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ORIGINAL PAPER

APLP1 promotes dFoxO-dependent cell death in *Drosophila*

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Abstract The amyloid precursor like protein-1 (APLP1) belongs to the amyloid precursor protein family that also includes the amyloid precursor protein (APP) and the amyloid precursor like protein-2 (APLP2). Though the three proteins share similar structures and undergo the same cleavage processing by α-, β- and γ-secretases, APLP1 shows divergent subcellular localization from that of APP and APLP2, and thus, may perform distinct roles in vivo. While extensive studies have been focused on APP, which is implicated in the pathogenesis of Alzheimer's disease, the functions of APLP1 remain largely elusive. Here we report that the expression of APLP1 in *Drosophila* induces cell death and produces developmental defects in wing and thorax. This function of APLP1 depends on the transcription factor dFoxO, as the depletion of dFoxO abrogates APLP1-induced cell death and adult defects. Consistently, APLP1 up-regulates the transcription of dFoxO target *hid* and *reaper*-two well known pro-apoptotic genes. Thus, the present study provides the first in vivo evidence that APLP1 is able to induce cell death, and that FoxO is a crucial downstream mediator of APLP1's activity.

Keywords APLP1 · dFoxO · Cell death · *Drosophila*

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Introduction

Human amyloid precursor like protein-1 (APLP1) belongs to a protein family which also contains amyloid precursor protein (APP) and amyloid precursor like protein-2 (APLP2) [1–5].The three proteins share similar structures with conserved N- and C-terminal domains, and can be cleaved by the same α-, β- and γ-secretases [6–10], implying that they might perform similar functions in development. Previous studies revealed that single knock-out mice of APP, APLP1 or APLP2 produced only subtle phenotypes, while double knock-out APP/APLP2 or APLP1/APLP2 mice died shortly after birth, implying a possible functional redundancy among the proteins [11–13].However, other studies suggest that these proteins may bear diverse functions rather than simply compensating for each other [14–16]. In particular, APLP1 exhibits distinct expression pattern and subcellular localization from that of APP and APLP2. While APP and APLP2 are ubiquitously expressed and predominantly distributed in intracellular compartments including the endosomes, ER and Golgi apparatus [5], APLP1 specifically concentrates in the nervous system but also shows a weak signal in other tissues such as heart, lung, liver and kidney of E15 mouse embryos observed by in situ hybridization [17]. However, the functions of APLP1 in the non-neuronal tissues remain unknown. APLP1 mainly localizes to the plasma membrane [5], indicating APLP1 may perform divergent in vivo functions from that of APP and APLP2. Though all APP family members are able to generate the intracellular domains (ICDs) through cleavage by γ- and ε-secretases [18–22], ICDs derived from APP and APLP2, but not APLP1,

interact with Tip60 and Fe65 to localize to the spherical nuclear AFT complexes, where they regulate target genes expression [23, 24].

APLP1 is shown to be transcriptionally regulated by p53 tumor suppressor [25], and forms oligomers on the plasma membrane via its E2 domain [26]. APLP1 is found in the senile plaques of Alzheimer's disease (AD) patients' brains [27, 28], suggesting a possible role of APLP1 in the progression of the disease. Consistent with this, APLP1 undergoes the processing by β- and γ-secretases to form short Aβ-like peptides [29], which is also reported to be altered in Down's Syndrome [30]. Besides, APLP1 is shown to regulate synapse formation, neuronal differentiation and synaptic plasticity [1, 4, 31–34]. However, compared with APP, the in vivo functions of APLP1 have remained largely unknown.

Drosophila has been used as a powerful model organism to investigate the in vivo functions and underlining mechanisms of human genes. Here in this report, we introduced APLP1 into *Drosophila* and found that expression of APLP1 induced dramatical cell death and developmental defects in various tissues. In addition, APLP1-induced cell death is mediated by the transcription factor dFoxO. Consistently, APLP1 up-regulates the expression of dFoxO target genes *hid* and *reaper*. To our knowledge, this study provides the first in vivo evidence that APLP1 triggers cell death in development.

Materials and methods

Drosophila strains

UAS-APLP1, *UAS*-APLP2 and *UAS*-APPL^{sd} [35] were kind gifts from Dr. Merders. *dfoxO*$^{\triangle 94}$ and *dfoxO*^{21} [36] were kind gifts from Dr. Partridge. *ptc*-Gal4, *en*-Gal4, *pnr*-Gal4, *sd*-Gal4, *UAS*-GFP, *UAS-dfoxO*-IR#1, *UAS-dfoxO*-IR#2, *hid*-LacZ [37] were previously described. *UAS*-LacZ, *dpp*-Gal4, *reaper*-LacZ, *APPL*-Gal4, *UAS*-p35 and *UAS*-DIAP1 were obtained from the Bloomington Stock center.

AO staining

Wing discs were dissected from the 3rd instar larvae in 1 % PBS buffer and stained for acridine orange as described [38]. Each genotype was dissected with 20 discs for statistics.

Light image

Freshly eclosed flies of indicated genotypes were collected and immediately frozen in −80 °C. Wings were dissected and mounted on the slide in the alcohol/glycerol (1:1) medium, and flies were mounted on the 1 % agarose plate in the alcohol/glycerol medium. Light images of wings were collected with Olympus microscope BX51, and light images of thoraxes were collected with OLYMPUS stereo microscope SZX16.

Immunohistochemistry

3rd instar larvae of indicated genotypes were collected and dissected in 1 % PBS buffer. And the antibody staining of imaginal discs was conducted as previously described [39]. The following antibodies were used: rabbit anti-cleaved caspase-3 (1:400, Cell Signaling and Technology), anti-rabbit-Alexa (1:1,000, Cell Signaling and Technology).

X-gal staining

Wing discs were dissected from the 3rd instar larvae in 1 % PBS buffer and stained for *β-galactosidase* activity as described [40].

Statistical analysis

For the statistics of anterior cross vein (acv), wings from freshly eclosed virgins were dissected and the presence of acv was counted. 20 wings were examined for each genotype. For the statistics of scutellum size, freshly eclosed virgins were collected and photoed, the area of the scutellum was measured with the software Cellsens of Olympus. Each genotype was tested with 10 flies.

Results

APLP1 induces caspase-dependent cell death in *Drosophila*

To characterize the in vivo functions of APLP1 in development, we expressed APLP1 in multiple tissues of *Drosophila*. Targeted expression of APLP1 along the anterior/posterior (A/P) compartment boundary in 3rd instar wing discs driven by the *patched*-Gal4 (*ptc*-Gal4) driver (Fig. 1a, b) [37, 41] initiated extensive cell death in the *ptc* domain, as revealed by acridine orange (AO) staining (Fig. 1d, g), compared with the *ptc*-Gal4 control (Fig. 1c, g), indicating the expression of APLP1 is sufficient to induce cell death in *Drosophila*. To examine whether APLP1-induced cell death is caspase dependent, we performed immunostaining against the cleaved caspase-3. Expression of APLP1 initiated strong cleaved caspase-3 staining along the A/P compartment boundary (Fig. 1i), compared with the *ptc*-Gal4 control (Fig. 1h), suggesting

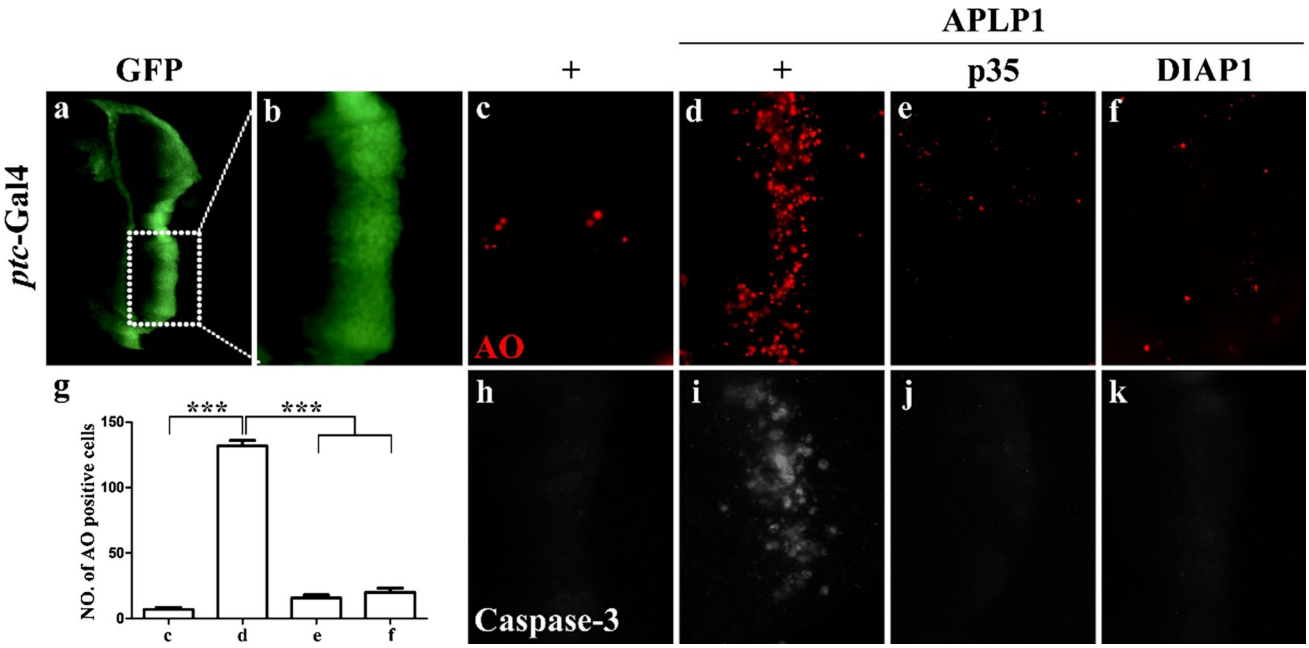

Fig. 1 APLP1 induces caspase-dependent cell death in *Drosophila*. Fluorescent images of GFP expression (**a**, **b**), or acridine orange staining (**c–f**), or anti-cleaved caspase-3 staining (**h–k**) of wing discs from 3rd instar larvae are shown. *ptc*-Gal4 was used as a control (**c**, **h**), or to drive the expression of GFP (**a**, **b**) or APLP1 (**d–f**, **i–k**). APLP1-induced cell death (**d**) and cleaved caspase-3 activation (**i**) are suppressed by the expression of p35 (**e**, **j**) or DIAP1 (**f**, **k**). **g** is the statistical analysis of acridine orange-positive cells in figures **c–f**. ***$P \leq 0.001$. Genotypes: *ptc*-Gal4 *UAS*-GFP/+ (**a**, **b**); *ptc*-Gal4/+ (**c**, **h**); *ptc*-Gal4/+; *UAS*-APLP1/+ (**d**, **i**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS*-p35 (**e**, **j**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS*-DIAP1(**f**, **k**)

APLP1 induces caspase activation in *Drosophila*. Consistent with this finding, expression of p35, a viral protein that inhibits effector caspases [42], or the *Drosophila* inhibitor of apoptosis protein1 (DIAP1) [43, 44], suppressed APLP1-induced cell death and cleaved caspase-3 activation (Fig. 1e–g, j, k).Together, these data suggest that APLP1 induces caspase-dependent cell death in *Drosophila*.

To further examine the role of APLP1 in regulating cell death in wing development, we used *engrailed*-Gal4 (*en*-Gal4) to drive APLP1's expression in the posterior compartment of wing discs (Fig. 2a), and observed evident cell death as compared with the *en*-Gal4 control (Fig. 2b–d). Interestingly, a loss of acv phenotype in the posterior compartment was noticed in wings of APLP1-expressing flies, but not those of *en*-Gal4 controls or the ones that express GFP (Fig. 2e–h), indicating APLP1 affects vein formation in *Drosophila*. Similarly, *ptc*-Gal4 driven expression of APLP1 resulted in strong cell death (Fig. 1d, g and Fig. 3c, h) and the complete loss of the acv in adult wings (Fig. 4c, f), while the *ptc*-Gal4 control (Fig. 4a, f) or expression of GFP (Fig. 4b, f) failed to generate such phenotype, further suggesting APLP1 affects vein development. Furthermore, expression of APLP1 driven by *dpp*-Gal4 along the A/P boundary (Fig. S1a) triggered strong cell death in wing discs (Fig. S1c, d), and generated the loss-of-acv phenotype in adult wings (Fig. S1g, h), compared with the controls (Fig. S1b, d, e, f, h).Finally, expression of APLP1 driven by *sd*-Gal4 (Fig. S2a) provoked extensive cell death in the wing pouch (Fig. S2c) and produced a blistered wing phenotype (Fig. S3b, d), compared with the controls (Fig. S2b, S3a).

To check if expression of APLP1 could induce cell death in other tissues, we expressed APLP1 in the thorax by *pnr*-Gal4, and observed a reduced scutellum phenotype (Fig. 5c, i) resulted from enhanced cell death in the notum tips of wing discs (Fig. S4b, d). As negative controls, neither *pnr*-Gal4 nor expression of GFP was able to induce cell death in wing discs and scutellum defect in adult flies (Fig. 5a, b, i; Fig. S4a, d). In addition, expression of APLP1 under the control of the pan-neuron *elav*-Gal4 driver (Fig. S5a) [37] provoked neuronal cell death in the ventral nerve cord (Fig. S5b–d), while expression of APLP1 in the eyediscs driven by *GMR*-Gal4 (Fig. S5e) induced strong cell death posterior to the morphogenetic furrow (Fig. S5f–h). Taken together, these results indicate that APLP1 induces cell death in a non-tissue specific manner in *Drosophila*.

Together, the above data indicate that APLP1 induces cell death and developmental defects in *Drosophila*. Consistent with our observation, the depletion of APLP1 in cultured neuroblastoma cells reduced stress-induced apoptosis while overexpression of APLP1 slightly enhanced stress-induced

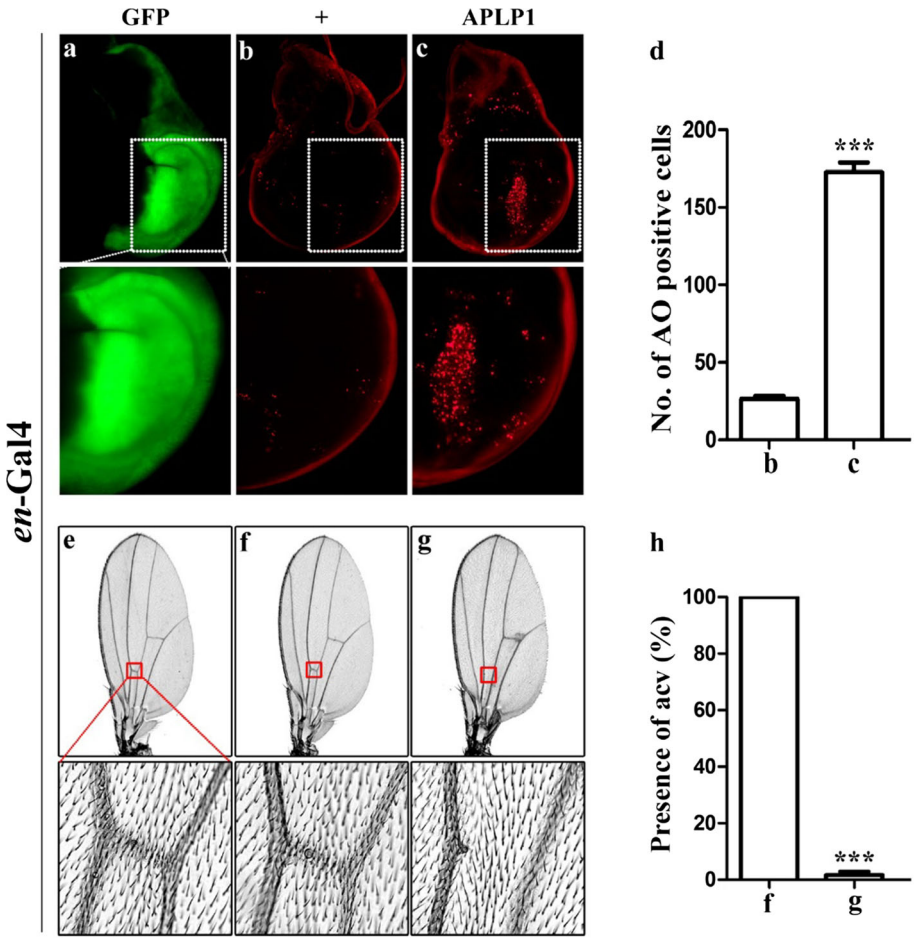

Fig. 2 APLP1 induces cell death and morphological defects in wing development. Fluorescent images of GFP expression (**a**) or acridine orange staining (**b**, **c**) of wing discs from 3rd instar larvae and light images of adult wing (**e–g**) are shown. *en*-Gal4 was used as a control (**b**, **f**), or to drive the expression of GFP (**a**, **e**) or APLP1 (**c**, **g**). The lower panels are high magnification of the boxed areas in the upper panels. **d** shows the statistical analysis of acridine orange-positive cells in the boxed areas in **b** and **c**, while **h** shows the statistical analysis of the acv presence in **f** and **g**. $***P \leq 0.001$. Genotypes: *en*-Gal4 *UAS*-GFP/+ (**a**, **e**); *en*-Gal4/+ (**b**, **f**); *en*-Gal4/+; *UAS*-APLP1/+ (**c**, **g**)

apoptosis [25]. However, expression of APLP1 alone failed to trigger apoptosis in these cells [25], suggesting APLP1 induces cell death in a cell context dependent manner. Therefore, our data not only demonstrate for the first time that APLP1 by itself is sufficient to induce cell death, but also provide the first in vivo evidence for this function of APLP1.

APLP1 belongs to a protein family which also contains amyloid precursor like protein-2 (APLP2) [1–5]. To check if APLP2 can induce cell death and produce similar phenotypes as that of APLP1, we also expressed APLP2 under the control of various Gal4 drivers in *Drosophila*. Interestingly, *ptc*>APLP2 promoted cell death along the A/P boundary in the wing disc (Fig. S6a–c) and loss of acv in the adult wing (Fig. S6d-f), *sd*>APLP2 triggered strong cell death in the wing pouch (Fig. S7a, b) that resulted in small blistered wings (Fig. S7c, d), and *pnr*>APLP2 produced dramatically diminished scutella (Fig. S7e, f). Thus, APLP2 is able to recapitulate the cell death phenotype of APLP1 in *Drosophila*.

In *Drosophila*, there is only one APP family member named amyloid precursor protein-like (APPL) protein [45], which plays a vital role in regulating the development of the *Drosophila* nervous system [46]. APPL regulates the synaptic growth [47], axon transport [48, 49] and is also a modulator of wnt PCP signaling [50]. Expression of APPL in the thorax resulted in loss of thoracic macrochaetes and a reduction in scutellum size [51], reminiscent of the thorax phenotypes of *pnr*>APLP1 in this study (Fig. 5c). Furthermore, expression of APPL in the wing disc induced caspase dependent cell death and produced a loss-of-acv phenotype [51], which is similar to that of *ptc*>APLP1 (Fig. 4c). To examine whether APPL could trigger cell death in the nervous system, we expressed APPL in the ventral nerve cord (Fig. S8a), and observed mild cell death compared with the control (Fig. S8b–d), suggesting APPL could induce neuronal cell death in *Drosophila.*

Previous work showed that expression of human APP or *Drosophila* APPL induced cell death in *Drosophila* [37]. This study indicated a similar function for human APLP1 and APLP2. Thus, the roles in regulating cell death have been conserved by all APP family members.

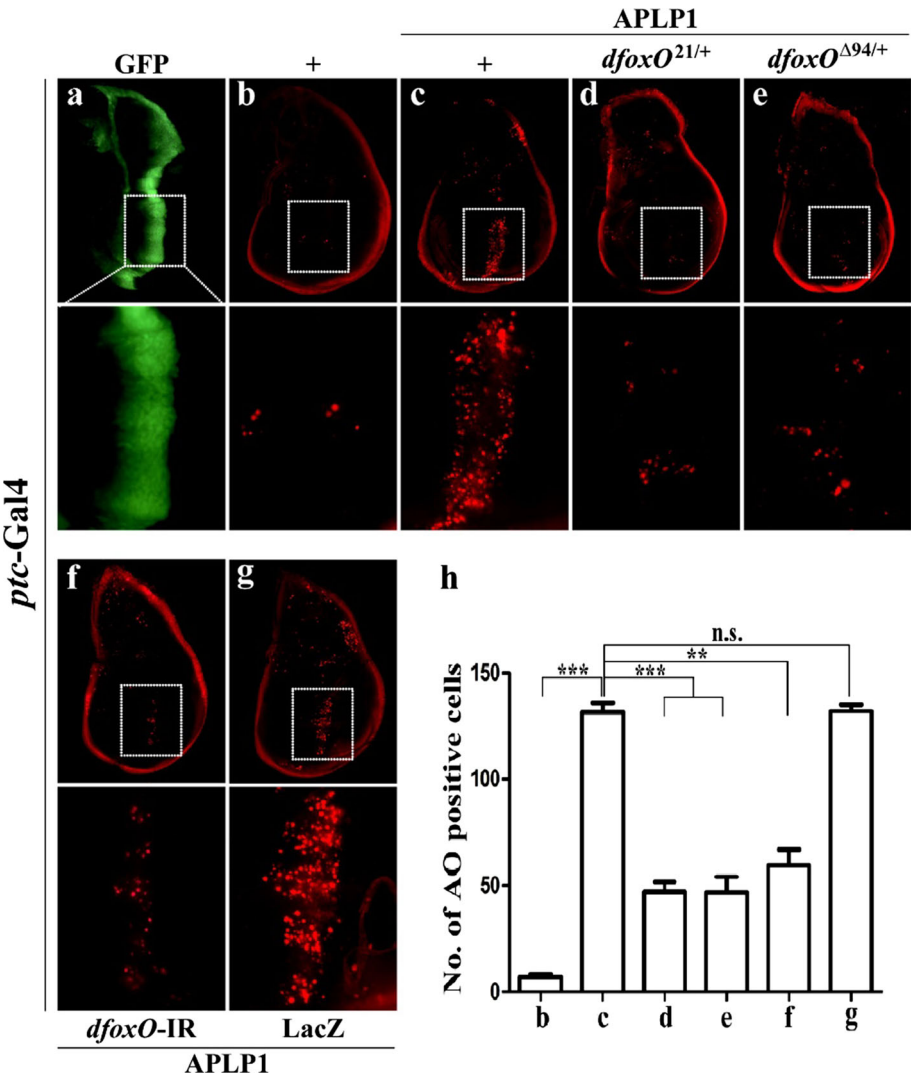

Fig. 3 APLP1 induces dFoxO-dependent cell death in wing discs. Fluorescent images of GFP expression (**a**) or acridine orange staining (**b–g**) of wing discs from 3rd instar larvae are shown. *ptc*-Gal4 was used as a control (**b**), or to drive the expression of GFP (**a**) or APLP1 (**c–g**).APLP1-induced cell death is suppressed by two *dfoxO* mutations (**d**, **e**) or expression of a *dfoxO* RNAi (**f**), but remains unaffected by the expression of LacZ (**g**). The lower panels are high magnification of boxed areas in the upper panels. **h** is the statistical analysis of acridine orange-positive cells in the lower panels of **b–g**. ***$P \leq 0.001$; **, $P < 0.01$; n.s., not significant. Genotypes: *ptc*-Gal4 *UAS*-GFP/+ (**a**); *ptc*-Gal4/+ (**b**); *ptc*-Gal4/+; *UAS*-APLP1/+ (**c**); *ptc*-Gal4/+; *UAS*-APLP1/*dfoxO*^{21} (**d**); *ptc*-Gal4/+; *UAS*-APLP1/*dfoxO*$^{\triangle 94}$ (**e**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS*-*dfoxO*-IR#1 (**f**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS*-LacZ (**g**)

Loss of dFoxO suppresses APLP1-induced cell death in *Drosophila*

To investigate the mechanism underlying APLP1-induced cell death, we called specific attention to the transcription factor dFoxO encoding the *Drosophila* homolog of mammalian FoxO3a, based on the following reasons. Firstly, both APLP1 and FoxO3a play pivotal roles in regulating stress-induced cell death [25, 52]. Secondly, both APLP1 and FoxO3a are required for p53-dependent apoptosis [25, 53]. Thirdly, both APLP1 and dFoxO are sufficient to induce cell death in *Drosophila* [54]. Finally, we previously noted that expression of dFoxO or human FoxO3a driven by *ptc*-Gal4 recapitulated the loss-of-acv wing phenotype of *ptc*>APLP1 (Fig. S9) [37].

To check whether dFoxO is required for APLP1-induced cell death, we expressed APLP1 in *Drosophila* with depleted *dfoxO* expression. In support of the hypothesis, we found that *ptc*>APLP1 induced cell death in wing discs was significantly suppressed by mutations in *dfoxO*(Fig. 3d, e, h) or RNAi-mediated knocking down of *dfoxO* (Fig. 3f, h), but not by the expression of LacZ (Fig. 3g, h). Consistently, *ptc*>APLP1 induced loss-of-acv phenotype in adult wings was also suppressed by loss of *dfoxO*, but remained unaffected by the expression of LacZ (Fig. 4c–f). Furthermore, *sd*>APLP1 triggered blistered wing phenotype was also suppressed in *dfoxO* mutants (Fig. S3c, d).

Consistent with the results obtained in the wing, we found that *pnr*>APLP1 induced cell death in the notum area of wing discs and the reduced scutellum phenotype in adult were considerably suppressed by mutations or RNAi-mediated down-regulation of *dfoxO* (Fig. 5e–i, Fig. S4c, d), but remained unchanged by the expression of LacZ (Fig. 5d, i).

Taken together, these data suggest that dFoxO is required for APLP1-induced cell death and developmental defects in the *Drosophila* wing and thorax.

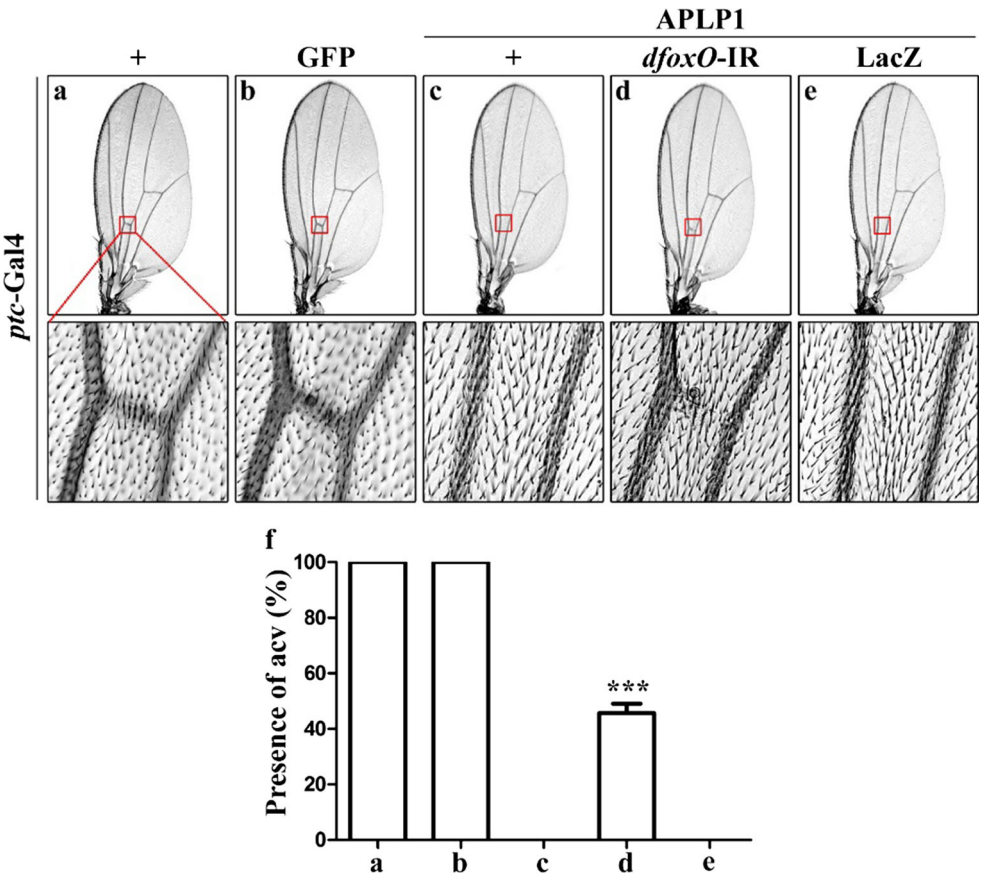

Fig. 4 APLP1 induces dFoxO-dependent loss-of-acv phenotype in adult wings. Light images of adult wings are shown (**a–e**). *ptc*-Gal4 was used as a control (**a**), or to drive the expression of GFP (**b**) or APLP1 (**c–e**). Expression of a *dfoxO* RNAi (**d**), but not LacZ (**e**), partially suppressed APLP1-induced loss-of-acv phenotype. The lower panels are high magnification of boxed areas (showing acv) in the upper panels. **f** is the statistical analysis of the acv presence in **a–e**. $***P \leq 0.001$. Genotypes *ptc*-Gal4/+ (**a**); *ptc*-Gal4 *UAS*-GFP/+ (**b**); *ptc*-Gal4/+; *UAS*-APLP1/+ (**c**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS-dfoxO*-IR#1 (**d**); *ptc*-Gal4/+; *UAS*-APLP1/*UAS*-LacZ (**e**)

APLP1 up-regulates dFoxO target gene expression

The above results highlight a role of dFoxO in mediating APLP1-induced cell death in *Drosophila*, which suggests that APLP1 may activated FoxO to initiate the downstream cell death machinery. To examine whether APLP1 activates dFoxO in vivo, we checked the expression of the pro-apoptotic genes *hid* and *reaper* (*rpr*), the well documented transcriptional targets of dFoxO [37, 54, 55]. Indeed, the expression of APLP1 in the wing pouch driven by *sd*-Gal4 resulted in dramatically up-regulated transcription of *hid* and *rpr*, as monitored by a *hid*-lacZ and a *rpr*-LacZ reporters, respectively (Fig. 6). Hence, the data demonstrate that APLP1 is able to activate dFoxO *invivo*, which provides a mechanism for APLP1-induced cell death in *Drosophila*. Yet, it remains unknown how APLP1 activates dFoxO in *Drosophila*, and whether APLP1 could trigger FoxO3a activation in mammalian cells. Further investigation will be required to address these important questions. Consistent with the observation that APLP2 phenocopied APLP1 in regulating cell death, expression of APLP2 can also activate dFoxO target genes *hid* and *rpr* transcription (Fig. S10). Collectively, these data indicate amyloid precursor like proteins are sufficient to activate FoxO mediated cell death machinery.

Discussion

Amyloid pecursor like protein-1(APLP1) is amammalian paralog of amyloid precursor protein (APP). While APP has been extensively studied for its involvement in the Alzheimer's disease, few studies have been directed to APLP1 and its in vivo functions remain largely unknown. In the present study, we investigated the in vivo functions of APLP1 using *Drosophila* as a model organism. We found that ectopic expression of APLP1 induced cell death and developmental defects in the nervous and non-nervous system. Our genetic study characterized the transcription factor dFoxO as a critical downstream factor that mediates APLP1's activity, for the depletion of dFoxO significantly suppressed APLP1-induced cell death in larval discs and associated phenotypes in adults. Further study confirmed that APLP1 was able to up-regulate the transcription of dFoxO target genes *hid* and *reaper*.

APLP1 was reported to function mainly in the nervous system, as high expression level of APLP1 was detected in the developing central and peripheral nervous systems, yeta weak expression signal of APLP1 was also observed in organs like heart, lung, liver and kidney in mouse embryos [17], implying a role of APLP1 in the development of non-

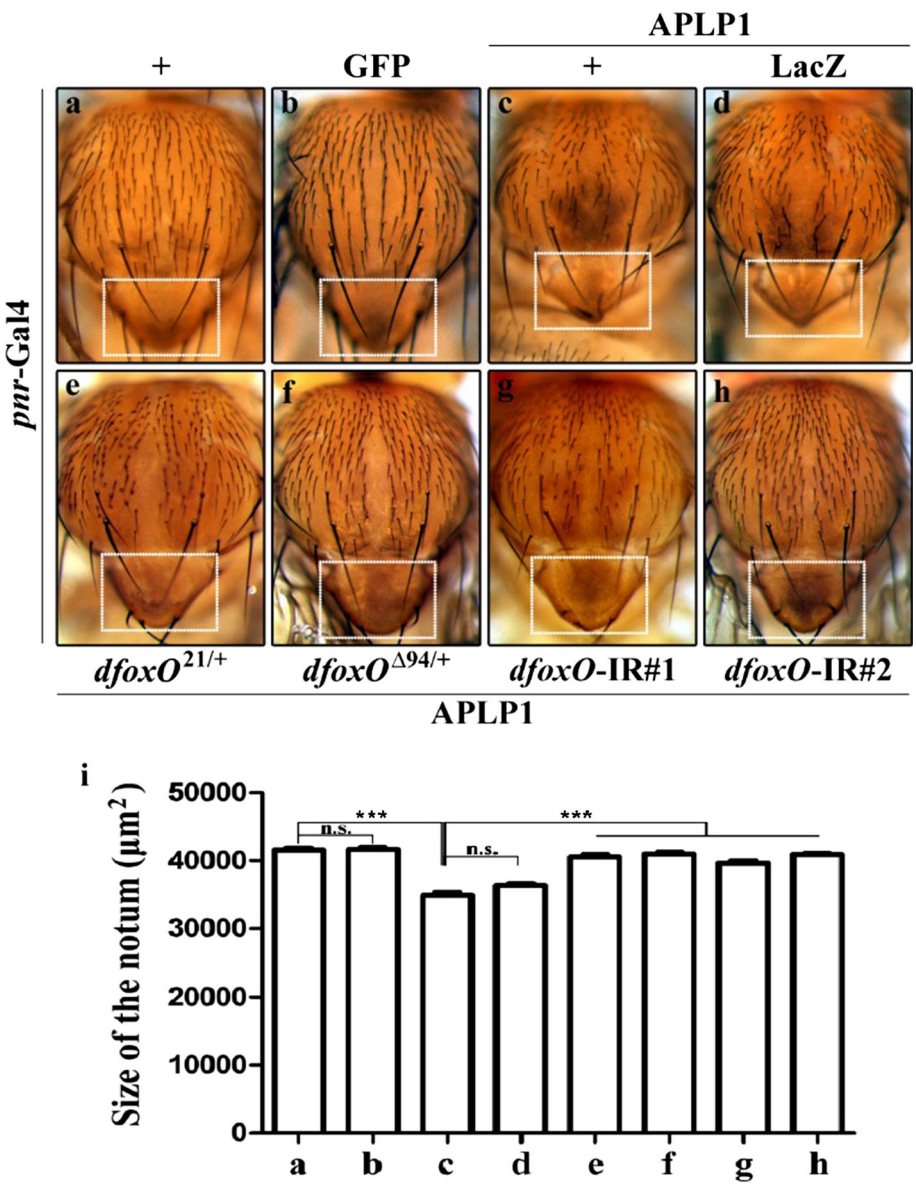

Fig. 5 Loss of *dfoxO* suppresses APLP1-induced small scutellum phenotype. Light images of *Drosophila* adult thoraxes are shown. Compared with the *pnr*-Gal4 control (**a**), expression of APLP1 (**c**) but not GFP (**b**) induced a small scutellum phenotype, which was suppressed by two *dfoxO* mutations (**e**, **f**) or expression of two independent *dfoxO* RNAi (**g**, **h**), but not that of LacZ (**d**). **i** is the statistical analysis of the size of scutellum in **a–h**. ***$P \leq 0.001$; *n.s.* not significant. Genotypes: *pnr*-Gal4/+ (**a**); *pnr*-Gal4 *UAS*-GFP/+ (**b**); *pnr*-Gal4 *UAS*-APLP1/+ (**c**); *pnr*-Gal4 *UAS*-APLP1/*UAS*-LacZ (**d**); *pnr*-Gal4 *UAS*-APLP1/$dfoxO^{21}$ (**e**); *pnr*-Gal4 *UAS*-APLP1/$dfoxO^{\triangle 94}$ (**f**); *pnr*-Gal4 *UAS*-APLP1/*UAS*-*dfoxO*-IR#1 (**g**); *pnr*-Gal4 *UAS*-APLP1/*UAS*-*dfoxO*-IR#2 (**h**)

neuronal tissues. Consistent with this explanation, RNAi mediated knockdown of APLP1 in WI-38 and MCF7 cells dramatically reduced the proliferation of these cells [25]. In the present study, we showed that expression of APLP1 could induce cell death and developmental defects in both neuronal and non-neuronal systems in *Drosophila*, and thus, providing further evidence for the function of APLP1 in non-neuronal cells.

Previous studies showed that loss of APLP1 diminishes stress induced apoptosis in neuroblastoma cells, whereas ectopic expression of APLP1 moderately enhances cell death upon stress stimulation [25]. However, expression of APLP1 alone is not sufficient to induce neuroblastoma cell death [25], suggesting APLP1 induces cell death in a context dependent manner. Our data not only demonstrate for the first time that APLP1 by itself is sufficient to induce cell death, but also provide the first in vivo evidence for this function of APLP1. APLP1 was reported to be a direct transcriptional target of the p53 tumor suppressor [25], which suggests a possible involvement of APLP1 in p53-induced cell death. p53 was known to interact with the transcriptional factor FoxO [56–58], and MDM2 was reported to act downstream of p53 to promote FoxO ubiquitination and degradation [58]. In the present study, we showed that FoxO mediates APLP1-induced cell death. The exact relationship between APLP1, FoxO and p53 in cell death will require further investigation. Overall, this study highlights a novel function of APLP1 in promoting FoxO-mediated cell death in vivo, which will shed light on the role of APLP1 in mammalian cells.

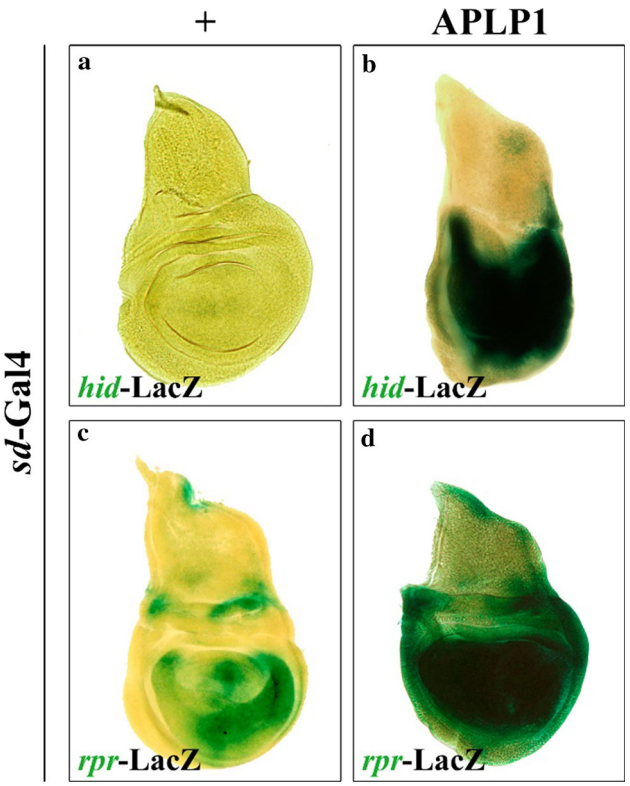

Fig. 6 Expression of APLP1 activates the transcription of FoxO target genes. Light images of *Drosophila* 3rd instar wing discs are shown. X-Gal staining of *hid*-LacZ and *reaper*-LacZ reporters in the wing pouch (**a**, **c**) are dramatically up-regulated by the expression of APLP1 (**b**, **d**)

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