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Drosophila jumu modulates apoptosis via a JNK-dependent pathway and is required for other processes in wing development

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Abstract
Previous studies in several model organisms have revealed that members of the Forkhead (Fkh) transcription factor family have multiple functions. *Drosophila* Jumeau (Jumu), a member of this family, participates in cardiogenesis, hematopoiesis and immune system homeostasis. Here, we show that loss of *jumu* function positively regulates or triggers apoptosis via a JNK-dependent pathway in wing development. *jumu* mutants showed reduced wing size and increased apoptosis. Moreover, we observed a loss of the anterior cross vein (ACV) phenotype that was similar to that observed in wings in which JNK signaling has been ectopically activated. The JNK signaling markers *puckered* (*puc*) and p-JNK were also significantly increased in the wing discs of *jumu* mutants. In addition, apoptosis induced by the loss of *jumu* was rescued by knocking down JNK, indicating a role for JNK in reducing *jumu*-induced apoptosis. Jumu could also control wing margin development via the positive regulation of *cut* expression, and the observed wing margin defect did not result from a loss of *jumu*-induced apoptosis. Further, *jumu* deficiency in the pupal wing could induce multiple wing hairs via a Rho1-mediated planar cell polarity pathway, but abnormal *Rho1* expression was not why *jumu* loss induced apoptosis via a JNK-dependent pathway in wing discs.

Keywords *Jumu* · Apoptosis · JNK pathway · Wing development

Introduction

Drosophila is a useful model system for studying the underlying molecular mechanisms of morphogenesis and patterning at both the cellular and tissue levels. In particular, much has been learned about the genetic basis of the development of the wing (the largest organ in *Drosophila*).

c-Jun NH_2-terminal kinase (JNK) belongs to the mitogen-activated protein kinases (MAPKs) superfamily and is activated primarily by cytokines and exposure to environmental stress [1, 2]. Previous studies have shown that the ectopic activation of JNK leads to small, rough eyes and abnormal wing phenotypes. The overexpression of *eiger* (*egr*) or *hemipterous* (*hep*) activates the JNK pathway in the wing discs, resulting in cell death, a loss-of-anterior cross vein (ACV) phenotype and small wings [3, 4]. Moreover, reducing the level of *puckered* (*puc*), a negative regulator of JNK activity, results in robust cell death in third-instar larval wing discs and a loss-of-ACV phenotype in adult wings [3, 5, 6]. Apoptosis is a major form of programmed cell death in which cells activate a self-destruct process. During development and under stress, organisms must remove excess or damaged cells to maintain tissue homeostasis [7]. The JNK pathway plays a critical role in regulating cell death via the core apoptotic pathway [2, 8]. Recent work has shown that apoptotic cells can induce neighbouring surviving cells to increase their proliferation to compensate for cell loss, a phenomenon termed apoptosis-induced proliferation (AiP) [9, 10]. In *Drosophila*, AiP is a JNK-dependent process that leads to the production of mitogens, including wingless (Wg), decapentaplegic (Dpp), and spitz (Spi), for tissue repair and regeneration [11, 12]. AiP can also cause tissue overgrowth, a process that has important implications for cancer biology. One study showed that impaired Hippo signaling induces JNK activation through Rho1 and causes tissue overgrowth, which

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indicates an essential role for the JNK pathway in Hippo-signaling-related tumourigenesis [13].

jumeau (*jumu*) encodes a 720-amino-acid (aa) nuclear protein and is a member of the winged-helix/forkhead (WH/FKH) family of transcription factor genes in *Drosophila*. *jumu* is well conserved among insect species and exhibits a broad spectrum of functions. In addition, Jumu shows similarity to the mammalian winged-helix nude (whn) protein near its C-terminus. *whn* is required for the regulation of tissue-specific transcription and cell fate in thymus and hair development [14]. Jumu also plays a role in cell fate decisions during differentiation in neurogenesis as well as in compound eye, wing and bristle development. During neuronal differentiation, Jumu is required for the generation of asymmetric sibling localization and the segregation of Pon/Numb [15]. The phenotypes of *jumu* homozygous mutants include bristle disorders, variegated eyes and defective posterior wing margins. Moreover, they exhibit severely diminished vitality and fertility [16]. A recent study showed that Jumu and its checkpoint suppressor homologue (CHES-1-like) control the division of cardiac progenitors via a Polo-dependent pathway during *Drosophila* cardiogenesis [17]. Moreover, in mutants lacking *jumu*, the expression levels of the gene *frizzled* (*fz*), which encodes a receptor of the Wnt signaling protein wingless (Wg), were significantly reduced in the mesoderm of embryos [18]. Our previous work demonstrated that Jumu is involved in *Drosophila* hematopoiesis, mediating the proliferation and differentiation of blood cells [19]. In addition, *jumu* overexpression induces the deposition of hemocytes and the formation of melanotic nodules by activating the Toll pathway [20]. In particular, *jumu* plays an important role in the control of hematopoietic progenitors in the *Drosophila* lymph gland [21]. In this study, we observed small wings, the loss of the ACV, defective wing margins and multiple wing hairs in double heterozygous *jumu* mutants and *jumu* knockdown flies. Furthermore, we detected cell death in late third-instar larvae after reducing the expression of *jumu*. Simultaneously, we observed increased levels of *puc* and p-JNK, which are factors in the JNK pathway. Moreover, the loss of *jumu* affected Wg-producing cells and led to decreased Cut levels. During the pupal stages, *jumu* positively regulated Rho1 to refine multiple bundles into a single growing hair. Therefore, we have identified previously unknown functions for Jumu in modulating JNK-dependent cell death and other processes in wing development.

Materials and methods

Fly stocks

The following transgenic lines were used in our study: *jumu* RNAi (v12610, *jumu-i*) was obtained from the Vienna *Drosophila* RNAi Stock Center (VDRC). $jumu^{P}$ (GE27806) was purchased from GenExel (Daejeon, South Korea). $jumu^{Df3.4}$ and *UAS-jumu* were gifts from Michelson [17, 22]. *dTAK1* RNAi (National Institute of Genetics, 5115R2), *hep* RNAi (VDRC, v47507), *dTRAF1* RNAi [23], *JNK* RNAi (VDRC, v34138), bsk^{DN} [23] and *UAS-puc* [24] were gifts from Xu. *MS1096-Gal4* [25, 26] was a gift from Liu [27]. *dTRAF1* RNAi *dTRAF2* RNAi and $DRONC^{DN}$ were gifts from Xue [3, 23, 28]. *en-Gal4* was obtained from the Tsinghua Fly Center. Other strains used in this study included $Rho1^{CA}$ [29], *UAS-p35* (BL5072) [30], *UAS-cut* (BL36496), *dpp-lacZ* [31], *puc-lacZ* [24] and w^{1118}. All genotypes were bred into the w^{1118} background.

Immunohistochemistry

Wing imaginal discs obtained from third-instar larvae were fixed in 4% paraformaldehyde for 30 min at room temperature. The wing discs were then placed in blocking buffer (PBS plus 0.1% Tween 20 and 5% normal goat serum) for 1 h at room temperature. For the preparation of pupal wings, late third-instar larvae were selected and allowed to develop for 24–36 h APF at 29 °C. Whole pupae were removed from their pupal cases and fixed in 3.7% formaldehyde for 2 h at room temperature. The pupal wings were dissected in 0.3% PBST (0.3% Triton X-100 in PBS) and incubated in blocking buffer (PBS containing 0.3% Triton X-100, 2% BSA, and 2% normal goat serum) for 1 h. The wings were incubated in primary antibodies overnight at 4 °C and then incubated with secondary antibodies according to standard methods. Finally, the wings were mounted in Vectashield fluorescent mounting medium (Vector Laboratories) or Prolong Diamond Antifade Mountant (Molecular Probes). The tissues were analyzed using an LSM 510 META confocal microscope (Zeiss) or an Axioskop 2 plus microscope (Zeiss). The following primary antibodies were used: mouse anti-β-gal (Promega), mouse anti-Wg, mouse anti-Cut, mouse anti-DE-cad, mouse anti-βPS (Developmental Studies Hybridoma Bank), rabbit anti-dMyc (Santa Cruz), rabbit anti-p-JNK, rat anti-Jumu [21], rabbit phospho-H3 (1:800, Upstate) and 7-AAD (Life Technologies). Secondary antibodies were conjugated with Alexa Fluor 488 and Alexa Fluor 568 (Molecular Probes) and used at 1:200 dilution. All of the experiments were independently repeated at least three times.

TUNEL assay for wing imaginal discs

TUNEL assays were performed using an In Situ Cell Death Detection Kit (Roche Applied Science) according to the manufacturer's instructions. Third-instar larval wing discs were dissected in ice-cold PBS and fixed in 4% paraformaldehyde in PBS for 30 min at room temperature. The samples were then washed 4 times in 0.4% PBST (0.4% TritonX-100 in PBS) and permeabilized by incubation in a PBS

containing 100 mM sodium citrate and 0.1% Triton X-100 for 3 min on ice. After extensive washing with PBS, the samples were submerged in a terminal deoxynucleotidyl-transferase-mediated dUTP nick-end labeling (TUNEL) reaction solution and incubated in a 37 °C chamber for 2 h. After being washed three times with PBS and mounted in mounting medium, the wing discs were observed using a confocal laser microscope (Carl Zeiss, Germany).

Statistical analysis

Images were acquired using a Zeiss fluorescence microscope. All numerical data, including wing size, cell number and intensity values, were analyzed using ImageJ software. The statistical analyses were performed using a two-tailed unpaired Student *t* test with Prism software (GraphPad 6.0). The results were considered statistically significant when $P < 0.05$. ****, ***, ** and * indicate $P < 0.0001$, $P < 0.001$, $P < 0.01$ and $P < 0.05$, respectively. "ns" indicates no significant difference. The error bars in the graphs indicate the SEMs.

Results

Depletion of *jumu* induces abnormal wing phenotypes in adult flies

Because all *jumu* homozygous null mutants died during embryogenesis, we examined the wings of *jumu* heterozygous mutant adults to further analyse the functions of Jumu in wing development. The wings of *jumu* heterozygous mutant adults showed obvious reductions in size (Fig. S1g–j). The wings of adult *jumu* heterozygotes showed a nearly 10% reduction in size, whereas *jumu* double heterozygous mutants showed wing size reductions of more than 14% (Fig. 1a, b, g, i). Accordingly, *jumu* double mutants displayed greatly reduced adult eye size (Fig. 1e, f, j). In addition, $jumu^{Df3.4}/jumu^{P}$ double heterozygous mutant adults exhibited normal wing vein locations (Fig. 1a, b), but unexpectedly, the wings of adults displayed a loss of wing margin structures (Fig. 1a′, b′). Notably, multiple hairs formed on the wings (Fig. 1a″, b″). These results indicate that changes in *jumu* expression substantially disturb the growth and development of the wings.

To determine whether the abnormal wing phenotypes were caused by autonomous loss of the Jumu protein, we used *MS1096-Gal4* to ubiquitously knock down *jumu* in the wing blade. The resulting flies displayed a 23% smaller wing size (Fig. 1h, i) and more cells with multiple hairs than the control flies (Fig. 1c″, d″). Accordingly, we observed a loss of wing margin tissue in *MS1096>jumu* RNAi mutants (Fig. 1c′, d′). Unexpectedly, *MS1096>jumu* RNAi flies exhibited a loss-of-ACV phenotype (Fig. 1d, over 55%). To provide further confirmation that Jumu is involved in wing development, we used transgenic *UAS-jumu* [17, 21, 22] flies in which the entire *jumu* coding region was ectopically expressed. The wing morphological defects detected in *MS1096>jumu* RNAi flies were clearly rescued by *jumu* overexpression (Fig. S1a, b, d, f, g). $jumu^{P}$ [21] contains a *P*-element insertion in the 5′ UTR region of *jumu* that encodes a Gal4-responsive enhancer. Similar to previous results, overexpressing $jumu^{P}$ using the *MS1096-Gal4* driver as the background for *jumu* RNAi rescued the abnormal wing phenotypes (Fig. S1a–c, e, g). These observations suggest that Jumu performs an important role in development, particularly in wing development. Furthermore, the loss-of-ACV phenotype in the *jumu* RNAi wings was similar to that observed in JNK signaling gain-of-function wings [3–6]. We therefore propose that Jumu may be involved in JNK signaling during *Drosophila* wing development.

Regenerative apoptosis-induced proliferation is triggered by loss of Jumu

To further investigate the biological function of Jumu during wing development, we first identified the expression patterns of the Jumu protein in larval and pupal wings by staining with anti-Jumu antibodies. In *wild-type* larvae, Jumu was expressed in the wing blade and showed high expression at the dorsal/ventral (D/V) wing margin (Fig. S2a, c). In addition, Jumu was expressed in intervein cells and localized to the nucleus during the pupal stages (Fig. S2f, f′). However, the signal was nearly abolished in *jumu* double heterozygotes and *MS1096>jumu* RNAi mutants (Fig. S2b, d). In *en>jumu* RNAi flies, Jumu was specifically depleted in the posterior compartment (Fig. S2e, e′). Adult wing size was reduced in the *jumu* mutant flies, which led us to speculate that the deletion of *jumu* resulted in cell death during the larval stage. We detected many 7-AAD-positive cells in the late third-instar larval wing discs of *jumu* mutants (Fig. 2a–d, m). This result suggested that the wing blade cells were dying or dead and had lost their normal functions. We also used TUNEL assays to detect apoptotic cells and found that many cells in the wing blade were apoptotic (Fig. 2e–h, n). To determine whether Jumu is involved in apoptosis in a cell-autonomous manner, in addition to its role in the regulation of tissue repair processes, we used the *en-Gal4* driver to knock down *jumu* in the posterior compartments of the wing discs. Only the posterior part of the wing disc, rather than the whole wing blade region, exhibited dead cells (Fig. S3d, e). Simultaneously, we analysed wing blade cell proliferation using anti-phospho-histone H3 antibodies (PH3), which stain dividing cells in M phase. There were significantly more PH3-positive cells in $jumu^{Df3.4}/jumu^{P}$ double heterozygous and *MS1096>jumu* RNAi mutant wing discs than in

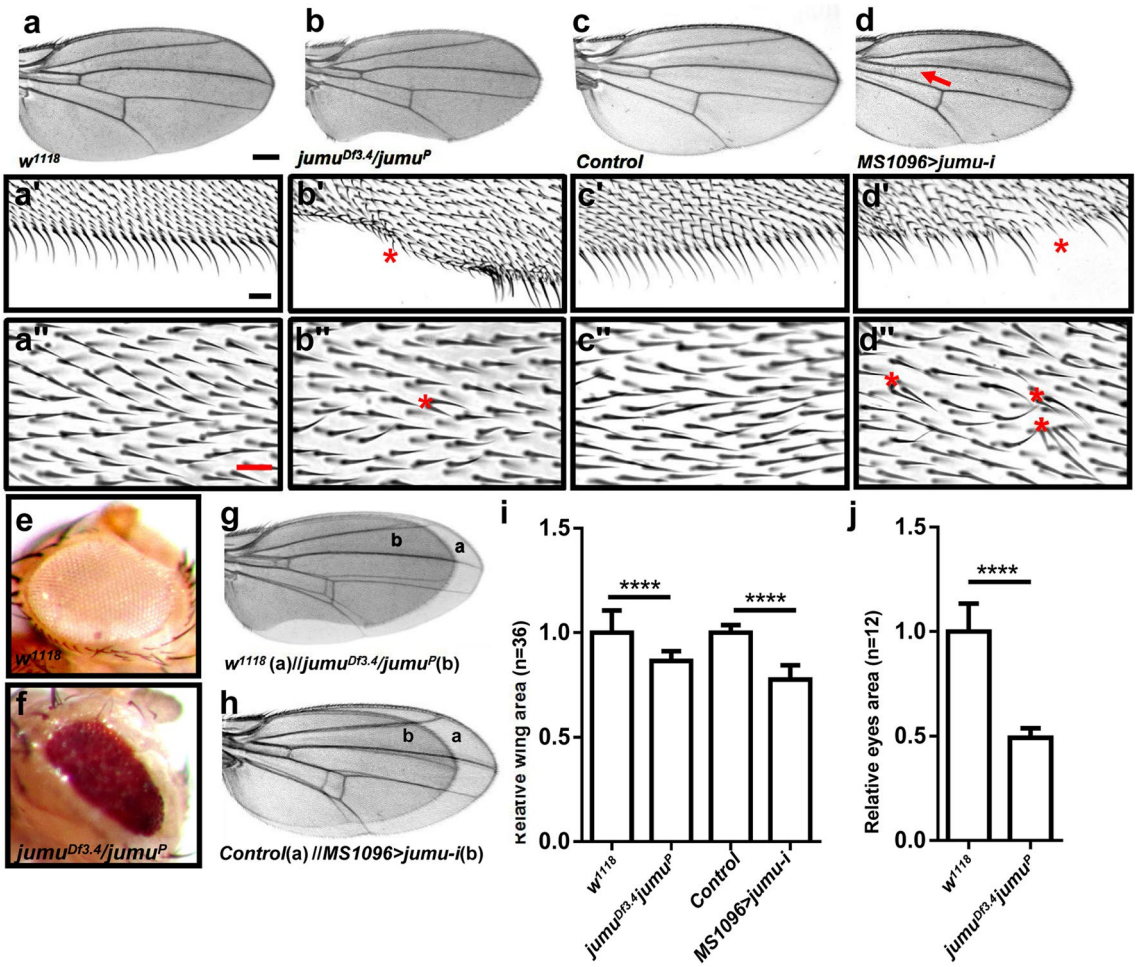

Fig. 1 Adult wing phenotypes of *jumu* mutants. **a, c** Control wings of a w^{1118} or *MS1096*/w^{1118} (*MS1096*>+, *control*) fly. Normal wing; note the prominent veins (structural struts) and intact wing edge or margin. **b, d, a′–d″** Loss of *jumu* resulted in abnormal wing phenotypes. **b** Wing from *jumu* double heterozygous mutant ($jumu^{Df3.4}/jumu^{P}$); note the nick at the wing margin. **d** RNAi-mediated downregulation of *jumu* in the wing blade area via *MS1096-Gal4* produced a loss-of-ACV (over 55%) phenotype in adult wings, indicated by the arrow. **a′–d″** Comparison with the control; reduced *jumu* expression resulted in a loss of the wing margin and in multiple hairs, as indicated with asterisks. **e, f** Micrographs showing adult *Drosophila* eyes. **f** The small, rough eye phenotype of the *jumu* mutant. **g, h** An analysis of the overlap of wings from adult females showed that *jumu* mutants had smaller wings than the controls. **i** Quantification of wing size in females based on the data in **g** and **h. j** Quantification of eye size in females based on the data in **e** and **f**. Scale bars: 200 µm (**a**–**d**), 50 µm (**a′–d′**) and 20 µm (**a″–d″**)

the controls (Fig. 2i–l, o). We further examined the late third-instar larval wing blade using anti-Wg antibodies and found that Wg expression levels were significantly increased (Fig. 2i′–l′, p). The depletion of *jumu* in the posterior half of the wing disc under *en-Gal4* control increased the number of PH^{+} cells and Wg intensity in the posterior compartment (Fig. S3a–c).

JNK signaling has been proposed to stimulate surviving cells neighbouring apoptotic cells, thereby inducing an increase in cell proliferation to compensate for cell loss [9, 10]. Reductions in *jumu* expression in larval wing discs caused ectopic cell proliferation and increased Wg levels. These results suggest that the mechanism through which Jumu controls tissue regeneration is mainly mediated by the regulation of cell proliferation by the Wg mitogen in the imaginal discs. Taken together, these findings suggest that Jumu may be involved in JNK signaling during *Drosophila* wing development.

Loss of *jumu* induces JNK pathway activation

To examine whether JNK signaling plays a role in the loss of *jumu*-induced cell death, we used a *lacZ* insert in the *puc* gene, a transcriptional target of the JNK pathway [24]. We found that knocking down *jumu* in the wing blade of the wing disc resulted in greater expression of *puc-lacZ* (Fig. 3b, l) than of the *MS1096-Gal4* control (Fig. 3a, l), suggesting that the loss of *jumu* promotes JNK pathway activation. The

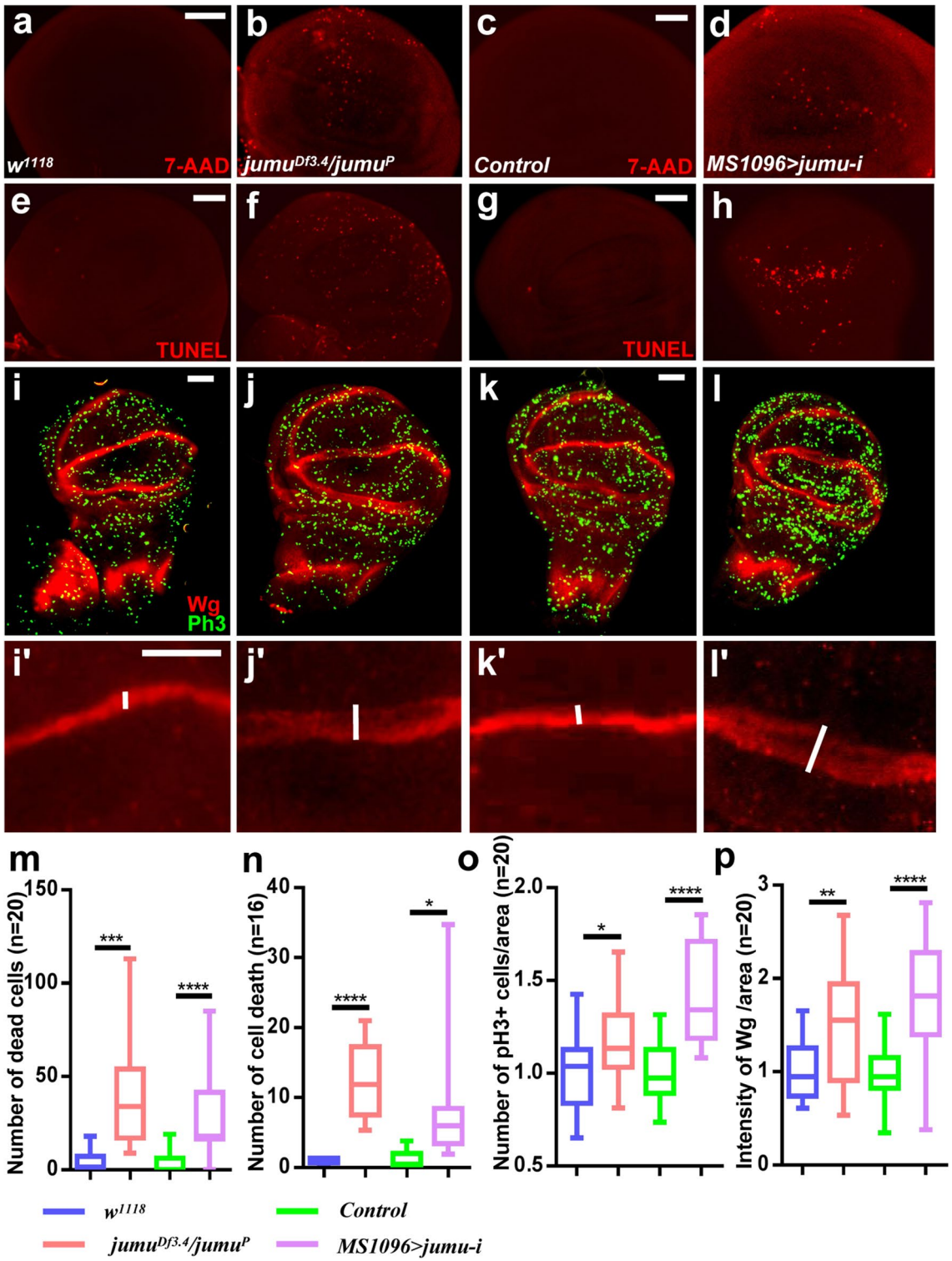

Fig. 2 Loss of *jumu* promotes cell death by affecting cell cycle progression. **a**–**d** Wing discs of the indicated genotypes analysed via 7-AAD staining. Red spots indicate dead cells with disrupted membrane integrity. **e**–**h** Wing discs of the indicated genotypes analysed via TUNEL staining. Red spots indicate apoptotic cells. **i**–**l** Analysis of mitosis and wing blade development in the indicated genotypes. Cell proliferation was monitored using an anti-PH3 antibody (green); wing blade morphology was detected via Wingless staining (Wg, red). The number of $PH3^+$ cells in the area containing the wing blade and the levels of Wg were significantly increased in the loss-of-*jumu* flies. **i′**–**l′** High magnification revealed a widespread Wg signal along the strip of the D/V boundary in *jumu* mutant wing discs compared with those of the controls. **m**–**p** Quantification of dead cells, apoptotic cells, $PH3^+$ cells and Wg labeling intensity in wing discs from females (fold difference from control). Scale bar: 50 µm

activity of *Drosophila* JNK was simultaneously assessed by immunostaining the discs with a phospho-specific antibody that recognizes the active form of JNK. The suppression of the *jumu* gene in the wing blade resulted in a significant increase in p-JNK levels (Fig. 3c, d, m). Moreover, the depletion of *jumu* in the posterior half of the wing disc under *en-Gal4* control increased the activity of the JNK pathway in the posterior compartment (Fig. S3j–m). These results collectively indicated that *jumu* is an inhibitor of JNK.

JNK is indispensable for apoptosis induced by the loss of Jumu

The remarkable contribution of Jumu to apoptotic cell death prompted us to conduct a genetic modifier screen that was designed to search for additional JNK pathway components mediating *MS1096>jumu* RNAi-induced apoptosis. To fully inhibit JNK activity, we utilized *UAS-puc, bsk^{DN}*, *JNK* RNAi, *hep* RNAi, *dTRAF1* RNAi, *dTRAF2* RNAi and *dTAK1* RNAi combined with *MS1096>jumu* RNAi (Fig. 4a–h, the percentage indicates the loss-of-ACV phenotype). As expected, in the reduced-JNK-signaling background, the *MS1096-Gal4*-driven decrease in *jumu* levels rescued the wing size and loss-of-ACV phenotypes. More importantly, in the *dTRAF2* RNAi and *dTAK1* RNAi mutant backgrounds, the *jumu*-knockdown-induced abnormal wing phenotypes were completely suppressed (Fig. 4g, h, m). This finding clearly demonstrated that the JNK pathway is indispensable for apoptosis induced by the loss of *jumu*. The wings of *UAS-puc, bsk^{DN}*, *JNK* RNAi, *hep* RNAi, *dTRAF1* RNAi, *dTRAF2* RNAi and *dTAK1* RNAi flies, driven by *MS1096-Gal4*, exhibited normal adult wing phenotypes (Fig. S4). We detected almost no dead cells in the late third-instar larval wing discs of *MS1096>dTAK1 RNAi* mutants with a *jumu* knocked-down background (Fig. S3h, i). Moreover, inhibiting apoptosis by suppressing *DRONC* or ectopically activating *p35* also rescued the abnormal wing phenotypes of *MS1096>jumu* RNAi flies (Fig. 4i–m). Considering these results, we concluded that *jumu* loss activates JNK and that this activation is essential for loss-of-Jumu-induced apoptosis in *Drosophila*.

Reducing *jumu* in *Drosophila* wings results in a loss of Wnt signaling responses

Compared with the control, the *jumu$^{Df3.4}$/jumu^{P}* double heterozygous and *MS1096 > jumu* RNAi mutant adults exhibited wing margin defects (Fig. 1a–d′). Originally, we speculated that these defects might be explained by apoptosis in the *jumu* mutant wing discs. However, the *MS1096>jumu* RNAi-induced wing margin defects were not rescued by knocking down JNK signaling or inhibiting cell death (Fig. 4). Additionally, there were no dead cells along the pupal wing margin (data not shown). Since such phenotypes can also arise from defective Wg signaling, we analysed the expression of a Wg target in the wing imaginal discs of late third-instar larvae. We observed the downregulation of the short-range Wg target gene *cut* in the *jumu* mutant wing discs (Fig. 3e–h, n). High magnification revealed a loss of the Cut signal along the strip of the D/V boundary (Fig. 3e′–h′). The depletion of *jumu* in the posterior half of the wing disc under *en-Gal4* control inhibited the level of *cut* in the posterior compartment (Fig. S3f, g). To further confirm the role of *cut* in the regulation of wing margin development in the *MS1096>jumu* RNAi mutants, we performed a rescue experiment with a *UAS-cut* transgenic line. As expected, the overexpression of *cut* effectively reduced the wing margin defects in the *MS1096>jumu* RNAi mutants (Fig. 3i–k). In *wild-type* discs, the majority of anti-Wg staining was localized close to the stripe of Wg-producing cells, and the concentration of Wg rapidly declined with the distance from the source of production (Fig. 2i′, k′). Unexpectedly, *jumu* loss resulted in the erosion of the Wg gradient, and high magnification of the anti-Wg staining revealed that Wg was widespread (Fig. 2j′, l′). Thus, the loss of *jumu* changes the way Wg-producing cells release Wg. This change apparently involves increased secretion and the production of a more-mobile form of Wg, affecting gradient formation. In addition, a previous study reported that Jumu can control the Wnt signaling pathway by regulating Fz levels during cardiac progenitor development [18]. Taken together, Jumu may positively regulate *cut* levels to affect *Drosophila* wing margin development through a Wnt-dependent pathway.

Jumu regulates *Drosophila* wing planar polarity through Rho1

Recent research has indicated that the Rho-family GTPases, including RhoA, Rac, and Cdc42, play a central role in JNK signaling during both morphogenesis and apoptosis [32]. In addition, Rho1, the *Drosophila* RhoA homologue, promotes apoptosis by activating the JNK pathway [33]. Rho1 also plays a role in wing planar polarity, and aberrant Rho1 activity leads to multiple wing hairs [34]. The results described above showed that the loss of *jumu* in the adult wing caused the formation of multiple hairs (Fig. 1b″, d″). This phenotype is related to wing planar polarity, as mutations in tissue polarity genes lead to hairs forming at alternative cellular locations and the formation of multiple hairs. Published images of developing wing hairs usually show fixed, phalloidin-stained (phalloidin specifically stains F-actin) wings in an intermediate stage of hair growth, while at later stages, only a single large region (bundle) of F-actin is observed, which appears to fill the hair (Fig. 5a). To further examine whether Jumu can regulate wing hair formation,

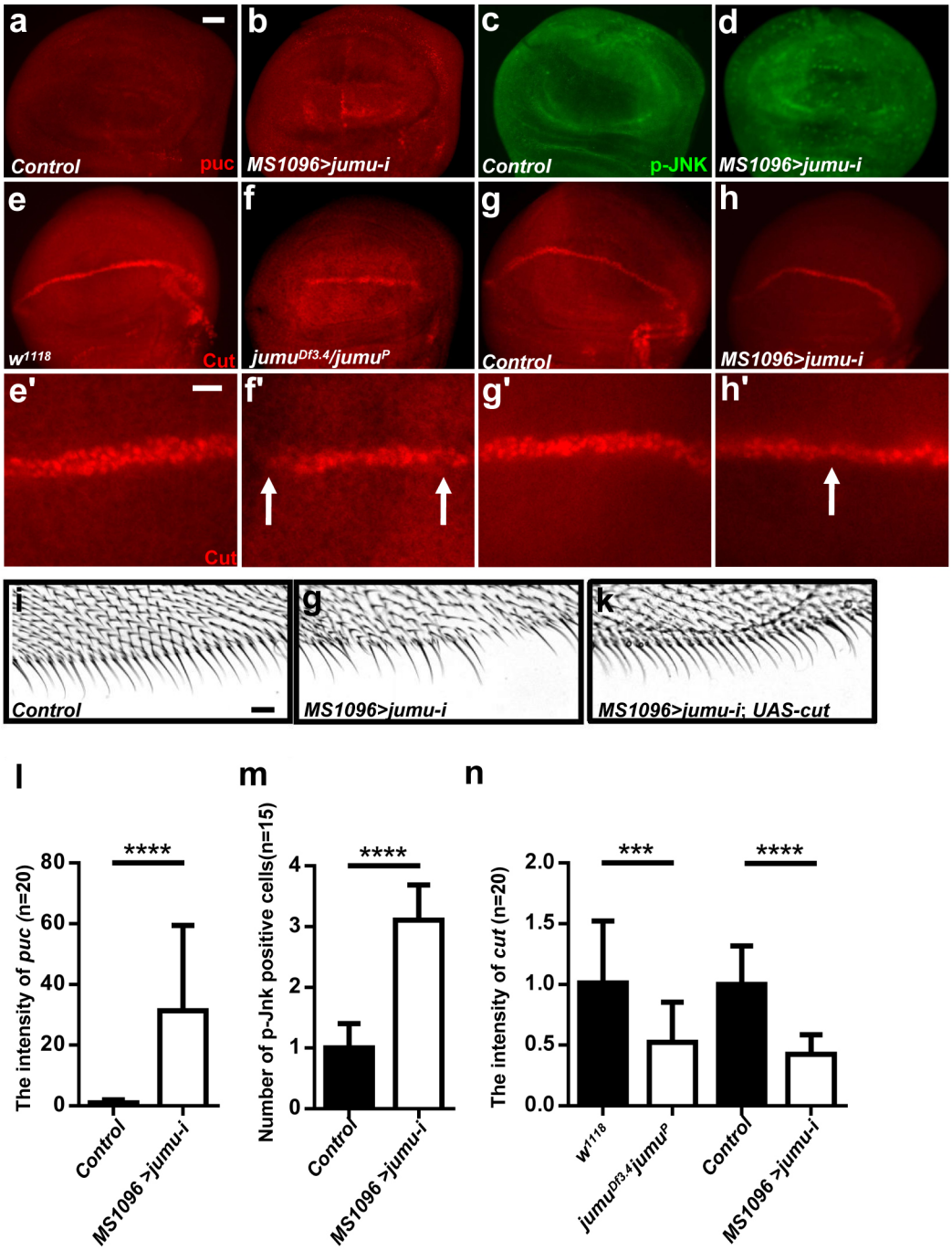

Fig. 3 Loss of *jumu* induces JNK pathway activation and reduces Cut levels during wing development. **a, b** The expression of the JNK target *puckered* (*puc*, labeled red) was determined using the *lacZ* reporter gene. **c, d** The activity of *Drosophila* JNK was assessed by immunostaining the discs with a phospho-specific antibody that recognizes the active form of JNK (p-JNK). **e–h** The levels of Cut (red) were examined via anti-Cut staining. **e′–h′** High magnification revealed a loss of the Cut signal along the D/V boundary in *jumu* mutant wing discs compared with those of the controls, as indicated with arrows. **i, g** Low *jumu* expression resulted in the wing margin defects, **k** which were significantly suppressed by the overexpression of *cut*. **l–m** Quantification of *puc* total intensity, p-JNK-positive cells and *cut* total intensity in the wing blade. Scale bars: 50 μm (**a–h, i–k**) and 10 μm (**e′–h′**)

we stained growing hairs for F-actin 32 h APF and consistently observed that the loss of *jumu* in *MS1096>jumu* RNAi pupal wings led to substantially more actin bundles than in the controls (Fig. 5b). Moreover, *en>jumu* RNAi mutant wings presented a stronger phenotype than that observed in the wings of *MS1096>jumu* RNAi flies (Fig. 5c, d, f,

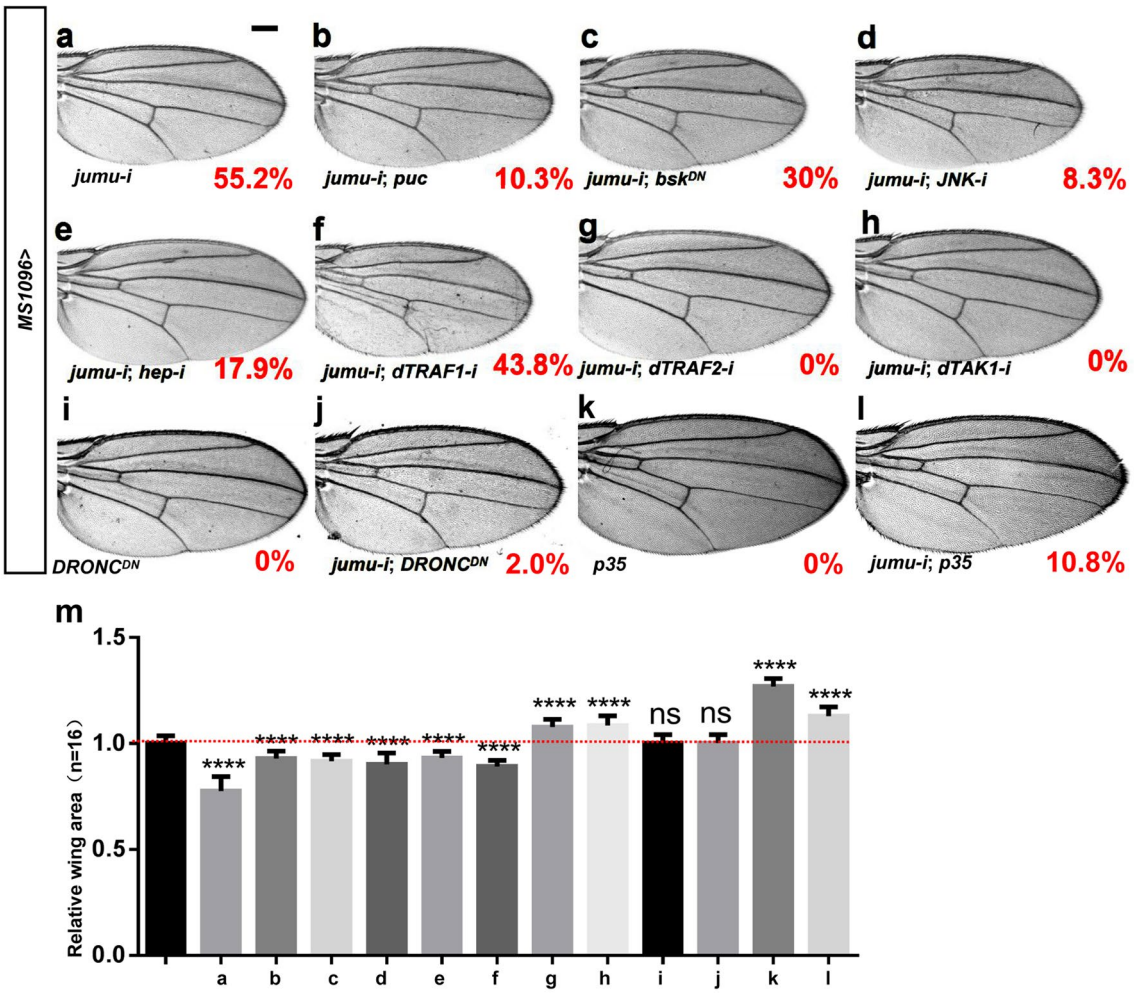

Fig. 4 *jumu* antagonizes the JNK signaling pathway, and its loss mediates caspase-dependent cell death. Light micrographs of adult *Drosophila* wings are shown (the percentage indicates the loss-of-ACV phenotype). **a** Low *jumu* expression resulted in the clear loss of the ACV (by 55.2%) and a reduction in wing size, **b–h, j, l** which were significantly suppressed by the loss of JNK signaling or the suppression of cell death. **g, h** The ACV-defective wing phenotype induced by *MS1096>jumu* RNAi was strongly suppressed by *dTRAF2-i* or *dTAK1-i*. **j, l** The suppression of apoptotic signals via $DRONC^{DN}$ or *p35* overexpression rescued wing size and the defective ACV. **i, k** The overexpression of $DRONC^{DN}$ or *p35* via *MS1096-Gal4* resulted in normal wing phenotypes. Quantification of observed wing sizes from female adults for all combinations is shown in **m** (fold difference compared with *MS1096>+*, red dotted line). Scale bar: 200 µm

h). One interesting finding was that the DE-cad-stained cell membrane showed a dramatic downregulation of DE-cadherin (Fig. 5a′, b′). Cadherins are central to the formation of adherens junctions (AJs) and are prominent markers of AJs [35]. In some cells, no cadherin staining remained, while others showed prominent gaps in DE-cadherin staining (Fig. 5b′, arrows). We also immunostained the wings of *en>jumu* RNAi flies that exhibited a specific, posterior compartment knockdown phenotype. The most strongly affected cells also showed gaps in DE-cadherin staining (Fig. 5c′, g, i) that appeared similar to what we observed following the knockdown of *jumu* expression driven by *MS1096-Gal4*. This result suggested that Jumu might also be involved in the maintenance of epithelial cell structure and AJs.

Based on the results described above, we identified a role for Jumu in wing planar polarity and found that Jumu had multiple functions. As expected, these phenotypes were similar to those observed in association with abnormal Rho1 activity in pupal wings [34]. Hence, we hypothesized that *jumu* affects wing planar polarity via Rho1. When we stained *jumu* knockdown pupal wings with anti-Rho1 antibodies, we found that *jumu* knockdown resulted in a dramatic downregulation of *Rho1* (Fig. 5a″–c″). These experiments show that Jumu functions upstream of Rho1, a *Drosophila* actin cytoskeleton regulator, to affect wing planar polarity.

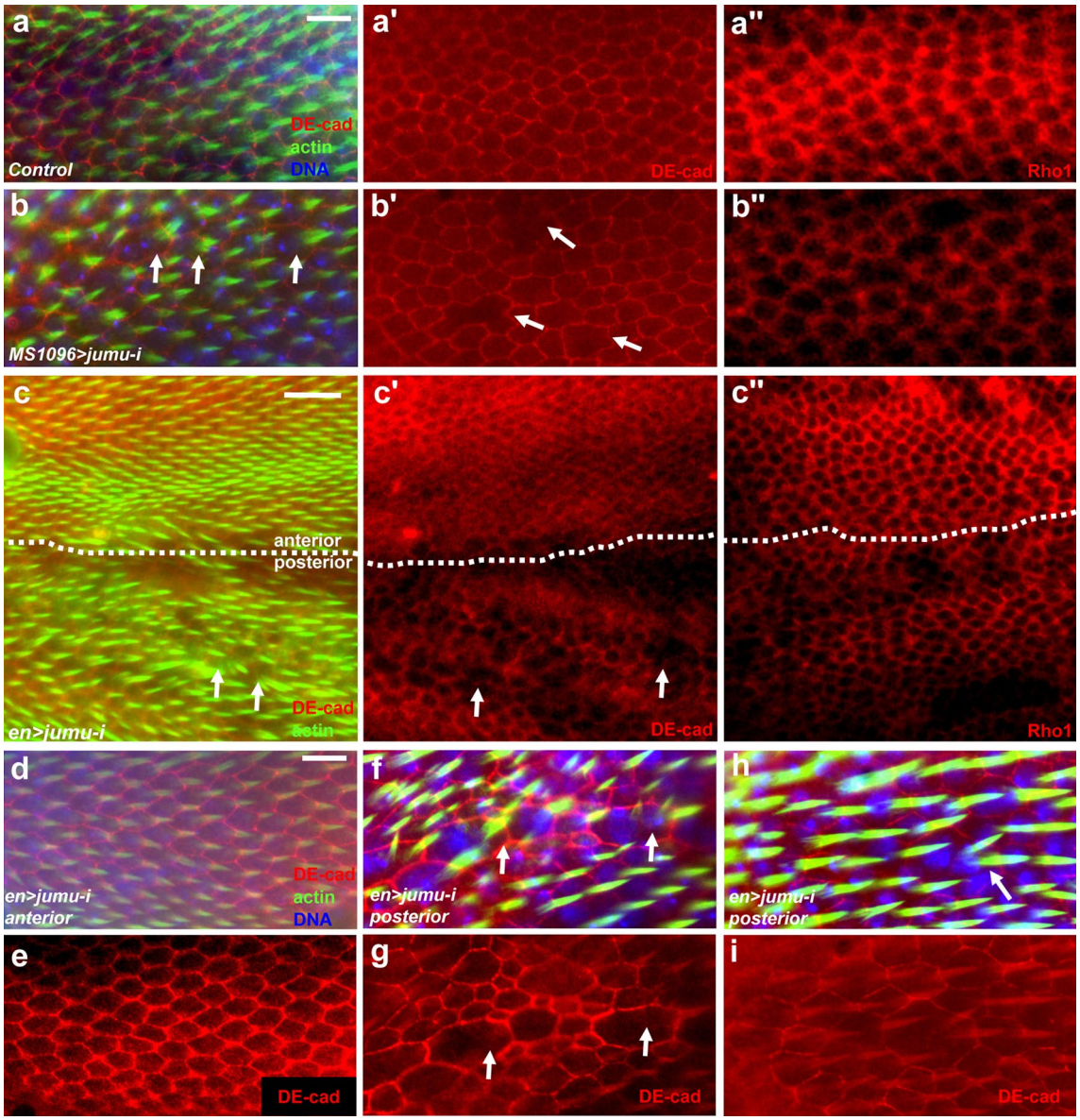

Fig. 5 Effects of *jumu* loss on pupal wings. **a, a′, d, e** Immunochemical staining for F-actin (green) and DE-cadherin (red) showing wing hairs and wing cell shape in a 32 h APF control pupal wing. **b, c, f, h** Arrows indicate cells with multiple hairs/actin bundles in *MS1096>jumu* RNAi or *en>jumu* RNAi flies. **b′, c′, g, i** Knocking down *jumu* resulted in large cells in pupal wings, where multiple hairs/actin bundles are marked by DE-cadherin and the levels of DE-cadherin were significantly decreased, as indicated with arrows. **a″–c″** Compared with the control, reduced *jumu* expression resulted in a greater reduction in Rho1 (red). **a–b″** and **d–i**, Scale bars: 20 μm. **c–c″**, scale bars: 50 μm

jumu is epistatic to *Rho1*

To further confirm the involvement of Rho1 in the regulation of wing planar polarity in the *MS1096>jumu* RNAi and *en>jumu* RNAi mutants, we performed a rescue experiment with a $Rho1^{CA}$ mutant. The overexpression of *Rho1* effectively reduced the increase in multiple hairs in the *MS1096>jumu* RNAi and *en>jumu* RNAi mutants (Fig. 6a–i, **a′**–h′); however, the elevation of *Rho1* expression did not rescue the small wing size or loss-of-ACV phenotypes of the *jumu* knockdown flies (Fig. 6d, h), and we did not detect any differences between the *jumu* knockdown compartment and the control in terms of *Rho1* levels (Fig. S5n, n′) during the larval stages. Taken together, these results suggest that a loss of *jumu* in wing development can decrease *Rho1* expression in the pupal wing blade cells and that insufficient amounts of Rho1 prevent the polymerization of multiple actin bundles, consequently inducing multiple wing hairs on adult wings. Rho1 plays an important role in regulating the formation of multiple hairs in *jumu* mutant

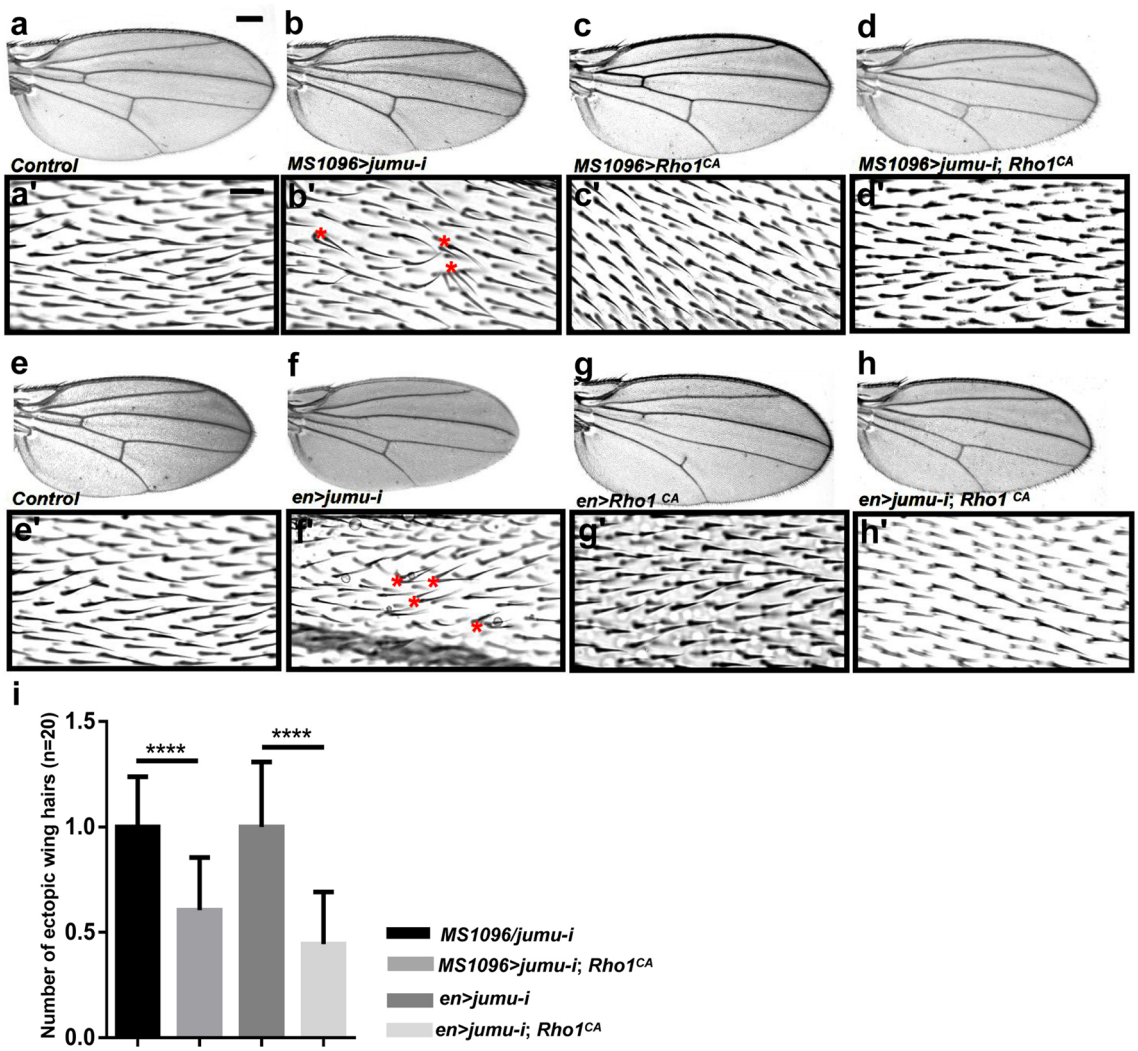

Fig. 6 The epistatic relationship between *jumu* and *Rho1* is related to multiple wing hairs. **a–a′, e–e′** Control wings of *MS1096>+* or *en>+*flies. **b–b′, f–f′** *MS1096>jumu* RNAi or *en>jumu* RNAi resulted in multiple wing hairs, as indicated by asterisks. **c–c′, g–g′** Wings of Rho1-overexpressing, *MS1096>Rho1*^{CA} or *en>Rho1*^{CA} flies. **d–d′, h–h′** *MS1096>jumu* RNAi; *Rho1*^{CA}, *en>jumu* RNAi; *Rho1*^{CA} suppressed the multiple wing hair phenotype in adult wings. **i** Quantification of wing cells with multiple hair cells. **a–h** Scale bars: 200 μm. **a′–h′** Scale bars: 20 μm

flies, independent of JNK signaling, and Jumu is important for refining multiple bundles into a single growing hair.

Discussion

In the present study, we used double heterozygous *jumu* mutants and *jumu* RNAi hairpins combined with the GAL4/UAS system to deplete *jumu* expression in the *Drosophila* wing. We present evidence that the impact of Jumu depletion on the size of the wing and the formation of the ACV, wing margin and hairs is moderate but consistent. Jumu depletion reduced the size of the adult wing. *jumu* mutants displayed autonomous apoptosis in the wing blade in an area similar to the region of Jumu activity. In addition, we observed many PH3-positive cells and increased Wg levels in *jumu* mutant flies. Moreover, the adult wing showed a loss-of-ACV phenotype, and target genes of JNK signaling were significantly upregulated. These results argue against a direct relationship between loss-of-Jumu-induced cell death and the JNK pathway within the developing wing blade. However, a decrease in Jumu levels in the wing discs induced a wing margin defect resulting from the loss of Wnt signaling responses. Furthermore, during pupal development, Jumu was found in intervein cell regions, where we suggest that it functions in wing hair polarity to promote actin polymerization and refine multiple bundles into a single growing hair via a Rho1-dependent pathway.

The loss of *jumu* throughout the wing disc caused a decrease in wing size in adults. Numerous factors participate in organ growth within developing tissues. Wing size depends on the conditions of wing disc growth during the larval stages. For example, cell proliferation and cell death mediate wing disc growth in different ways. The wing disc has a distinctive shape and size, indicating that its growth is regulated before the onset of morphogenesis [36]; hence, Dpp and Wg signaling can induce the formation of an additional wing blade [37]. The morphogen Dpp is expressed along the anterior/posterior (A/P) compartment boundary, where it forms a gradient during wing disc development [38–40]. Dpp has long been thought to exclusively promote growth and proliferation. This view is based on observations that the overexpression or enhancement of *dpp* in wing discs leads to overgrowth or enlarged wings in adult flies [25, 41–43]. Knocking down Dpp signaling leads to the development of small discs, reducing adult wing size [44, 45]. The Wg morphogen is secreted across two to three cell widths straddling the D/V boundary of the wing disc and forms a gradient that helps to regulate adult wing development [46–48]. Reduced Wg signaling leads to the development of small wings and defective wing margins [10]. We observed a marked increase in the number of PH3-positive cells and the level of Wg in *jumu* mutant flies, which indicates that Jumu opposes Dpp or Wg to inhibit cell proliferation in wing discs. However, the levels of Dpp were not significantly altered and were similar to the levels observed in the controls (Fig. S5o, p). The involvement of Wg signaling in compensatory proliferation has been explored by examining its expression in apoptotic cells, and in these cells, ectopic Wg signaling induces compensatory proliferation during mitosis [10, 49]. Based on this information, we examined *jumu* mutant wing discs and found many dead cells via 7-AAD staining (Fig. 2a–d). As expected, TUNEL assays revealed that the number of apoptotic cells significantly increased when the Wg intensity increased in $jumu^{Df3.4}/jumu^{P}$ double heterozygous and *jumu* RNAi-induced wing discs. Moreover, we also found that the cell size in *jumu*-knockdown pupal wings was clearly larger than that in the controls (Fig. 5e, g, i). These results demonstrate that the observed small wing size is related to apoptotic signaling (Fig. 2e–h). The JNK pathway is closely linked to cell death regulation in the core apoptotic pathway. When cell death is induced by JNK, apoptotic cells secrete Wg, Dpp or Spi, which are involved in regenerative responses to maintain tissue homeostasis [11, 12]. Therefore, we analysed the downstream transcriptional targets of the JNK pathway in *jumu* knockdown flies and found that the levels of *puc* and p-JNK were clearly increased (Fig. 3a–d). These data further confirm that *jumu* likely induces apoptosis and proliferation via a JNK-dependent pathway.

Several factors are involved in regulating the size and shape of an organ. Previous studies have suggested that Myc plays pivotal roles in promoting cell growth, proliferation and apoptosis in organ development [50, 51]. The loss of dMyc leads to the development of smaller wings and delayed patterning [52]. Moreover, our recent work demonstrated that Jumu could control the proliferation of lymph gland cells by regulating *dMyc* expression [21]. In this study, we analysed dMyc levels in wing discs via immunohistochemical staining. However, we did not observe any differences between the *jumu* knockdown and control wing discs (Fig. S5a–c′), indicating that the small wing size of the *jumu* RNAi flies was not mediated by *dMyc*.

Members of the Rho GTPase family control the polymerization of actin and the assembly of focal complexes at the plasma membrane in response to extracellular signals [53, 54]. Several studies have indicated that *Rho1* uniquely and specifically regulates apoptosis-induced compensatory proliferation in *Drosophila* epithelia through a JNK-dependent pathway. Previous observations showed that reducing the expression of *Rho1* is sufficient to activate the JNK pathway and that overexpressing *Rho1* induces apoptosis in imaginal wing disc epithelia [33, 55, 56]. The specific disruption of the Cdc42/Par6/aPKC polarity complex leads to Rho1-JNK-dependent growth [57]. Rho1-induced apoptosis is generally coupled to effects on cell adhesion. Studies have shown that abnormal levels of Rho1 affect the organization of F-actin [58]. In *Rho1* mutants, DE-cadherin is downregulated or shows a disturbed pattern of localization [34, 59]. We analysed *Rho1* levels in wing discs via immunohistochemical staining. However, we did not observe any differences between *jumu* knockdown and control wing discs (Fig. S5n, n′), indicating that the apoptosis observed in *jumu* RNAi flies is not caused by alterations in *Rho1* levels. We also detected F-actin, βPS and DE-cadherin in the wing discs of *jumu* knockdown flies, all of which were found to retain a normal pattern (Fig. S5d–m′). Thus, the results demonstrated that *Rho1* downregulation due to reduced levels of *jumu* only occurred during the pupal stages.

In summary, *jumu* has multiple functions in *Drosophila* wing development. Loss-of-Jumu-induced apoptosis causes compensatory cell proliferation in *Drosophila* epithelia through a JNK-dependent pathway. In addition, a decrease in Jumu levels in the wing discs induces a wing margin defect due to a loss of Wnt signaling responses. *jumu* appears to function as a positive regulator of hair morphogenesis. These multiple functions of Jumu lead to the wide range of phenotypes seen in *jumu* mutants.

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Compliance with ethical standards

Competing interests The authors declare no competing or financial interests.

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