of the 90-kD fragment was increased (Fig. 6 E, single asterisk) without affecting the production of the 100-kD fragment (Fig. 6 E, arrowhead) or the 60-kD fragment (Fig. 6 E, double asterisk) in 293T cells. Because the requirement of an Asp at the P1 position is recognized as a key factor for caspase cleavage (Thornberry et al., 1997), this result suggests that Flightless-I is an unusual substrate for caspase-1.

To further characterize the requirement of Asp526 for caspase-1-dependent cleavage, we generated a D526A Flightless-I mutant. Interestingly, in the *in vitro* cleavage assay, the Flightless-I (D526A) mutant had a significantly reduced level of the 90-kD cleavage band (Fig. 6 F, single asterisk) but an enhanced level of 100-kD cleavage band (Fig. 6 F, arrowhead). Consistent with the *in vitro* result, the expression of D526A Flightless-I mutant with caspase-1 in 293T cells completely abolished the appearance of the 90-kD fragment, whereas that of the D526E Flightless-I mutant led to an increased level of the 90-kD fragment compared with that of the wt (Fig. 6 G, single asterisk).

To determine the requirement of Asp526 for Flightless-I to inhibit caspase-1 activation, we coexpressed wt, D526A, and D526E Flightless-I with caspase-1 in 293T cells. Both Flightless-I D526A and D526E mutants were less efficient in inhibiting caspase-1 activation than wt Flightless-I (Fig. 6 H). The Flightless-I D779E mutant, which did not affect caspase-1-dependent Flightless-I cleavage (Fig. 6, F and G), had a similar inhibitory effect on caspase-1 activation as wt Flightless-I (Fig. 6 H). These results

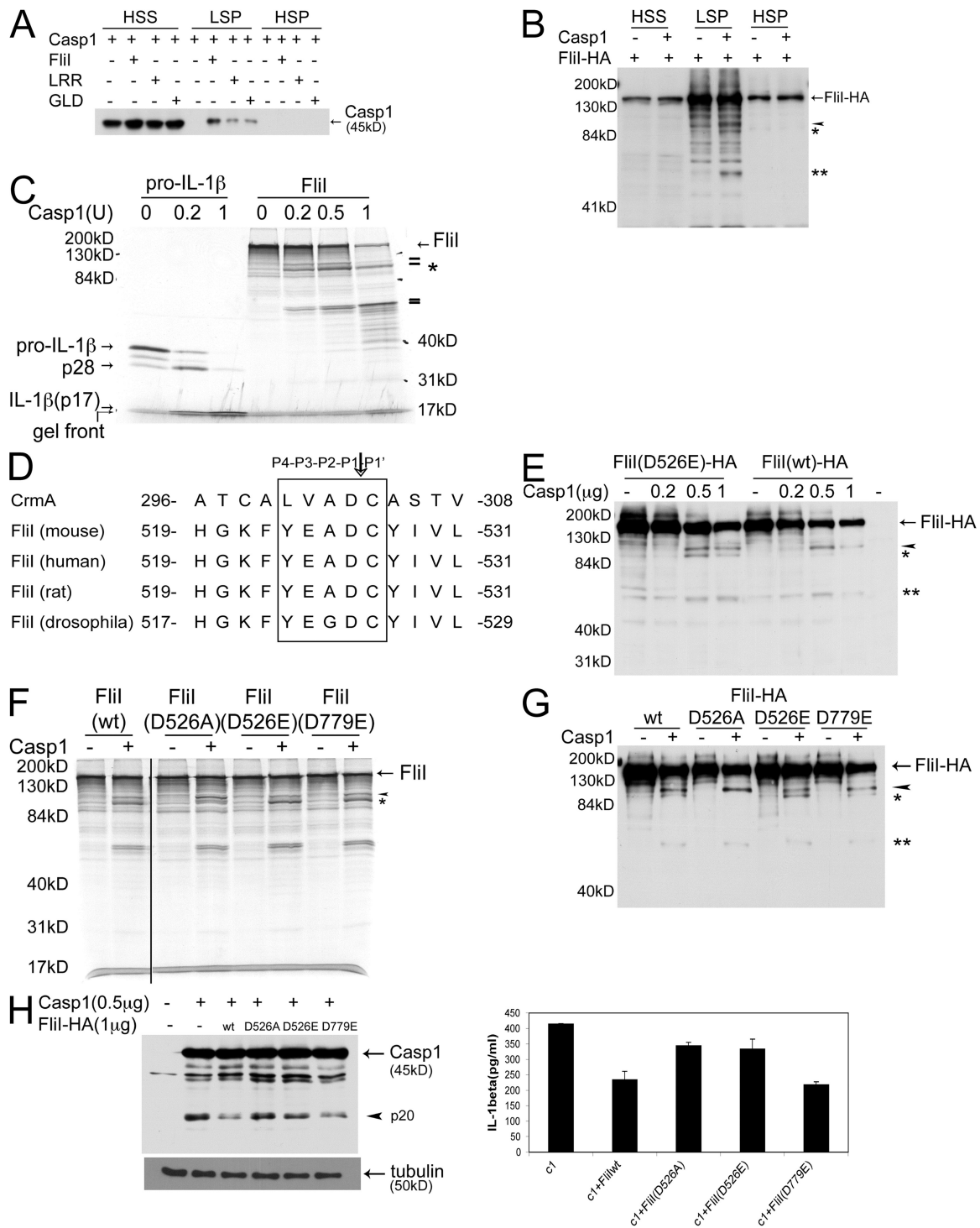


Figure 6. Flightless-I inhibits caspase-1 as a pseudosubstrate. (A) Overexpression of full-length Flightless-I or the LRR domain and the GLD domain of Flightless-I increased the distribution of caspase-1 in the Triton X-100-insoluble LSP fraction. The experimental procedure was the same as in Fig. 2 D. A Western blot of the proteins in each fraction was probed with anti-caspase-1 antibody. (B) Overexpression of caspase-1-induced Flightless-I cleavage in the Triton X-100-insoluble LSP fraction. The cell lysates of 293T cells expressing C-terminal HA-tagged Flightless-I were fractionated as in Fig. 2 B. A Western blot of the proteins in each fraction was probed with an anti-HA antibody. (C) Caspase-1 cleaves Flightless-I in vitro. Recombinant caspase-1 was incubated with [³⁵S]Met labeled in vitro translated pro-IL-1 β or Flightless-I at 30°C for 1.5 h in 20- μ l volume. Cleavage of pro-IL-1 β or Flightless-I was visualized by autoradiography. The cleavage products of Flightless-I are indicated with solid lines. (D) Sequence alignment of Flightless-I and CrmA at caspase-1 recognition sites. Potential cleavage is between the P1 and P1' positions. (E) Mutation of Asp526 to Glu526 on Flightless-I-enhanced caspase-1-dependent cleavage. Expression vectors of C-terminal HA-tagged Flightless-I (wt) and Flightless-I (D526E) mutant at 1 μ g were cotransfected with the indicated amount of caspase-1 plasmid into 293T cells. Western blots of the proteins in the Triton X-100-insoluble LSP fraction were probed with an anti-HA antibody. (F) Asp526 on Flightless-I is a caspase-1 cleavage site in vitro. Recombinant caspase-1 (0.5 U) was incubated with [³⁵S]Met labeled in vitro translated wt or indicated point mutants of Flightless-I at 30°C for 1.5 h in 20- μ l volume. The cleavage of Flightless-I was visualized by autoradiography. The black line indicates that intervening lanes have been spliced out. (G) Asp526 on Flightless-I is a caspase-1-dependent cleavage site in vivo. C-terminal HA-tagged wt and indicated point mutant of Flightless-I (1 μ g cDNA plasmid) was coexpressed in 293T cells with caspase-1 (0.5 μ g). 24 h after transfection, cells were directly lysed in

suggest that the caspase-1 recognition site (⁵²³YEADC⁵²⁷) on Flightless-I, but not the cleavage per se, is a critical factor in determining the ability of Flightless-I to inhibit caspase-1 because converting it to noncleavable site (D526A) reduced its inhibitory effect. However, converting it to a better substrate (D526E) also reduced the inhibitory effect of Flightless-I on caspase-1. These unusual properties of Flightless-I as a substrate and inhibitor of caspase-1 are reminiscent of what has been described for CrmA (Komiya et al., 1994), which suggests that Flightless-I might function as an inhibitor of caspase-1 in living cells through a mechanism similar to that of CrmA.

Discussion

We described here the interaction of a gelsolin family actin-remodeling protein, Flightless-I, with proinflammatory caspases caspase-11 and caspase-1. A connection between caspase-11 and actin cytoskeleton remodeling has been demonstrated before in our previous study about caspase-11 in promoting Aip1-dependent actin depolymerization and cell migration (Li et al., 2007). Our study predicted that by interacting with the cytoskeletal network in the lamellopodia, caspase-11 might be present at the leading edge to regulate cell migration. In this study, we show that Flightless-I interacts with the catalytic domain (p30 domain) of caspase-11. The interaction of caspase-11 with Flightless-I increases the partition of caspase-11 into the Triton X-100-insoluble actin bundle fraction, where Flightless-I preferentially exists, and also increases the distribution of caspase-11 at the F-actin-rich region of the cell leading edge (Fig. 2). Therefore, the interaction of caspase-11 and Flightless-I provides a potential mechanism for targeting caspase-11 to the actin network at the cell front to regulate cell movement.

We show that Flightless-I also interacts with caspase-1. Flightless-I inhibits caspase-1 activation and IL-1 β maturation, whereas its interaction with caspase-11 has no effect on caspase-11 activity (Figs. 4 and 5). Both the LRR domain and the GLD domain of Flightless-I could interact and inhibit caspase-1 activation (Fig. 3), which suggests that Flightless-I might act as an inhibitory “clamp” on caspase-1. The GLD domain of Flightless-I has also been shown to be able to bind the toll-interleukin 1 receptor domains of MyD88 and TLR4, thereby disrupting the MyD88-TLR4 complex formation (Wang et al., 2006). Therefore, the functions of the GLD domain of Flightless-I might have been expanded significantly beyond the “gelsolin-like” actin remodeling.

In response to LPS stimulation, we observed full-length active caspase-1 and caspase-11 as indicated by the labeling of biotinylated caspase substrate (Fig. 5 H) associated with inefficient IL-1 β secretion (Fig. 5 G). After LPS plus ATP treatment, caspase-1 is cleaved and IL-1 β secretion is dramatically increased (Figs. 5 G and S3). We propose that caspase-1

and caspase-11 may be activated without cleavage and cell death, whereas caspase-1 cleavage and secretion of mature IL-1 β follow cell death. This is consistent with the activation of other caspases with long prodomains such as caspase-2, -8, -9, and -10, which can be achieved by dimerization and oligomerization, and the cleavage of initiator caspases stabilizes the active enzymes (Acehan et al., 2002; for review see Boatright and Salvesen, 2003; Chang et al., 2003; Huang et al., 2007).

The protein inhibitors of caspase-1 so far are divided into two categories. The first category binds to the CARD domain of the caspase-1 and interferes with caspase-1 oligomerization, such as Iceberg and COP, which are nonviral but induced by inflammatory stimuli and thus regulated in a signal-dependent fashion (Humke et al., 2000; Druilhe et al., 2001; Lee et al., 2001). The second category contains a caspase-1 substrate recognition sequence and functions as an inhibitory substrate, such as CrmA and p35, which are both virally derived. CrmA (a cowpox virus product) was the first caspase-1 inhibitor identified (Ray et al., 1992). Cowpox viruses use CrmA to bind active caspase-1 to reduce the defensive inflammatory response triggered by IL-1 β . The pseudosubstrate recognition region of CrmA has the residues LVADC (P4-P1-P1'). CrmA remains intact after binding to caspase-1 to form an inhibitory complex (Komiya et al., 1994). PI-9 is a close mammalian homologue of CrmA based on the sequence. The pseudosubstrate region has residues VVAEC (P4-P1-P1'), and the acidic residue (P1) in its pseudosubstrate region is a glutamate residue rather than the aspartate found in CrmA (Annand et al., 1999). p35 is a baculoviral protein that can block the defensive apoptotic response of insect cells to viral infection (Clem et al., 1991). The pseudosubstrate region of p35 has the residues DQMDG (P4-P1-P1'). After it is cleaved after its P1 residue by caspases, the cleaved subunits of p35 remain in an inhibitory complex with the caspases (Zhou et al., 1998). Flightless-I inhibits caspase-1 in a very similar way to CrmA. The pseudosubstrate region has the residue YEADC (P4-P1-P1'). Both converting it to a poorer or better substrate of caspase-1 reduced the ability of Flightless-I to inhibit caspase-1 (Fig. 6). Flightless-I could inhibit caspase-1 activation at the dose when Flightless-I was not cleaved by caspase-1 (Figs. 3 C and 6 E), which suggests that the cleavage of Flightless-I is not required for its inhibitory activity. The residues ⁵²³YEADC⁵²⁷ lie in the GLD domain of Flightless-I. The LRR domain of Flightless-I alone (aa 1–400) also contains the inhibitory activity (Fig. 3 C), although it is weaker than the GLD domain alone (aa 401–1,272). How the LRR domain inhibits caspase-1 activation remains to be clarified. Overall, the interaction between Flightless-I and proinflammatory caspases identified in this study demonstrates a novel mechanism by which caspases are regulated in living cells.

2 \times SDS loading buffer. A Western blot of the total cell lysate was probed with anti-HA antibody. (H) Asp526 on Flightless-I is critical for the inhibitory effect of Flightless-I on caspase-1 activation. 293T cells were transfected and lysed as in G. Western blot of the total cell lysate was probed with an anti-caspase-1 antibody. IL-1 β secretion was measured as in Fig. 3 D except that 0.05 μ g of pro-IL-1 β plasmid and 0.5 μ g of caspase-1 plasmid were used for transfection. Error bars represent SD of duplicates; *, 90-kD Flightless-I fragment; **, 60-kD Flightless-I fragment; arrowhead, 100-kD Flightless-I fragment.

Materials and methods

Reagents

Antibodies used were anti-Flightless-I (Santa Cruz Biotechnology, Inc.), anti-HA (Covance), anti-pro-IL-1 β (R&D Systems), and anti-caspase-1 and anti-caspase-11 (Wang et al., 1998). Anti-Flag M2 agarose beads were obtained from Sigma-Aldrich. LPS was obtained from Sigma-Aldrich. Ac-YVAD-pNA and Ac-LEHD-pNA were obtained from Axxora, LLC. Recombinant caspase-1 was obtained from Millipore.

Flag-tagged affinity purification and mass spectrometry analysis

Experiment details have been described previously (Li et al., 2007). Peptide sequences identified from the 50-kD band in the J774-C11-C254G lane (Fig. 1 A) corresponding to mouse LRRFIP2 were KLAGKDELLSQIRK, NDGMSGDLAQLQNGSDLQFIEMQR, AEQDIATLEQSISR, TAAENAEKIE-DELKAER, TAQDKIEMEMTNSHLAK, DIYDLKDKQIHDVEGR, ESLSEVEEK, AMVSNAQLDNEKNNLIYQVDTLK, and DVIEEQEEQMAEFYRENEEK.

Construction of mouse Flightless-I, LRRFIP2, and caspase-11 expression plasmids

Plasmid containing cDNA encoding full-length mouse Flightless-I was obtained from the American Type Culture Collection (IMAGE clone ID, 5040746). The cDNA fragments encoding the full-length, LRR (aa 1–400), and GLD (aa 398–1,272) of Flightless-I were PCR amplified from the IMAGE clone and inserted into a pCDNA3-HA vector at HindIII-XhoI sites. Flightless-I D526A, D526E, and D779E mutant constructs were generated from an HA-tagged wt Flightless-I plasmid using the site-directed mutagenesis kit (Stratagene). A plasmid containing full-length mouse LRRFIP2 cDNA was obtained from the American Type Culture Collection (IMAGE clone ID, 4217995). The open reading of full-length LRRFIP2 cDNA was PCR amplified from the IMAGE clone and inserted into a pCDNA3-HA vector at EcoRI-NotI sites. Generation of the full-length and truncated mutant constructs of caspase-11 has been described previously (Li et al., 2007). The EGFP-tagged caspase-11 construct was generated in EGFP-N2 vector with the full-length caspase-11 open reading frame inserted at EcoRI-BamHI sites.

Fractionation of actin pools

Actin pools were assayed by differential centrifugation of Triton X-100 cell extracts (Yamamoto et al., 2001). Cells were lysed in Triton buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, and 1% Triton X-100) with protease inhibitor (Roche). Lysates were centrifuged sequentially at 15,900 g for 2 min and then at 366,000 g for 20 min. The pellets from each centrifugation step were resuspended into the original lysate volume. Samples were boiled in SDS gel sample buffer and equal fractions of each pool were analyzed by SDS-PAGE.

Immunostaining

$\sim 2.5 \times 10^4$ HeLa or COS cells were seeded on 18-mm-diameter coverslips in a 12-well plate overnight. 1 μ g of total DNA was transfected using TransilT1 reagent (Mirus). Cells were fixed 24 h after transfection with 4% paraformaldehyde for 20 min. After permeabilization and blocking with 10% FBS/0.1% Triton X-100/PBS for 30 min, cells on coverslips were incubated with anti-HA mouse or rat monoclonal antibody (1:500 dilution) in 1% FBS/0.1% Triton X-100/PBS at 4°C overnight. Cells were incubated with goat anti-mouse-IgG-Alexa 594, goat anti-mouse-IgG-Alexa 647, or goat anti-rat-IgG-Alexa 488 (Invitrogen; $\sim 1:250$ – $1:500$ dilution) with or without rhodamine-phalloidin (Invitrogen; 1:200 dilution) for 45 min. After cell nuclei were stained with Hoechst 33334 for 10 min, coverslips were mounted onto glass slides with 1 mg/ml p-phenylalanine diamine/90% glycerol/PBS. Images were taken using a fluorescence microscope (E800; Nikon) with a 40x NA 0.75 objective (Nikon) and Spot Advanced software (Diagnostic Instruments, Inc.; Fig. 3 E) or a microscope (TE2000; Nikon) with a C1 confocal laser scanning head with a 60x NA 1.4 oil objective and EZ-C1 3.50 software (Nikon; Fig. 2, E–G).

Assay for IL-1 β secretion

$\sim 3.5 \times 10^5$ per well 293T cells were seeded in 6-well plates overnight. cDNA constructs of pro-IL-1 β , caspase-1, caspase-11, and Flightless-I were transfected using the calcium phosphate precipitation method (Wang et al., 1996). In some cases, 100 μ M zVAD-fmk was added to the culture media after the addition of transfection mixture. 24 h after transfection, the cell lysate was collected for Western blotting. IL-1 β in the culture media was measured by an ELISA kit (R&D Systems).

In vitro caspase-1 cleavage assay

Pro-IL-1 β and Flightless-I were in vitro synthesized using TNT Quick coupled transcription/translation systems (Promega) with [35 S]Met at 30°C for 1.5 h.

An in vitro cleavage reaction was performed in the buffer containing 20 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and 10 mM DTT at 30°C for 1.5 h. The cleavage was visualized by autoradiography.

Cleavage of 200 μ M of colorimetric substrate Ac-YVAD-pNA (for caspase-1) and Ac-LEHD-pNA (for caspase-11) were performed in a buffer containing 25 mM Hepes, pH 7.4, 0.1% CHAPS, 10% glycerol, 1 mM EDTA, and 10 mM DTT at 37°C and measured at an OD of 405 nm on a Victor plate reader (PerkinElmer).

Biotinylated substrate labeling of active caspases

The experimental methods were performed according to Marsden et al. (2002) with minor modifications. J774 cells were treated with 1 μ g/ml LPS for 12 h. Lysates were made in 10 mM Hepes-KOH, pH 7.4/2 mM EDTA/0.1% CHAPS/5 mM DTT/1 mM PMSF. To label active caspases, 20 μ M biotin-YVAD-cmk (EMD) was added to lysates, which were incubated for 45 min at 37°C. Lysates were then incubated with Neutravidin beads (Thermo Fisher Scientific) for 2 h at 4°C. After extensive washing, biotinylated polypeptides were eluted by directly boiling the beads in 2x SDS sample buffer.

Protein purification

The Flightless-I open reading frame was PCR amplified and subcloned into a pGEX-6P-1 vector at BamHI-XhoI sites. Recombinant GST-Flightless-I was purified as described previously (Frangioni and Neel, 1993). His-tagged caspase-11 was purified as described previously (Kang et al., 2000).

Flightless-I RNAi in J774 cells

Synthesized siRNA oligos targeting Flightless-I (siGENOME set of four duplexes) and nontargeting control oligos were obtained from Thermo Fisher Scientific. siRNA oligos were electroporated into J774 cells using a nucleofector machine (Amaxa) according to the manufacturer's instructions. The Flightless-I level was measured by Western blotting 48 h after transfection. The sequences were: siRNA No. 1, 5'-GAAGAUACACACUAUGUUUUU-3' (sense) and 5'-PUAACAUAGUGUGUAUCUUCUU-3' (antisense); siRNA No. 3, 5'-CAACAUUGACUUCUCGCUAUU-3' (sense) and 5'-PUAGCGAGAAGUCAUUGUUUUU-3' (antisense); siRNA No. 4, 5'-AAACAAGAAUGAGCGGAAUUU-3' (sense) and 5'-PUUUCGCUCAUUCUUGUUUUU-3' (antisense); and nontargeting control siRNA, 5'-UAGCGACUAAACACAUCAA-3' (sense).

Oligonucleotides corresponding to the sense 5'-GAAGATACACACTATGTA-3' and antisense 5'-TAACATAGTGTATCTTC-3' (FliishR1) for mouse Flightless-I were annealed and inserted into an pSRP-puro vector at BglII-XhoI sites. pSRP-FliishR1 or pSRP vector plasmids were transfected into 293T cells for retrovirus production. J774 cells were infected with the virus and selected with 2 μ g/ml puromycin for 1 wk to generate stable knockdown cells.

Online supplemental material

Fig. S1 shows the interaction between endogenous caspase-1 and Flightless-I and the binding specificity of Flightless-I with caspase-1/caspase-11. Fig. S2 shows the inhibitory activity of CrmA on caspase-1. Fig. S3 shows the full gel scans of caspase-1 and pro-IL-1 β expression and cleavage patterns in cell lysates and culture media of Flightless-I knockdown cells. Fig. S4 shows caspase-1-like protease activity in Flightless-I knockdown cells, caspase-1 localization in COS cells, and the in vitro cleavage of Flightless-I (wt) and Flightless-I (D526E) mutant. Table S1 shows caspase-11-induced cell death in HeLa cells with or without Flightless-I coexpression. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200711082/DC1>.

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