

RESEARCH ARTICLE

Loss of *putzig* Activity Results in Apoptosis during Wing Imaginal Development in *Drosophila*

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Abstract

The *Drosophila* gene *putzig* (*pzg*) encodes a nuclear protein that is an integral component of the Trf2/Dref complex involved in the transcription of proliferation-related genes. Moreover, Pzg is found in a complex together with the nucleosome remodeling factor NURF, where it promotes Notch target gene activation. Here we show that downregulation of *pzg* activity in the developing wing imaginal discs induces an apoptotic response, accompanied by the induction of the pro-apoptotic gene *reaper*, repression of *Drosophila* inhibitor of apoptosis protein accumulation and the activation of the caspases Drice, Caspase3 and Dcp1. As a further consequence 'Apoptosis induced Proliferation' (AiP) and 'Apoptosis induced Apoptosis' (AiA) are triggered. As expected, the activity of the stress kinase Jun N-terminal kinase (JNK), proposed to mediate both processes, is ectopically induced in response to *pzg* loss. In addition, the expression of the mitogen *wingless* (*wg*) but not of *decapentaplegic* (*dpp*) is observed. We present evidence that downregulation of *Notch* activates Dcp1 caspase and JNK signaling, however, neither induces ectopic *wg* nor *dpp* expression. In contrast, the consequences of *Dref*-RNAi were largely indistinguishable from *pzg*-RNAi with regard to apoptosis induction. Moreover, overexpression of *Dref* ameliorated the downregulation of *pzg* compatible with the notion that the two are required together to maintain cell and tissue homeostasis in *Drosophila*.



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Introduction

Cellular and tissue homeostasis describes a complex process ensuring the survival and correct development of an organism. Apoptosis, the major form of programmed cell death, contributes to tissue homeostasis by eliminating aberrant, surplus or malignant cells during 'normal' development and in response to stress induced conditions. This safeguarding system demands a fine-tuned control as an unregulated apoptosis has been connected to various human diseases including cancer (reviewed in [1–4]). Research in several model organisms including *Drosophila* has expanded our knowledge on the molecular mechanisms underlying the well conserved apoptotic execution program in metazoans: Under normal conditions, cell survival is

guaranteed by the Inhibitors of Apoptosis Proteins (IAP, DIAP1 in *Drosophila*), which bind and inhibit caspases, the key executing enzymes of apoptosis (reviewed in [4–6]). In contrast, if cell death is triggered, e.g. under cellular stress conditions, pro-apoptotic gene activity is induced in *Drosophila*: Pro-apoptotic gene products include the DIAP1-antagonists Hid, Rpr and Grim, which themselves mediate the ubiquitin dependent degradation of DIAP1 thereby enabling caspases to provoke the death of the cell [7–13].

Although diverse stress signals provoke a strong apoptotic answer in the respective tissue, organisms can often compensate this cell loss allowing them to survive with no or only minor consequences on the final tissue or body size. This intriguing fact has been designated Apoptosis-induced Proliferation (AiP) and describes the striking property of dying cells to stimulate proliferation of adjacent surviving cells, and therefore tissue regeneration (reviewed in [14–17]). The model system *Drosophila* with its sophisticated genetic methods offers the opportunity to study the mechanisms underlying the communication between damaged cells and the surrounding tissues. Here, cell death was experimentally induced in cells of larval imaginal discs and, as a consequence, the ectopic induction of mitogens like *wingless* (*wg*), *decapentaplegic* (*dpp*) or *hedgehog* (*hh*) was often observed in the apoptotic cells [18–21]. These mitogens, the key players in the regulation of morphogenesis and growth in the course of *Drosophila* development [22,23], provide a well-founded explanation for the compensatory proliferation of neighboring cells. However, since the genuine dying cells are often rapidly eliminated, much of our current knowledge on AiP was deduced from a special type of apoptotic cells referred to as 'undead' cells. These cells are experimentally obtained by provoking cell death while expressing the caspase inhibitor p35 at the same time. p35 specifically blocks the function of the effector caspases Drice and Dcp-1 without affecting the activity of the initiator caspase Dronc [19,24,25]. Therefore, these cells are trapped in the execution of cell death, but fail to complete the process due to the blocked function of effector caspases [18,19,26]. This experimental approach led to the conclusion that the Jun N-terminal kinase (JNK) acts as a central player of AiP in the dying cells. A robust activity of this stress kinase is associated with diverse aspects of tissue regeneration, including the expression of the aforementioned mitogens and the delay of larval development that keeps the animal in the growth phase ([27–28]; reviewed in [29]). Moreover, as dying cells lose their epithelial integrity, JNK-signaling enforces the restitution of an intact epithelium including the formation of actin cables and filopodia in accordance with its well-defined role in the healing of epidermal wounds [30–33].

Intriguingly, JNK-signaling activity is not only associated with emanating proliferative signals from the apoptotic cells, but also with the non-autonomous induction of secondary cell death at a considerable distance from the primary cell death source. This phenomenon was recently termed Apoptosis induced Apoptosis (AiA) and might be the mechanistic explanation for the systemic cell death occurring both in normal development of metazoans as well as in some human pathologies like e.g. neurodegenerative disorders ([34]; reviewed in [35]). Obviously, tissue homeostasis is coordinated by a cross-regulatory relationship of widespread signaling molecules keeping proliferation and apoptosis in a balanced ratio.

The DNA replication-related element-binding factor (Dref)-complex as well as the Notch (N) signaling pathway are both suitable candidates for being part of such a regulatory network, as they are known to govern numerous developmental processes including cell proliferation and apoptosis (reviewed in [36–38]). Dref acts as a transcription factor in *Drosophila* and is proposed to regulate the expression of a multitude of genes required for cell proliferation such as cell cycle regulators, growth factors or DNA replication factors (reviewed in [37]). A similar pleiotropic influence is mediated by the highly conserved N signaling pathway, being reiteratively used during the development of a variety of tissues in higher eumetazoa. Depending on the cellular context, N can either promote or inhibit growth processes emphasizing the

importance of a tight and fine-tuned regulation of the signaling cascade (reviewed in [38,39]). In *Drosophila*, both signaling networks depend on *putzig* (*pzg*), an essential positive regulator of the Dref and N signaling cascades [40,41]. Pzg is an integral component of the Trf2/Dref protein complex that regulates proliferation-related genes [40]. Moreover, Pzg acts Dref independently and promotes N target gene activation via the Nucleosome remodeling factor (Nurf), implying a Pzg-mediated epigenetic influence on N target gene activation [41].

Here, we show that reduction of *pzg* activity during larval wing development results in the induction of genuine dying cells which initiate AiP mechanisms and enable AiA. As expected, ectopic JNK-signaling activity is induced autonomously and non-autonomously, likely to mediate the systemic response. This spectrum of consequences is not mimicked by a downregulation of N receptor activity: Though apoptosis and proliferation are induced, the latter is not mediated by the ectopic induction of *wg* in the genuine dying cells, unlike in the *pzg* depleted cells. In contrast, a downregulation of *Dref* activity does not only provoke apoptosis but also AiP, mediated by the induction of *wg*, similar to the effects observed after *pzg* knockdown. We conclude that Pzg is fundamental for the fine-tuned homeostasis of cell survival and proliferation via its influence on important signaling networks during the development of *Drosophila*.

Materials and Methods

Fly stocks, genetics and work

The following fly stocks were used:

Gal4/UAS lines. EP-*pzg* (EP-756; Exelixis stock collection, USA). Gal4-lines: *en-Gal4* UAS-GFP [42]; *en-Gal4* UAS-GFP UAS-*pzg*-RNAi/CyO [40]; *Gmr-Gal4* [43]; *Gmr-grim/TM3Sb* [9]; *Gmr-hid/CyO* [8]; *Gmr-rpr/TM6B* [44]; *Omb^{md65}-Gal4/FM7* [45]. UAS-lines: UAS-*Dref* [46]; gift from D. Bohmann); UAS-*Dref*-RNAi (BL31941); UAS-*lacZ* (BL8529); UAS-*H*-RNAi [47]; UAS-*N*-RNAi (BL7078); UAS-*p35* (BL6298); UAS-*pzg*-RNAi [40]; UAS-*pzg*-RNAi (VDRC v25542).

Reporter-strains. *dpp-lacZ* [48]; *puc-lacZ* [49]; *rpr-lacZ* [50].

Flies and crosses were raised on standard fly food supplemented with fresh yeast paste at 25°C. Crosses with UAS-RNAi lines were cultured at 29°C to ensure strong RNAi induction.

Antibody staining and documentation with confocal microscopy

Antibody staining on wing imaginal discs was done according to Müller *et al.* [51]. The following antibodies were used: guinea-pig anti-Hairless (H) (1:500) [52]; guinea-pig anti-Pzg (1:500) [40]; mouse anti-Arm (1:50; DSHB); mouse anti-beta Galactosidase (1:50; DSHB); mouse anti-Notch (ICN) (1:25; DSHB); mouse anti-Wg (1:50; DSHB) all obtained from the Developmental Studies Hybridoma Bank, Department of Biological Science, University of Iowa City, IA 52242, USA; mouse anti-DIAP1 (1:400) [13]; rabbit anti-activated Caspase 3 (1:200; Cell Signaling, Germany); rabbit anti-activated Dcp-1 (1:200; Cell Signaling, Germany), rabbit anti-activated Drice (1:250 [13]; rabbit anti-GFP (1:100; Santa Cruz, USA); rabbit anti-Phospho-Histone H3 (PH3) (1:50; Cell Signaling, Germany); rat anti-Dilp8 (1:500 [53]; gift from P. Léopold, Nice, France). Secondary antibodies coupled to fluorescein, Cy3 or Cy5 were purchased from Jackson Laboratories (Dianova, Germany). Dissected tissues were mounted in Vectashield (Vector Laboratories, USA).

For labeling cells undergoing DNA synthesis, the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, Eugene, Oregon, USA) was used. After dissection in ice-cold PBS, larval tissues were incubated for two hours at room temperature in M3 insect medium including 10 mM EdU stock solution. A 4% para-formaldehyde fixative was added for 25 minutes, removed, and washed at least four times with PBS, followed by a 20 minutes incubation with PBX (PBS with

0.3% Triton X-100) and two washing steps for 10 minutes each. The Click-iT reaction cocktail was added containing 443.5 μ l H₂O, 21.5 μ l 10X Click-iT reaction buffer, 10 μ l CuSO₄, 0.5 μ l Alexa Fluor Azide and 25 μ l reaction buffer additive, and the discs were incubated for 30 minutes in the dark. After removal of the reaction cocktail solution, the tissues were washed with PBS two times, prepared and embedded in Vectashield (Vector Laboratories, USA).

Confocal images were acquired with a Zeiss Axioskop linked to a Bio-Rad MRC1024 scan-head by using Bio-Rad Laser Sharp 3.1 software.

Documentation of adult eyes and statistical quantification of eye size

Adult eyes of females were documented with an ES120 camera (Optronics, Goleta CA, USA) using Pixera viewfinder software version 2.0. For quantification, eye size of five females each was measured with Image J and eye area was calculated. Statistical significance of probes was determined according to Student's T-test (<http://www.physics.csbsju.edu/stats/t-test.html>) and p-value was scaled accordingly: $p > 0.05$ (not significant, n.s.); $p < 0.05$ (weakly significant; *); $p < 0.01$ (significant; **); $p < 0.001$ (highly significant; ***).

Results

Pzg genetically interacts with pro-apoptotic genes and is required for cell survival

Knock down of *pzg* gene activity by *pzg*-RNAi induction during larval development results in tissue size reduction (Fig 1A–1C). Previously, we have shown that *pzg*-RNAi effects are negatively correlated with cell cycle progression, thereby influencing cell proliferation and cell growth [40]. In order to test whether tissue loss and size reduction might also be a consequence of the induction of programmed cell death, we performed genetic interaction assays with the well defined apoptosis-inducing factors *hid*, *rpr* and *grim*, whose transcriptional activation is an essential step in the execution of most apoptotic events in the development of *Drosophila* [54–57] (reviewed in [58]). It is well established that ectopic expression of pro-apoptotic genes in the developing eye imaginal disc using the *Gmr* promoter causes cell killing and therefore conspicuously small eyes in the adults (Fig 1D–1F). Eye size reduction was considerably suppressed by additional expression of *Pzg* (Fig 1G–1I and S1 Fig), whereas it was enhanced by *pzg*-RNAi induction within the affected tissue (Fig 1J–1L and S1 Fig).

To address this phenomenon in more detail, we examined the expression of several components of the cell death machinery in wing imaginal discs upon *pzg* depletion. We have chosen the wing disc tissue for our analyses, as there the endogenous level of apoptosis is comparatively low [18,59]. Thus higher levels of apoptosis can be attributed to a reduced *pzg* activity.

Pzg activity was downregulated with the UAS-*pzg*-RNAi shown before to efficiently reduce *Pzg* protein levels [40]. *Pzg*-RNAi induction either in the posterior compartment (using *en*-Gal4) or in a more central area (using *omb*-Gal4) of the wing disc triggered the execution of the apoptotic program: Pro-apoptotic gene activity, visualized by the activation of a *rpr*-lacZ reporter (Fig 2A–A" and S2A–A" Fig), a reduced level of the anti-apoptotic protein DIAP1 (Fig 2B–B" and S2B–B" Fig) and finally the accumulation of activated initiator and effector caspases Drice, Caspase-3 and Dcp-1 were observed in the *pzg* mutant area of the wing disc (Fig 2C–E" and S2C–E" Fig). To exclude, however, off-target effects, a second independent RNAi-line (VDRC v25542) was included in the analysis of apoptosis induction and gave the same overall results. These data show that an impaired *pzg* activity leads to an increase in apoptosis.

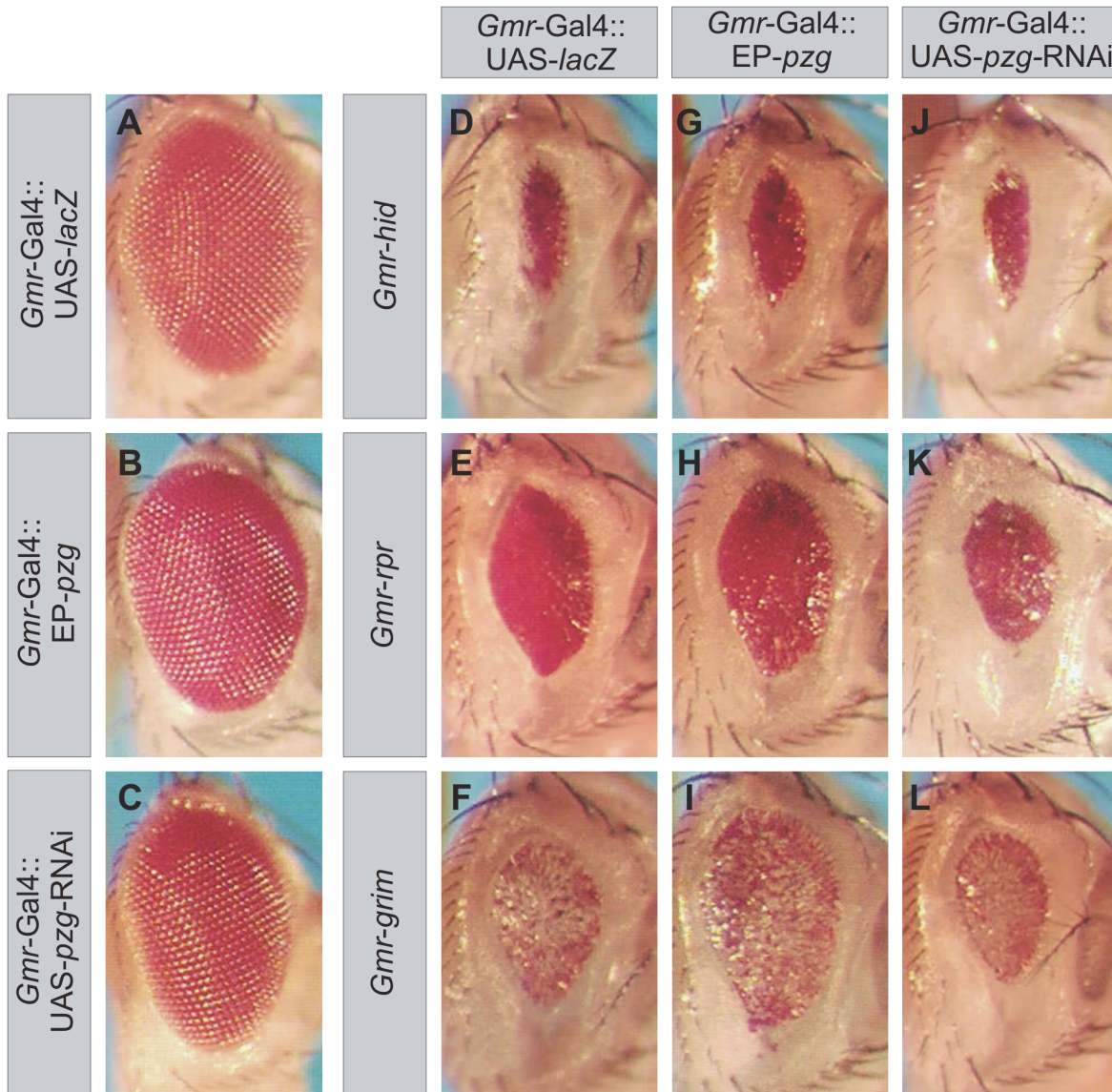


Fig 1. *pzg* genetically interacts with pro-apoptotic genes. (A-C) Control eyes after overexpression of *lacZ* and *pzg*, as well as *pzg*-RNAi induction. (D-F) Overexpression of *hid*, *rpr* and *grim* during eye development causes small and rough eyes in the adults. (G-I) Overexpression of *pzg* ameliorates the small eye size resulting from ectopically expressed pro-apoptotic genes, whereas depletion of *pzg* activity by RNAi enhances the phenotypes (J-L). Genotypes analyzed: *Gmr-Gal4*^{+/+}; *UAS-lacZ*^{+/+}. *Gmr-Gal4*^{+/+}; *EP-pzg*^{+/+}. *Gmr-Gal4*^{+/+}; *UAS-pzg-RNAi*^{+/+}. *Gmr-hid*/*Gmr-Gal4*; *UAS-lacZ*^{+/+}. *Gmr-Gal4*^{+/+}; *Gmr-rpr* or *Gmr-grim*/*UAS-lacZ*. *Gmr-hid*/*Gmr-Gal4*; *EP-pzg*^{+/+}. *Gmr-Gal4*^{+/+}; *Gmr-rpr* or *Gmr-grim*/*EP-pzg*. *Gmr-hid*/*Gmr-Gal4*; *UAS-pzg-RNAi*^{+/+}. *Gmr-Gal4*^{+/+}; *Gmr-rpr* or *Gmr-grim*/*UAS-pzg-RNAi*.

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pzg silencing triggers JNK-mediated stress responses

A key factor known to convey apoptosis in *Drosophila* and mammals alike is the c-Jun N-terminal kinase (JNK) pathway (reviewed in [60,61]). To measure JNK signaling activity in *pzg* mutant cells, we made use of the JNK downstream effector *puckered* (*puc*) and monitored the expression of a *puc-lacZ* enhancer trap line [62]. In wild type third instar wing discs, *puc-lacZ* expression is detected in the small stalk region attaching the disc to the larval epidermis [62]. Knock down of *pzg* robustly activated the *puc-lacZ* reporter gene within the depleted area and weakly in adjacent regions notably in older wing discs (Fig 3A-B" and S2F-F" Fig), consistent

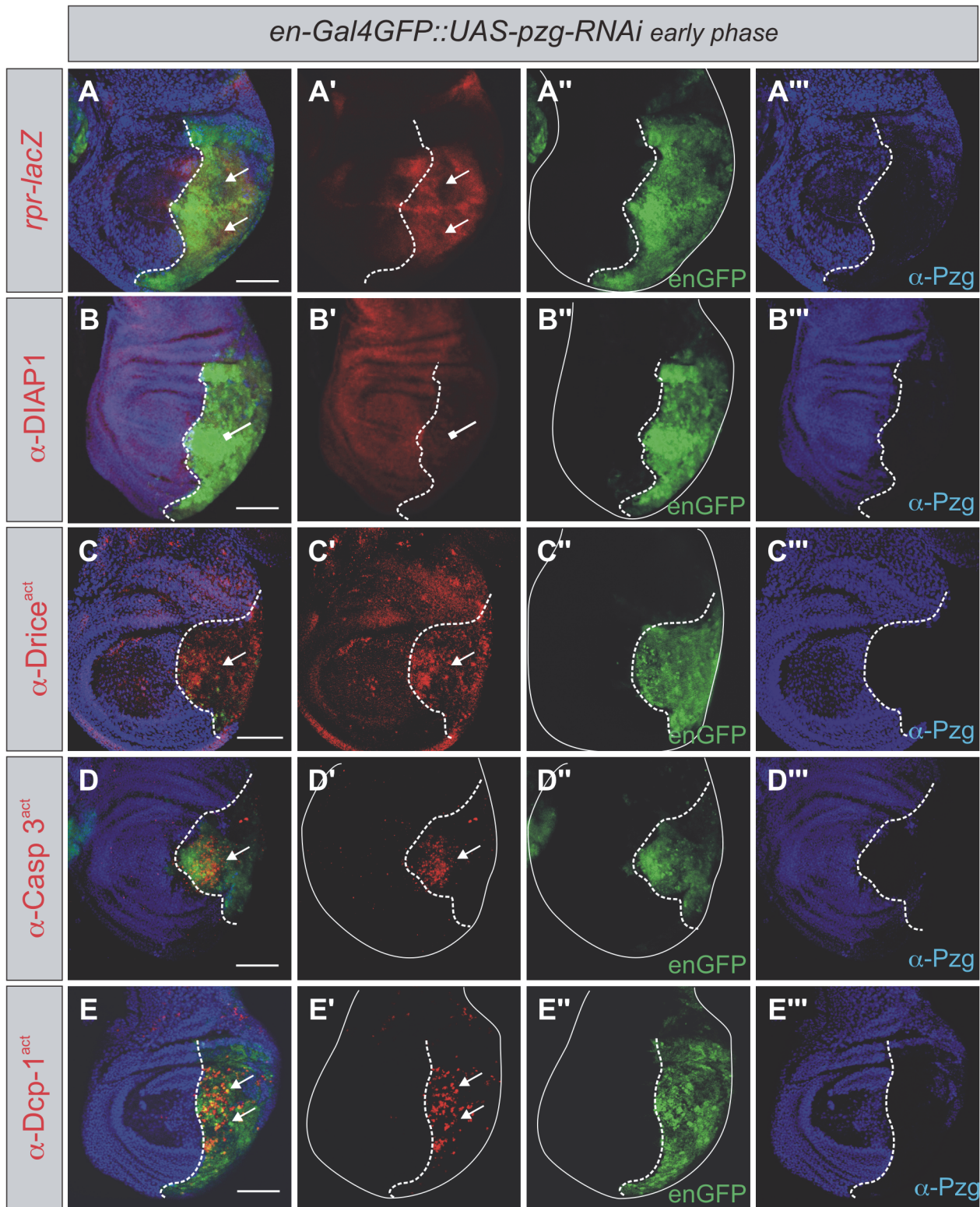


Fig 2. *pzg* depletion autonomously triggers the apoptotic signaling cascade in wing imaginal discs. RNAi mediated depletion of *pzg* was induced in the posterior part of the wing disc using *en-Gal4*. *Rpr-lacZ*, DIAP1 and caspase activity (in red) was monitored as indicated. (A-A''') A strong activation of the pro-apoptotic gene *rpr* (arrows) as well as the *Drosophila* activated caspases Drice^{act} (C-C''', arrow), Caspase 3^{act} (D-D''', arrow) and Dcp-1^{act} (E-E''', arrows) is detected in the posterior half of the disc, whereas DIAP1 protein level is reduced (B-B''', repressive arrow). (A-A''') *en-Gal4* UAS-GFP UAS-*pzg*-RNAi/+; *rpr-lacZ*/+. (B-E''') *en-Gal4* UAS-GFP UAS-*pzg*-RNAi/+. Pzg protein is shown in blue (anti-Pzg, A-E and A'''- E'''); GFP in green (*en-Gal4* GFP) marks

the posterior compartment (A-E and A''-E''). Posterior is to the right and dorsal up. The antero-posterior compartment boundary is marked with a dashed line. Scale bars: 100 μ m.

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with the induction of JNK-mediated cell death. Great experimental insights were gained in the recent years demonstrating that JNK-mediated cell death in *Drosophila* is crucial for eliminating aberrant cells, thereby ensuring further development and morphogenesis (reviewed in [16,29,61]). This includes also the induction of a developmental delay and inhibition of metamorphosis, allowing the larva to compensate growth deficits and repair injured tissue. The gene encoding Dilp8, a member of the insulin-relaxin peptide family, was found to be upregulated in response to JNK signaling and is thought to delay metamorphosis by inhibiting ecdysone biosynthesis [53,63]. Tracing Dilp8 in wing discs, where *ptg*-RNAi was induced in the posterior half, revealed no obvious accumulation in younger discs (approximately 96 h AEL; Fig 3C-C''), whereas Dilp8 was highly enriched in later stages (approximately 120 h AEL; Fig 3D-D'').

Interestingly, the older *ptg* mutant wing discs appeared more crumpled and folded than the younger ones (compare Fig 3A and 3C with 3B and 3D). This phenomenon is reminiscent of regenerative growth during larval development experimentally induced with non-surgical tissue damage, e.g. pro-apoptotic gene activity ([64–65]; reviewed in [66]). Moreover it has been shown that cell-adhesion is reduced by the caspase dependent cleavage of Armadillo (Arm) [67,68]. Indeed, we observed a strong reduction of membrane-associated Armadillo (Arm, beta-Catenin) in the *ptg* silenced area of the wing disc (Fig 3E-E''). In a wild type wing disc, the antero-posterior compartment border is straight and defined. In contrast we observed 'finger-like' structures notably in older *ptg*-silenced discs (Fig 3F-F''). Apparently, *ptg*-depletion enabled the mutant cells to invade the anterior compartment while still retaining posterior identity (Fig 3F-F'').

Loss of *ptg* activity results in 'genuine' dying cells and consequently AiP

Genetic studies in *Drosophila* have shown that different mechanisms are triggered in apoptotic cells leading to an increase in proliferation and cell division rates of adjacent cells, a process which is referred to as Apoptosis induced Proliferation (AiP) (reviewed in [14,17,69,70]). In order to investigate the consequences of *ptg* depletion with regard to AiP, we monitored the proliferation rates in wing discs, where *ptg*-RNAi was induced in the posterior compartment. As *ptg* was shown to be required for the activation of cell cycle related genes [40], the autonomous decrease of actively dividing cells upon *ptg* depletion compared with controls was expected (Fig 4A-B'''). In addition we noted an elevated number of cells undergoing DNA synthesis (EdU labeled) as well of mitotic cells (PH3 labeled) abutting the *ptg*-RNAi mutant territory at the anterior, implying the induction of a non-autonomous cell division response (Fig 4A-B''').

Apoptotic cells ectopically activate morphogenetic genes like *wingless* (*wg*) or *decapentaplegic* (*dpp*), responsible for the proliferation stimulus in directly adjacent cells [18,19]. To test if this is also a response to *ptg* loss, we firstly analyzed the distribution of Wg protein in wing discs, where *ptg*-RNAi was induced in the posterior compartment. At later stages (approximately 120 h AEL) we observed an ectopic expression of Wg in areas of the posterior compartment (Fig 4C', arrows), whereas the wild type expression along the dorso-ventral boundary in the posterior compartment of the disc was interrupted (Fig 4C', arrowhead). As *wg* is a well known target of N signaling, a reduction of wild type Wg accumulation along the dorso-ventral boundary was expected [40]. Intriguingly, no ectopic induction of *dpp* was observed in *ptg* silenced cells, inferred from the normal expression levels of a *dpp*-lacZ reporter along the

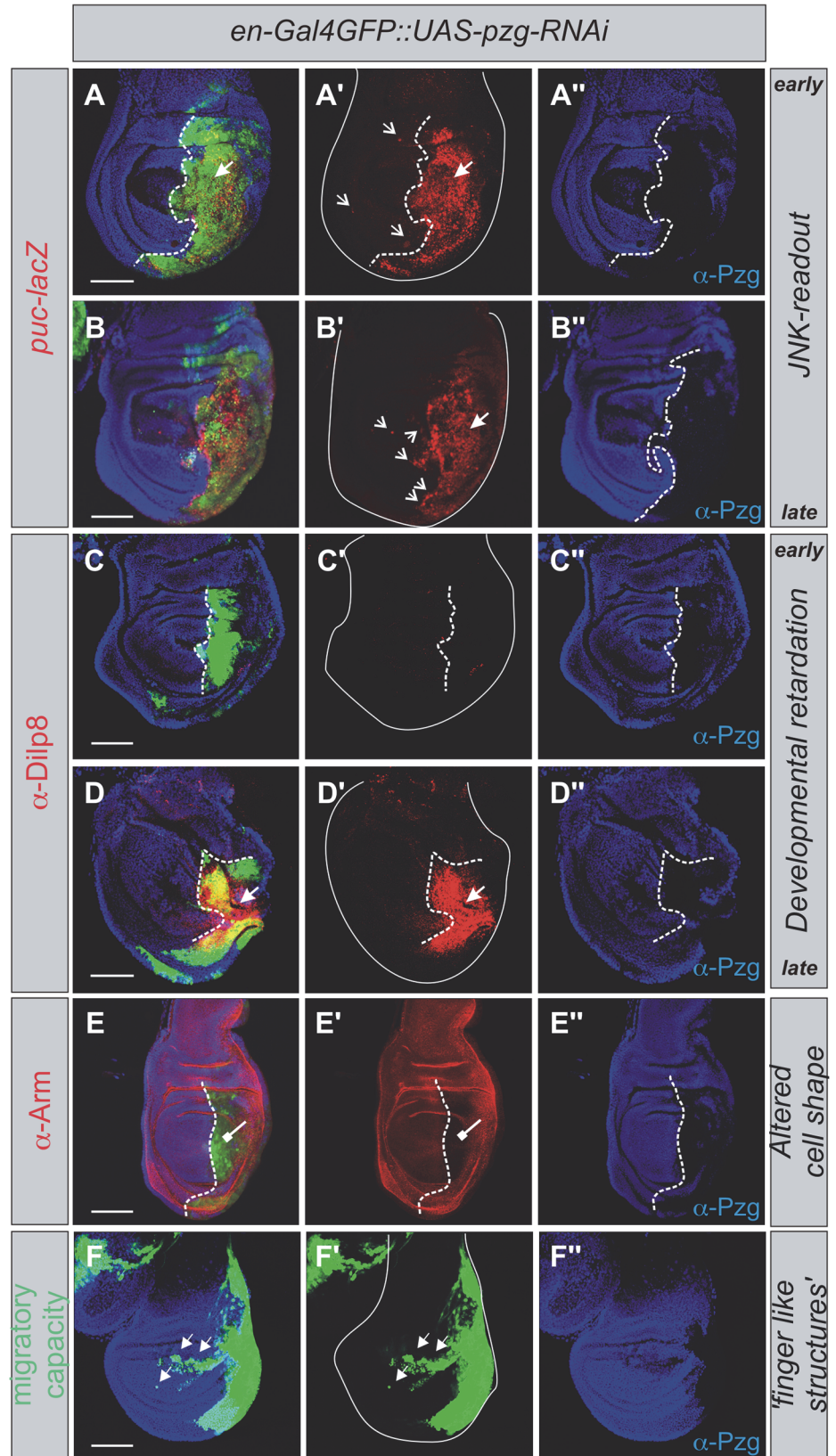


Fig 3. *pzg* depletion results in an inappropriate JNK-signaling activation. *pzg* was downregulated in the posterior part of the wing disc (Pzg protein is shown in blue). (A-B'') *puc-lacZ* activity is induced in *pzg*-RNAi mutant cells reflecting JNK-signaling activity (red in A-A', B-B', arrows) (*en-Gal4 UAS-GFP UAS-pzg-RNAi/+; puc-lacZ/+*). In addition, especially in late third instar larval discs, a non-autonomous activity can be detected in the anterior compartment (open arrows in A', B'). (C-F'') Further consequences of JNK-mediated developmental apoptosis induction can be observed: (C-D'') Dilp8 protein is secreted in the *pzg* depleted cells, however not before late third larval instar (red in D, D', arrow). (E-E'') In *pzg*-RNAi mutant cells, Arm protein accumulation is disturbed (red, repressive arrow). (F-F'') *pzg* mutant cells penetrate into the anterior compartment while retaining their posterior identity (green, arrows in F, F'). (C-F'') *en-Gal4 UAS-GFP UAS-pzg-RNAi/+*. The A/P compartment boundary is marked with a dotted line. Scale bars: 100 μ m.

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antero-posterior compartment boundary (Fig 4D-D''). This demonstrates that *pzg* mutant cells behave like 'genuine' apoptotic cells with respect to the induction of ectopic *wg*, and that they induce AiP without the involvement of ectopic *dpp* activity.

Loss of *pzg* activity is accompanied by Apoptosis induced Apoptosis (AiA)

Interestingly, cells doomed to die not only trigger AiP but also induce non-autonomous secondary apoptosis, abbreviated AiA (Apoptosis induced Apoptosis) [34]. To examine if such an effect is also observed in *pzg*-RNAi mutant cells we followed the activation of Caspase3 and Dcp1 in late third instar larval wing discs. Under these conditions, a strong caspase activity was observed in the anterior half of the disc, either visualized by staining for cleaved Caspase-3 or Dcp-1 (Fig 5A-A'' and 5C-C''). The co-expression of the baculovirus caspase inhibitor *p35* in the *pzg*-RNAi mutant cells strongly enhanced this effect and triggered tumorous overgrowth of the wing discs and non autonomous AiA (Fig 5B-B'' and 5D-D''). This can be explained by the induction of the apoptotic machinery but the prevention of execution through inhibition of effector caspases by *p35*, resulting in so-called 'undead' cells [18,19,26,71]. Altogether, these data indicate that loss of *pzg* activity results in apoptosis followed by AiP and AiA.

Apoptotic consequences of N depletion in wing discs

As Pzg was shown to be required for efficient N signaling to occur, we asked whether the observed apoptotic outcome after *pzg*-RNAi induction might be the consequence of an impaired N signaling activity [40,41]. It is well established that a reduction in N signaling activity during imaginal development is correlated with tissue loss and apoptosis [51,72]. Therefore, we induced *N*-RNAi in the posterior compartment of the wing disc to compare the effects with those obtained after *pzg* depletion. To this end, we used the same *N*-RNAi line that has been shown by others to provide RNAi-mediated knock-down of N activity in wing imaginal discs [38,73]. We observed an accumulation of activated Dcp-1 caspase autonomously in *N* mutant cells and also non-autonomously in the anterior compartment, indicating that a downregulation of N signaling contributes to AiA (Fig 6A-B''). Moreover, a robust induction of *puc-lacZ* was detected in both compartments, indicating that JNK-mediated activity was induced as well (Fig 6C-C''). In contrast, cell proliferation, visualized with EdU labeling, was different from *pzg*-RNAi depleted cells: Cells within the *N*-RNAi depleted compartment were still able to cycle through the cell cycle concluded from EdU incorporation (Fig 6D-D''). Based on the modest reduction of EdU signals, less cells however, appeared to enter the S-phase. Moreover, AiP was only weakly observed as EdU labeled cells abutting the *N*-deficient area appeared only more densely spaced (Fig 6D-D''), and no ectopic induction of the mitogens *Wg* (Fig 6E-E'') or *dpp-lacZ* (Fig 6F-F'') was detected.

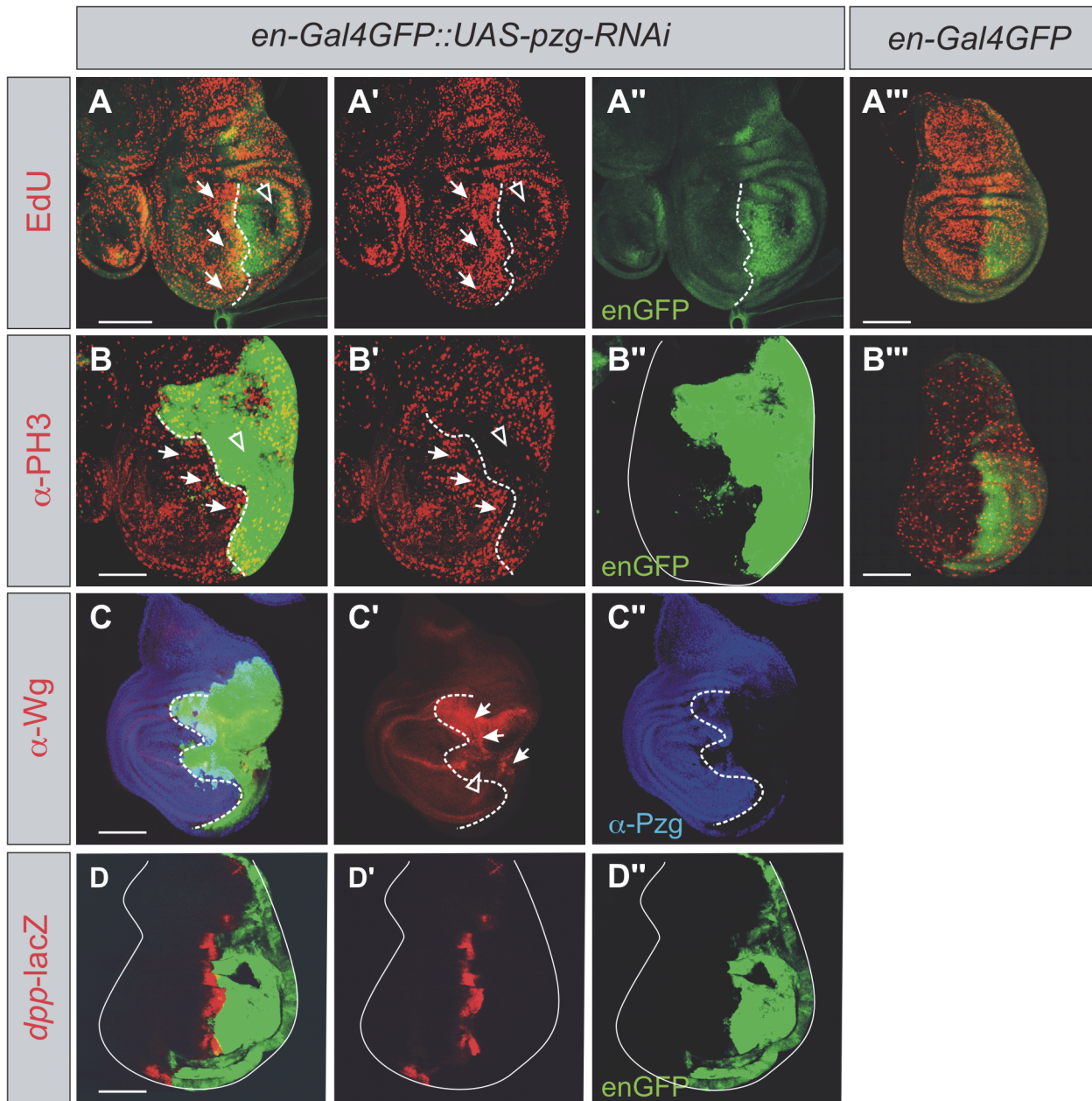


Fig 4. *pzig* mutant cells show characteristics of genuine dying cells and induce AiP. *pzig* was downregulated in the posterior part of the wing disc. Pzig protein is shown in blue (C, C''), the posterior compartment is marked in green with GFP (A-D; A'-D'; A''-B'''). (A-A''') DNA-synthesis visualized with EdU-labeling (red) is amplified anteriorly along the A/P compartment boundary (arrows) upon *pzig* depletion (A-A') compared to *en-Gal4* UAS-*GFP* control (A'''), whereas a loss can be observed within the posterior compartment (open arrow, A-A'). (B-B''') Cell division was visualized with anti-PH3 (red). Compared to the control (B'''), many cells in the posterior domain lost this marker upon *pzig* depletion (arrowhead, B-B'), whereas a strip of cells anterior to the A/P boundary shows stronger PH3 labeling (arrows, B-B'). (C-C'') Expression of Wg protein was monitored (red): it is interrupted at the dorso-ventral boundary in response to *pzig*-RNAi depletion (arrowhead in C'), whereas ectopic induction of Wg is observed in the posterior compartment outside the normal Wg expression domain (arrows, C'). (D-D'') *dpp-lacZ* expression (red) is unchanged by *pzig* depletion in the posterior compartment. Genotypes: (A''-B''') *en-Gal4* UAS-*GFP*+/+. (A-C'') *en-Gal4* UAS-*GFP* UAS-*pzig*-RNAi/+. (D-D'') *en-Gal4* UAS-*GFP* UAS-*pzig*-RNAi/++; *dpp-lacZ*+/+. Wing discs are oriented posterior rightwards and dorsally upwards. The A/P compartment boundary is marked with a dotted line. Scale bars represent: (A, B, D) 100 μ m, (C) 50 μ m.

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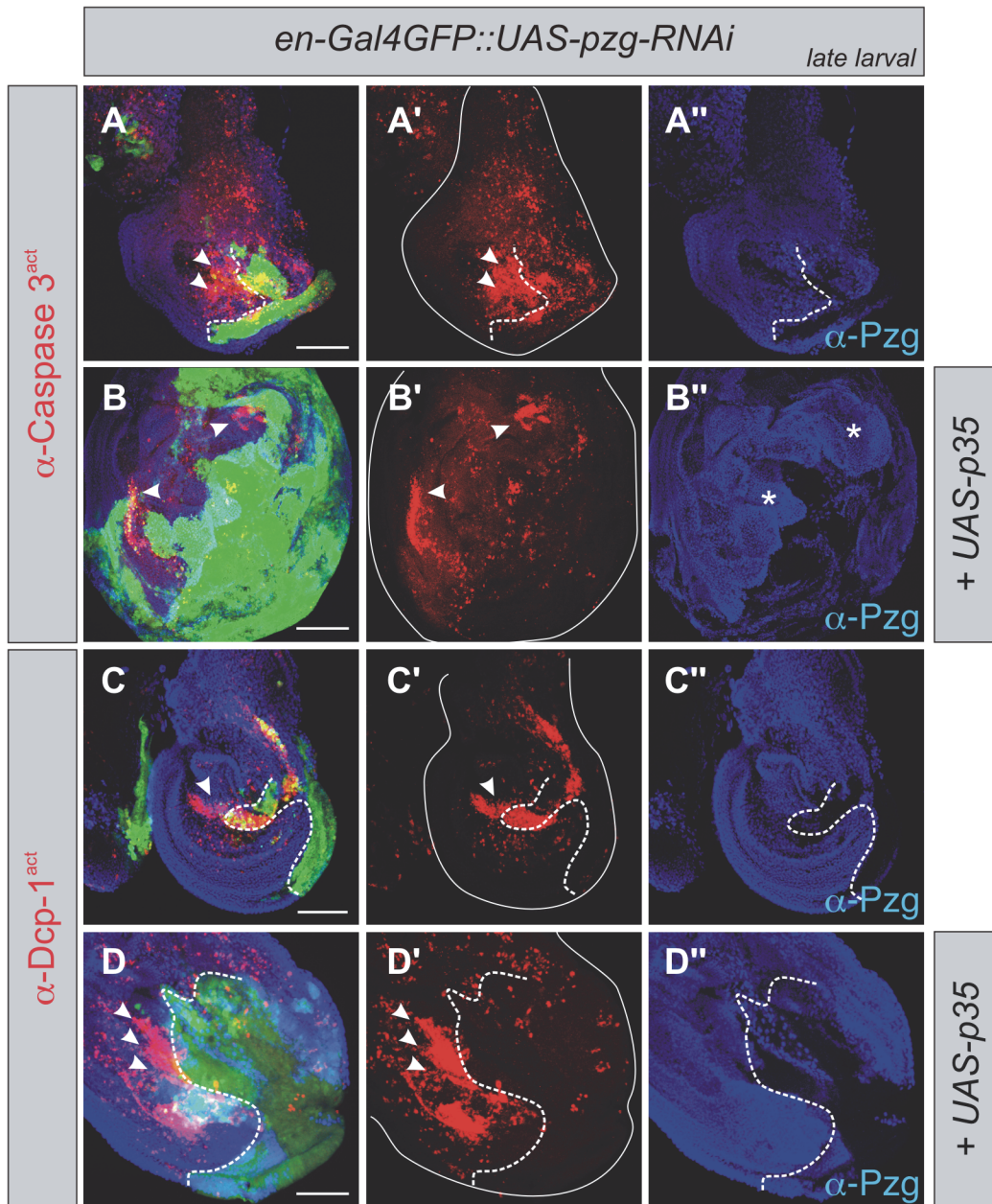


Fig 5. *pzg*-RNAi induces AiA in late larval stages. (A-D'') Strong caspase activity, either visualized with anti-Caspase 3^{act} (red in A-A', B-B') or anti-Dcp-1^{act} (red in C-C', D-D'), can be detected non-autonomously in late larval wing disc in the anterior compartment (arrowheads in A-D'). Preventing cell death execution with p35 amplifies the overgrowth effect (asterisks in B'') but still induces AiA in the anterior half of the disc (B-B'', D-D'', arrowheads). Genotypes: (A-A'') and (C-C'') *en-Gal4 UAS-GFP UAS-pzg-RNAi/+*. (B-B'') and (D-D'') *UAS-p35; en-Gal4 UAS-GFP UAS-pzg-RNAi/+*. The A/P compartment boundary is marked with a dotted line. Scale bars: 100 μ m.

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In order to investigate, whether an upregulation of N signaling activity could ameliorate the apoptotic consequences of *pzg* loss, we initially sought to overexpress the activated form of the N receptor in *pzg*-RNAi depleted cells. However probably due to the immensely hyper-activated N response, we failed to obtain any viable larvae of this genotype. A more gentle method to increase N signaling activity is to reduce the activity of the general antagonist Hairless (H), e.g. by inducing a *H*-RNAi construct proven to reduce *H* activity in the fly [47]. Reduction of *H*

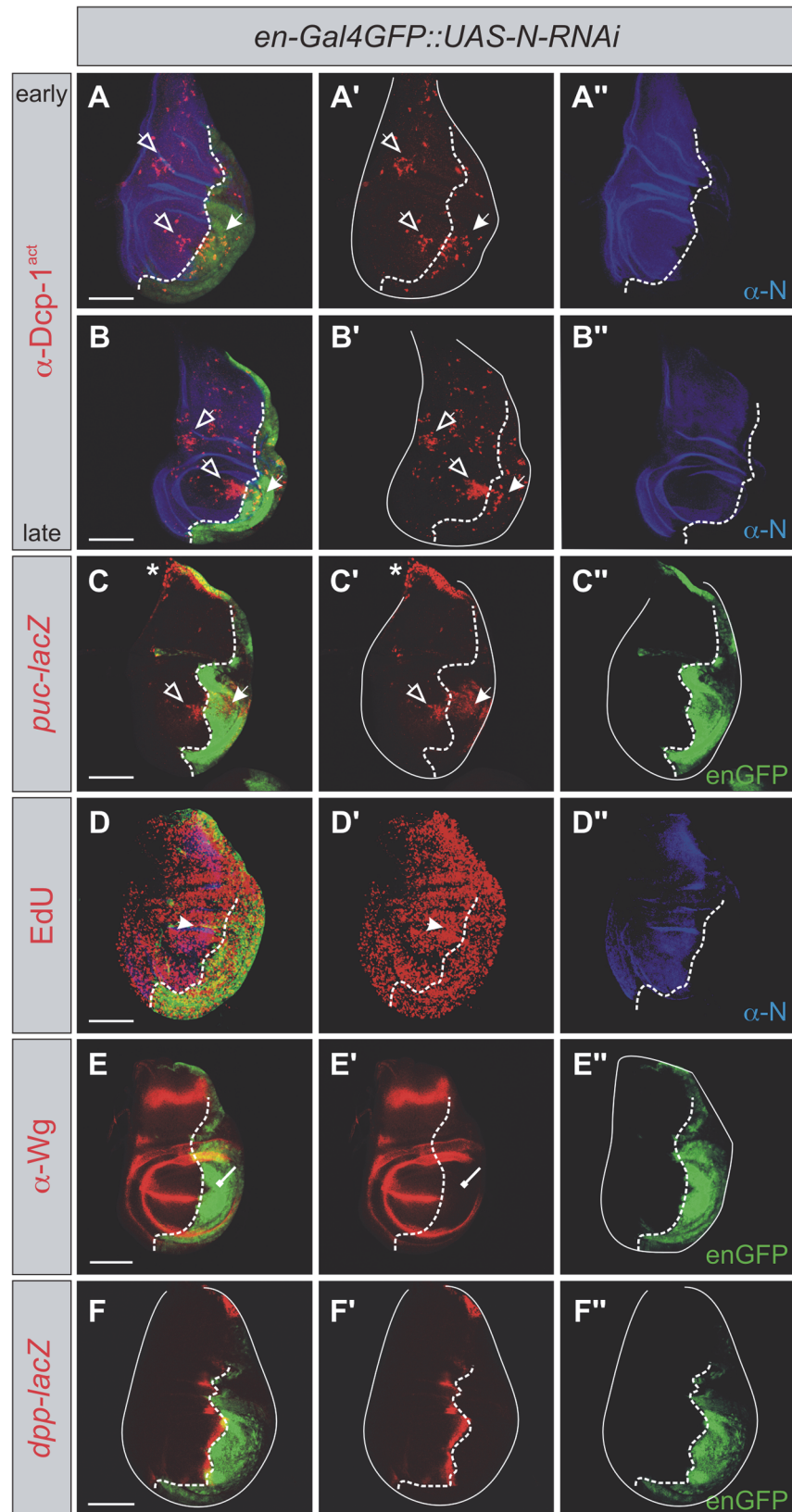


Fig 6. Apoptotic consequences of Notch depletion in wing imaginal discs. *N*-RNAi was induced in the posterior compartment of the wing disc, *N* protein is shown in blue (A, B, D and A', B', D'), the posterior compartment is marked in green with GFP (A-F and C', E', F'). (A-B'') Caspase activity, visualized with anti-Dcp-1act (red in A, A' and B, B') is detectable in early (96 h AEL) and late third instar wing discs (120 h AEL) autonomously (arrows in A, A' and B, B') and non-autonomously (open arrows in A, A' and B, B'). The effect of AiA is enhanced in later phases of development (B, B'). (Genotype: UAS-*N*-RNAi; *en*-Gal4 UAS-GFP/+). (C-C'') JNK-signaling readout visualized with *puc*-lacZ expression is seen in both compartments (red in C, C' arrows; UAS-*N*-RNAi; *en*-Gal4 UAS-GFP/+; *puc*-lacZ/+). The wild type expression of *puc*-lacZ at the stalk region is marked with an asterisk (C, C'). (D-D'') Cells within S-phase are labeled with EdU (red); a minor autonomous reduction and weak increase in most central cells abutting the A/P compartment boundary is observed (arrow in D, D'; genotype as in A). (E-E'') The expression of *Wg* is lost at the dorso-ventral boundary in the posterior compartment (red in E, E', repressive arrow; genotype as in A). (F-F'') *dpp* expression is not affected by downregulation of *N* in the posterior compartment (red in F, F'; UAS-*N*-RNAi; *en*-Gal4 UAS-GFP/+; *dpp*-lacZ/+). The A/P compartment boundary is marked with a dotted line. Scale bars: 100 μ m.

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activity in *pzg*-RNAi mutant cells of the wing disc did not considerably abrogate the *pzg*-RNAi induced apoptosis defects (S3 Fig). These data suggest that the cell death resulting from loss of *pzg* is not primarily triggered by an inappropriate *N* signaling activity.

Apoptotic consequences of Dref depletion in wing discs

Apart from its role in *N* target gene activation, *Pzg* is important for cell proliferation as a member of the Trf2/Dref complex in *Drosophila* [40,74]. The transcription factor Dref regulates the expression of many proliferation-related genes, and has been described as a master key factor for cell proliferation (reviewed in [37]). We hence examined the consequences of *Dref*-RNAi depletion on apoptosis, AiP and AiA. To this end the UAS-*Dref*-RNAi line was used that has been shown before to specifically target *Dref* activity [75]. Inducing *Dref*-RNAi in the posterior half of the wing disc indeed triggered a strong apoptotic response, including robust Dcp1 caspase activation in the anterior half of late third instar wing discs (appr. 120 h AEL), indicative of AiA (Fig 7A-B''). Moreover, JNK signaling, i.e. *puc*-lacZ, was induced autonomously in the affected compartment, and to a lesser degree also in the anterior compartment of the wing disc (Fig 7C-C''). As expected, reduction of *Dref* activity was correlated with impaired cell proliferation as demonstrated by reduced EdU labeling in these areas (Fig 7D-D''). Proliferation rates were, however, significantly increased in the tissue abutting the *Dref* mutant cells (Fig 7D-D''). Moreover, similar to the effects of *pzg*-RNAi depletion, ectopic *Wg* induction was detected in the *Dref*-RNAi mutant tissue (Fig 7E-E''), whereas *dpp* activity appeared unaffected (Fig 7F-F''). As expected from our earlier work, *Wg* expression along the dorso-ventral boundary was not affected by the depletion of Dref, in support of the notion that the effects of *Pzg* on *N* regulation are Dref independent [40, 41]. Overall, induction of apoptosis with all its consequences is very similar upon the depletion of either Dref or *Pzg* during larval wing development. We therefore asked whether an increase of *Dref* activity might ameliorate the apoptotic consequences of *pzg* depletion. To this end we overexpressed UAS-*pzg*-RNAi together with UAS-*Dref* in the posterior wing disc compartment. To account for possible titration effects of Gal4 by the extra UAS-dose, we compared the effects of *Dref* overexpression with those obtained in a *pzg*-RNAi plus UAS-*lacZ* background. We observed a weaker apoptotic response in the UAS-*lacZ* background, (Fig 8). However, a concomitant increase of *Dref* activity ameliorated the *pzg*-RNAi proliferation defects much more robustly and strongly attenuated the induction of activated Dcp-1 caspase (Fig 8). Therefore, although *Pzg* and Dref are part of a large multi-subunit complex, increasing the amount of Dref protein helps to counterbalance the apoptotic effects resulting from *pzg* loss.

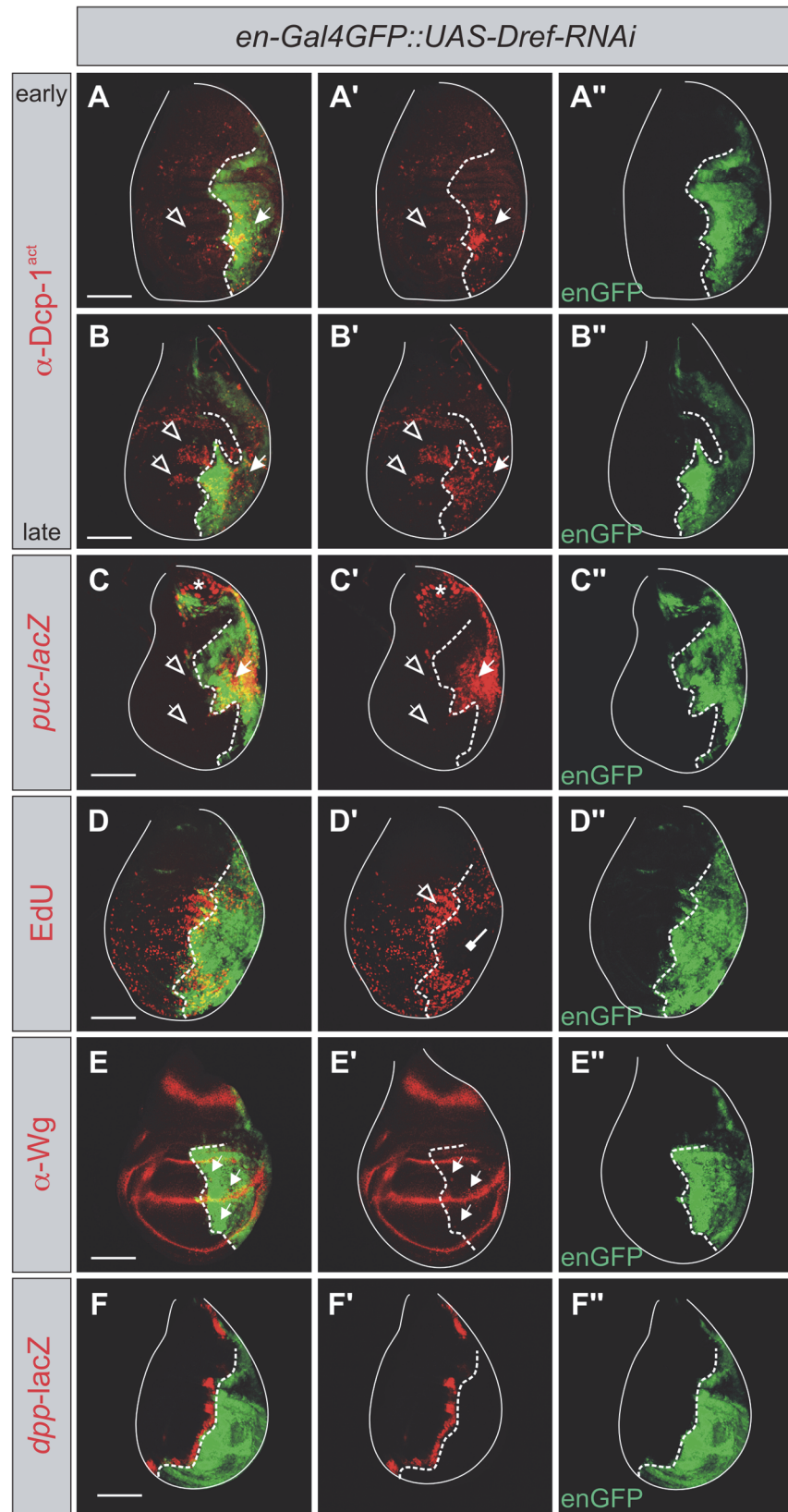


Fig 7. Loss of *Dref* activity entails cell cycle arrest, cell death and AiP. *Dref* was downregulated in the posterior compartment of the wing disc, which is marked in green with GFP (A-F; A''-F''). (A-B'') *Dref*-RNAi induction in the posterior compartment of the wing disc is correlated with an autonomous (arrow) and non-autonomous (open arrow) cell death induction, monitored with anti-Dcp1^{act} (red in A, A' and B, B'). Although AiA can be detected already in early third instar wing discs (96 h AEL, A' open arrow), the amount of non-autonomous cell death is strongly increased in later stages (120 h AEL, B' open arrows). (C-C'') *puc*-lacZ is ectopically induced in both compartments (red in C, C'; closed and open arrows). Asterisk highlights the wild type expression in the stalk region of the disc. (D-D'') Replication is disturbed autonomously after *Dref*-RNAi depletion, visualized with EdU staining (red in D, D', repressive arrow), whereas enhanced proliferation is induced anteriorly (open arrow). (E-F'') Ectopic induction of Wg (red in E, E') can be detected in the *Dref*-depleted compartment (arrows in E, E'), whereas *dpp*-lacZ expression appears unchanged (red in F-F'). Genotypes: (A-B'') and (D-E'') *en-Gal4 UAS-GFP/+; UAS-Dref-RNAi/+*. (C-C'') *en-Gal4 UAS-GFP/+; UAS-Dref-RNAi/puc-lacZ*. (F-F'') *en-Gal4 UAS-GFP/+; UAS-Dref-RNAi/dpp-lacZ*. Posterior is right, dorsal upwards. The dashed line marks the A/P compartment boundary. Scale bars: 100 μm.

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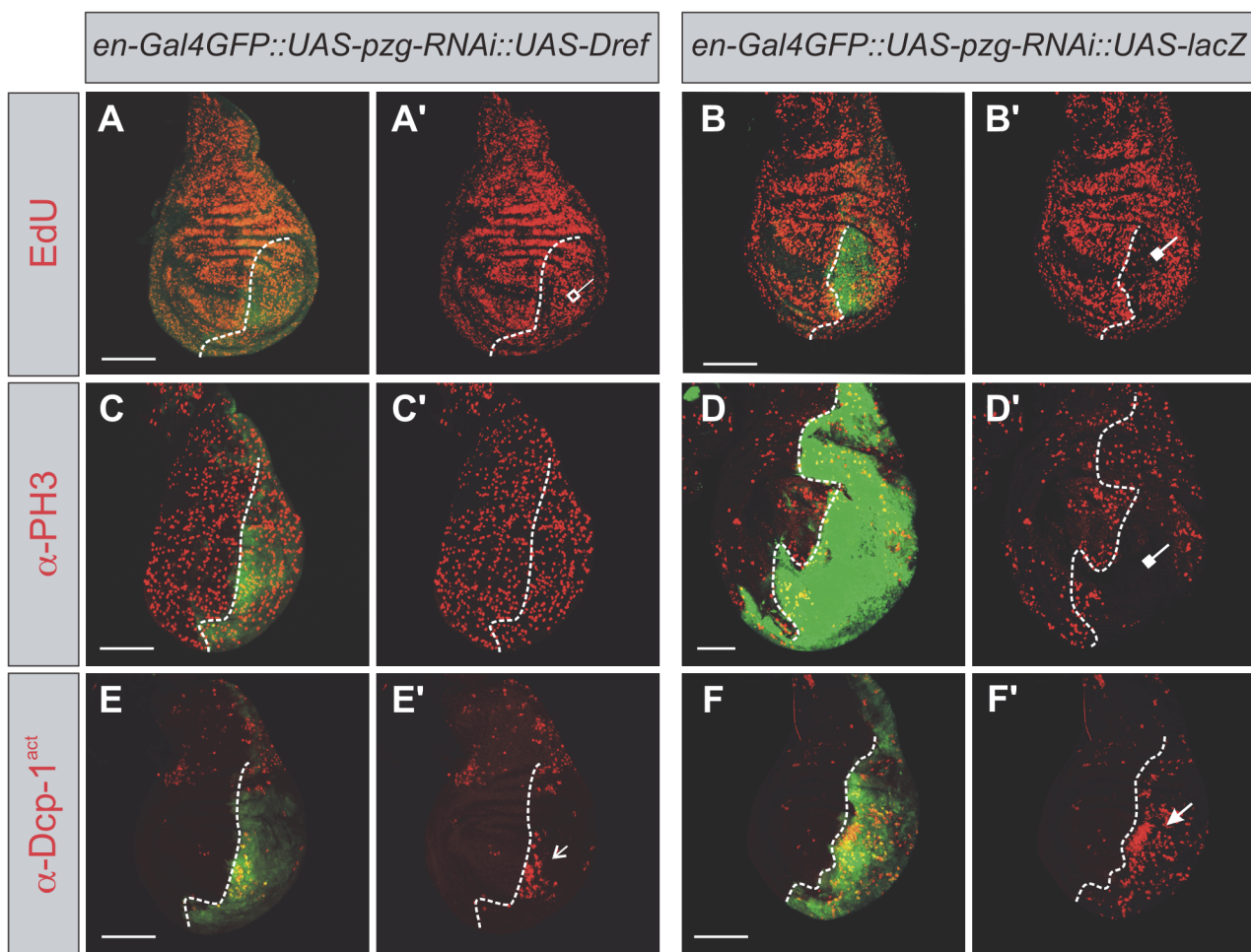


Fig 8. *Dref* overexpression ameliorates *pzg*-RNAi apoptosis defects. Comparison of the apoptotic consequences of *pzg* depletion in the presence (A-E') or absence (B-F') of ectopic *Dref* expression. (A-D') Compared to the control (B,B',D,D'), overexpression of *Dref* in *pzg*-RNAi depleted cells ameliorates the cell cycle arrest defect (A,A',C,C'), visualized either with EdU (S-phase, red in A-B') or anti-PH3 (M-phase, red in C-D'). Repression of cell division by *pzg* depletion (repressive arrow in B', D'), is absent (C') or less pronounced in the *Dref* overexpression background (open repressive arrow in A'). Activation of Dcp-1 (anti-Dcp-1^{act}, red in E-F') upon *pzg* downregulation (arrow in F'), is much weaker when *Dref* is overexpressed (open arrow in E'). Genotypes: (A, A', C, C'; E, E') *en-Gal4 UAS-GFP UAS-pzg-RNAi/+; UAS-Dref/+*. (B, B'; D, D'; F, F') *en-Gal4 UAS-GFP UAS-pzg-RNAi / UAS-lacZ*. Posterior is right and dorsal up. The A/P compartment boundary is marked with a dashed line. Scale bars: 100 μm.

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Discussion

In this work we show that loss of *pzg* induces apoptosis, including the autonomous activation of *rpr* and of various Caspases, of JNK and of Dilp-8 expression. Moreover, we observed non-autonomous apoptosis induced proliferation (AiP) including the induction of Wingless as well as apoptosis induced apoptosis (AiA). Apparently, *pzg* is required for the survival of the cell which in its absence undergoes apoptosis with all its consequences.

Pzg is a nuclear protein found in different multimeric complexes including the Trf2/Dref complex and the NURF complex. The Pzg/NURF complex has been implied in the epigenetic regulation of N and EcR target genes [41,76], whereas Dref is involved in the regulation of replication and proliferation related genes (reviewed in [37]). Both, downregulation of N as well as of Dref induced apoptosis. Whereas the former was expected [51,72], the latter was not since overexpression rather than downregulation of Dref was associated with apoptosis so far [77,78], presumably because Dref overexpression is sufficient to drive terminally differentiated cells into a new cell cycle [78]. Very similar to *pzg* RNAi, downregulation of Dref not only resulted in autonomous JNK- and Caspase activation, but also induced ectopic expression of Wingless, which was not observed as a consequence of N depletion. Moreover, unlike the activation of N signaling activity, overexpression of Dref was sufficient to ameliorate the effects of *pzg* induced apoptosis. Overall we conclude that the apoptosis induced by a depletion of *pzg* is primarily triggered by a disturbance of the Pzg/Trf2/Dref complex. In this case, at least part of the apoptotic consequences may result from a dysregulation of Pzg/Dref target genes involved in cell survival or cell death. For example, the failure to activate anti-apoptotic factors in the *pzg*- and *Dref*-RNAi mutant background might directly induce apoptosis. Amongst the plethora of Dref target genes is the *Drosophila* proto-oncogene *raf* [79]. Indeed it was shown that overexpression of Dref stimulates MAPK signaling activity [46]. MAPK, however, is an important negative regulator of pro-apoptotic gene activity in *Drosophila* [55,80], easily explaining the pro-apoptotic effects of a Dref loss. Moreover, inspection of the promoter region of the *Drosophila* inhibitor of apoptosis (*DIAP1*) gene reveals the presence of several potential DRE sites (S4 Fig). Hence, Dref may support cell survival by activating *DIAP1* under normal circumstances. Future will show whether Dref acts together with Pzg, i.e. whether Pzg binds to the promoters of cell death regulators as well. Dref, however, not only stimulates the expression of anti-apoptotic or survival factors, but it also promotes the transcriptional activation of the apoptosis inducer *dmp53* [81]. Similar to the apparent disparate role of Dref in the regulation of apoptosis, Dref not only promotes cell proliferation by the transcriptional activation of proliferation related genes like Pol alpha or PCNA, but Dref also inhibits proliferation as different members of the Hippo signaling pathway are Dref target genes as well [82,83].

Alternatively to a direct role of Dref and Pzg in the transcriptional regulation of genes involved in cell survival, cell death may arise as a consequence of conflicting signals that result from a collective dysregulation of the numerous Dref target genes. Depletion of *pzg* or *Dref* presumably affects a wide range of Dref-target genes. Dref target sequences have been found in more than 200 *Drosophila* genes, including replication and proliferation related genes, genes involved in growth, development and differentiation as well as components of protein biosynthesis or RNA binding proteins (reviewed in [37]; [84]). Therefore, it appears likely that crippling *pzg* activity, impairs the Dref-mediated cellular homeostasis, and hence the balance between survival and death decisions.

Recent molecular studies in *Drosophila* demonstrated that Pzg protein can be specifically detected at the telomeres of the chromosomes [85]. In *Drosophila*, the maintenance of telomeres is realized by a repeated transposition of retrotransposons instead of the telomerase-dependent extension of other eukaryotes (reviewed in [86,87]). Despite this difference, mutations

that cause dysfunctions of the telomeres give rise to chromosome- and DNA-damage in all eukaryotes, and consequently result in apoptosis and in an increased lethality ([88,89]; reviewed in [90]). *Pzg* mutant animals were shown to suffer from moderate telomere instability, inferred from a significant increase in the in vivo incidence of telomere fusions in anaphase neuroblasts. Telomere fusions were attributed to major chromatin changes causing altered transcriptional activity of the retrotransposon *Het-A* due to the loss of *pzg* activity [91]. In accordance with a functional relationship between *Pzg* and *Dref* in this developmental context, *Dref* mutants show similar alterations in retrotransposon expression [92]. Moreover, specific *Dref* target sequences were identified in the promoters of several retrotransposons in *Drosophila*, implying a direct regulatory function of *Dref* on the retrotransposon activity and telomere elongation [92]. Therefore, apoptosis induction observed in *pzg* and *Dref* mutant tissues might involve excessive retrotransposon activity, destroying the fine-tuned genomic stability.

Supporting Information

S1 Fig. Quantification of eye sizes. Eye size of flies was determined in five females of each combination shown in Fig 1. Average eye area is shown in each column. The ordinate shows the percentage of eye area relative to the respective control (left column each, light grey, 100%). Error bars denote standard deviation. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; ns: not significant according to Student's T-test.

(DOC)

S2 Fig. Induction of *pzg*-RNAi with *omb*-Gal4 provokes cell death. *pzg*-RNAi application in the most central part of the wing disc with *omb*-Gal4 induces *rpr*-lacZ (red in A, A', arrows), activated Drice (red in C, C', arrows), activated Caspase 3 (red in D, D', arrow), Dcp-1^{act} (red in E, E', arrows) and *puc*-lacZ (red in F, F', arrows). In contrast, the level of the anti-apoptotic protein DIAP1 is reduced (red in B, B', repressive arrows). (A-A'') *omb*-Gal4; UAS-*pzg*-RNAi/+; *rpr*-lacZ/+, (B-E'') *omb*-Gal4; UAS-*pzg*-RNAi/+; (F-F'') *omb*-Gal4; UAS-*pzg*-RNAi/+; *puc*-lacZ/+. Anti-Putzig staining is shown in green. Posterior is right and dorsal up. The affected area is outlined. Scale bars: 100 μ m.

(DOC)

S3 Fig. Reduced *H* activity still induces apoptotic effects in *pzg*-RNAi mutant cells. Reducing the activity of the N repressor *Hairless* (*H*) formally enhances N activity but does not rescue the apoptotic consequences observed in *pzg*-RNAi mutant cells. (A-B'') Autonomous induction of Dcp-1^{act} (red in A, A', arrow) can be detected in wing discs app. 96 h AEL, whereas additional non autonomous Dcp-1^{act} activity is provoked in later stages (B, B', open arrows). (C-D'') Cell cycle progression is still autonomously impeded in *pzg*-RNAi depleted cells (cells in S-phase marked with EdU-labeling red in C, C', repressive arrow and cells in M-phase depicted with anti-PH3, red in D, D' repressive arrow). Enhanced proliferation in cells directly abutting the posterior compartment is still observed (open arrows in C' and D'). Anti-H staining is shown in blue (A'', B'', C'', D'') depicting loss of H protein by induction of *H*-RNAi. Posterior is right and dorsal up. The dashed line assigns the A/P compartment boundary. Scale bars: 100 μ m.

(DOC)

S4 Fig. Potential DRE sites in *Diap1*. According to flybase (R6.03; FB2014_06, released November 12th, 2014), there are 6 strongly supported transcripts *Diap1* RA-RE, transcribed from 5 different promoters (<http://flybase.org/cgi-bin/gbrowse2/dmel/?Search=1;name=FBgn0260635>). Potential DRE sites are marked with arrows and listed below. Transcript RB starts only 363 bp downstream of RE; they may share the DRE sites. No DRE sites were found

in the proximity of the RA/RE transcription start. Dref regulation of RC appears less likely due to sequence divergence and distance of DRE.
(DOC)

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Author Contributions

Conceived and designed the experiments: ACN SJK. Performed the experiments: MZ SJK AS ACN. Analyzed the data: MZ SJK ACN. Contributed reagents/materials/analysis tools: MZ SJK AS ACN. Wrote the paper: ACN.

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