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Genetic Models of Apoptosis-Induced Proliferation Decipher Activation of JNK and Identify a Requirement of EGFR Signaling for Tissue Regenerative Responses in Drosophila

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Abstract
Recent work in several model organisms has revealed that apoptotic cells are able to stimulate neighboring surviving cells to undergo additional proliferation, a phenomenon termed apoptosis-induced proliferation. This process depends critically on apoptotic caspases such as Dronc, the Caspase-9 ortholog in Drosophila, and may have important implications for tumorigenesis. While it is known that Dronc can induce the activity of Jun N-terminal kinase (JNK) for apoptosis-induced proliferation, the mechanistic details of this activation are largely unknown. It is also controversial if JNK activity occurs in dying or in surviving cells. Signaling molecules of the Wnt and BMP families have been implicated in apoptosis-induced proliferation, but it is unclear if they are the only ones. To address these questions, we have developed an efficient assay for screening and identification of genes that regulate or mediate apoptosis-induced proliferation. We have identified a subset of genes acting upstream of JNK activity including Rho1. We also demonstrate that JNK activation occurs both in apoptotic cells as well as in neighboring surviving cells. In a genetic screen, we identified signaling by the EGFR pathway as important for apoptosis-induced proliferation acting downstream of JNK signaling. These data underscore the importance of genetic screening and promise an improved understanding of the mechanisms of apoptosis-induced proliferation.

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Introduction
Apoptosis is the major form of programmed cell death. It is used during development and under stress conditions to remove excess, unwanted or damaged cells. Deregulated apoptosis can give rise to malignancies including cancer and neurodegeneration [1]. A central step for the execution of apoptosis is the activation of caspases, a family of cysteine-proteases that are ubiquitously expressed as inactive zymogens [2]. There are two different types of caspases. Initiator caspases are incorporated into multimeric complexes such as the apoptosome [3] in response to developmental signals, cellular stress and injury. The initiator caspase complex cleaves and activates effector caspases which then proteolytically process a large number of cellular proteins inducing the death of the cell.

Caspases are very well conserved in the animal kingdom. Of the seven caspases in Drosophila, only the initiator caspase Dronc and the two effector caspases DrICE and Dcp-1 have been implicated in apoptosis in imaginal discs [4–12]. Caspases are negatively regulated by inhibitor of apoptosis proteins (IAP) which directly bind to processed caspases and inhibit their activity [13]. Drosophila IAP1 (Diap1) binds to and inhibits Dronc, DrICE and Dcp-1 [14,15]. In cells committed to die, IAP-antagonists such as Reaper, Hid and Grim [16–18] promote ubiquitin-mediated degradation of Diap1, thus releasing Dronc, DrICE and Dcp-1 from Diap1 inhibition [19–23]. Dronc associates with the scaffolding protein Ark (ApaF-1 related killer) to form the apoptosome which triggers activation of DrICE and Dcp-1.

Developing organisms have the ability to compensate for massive apoptotic cell loss by inducing compensatory proliferation. For example, developing Drosophila imaginal discs can form a normal-sized and patterned organ even after more than 50% of their cells have been killed by X-ray treatment due to compensatory proliferation [24]. Surprisingly, work in Drosophila, and later in hydra, Xenopus, planarians, newt and mice, has revealed that apoptotic caspases may be the driving force for compensatory...
proliferation in apoptotic tissue [12,25–35] (reviewed in [36–38]). Because this regenerative proliferation requires apoptotic caspases, it has been termed Apoptosis-induced Proliferation, henceforth referred to as AiP [38,39].

There are two commonly used experimental models that study AiP in larval imaginal discs, usually wing and eye discs in Drosophila. The first type of model takes advantage of the fact that another caspase inhibitor, the P35 protein from Baculovirus, specifically referred to as AiP [38,39]. It has been termed Apoptosis-induced Proliferation, henceforth referred to as AiP [38,39].

Much of what we know about AiP came from studies of ‘undead’ cells. In ‘undead’ cells, Dronc activates in ‘undead’ cells and can fulfill non-apoptotic roles including AiP which triggers overgrowth [12,25–28] which may be relevant for tumorigenesis. More recently, P35-independent models of AiP have been described [42–44]. In these models, apoptosis is temporally induced followed by analysis of the events leading to replacement of the dying tissue. Because they mimic the conditions of normal regenerative growth, we referred to them as ‘genuine’ AiP models [45].

The ey>hid-p35 model

eyless (ey) is a regulatory gene for eye development and is expressed during the growth phase of eye imaginal discs [57]. With the move of the morphogenetic furrow (MF) in 3rd instar larvae, ey expression ceases in and posterior to the MF [57]. Therefore, co-expression of hid and p35 during the growth phase of the eye disc using ey-Gal4 (referred to as ey>hid-p35) may provide a convenient model to induce AiP. Correspondingly, in eye imaginal discs, the anterior portion of the eye disc is overgrown compared to controls forming an expanded head capsule due to increased cell proliferation [29] (Figure 1A,B,C,D). Additional ocelli and bristles are observed (Figure 1D, arrow). The anterior overgrowth is at the expense of posterior tissue (Figure 1A,B) which specifies the retina. As a result, eyes are smaller than wild-type and often absent (Figure 1E,F). In eye discs, we use ELAV labeling which labels photoreceptor neurons, as a marker to assess the extent of anterior overgrowth and distortion of the eye disc (Figure 1B,G). We refer to these phenotypes as AiP phenotypes. The small eye tissue is likely due to the expansion of Wg expression anterior to the MF (Figure 1G,H) which is an inhibitor of MF progression [58]. We also observed anterior expansion of dpp-lacZ expression [29]. Finally, expression of the JNK marker puc-lacZ and TRE-Red [59] are strongly expanded anterior to the MF (Figure 1I,J; Suppl. Figure S1). Therefore, known markers of AiP are induced in the ey>hid-p35 model which therefore may represent a convenient AiP model for genetic screening.

The ey>hid-p35 model requires the apoptosome components dronc and ark, but is independent of effector caspases

To test the feasibility of the ey>hid-p35 model for genetic screening, we first examined if mutants and RNA interference
(RNAi) of caspases and ark genetically modify the AiP phenotype. Heterozygosity of dronc and dronc RNAi strongly suppressed the AiP phenotype (Figure 2A,B). Under these conditions, more than 95% of the flies display completely normal eye and head morphology. Heterozygosity of the apoptosome component ark also suppresses the AiP phenotype to a similar extend (Figure 2C). Therefore, these data demonstrate that the ey>hid-p35 model is sensitive to genetic alterations and extend previous findings that AiP not only requires dronc, but also ark, i.e. a functional apoptosome.

Because this type of AiP is dependent on effector caspase inhibition by P35, it was inferred that it does not require effector caspases [25,27,28]. However, it was recently suggested that despite P35 inhibition, effector caspases may still be active at low levels in ‘undead’ cells [60]. This low level effector caspase activity may be insufficient to induce apoptosis, but sufficient to trigger non-apoptotic functions such as invasive behavior of ‘undead’ cells [60]. To test this possibility for AiP, we further reduced DrICE and Dcp-1 activity by double RNAi due to the redundancy of these two effector caspases [8]. However, in contrast to the invasive behavior of ‘undead’ cells [60], the AiP phenotype was not suppressed by dcp-1;drICE double RNAi (Figure 2D). The RNAi stocks used are functional as dcp-1;drICE double RNAi suppresses hid activity in a different apoptotic model, GMR-hid (Suppl. Figure S2). In summary, the overgrowth of the ey>hid-p35 model is dependent on the apoptosome.
components Dronc and Ark, but independent of effector caspases.

Identification of JNK pathway components involved in AiP

It is unknown how the apoptosome induces JNK activity for AiP. To obtain further insight into this question, we tested components of the JNK pathway in a pilot RNAi screen for modification of the ey\textsuperscript{>hid-p35} model. As expected, RNAi targeting bsk, the JNK ortholog in Drosophila, completely suppresses the AiP phenotypes in more than 90% of the flies (Figure 2E,F,G). Downstream of JNK, RNAi knockdown of the components of the AP1 transcription factor, jun-related antigen (jra) and the Fos ortholog kayak (kay), also suppressed the ey\textsuperscript{>hid-p35} AiP phenotypes, although to a lesser extent (Figure 2F,G) suggesting that they are at least partially required for AiP.

To identify upstream components in the JNK pathway involved in AiP, we tested RNAi lines targeting all known components in the JNK pathway [52]. Interestingly, only a subset of them were found to suppress the AiP phenotypes (Figure 2F,G). This includes

Figure 2. Modification of the ey\textsuperscript{>hid-p35} phenotype by JNK pathway components. (A–E) dronc (A) and ark (C) heterozygosity strongly suppresses the ey\textsuperscript{>hid-p35} phenotype (compare to Figure 1D). RNAi targeting dronc (B) and bsk (E) also strongly suppresses it. Double RNAi targeting dcp-1 and drICE (D) has no effect. (F) Results of the suppression of ey\textsuperscript{>hid-p35} using RNAi targeting components of the Bsk/JNK pathway in Drosophila. Only select members of the Bsk/JNK pathway (dTRaf2, Rho1, dTAK1, dMKK4, Bsk and to a weaker extent hep, Jra and kay) show suppression. Each RNAi analysis was repeated at least twice with scoring more than 50 ey\textsuperscript{>hid-p35}/dsRNA adult flies. (G) Schematic summary of the suppression analysis of the Bsk/JNK pathway. Pathway components highlighted in red show RNAi-mediated suppression and are thus required for ey\textsuperscript{>hid-p35}-induced proliferation. (H–J) The GMR\textsuperscript{>eiger}-induced eye ablation phenotype (H) is strongly suppressed by dTRAF2 RNAi (I), but not by Rho1 RNAi (J). (K) GMR-Gal4 driven RNAi targeting Rho1 does not cause an eye ablation phenotype. This control experiment shows that failure of Rho1 RNAi to suppress GMR\textsuperscript{>eiger} (J) is not due to a secondary effect.

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the JNKKK dTak1 and the JNKKs hemipterous (hep) and Mkk4 (Figure 2F,G). The non-redundant functions of hep and Mkk4 for AiP is puzzling, but has been previously reported in different contexts [61,62]. Further upstream in the JNK signaling pathway, we only identified Traf2 (also known as Traf6) as AiP suppressor (Figure 2F,G). Another regulator of JNK signaling, the small GTPase Rho1 [63–66], was also identified as AiP suppressor. In contrast, the two ligand/receptor systems known to activate JNK, Eiger/Wengen and Pvf/PVR, do not suppress AiP (Figure 2F,G).

The RNAi lines against these genes are functional as shown in Suppl. Figure S3 and in [67–69]. Theoretically, it is possible that the suppression of AiP by these RNAi transgenes is an indirect result of suppression of apoptosis, as observed in the case of dorne and ark mutants or RNAi (Figure 2B,C; Suppl. Figure S4B). To exclude this possibility, we labeled ey>hid-p35 eye imaginal discs expressing these RNAi constructs with cleaved Caspase-3 (C3a) antibody, a marker of Drone activity [70], and ELAV antibody to evaluate rescue of disc morphology. Despite the rescue of disc morphology, C3a labeling is not significantly suppressed by these RNAi constructs (Suppl. Figure S4C–H) suggesting that the suppression of the AiP phenotype by reducing JNK activity is not due to suppression of caspase activity.

Because Rho1 is the least well characterized regulatory component in the JNK pathway, we examined the effect of Rho1 knockdown on JNK activity in the AiP model. Loss of Rho1 suppresses puc-lacZ in ey>hid-p35 eye discs (Suppl. Figure S5A,B). Rho1 RNAi also suppresses the AiP marker Wg (Suppl. Figure S5C,D). These data show that Rho1 acts genetically upstream of JNK in the AiP model consistent with previous reports [63–66].

To further place Rho1 into the AiP pathway and to relate it to Traf2, we examined the ability of Rho1 and Traf2 to suppress GMR-eiger, a known inducer of JNK activity causing a strong eye ablation phenotype (Figure 2H) [71,72]. Interestingly, while Traf2 knockdown effectively suppresses GMR-eiger as reported [73], Rho1 RNAi does not (Figure 2I,J). It is possible that Rho1 RNAi disrupts eye development by itself and that may be the reason for the failure to suppress GMR-eiger. However, Rho1 RNAi does not disrupt eye development (Figure 2K). These observations suggest that the role of Rho1 for JNK activation is independent of Eiger which is also consistent with the observation that Eiger knockdown does not suppress AiP (Figure 2F,G). Furthermore, these data raise the possibility that Traf2 serves as an integration point of both Eiger signaling and AiP for JNK activation. For these reasons, we place Rho1 upstream of Traf2 in the AiP pathway (Figure 2G), but there may also be other ways by which Rho1 controls JNK activation.

Activation of JNK signaling in ‘undead’/dying cells and neighboring, surviving cells

The lack of a requirement of Eiger/Wengen and Pvl/PVR in our AiP model (Figure 2F,G) may suggest that activation of JNK occurs in dying cells. However, conflicting data have been reported about the location of JNK activity in various AiP models. Initially, JNK signaling was observed in ‘undead’ cells [27]. In a p35-independent regeneration model, it was reported that JNK signaling occurs only in neighboring surviving cells [43]. More recently, JNK activity was reported to be both in dying and neighboring surviving cells [44]. While there are experimental differences between these studies, none of them used a mosaic approach to determine the location of JNK activation. Therefore, to clarify this issue, we re-examined both ‘undead’ and ‘genuine’ AiP models for location of JNK activity.

In a mosaic ‘undead’ model, we expressed hid and p35 in clones in eye and wing discs using a FLP-out approach and analyzed puc-lacZ expression as JNK reporter. GFP was used to mark hid;p35-expressing clones. Using this approach, puc-lacZ is predominantly expressed in hid/p35 expressing cells (Figure 3A,B; arrows). However, we also noted a few examples where puc-lacZ was expressed in GFP+ tissue (Figure 3A,B; arrowheads). These observations suggest that JNK activation occurs largely in ‘undead’ cells, but also in neighboring, normal cells.

To address this question in a ‘genuine’ (p35-independent) AiP model, we repeated the experiments by Bergantinos et al. (2010) [43] in wing imaginal discs. These authors reported JNK activity in neighboring surviving cells only. We induced hid in a temporally and spatially controlled manner using ptc-Gal4 and tub-Gal80° (ptc>hid) by temperature shifts for various times. In control experiments, just expressing GFP in the ptc domain does not affect the puc-lacZ pattern (Figure 3C,C'). However, when hid expression was induced, depending on the conditions, different results were obtained regarding the location of JNK activity. In response to a short pulse (6 hours) of hid expression followed by a 6 hours recovery period (ts6hR0h), an elevation of puc-lacZ activity was detected in dying cells and neighboring, surviving cells (Figure 3E', F', G'), i.e. in the ptc domain which had been exposed to hid 6 hours earlier, but have survived for unknown reasons. Similar results were observed in Figure 3E: the surviving cells inducing puc-lacZ are located in the GFP region, i.e. in the ptc domain (Figure 3E', F', G'; arrowheads). Thus, it is not clear whether JNK activity in surviving cells is induced autonomously in response to hid expression, or by a signaling event from the dying Cas3* positive cells. In any case, these data show that both in ‘undead’ and ‘genuine’ AiP models, JNK activity can be detected in ‘undead’/dying cells as well as in neighboring, surviving cells.

Identification of spi as AiP suppressor

A systematic mutagenesis screen for genes involved in AiP has not been performed to date due to absence of a convenient screening assay. However, the data presented in Figure 2 demonstrate that suppression of ey>hid-p35-induced AiP provides a convenient assay for genetic screening. Therefore, as proof of principle, we screened a total of 106 chromosomal deficiencies deleting segments on the left arm of chromosome 2 (2L) for modification of the AiP phenotype and identified four chromosomal segments as dominant AiP suppressors and seven deficiencies as dominant AiP enhancers (Table 1; Suppl. Table S1), validating the deficiency approach. Enhancers display an even stronger AiP phenotype with severely overgrown head cuticle and strong semi-lethality.

To identify the genes in the deficiencies that dominantly cause the suppression of AiP, we tested available mutants and UAS-RNAi stocks against all genes that map to these deficiencies. This approach has been completed for Df(2L)ED1303 (Table 1) and led to the identification of spi (spi) as a potential regulator of AiP (compare Figure 4C–F with Figure 4A,B). spi encodes the EGF ortholog in Drosophila [74]. Therefore, our deficiency screen raises
Figure 3. Location of Bsk/JNK signaling in ‘undead’ and ‘genuine’ AiP models. puc-lacZ was used as JNK activity marker. hid expressing areas are marked by GFP. Arrows indicate JNK activity in ‘undead’/dying cells, while arrowheads mark JNK activity in surviving cells. A double arrow in (C–F) marks the ptc domain. (A,B) Location of JNK signaling in ‘undead’ AiP models by mosaic analysis. Clones expressing hid and p35 were induced by FLP-out technology. In eye (A) and wing (B) imaginal discs, puc-lacZ expression is mostly induced in hid/p35-expressing clones (arrows in A’ , A”, B’, B”, B”). However, a few examples of puc-lacZ expression are noted in cells outside of hid/p35-expressing clones (arrowheads). (C–F) Location of JNK activity in a ‘genuine’ AiP model in wing imaginal discs. hid expression was under control of ptc-Gal4 and tub-Gal80ts (ptc;;ts). hid expression was under control of ptc-Gal4 and tub-Gal80ts (ptc;;ts). A temperature shift (ts) to 30°C for the indicated amount of time during 3rd larval instar induced hid expression. After the indicated recovery period (R), discs were labeled for GFP (to visualize the ptc domain), Cas3* (the death domain) and b-Gal (puc-lacZ, i.e. JNK activation). The ptc domain is outlined by white, dotted lines. Note that the death domain does not completely overlap with the ptc domain (see for example E9, E900 and F9, F900). (C–C900) A control disc just expressing GFP under the experimental conditions to visualize the normal puc-lacZ pattern (b-Gal). (D–D900) An experimental disc that was dissected immediately after a 6 hours pulse of hid expression without recovery (ts6hR0h). While caspase activity has been induced (D9), the puc-lacZ pattern is mostly unaffected (D90). (E–E900) An experimental disc that was allowed to recover for 6 hours after a 6 hours pulse of hid expression (ts6hR6h). Although apoptosis is now strongly induced (F9), it is not detectable in the entire ptc domain (GFP, F9). Nevertheless, there is an increase of puc-lacZ expression in cells outside of the death domain (F”; arrowheads). However, these cells reside in the ptc expression domain (F”;F”). Genotypes: (A,B) hs-FLP/UAS-hid; UAS-p35/act-γ”>Gal4 UAS-GFP; puc-lacZ/+; (C) ptc-Gal4 tub-Gal80ts/+; UAS-GFP/puc-lacZ. (D–F) UAS-hid/+; ptc-Gal4 tub-Gal80ts/+; UAS-GFP/puc-lacZ.

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the hypothesis that the EGFR pathway regulates AiP. Consistently, heterozygosity of Egfr suppresses the ey>hid-p35 phenotype in eye discs (compare Figure 4G–I with Figure 4A,B). We also found that Egfr RNAi suppresses an AiP model in wing imaginal discs (F46) (Suppl. Figure S6). Downstream of EGFR, mutant alleles of the Drosophila ortholog of Ras (Dra) and MAPK (rolled (r)) act as dominant suppressors of ey>hid-p35 (Figure 4I) suggesting that MAPK activity is required for AiP. These data imply that EGFR/Ras/MAPK signaling is essential for AiP in both eye and wing discs. These findings are exciting giving the controversy of the role of Wg and Dpp for AiP (see Introduction)

Table 1. Deficiencies that modify the ey>hid-p35-induced AiP phenotype as suppressors or enhancers.

| Suppressors of ey>hid-p35- induced Overgrowth | Chromosomal Location | Enhancers of ey>hid-p35- induced Overgrowth | Chromosomal Location |
|---------------------------------------------|----------------------|-------------------------------------------|----------------------|
| Df(2)L1C14A, Df(2)L1D136                   | 22F4-23A2            | Df(2)L1D123                                | 22D1-22D3            |
| Df(2)L1D206, Df(2)L1S17                   | 23C4-23C5            | Df(2)L1B56                                | 26D3-26E1            |
| Df(2)L1E7014, Df(2)L1B5C28               | 23C4-23C5            | Df(2)L1B56                                | 26D3-26E1            |
| Df(2)L1B5C31                               | 23E5-23F3            | Df(2)L1D508                                | 28C1-28C4            |
| Df(2)L1W137                               | ?                    | Df(2)L1E611                                | 29B4-29C3            |
| Df(2)L1E7013, Df(2)L1D1272               | 37F2-38A2            | Df(2)L1E7048                               | 31E3-31F4            |
|                                           |                      | Df(2)L1E1050                              | 35C1-35D1            |
|                                           |                      | Df(2)L1E7080                               | 38F5-39A2            |

The indicated chromosomal location is the smallest overlap of overlapping deficiencies. Df(2)L1W137 is marked with a ’?’ because other overlapping deficiencies do not suppress AiP (see Suppl. Table S1) indicating that the Df(2)L1W137 chromosome carries a suppressor mutation independent of the deficiency.

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Characterization of ‘genuine’ AiP in the eye imaginal disc: the DE9>hid model

Finally, we tested if genes identified in the ‘undead’ (P35-dependent) AiP model are also involved in ‘genuine’ (P35-independent) regeneration in the eye disc. To accomplish this we used a similar approach as previously described in wing discs [42–44]. hid expression was spatially restricted to the dorsal half of the eye disc by dorsal eye-Gal4 (DE-Gal4) [77] and controlled by Gal80+ [78] by a transient temperature shift (ts) to 30°C for 12 hours (Figure 6E). We refer to this system as DE9>hid. This model also induces GFP to label hid-expressing cells. Before and after the temperature shift, animals were incubated at 18°C (Figure 6E) to inhibit Gal4 activity and therefore hid and GFP expression. Note that although GFP is expressed only during the 30°C pulse, it is a rather stable protein and can be detected in control discs 72 h later (Figure 6D).

In experimental discs immediately after the 30°C pulse (recovery 0 hours – R0 h), a strong apoptotic response is detectable (Figure 6A′) which causes tissue loss and disruption of the bilateral symmetry of the disc 24 hours later (R24 h). In extreme cases, this treatment can result in ablation of the entire dorsal half (Figure 6B, asterisk), but usually some dorsal tissue remains. At that time, many cells are still Cas3*-positive [+], have been replaced by new, JNK-activating cells. This assumption is directly confirmed by the observation that kek-lacZ activity, a downstream marker of EGFR signaling, and Cas3+ labeling as apoptotic marker do not overlap (Figure 5F, arrows). In summary, these data imply that spi expression occurs downstream of Bsk/JNK activity and that EGFR signaling acts in signal-receiving, proliferating cells.

To determine the position of Spi/EGFR signaling in the AiP pathway, we performed epistasis experiments between spi and bsk. Heterozygosis of spi dominantly suppresses the adult AiP phenotype of ey>hid-p35 (Figure 4C,D,I). This suppression can also be visualized by the normalization of the ELAV pattern in ey>hid-p35 eyes (Figure 5A,B). In addition, we found that a target gene of the EGFR pathway, kekkan-1 (kek) [76] is induced during AiP (Figure 5D,E,F).

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The ‘escape’ response of these ‘genuine’ apoptotic cells is reminiscent of the invasive behavior of ‘undead’ cells in wing discs which move out of the

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First, we examined if JNK activity is induced in the DE<sup>u</sup>/hid model. Consistent with the ‘genuine’ AiP models in the wing [42–44], this was indeed observed. TRE-dsRed as marker of JNK activity [59] peaked at 6 h after recovery (R6 h) and is still detectable at R12 h (Suppl. Figure S7B,C). It is mostly gone after 24 h recovery (Suppl. Figure S7D). TRE-dsRed is confined to the GFP<sup>+</sup> area, i.e. in the death domain (Suppl. Figure S7B,C).

Second, we determined if bsk is genetically required for tissue regeneration in the DE<sup>u</sup>/hid model. We used the photoreceptor pattern (ELAV) as a marker to reveal disc outline and thus assess the degree of regeneration. Control discs (DE<sup>u</sup>/hid) at R72 h had completely regenerated (Figure 7A; n = 30). However, if bsk was inactivated by RNAi during the apoptosis-inducing phase (Figure 6E), about 35% (9 of 25 discs) of the discs show incomplete regeneration (Figure 7B). The incomplete regeneration after bsk RNAi is weak, presumably because the 12 h down-regulation of bsk during the temperature shift is not sufficient to completely remove Bsk activity. It is also possible that Bsk is resynthesized quickly during the recovery period. Nevertheless, the incomplete regeneration after bsk RNAi suggests that Bsk is at least partially required for tissue regeneration after DE<sup>u</sup>/hid-induced tissue loss.

Next, we examined whether Spi/EGFR signaling is activated in the DE<sup>u</sup>/hid model. spi-lacZ expression is induced in the ablated GFP<sup>+</sup>-expressing dorsal domain of the disc compared to controls (Figure 7F,F' G,G'). kck-lacZ as EGFR signaling marker is also strongly induced in the dorsal domain compared to controls (Figure 7H,H' L,L'; arrow).

To determine if spi is genetically required in the DE<sup>u</sup>/hid regeneration assay, we inactivated it by RNAi during the 30°C temperature shift, following the protocol in Figure 6E. In a control experiment, because spi is required for photoreceptor differentiation posterior to the morphogenetic furrow [79,80], we tested if a 12 h spi RNAi treatment followed by 72 h recovery (R72 h) affects normal photoreceptor differentiation. However, eye discs treated in this way have a normal ELAV pattern (Figure 7C,C'; n = 20). After this control experiment, we tested for a genetic requirement of spi for regeneration of lost tissue due to hid expression. Strikingly, the regeneration response as judged by ELAV labeling was partially impaired when spi was inactivated by RNAi during hid induction (Figure 7D,D'; arrow). All experimental discs (n = 30) showed incomplete regeneration. The regeneration is only weakly affected, likely because spi is inactivated by RNAi only during the 30°C pulse during hid expression (Figure 6E) and is likely restored soon after reducing the temperature to 18°C. Nevertheless, in a heterozygous spi condition, the discs also incompletely regenerated after DE<sup>u</sup>/hid treatment (Figure 7F,F'; N = 20). In summary, because spi RNAi and spi heterozygosity cause a partial failure to regenerate, these data imply a requirement of spi for regeneration in the ‘genuine’ DE<sup>u</sup>/hid AiP model. Furthermore, these data support the notion that genetic screening using the simpler ‘undead’ AiP model can lead to identification of genes that may also have important roles for regeneration in ‘genuine’ AiP.

**Discussion**

Apoptosis-induced proliferation (AiP) appears to be a mechanism by which developing organisms replace dying cells under stress conditions and initiate regenerative responses [reviewed by [37,45]]. In this paper, we described two AiP models in the developing Drosophila eye. The ‘undead’ ey<i>&gt;</i>hid-p35 model generates a hyperplastic overgrowth phenotype. To date this is the only known phenotype that provides a robust and convenient assay for genetic screening and identification of novel regulators of
Figure 5. Epistasis analysis of *spi* and *bsk*. Arrowheads indicate the morphogenetic furrow (MF) which separates the anterior (left) from the posterior eye tissue visualized by ELAV labeling. (A) *spi-lacZ* pattern (β-Gal; red in A; gray in A') in *ey>*-p35 control discs. Note there is little expression anterior to the MF. (B,C) Because the *spi-lacZ* allele (*spi*01068) is a suppressor of *ey>*-hid-p35 adult overgrowth phenotype (Figure 4I), there is variation in the β-Gal pattern. About 25% of the eye discs show strong induction of *spi-lacZ* in the anterior portion of the eye disc (B,B'; arrows) with a strong reduction of the eye field (ELAV). The remaining 75% of the eye discs show a suppressed, largely normal β-Gal and ELAV pattern in *ey>*-hid-p35 larvae (C,C'). This ratio corresponds to the suppression of the adult overgrowth phenotype (Figure 4I). (D,E) Strong induction of *kek-lacZ* (β-Gal; red in D,E; gray in D,E') in *ey>*-p35 eye discs (E; arrows) compared to *ey>*-p35 control discs (D). (F,F') *kek-lacZ* (β-Gal; red in F, gray in F') is preferentially induced in patches of tissue adjacent to areas with high levels of active caspases (arrows, Cas3* in green). (G,G') Heterozygosity of *spi* normalizes the eye field (ELAV, green), but does not suppress ectopic *puc-lacZ* expression (β-Gal; red in G, gray in G') in *ey>*-hid-p35 eye discs (arrows, compare to Figure 1J). Dotted white lines outline the region anterior to the MF. (H,H') Expression of *bsk* in *ey>*-hid-p35 discs normalizes the eye field (ELAV, green) and suppresses ectopic *kek-lacZ* expression (β-Gal; red in I, gray in I'); compare to E). The analysis in G, H and I strongly suggests that *spi* acts genetically downstream of *bsk*. doi:10.1371/journal.pgen.1004131.g005

Figure 6. Characterization of ‘genuine’ AIP in the eye imaginal disc: the *DEts-green* model. *hid* expression was under control of dorsal eye-(DE-)Gal4 and tub-Gal80<sup>ts</sup> (DEts-hid). A temperature shift (ts) to 30°C for 12 h during 2<sup>nd</sup> larval stage induced *hid* expression (E). After the indicated recovery period (R), discs were labeled for GFP (to visualize the DE expression domain), Cas3* (the death domain) and ELAV (to outline the shape of the disc). (A–C) DEts>*hid* experimental discs. *hid* expression induces a strong apoptotic response (A) causing strong tissue loss after 24 h recovery in some discs (panel B; R24 h, asterisk). After 72 h recovery (R72 h), the disc has fully recovered and has a normal photoreceptor pattern as judged by ELAV labeling (C). Please note the strong reduction of GFP intensity which suggests that most of the GFP<sup>+</sup> cells have been replaced by new GFP<sup>+</sup> cells. Arrow points to the region of new cells which are moving to the center of the disc. (D) A control disc 72 h after DEts-induced GFP expression. Please note that GFP is a very stable protein that can still be detected 72 h after synthesis. (E) The protocol of the DEts>*hid*-induced tissue ablation followed by recovery periods. (F,F',F'';G,G',G'') PH3-labeling of control (DEts>*GFP, F,F') and experimental discs (DEts>*hid, G,G'). GFP marks the outline of the DE domain (F',G'). (H) Quantification of the number of PH3-positive cells in dorsal and ventral compartments of control (F) and experimental discs (G). n = 40 for each genotype. doi:10.1371/journal.pgen.1004131.g006
AiP. In contrast, we have not identified a similar robust and convenient phenotype that would allow direct screening for genes involved in ‘genuine’ (p35-independent) AiP and regeneration. Nevertheless, we developed the DEts\hid model to verify genes identified in the ‘undead’ screen as being involved in ‘genuine’ AiP and regeneration.

Although the use of p35 to keep dying cells in an ‘undead’ condition may be considered as unphysiological and artificial, to date all genes identified under p35-expressing conditions such as JNK, Wg and Spi, were also found to be involved in AiP in p35-independent models [42,43] (this study). Furthermore, cancer cells may resemble ‘undead’ cells. They often initiate, but cannot execute the apoptotic program due to genetic loss or inactivation of effector caspases or other apoptotic components [81–84]. Such ‘undead’ cancer cells may contribute to tumor growth. Therefore, our p35-expressing AiP model could provide insights into new regulators of AiP as well as how impaired apoptosis may promote tumor growth.

Apoptotic caspases play a critical role for AiP. In Drosophila, the initiator caspase Dronc is required for activation of JNK activity which triggers AiP. However, it is unknown how Dronc activates JNK for AiP. Using RNAi, a specific subset of components in the JNK pathway were identified as required for AiP. The most upstream genes in the JNK pathway are Rho1 and Traf2. Traf2 appears to be an integration point for Eiger- and AiP-induced JNK activation, the latter one being mediated through Rho1 (Figure 2G). However, it is unknown how Dronc triggers Rho1 activation. It is unlikely that Dronc proteolytically cleaves Rho1 for two reasons. First, Rho1 does not contain a putative Dronc cleavage site [40,85]. Second, a proteolytic cleavage is likely to destroy Rho1; however, our genetic analysis implies that Rho1 function is required for AiP (Figure 2, Suppl. Figure S5). Therefore, it remains unknown how Dronc triggers Rho1 and thus JNK activation.

Interestingly, extracellular signaling pathways (Eiger/Wengen and Pvf/PVR) known to activate JNK [52] did not score as

Figure 7. Requirement of bsk and spi for complete regeneration in the ‘genuine’ AiP model DEts\hid. (A, A’) DEts\hid discs treated following the protocol in Figure 6E fully recover after 72 h (R72H). n = 30. (A’) shows the ELAV-only channel. (B, B’) About 35% of DEts\hid discs expressing UAS-bsk RNAi do not completely recover after 72 h. n = 25. The arrow in (B’) highlights the incomplete ELAV pattern on the dorsal half of the disc indicating that the regeneration response was partially impaired by reduction of bsk activity. Please note that this disc has also been labeled for GFP. (C, C’) A control eye disc expressing UAS-spi RNAi under DEts-control following the protocol in Figure 6E. After 72 h recovery, the obtained ELAV pattern in the dorsal half of the eye disc is largely normal (red in C, gray in C’), n = 20. (D, D’) An experimental DEts\hid eye disc that was simultaneously treated with spi RNAi. The arrow in (D’) highlights the incomplete ELAV pattern on the dorsal half of the disc indicating that the regeneration response was partially impaired by reduction of spi activity. 30 out of 30 discs show incomplete regeneration. Please note that this disc has also been labeled for GFP. (E, E’) An experimental DEts\hid eye disc that was heterozygous for spi01068. Similar to (D), the ELAV pattern is incomplete on the dorsal half of the disc (E’, arrow), n = 20. (F, F’, G, G’) spi-lacZ pattern in control (DEts\GFP; red in F, grey in F’) and experimental discs (DEts\hid red in G, grey in G’) at 24 h after recovery. The arrow in (G’) points to the increased β-Gal pattern in the dorsal half of the disc. Blue is Cas3*. (H, H’, I, I’) kek-lacZ pattern in control (DEts\GFP; red in H, grey in H’) and experimental discs (DEts\hid; red in I, grey in I’) at 30 h recovery. The arrow in (I’) points to the increased β-Gal pattern in the dorsal half of the disc. Blue is Cas3*.

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suppressors of AiP, suggesting that Drone may autonomously activate JNK activity. This is also consistent with our observation that JNK activity occurs largely in hid- and p35-expressing clones (Figure 3). Nevertheless, it is also possible that a third extracellular signal is generated by ‘undead’ cells in a Drone-dependent manner that triggers JNK activity in an autocrine and/or paracrine manner. The observation that in both ‘undead’ and ‘genuine’ AiP models, JNK activity is also detectable in neighboring surviving cells (Figure 3) may support such a mechanism. Further work is necessary to reveal the exact mode of JNK activation by ‘undead’/dying cells.

In the ‘genuine’ (p35-independent) AiP model (ptc>hid), JNK activity is detectable in both dying and surviving cells. However, the surviving cells with increased JNK activity are also present in the ptc domain (Figure 3E,F) which was exposed to hid expression during the temperature shift. JNK activity is also restricted to the death domain in the DE>hid model (Suppl. Figure S7). Therefore, it is unclear whether a signaling mechanism from dying cells induces JNK activity in surviving cells, or whether the previous hid induction accounts for the JNK activity in surviving cells. It is also unclear how these cells survive. Even after a 16 h pulse of hid induction causing a strong apoptotic response in a large fraction of cells in the ptc domain, some cells survive (Figure 3F). They may receive survival signals from cells outside of the ptc domain, but that needs to be determined. These are interesting questions to be addressed in the future.

We have tested signaling pathways known to be involved in growth control for modification of the ey>hid-p35 model. One example is the Hippo/Warts/Yorkie pathway [96,87]. However, neither mutants of this pathway nor transcriptional reporters (e.g., lacZ) scored positive in the ey>hid-p35 model (data not shown). Therefore, at least in the eye disc, not every pathway involved in growth control is also involved in AiP. These observations stress the necessity to perform unbiased genetic screens aimed at identifying the genes and mechanisms involved in AiP.

Therefore, we performed a pilot screen for modifiers of the ey>hid-p35 AiP model using deficiencies of chromosome arm 2L. We identified four deficiencies as suppressors and three as enhancers (Table 1). Identification of AiP enhancers implies that there is also negative regulation of AiP. In one case we identified spi, encoding the Drosophila EGF ortholog, as a suppressor of AiP suggesting an involvement of EGFR signaling for AiP. This is further confirmed by the strong transcriptional induction of spi and an EGFR target gene, kekkon, in our AiP model. We also found that EGFR signaling is involved in an ‘undead’ AiP model in the wing and – more importantly – in the ‘genuine’ DE>hid regeneration model in the eye. The latter finding is crucial as it demonstrates that genes identified in the ‘undead’ screen may be relevant players for ‘genuine’ regeneration in response to apoptotic tissue loss. An involvement of EGFR and MAPK for regeneration is not ‘genuine’ regeneration in response to apoptotic tissue loss. An involvement of EGFR and MAPK for regeneration is not.

Mosaic analysis

Larvae of the following genotype were heat shocked for 15 min at 37°C, raised at room temperature for 48 h before they were analyzed at the late 3rd instar larval stage. Genotype: hs-FLP/UAS-hid; UAS-p35/act>ptc-hid+; tub-Gal80d+/+. These larvae were scored positive for hid expression by induced temperature shift to 30°C for the indicated amount of time (Figure 3) or for 12 hours (Figure 6E). After the indicated recovery periods at 18°C, discs were dissected and analyzed as indicated in the panels.

Identification of Spi/EGFR signaling as suppressor of AiP was unexpected because EGFR signaling negatively regulates the apoptotic activity of hid [91,92]. Thus, by reducing EGFR activity, hid has increased apoptotic activity which is expected to induce even more AiP. Therefore, the AiP phenotype should be enhanced by heterozygosity of EGFR pathway components. However, the identification of spi, Egr, Dras and it as suppressor of AiP suggests that EGFR signaling is also required for AiP. One possibility to explain these two opposing functions of EGFR (negative regulation of hid and positive requirement for AiP) may be the exclusive appearance of Cas3-positive areas and areas with EGFR activity (Figure 3F). Accordingly, while the Spi signal is generated in Cas3-positive, apoptotic areas, it signals to neighboring Cas3-negative, surviving areas to inactivate Hid and promote proliferation.

The identification of Spi/EGFR signaling may help to resolve a controversy about the signaling pathways involved in AiP. The exact roles of Wg and Dpp for AiP are unclear [26,44,46] and signaling by the EGFR pathway may contribute to the proliferative response in AiP.

Recently, a genetic screen has been reported aimed at identification and characterization of genes required for compensatory growth [93]. These authors induced apoptosis conditionally using a temperature-sensitive cell lethal mutant (sec3). Under normal conditions, the ablated tissue is replaced by new tissue due to compensatory proliferation. The authors then screened for mutants that fail to renew the lost tissue [93]. This was done in a clonal screen for chromosome arm 2L, the same chromosome arm we screened in the deficiency screen in our model. However, the genes identified in the sec3 screen [93] do not map to the deficiencies that we have identified in our analysis (Table 1). Because it is unknown if sec3-induced compensatory proliferation requires caspase activity in apoptotic cells [93], it is not clear if this is a model of apoptosis-induced proliferation.

In summary, we have developed and tested the feasibility of the ey>hid-p35 model for genetic screening. We are confident that this model will close gaps in our understanding of AiP regulation under p35-expressing conditions and in p35-independent regeneration. Finally, it will have implications for the understanding of tumorigenesis by ‘undead’ as well as ‘genuine’ apoptotic tumor cells [94].
PH3 labelling and statistics in DEts>hid model

Two rounds of experiments (ts12hR24h, at least 20 discs were analyzed each round) were done for both DEts>GFP (control) and DEts>hid. Increase of PH3-positive cells in dorsal eye disc portions of DEts>hid animals are consistently observed. PH3-positive cell numbers were counted in dorsal (GFP+) and ventral eye disc portions in selected discs. Size of the dorsal (GFP+) and ventral eye disc portions were measured through the “histogram” function in Adobe Photoshop CS. To compare the density of PH3+ cells in each disc portion, number of PH3+ cells were divided by size (in pixels) of the corresponding tissue which is used to calculate the number of cells in 100,000 pixels (density). Such normalized density of PH3+ cells in various portions of eye discs (mean ± SD) were used for the statistical chart. PH3+ cell numbers in 100,000 pixels is on average 88 in DEts>GFP and 96 in control discs. Their statistical significance was evaluated through a two-tailed, unpaired Student’s t-Tests (P < 0.04). In contrast, the number of PH3+ cells are comparable in ventral disc portions of each genotype suggesting that increased proliferation mostly occurred in the dorsal part of the disc (at least at the time point of R24 h).

Immunohistochemistry

Imaginal discs were dissected from late 3rd instar larvae and stained using standard protocols. Antibodies to the following primary antigens were used: PH3 (Upstate), anti-cleaved Caspase-3 (Cell Signaling), β-GAL (Promega), ELAV and Wg (DSHB). Secondary antibodies were donkey Fab fragments from Jackson ImmunoResearch. Images were taken with either a Zeiss Axioslanger or a confocal microscope.

Supporting Information

Figure S1 The JNK activity marker TRE-dsRed is induced in ‘undead’ ey>hid-p35 cells. Shown are (A) wild-type (wt), (B) ey>p35 and (C) ey>hid-p35 eye imaginal discs labeled for dsRed (JNK marker, red in A-C; grey in A’-C’) and ELAV (photoreceptor neurous, green in A-G; grey in A’-C’). Only ey>hid-p35 discs induce TRE-dsRed expression (C, C’; arrow) and disrupt the ELAV pattern (C’). (TIF)

Figure S2 The UAS-dp-1RNAi and UAS-drICE-RNAi stocks are functional. Combined expression of UAS-dp-1RNAi and UAS-drICE-RNAi stocks suppresses both TUNEL-positive apoptosis (A,B) and eye-ablation of GMR-hid (C,D) suggesting that these stocks contain functional RNAi transgenes targeting dp-1 and drICE. (TIF)

Figure S3 Several UAS-RNAi transgenes of the JNK pathway suppress GMR-ey. (A) The unmodified GMR-Gal4 UAS-eiger (GMR>ey) eye ablation phenotype. (B-H) RNAi transgenes targeting the genes indicated above the panels suppress the eye ablation phenotype induced by GMR-Gal4 UAS-eiger (GMR>ey) suggesting that they are functional. (TIF)

Figure S4 Inactivation of JNK pathway genes in ey>hid-p35 eye discs does not affect caspase activity. (A) A ey>hid-p35 disc labeled for Cas3* and ELAV. (B) dronc RNAi suppresses Cas3* and normalizes the ELAV pattern in ey>hid-p35 discs. (C-H) RNAi transgenes targeting the indicated JNK pathway components normalize the ELAV pattern, but fail to suppress Cas3* activity in ey>hid-p35 discs suggesting that they suppress AIP downstream of caspase activation. (TIF)

Figure S5 Rho1 acts upstream of JNK in the ‘undead’ AIP model. (A,A’,C,C’) ey>hid-p35 discs are characterized by strong puc-lacZ (A,A’) and ugo (C,C’) expression as well as disrupted photoreceptor pattern (ELAV). (B,B’,D,D’) RNAi targeting Rho1 suppresses puc-lacZ (B,B’) and ugo (D,D’) expression as well as normalizes the ELAV pattern in ey>hid-p35 discs. Caspase activity is not affected suggesting that Rho1 acts downstream of Drone and upstream of JNK. (TIF)

Table S1 Chromosomal deficiencies tested in the AiP screen on 2L. Listed are the names of the deficiencies, the extent of the chromosomal deletions and the score in the AiP screen. Green marks suppressors and yellow marks enhancers. Deficiencies marked with * could not be scored, because they caused lethality in the ey>hid-p35 background. (PDF)

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Author Contributions

Conceived and designed the experiments: YF AB. Performed the experiments: YF SW JH VBY GH JLL CEF. Analyzed the data: YF SW AB. Contributed reagents/materials/analysis tools: YF AB. Wrote the paper: AB YF.
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