Autophagy-independent function of Atg1 for apoptosis-induced compensatory proliferation

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Abstract

Background: ATG1 belongs to the Uncoordinated-51-like kinase protein family. Members of this family are best characterized for roles in macroautophagy and neuronal development. Apoptosis-induced proliferation (AiP) is a caspase-directed and JNK-dependent process which is involved in tissue repair and regeneration after massive stress-induced apoptotic cell loss. Under certain conditions, AiP can cause tissue overgrowth with implications for cancer.

Results: Here, we show that Atg1 in Drosophila (dAtg1) has a previously unrecognized function for both regenerative and overgrowth-promoting AiP in eye and wing imaginal discs. dAtg1 acts genetically downstream of and is transcriptionally induced by JNK activity, and it is required for JNK-dependent production of mitogens such as Wingless for AiP. Interestingly, this function of dAtg1 in AiP is independent of its roles in autophagy and in neuronal development.

Conclusion: In addition to a role of dAtg1 in autophagy and neuronal development, we report a third function of dAtg1 for AiP.

Keywords: Apoptosis-induced proliferation, Atg1, ULK1/2, Autophagy, Jun-N-terminal kinase signaling

Background

Autophagy-related gene 1 (Atg1) in yeast, dAtg1 in Drosophila, uncoordinated-51 (unc-51) in C. elegans, and Unc-51-like kinase 1 and 2 (ULK1/2) in mammals are members of the evolutionary conserved Uncoordinated-51-like kinase (ULK) protein kinase family that play critical roles in organelle turnover and neuronal development (reviewed in [1, 2]). Autophagy is a catabolic process engaged during starvation and other stress conditions [3]. A critical step in autophagy is the formation of autophagosomes which trap cytosolic cargo for degradation after fusion with lysosomes [3]. Genetic studies in yeast identified Atg1 as an essential gene required for the initiation of autophagy [3–5]. This function of ULK proteins is conserved in evolution [6–9]. For this process, ATG1 forms a protein complex composed of ATG1/ULK1, ATG13, and ATG17 (FIP200), and in mammalian cells also ATG101 [10–15]. The ATG1/ULK complex phosphorylates several substrates including ATG9 [16, 17] and the Myosin light chain kinase (ZIP kinase in mammals, Sqa in Drosophila) [18], which are required for the formation of autophagosomes. Activation of the ATG1/ULK complex is also required for the recruitment of the ATG6/Beclin protein complex to the pre-autophagosomal structure (PAS) [3]. The ATG6/Beclin complex is composed of ATG6 (Beclin-1 in mammals), the type III PI3-K VPS34, as well as ATG14 and VPS15. Maturation of the PAS to autophagosomes requires lipidation of the ubiquitin-like ATG8/LC3 protein, which is mediated by two ubiquitin-like conjugation systems, ATG7 and ATG8/LC3 [3]. Critical components in these ubiquitin-like conjugation systems are ATG7 (E1), ATG10 and ATG3 (E2s), as well as another protein complex, ATG5–ATG12–ATG16, which serves as an E3 ligase for ATG8/LC3.

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lipidation [3, 19–21]. Finally, autophagosomes fuse with lysosomes for degradation of cargo.

In addition to a critical role in autophagy, ATG1 also has functions outside of autophagy, most notably in neuronal development. This was initially observed in mutants of the ULK ortholog unc-51 in C. elegans, which display uncoordinated movement with an underlying axonal defect [22–28]. A neuronal function of ULK orthologs was subsequently also reported in Drosophila, zebrafish and mammals [27, 29–33]. This autophagy-independent function of ULK proteins does not appear to involve other canonical autophagy proteins, including components of the ATG1/ULK protein complex such as ATG13 and FIP200 [34, 35]. Instead, the neuronal function of ULK proteins is dependent on different sets of proteins that include – depending on the organism analyzed – UNC-14, VAB-8 and PP2A (C. elegans), UNC-76 (Drosophila), and Syntegen and SynGAP (mammals) several of which are phosphorylated by ULKS [26–28, 33, 36–41]. Thus, the two known functions of ULK proteins in autophagy and neuronal processes involve different sets of proteins.

Apoptosis-induced proliferation (AiP) is a specialized form of compensatory proliferation that occurs after massive cell loss due to stress-induced apoptosis [42–45]. Initially described in Drosophila where it can compensate for the apoptotic loss of up to 60 % of imaginal disc cells [46], AiP has since been observed in many organisms, including classical regeneration models such as hydra, planarians, zebrafish, xenopus, and mouse [47–50]. Interestingly, AiP is directly dependent on a non-apoptotic function of caspases that otherwise execute the apoptotic program in the dying cell. In Drosophila, the initiator caspase Dronc triggers activation of Jun-N-terminal kinase (JNK) signaling, which leads to the production of mitogens including Wingless (Wg), Decapentaplegic (Dpp), and the EGF ligand Spitz for AiP [51–59].

However, many mechanistic details of AiP are still unknown. Therefore, we and others have developed several AiP models in eye and wing imaginal discs in Drosophila [52, 57, 58, 60–64]. In the first set of AiP models, apoptosis is induced upstream by expression of cell death-inducing factors such as hid or reaper, but blocked downstream by co-expression of the effector caspase inhibitor p35, generating ‘undead’ cells [52, 55, 56, 62]. Because undead cells do not die and P35 does not inhibit the initiator caspase Dronc, Dronc continues to generate the signals for AiP, which causes tissue overgrowth. For example, ey-Gal4-driven UAS-hid and UAS-p35 (ey > hid-p35) cause overgrowth of head capsules with ectopic sensory organs such as bristles and ocelli, and in severe cases forms amorphic head tissue (Fig. 1a–d) [52]. The ey > hid-p35 undead model is the only known overgrowth-promoting AiP model in which adult animals survive [52]. Other undead AiP models, mostly in the wing, such as nub > hid-p35 or hh > hid-p35 produce enlarged larval wing imaginal discs, but do not allow adult animals to eclose. Thus, the ey > hid-p35 undead AiP model is a convenient tool for genetic screening to identify genes involved in AiP by scoring adult flies.

The second type of AiP models does not involve the use of p35 and has been referred to as genuine or regenerative AiP [43, 52, 60, 61, 63]. These models take advantage of Gal80ts, a temperature-sensitive inhibitor of Gal4, which allows temporal control of UAS-transgene expression by a temperature shift to 29 °C [65]. Because these AiP models are p35-independent, cells complete the apoptotic program and we score the ability of the affected tissue to regenerate the lost cells by new proliferation. In our genuine/regenerative AiP model, we express the pro-apoptotic factor hid for 12 h under control of dorsal-eye-Gal4 [66] (referred to as DEts-hid) in eye imaginal discs during second or early third larval instar [52]. This treatment causes massive tissue loss which is regenerated by AiP within 72 h after tissue loss.

Here, we report the identification of dAtg1 as a suppressor of the overgrowth phenotype of the undead ey > hid-p35 AiP model. dAtg1 is also required for complete regeneration in the DEts-hid AiP model. Furthermore, we show that dAtg1 is genetically acting downstream of JNK activation, but upstream of mitogen production such as Wg. Consistently, dAtg1 is transcriptionally induced by JNK activity during AiP. Interestingly, the involvement of dAtg1 in AiP is independent of other dAtg genes, including dAtg13, dAtg17/Fip200, dAtg6, vps15, vps34, dAtg7, and dAtg8. These findings suggest that dAtg1 has an autophagy-independent function in AiP. Finally, dAtg1 is not employing the mechanism used during neuronal development as targeting unc-76 did not affect AiP. Therefore, in addition to a role of dAtg1 in autophagy and neuronal development, we define a third function of dAtg1 for AiP.

**Results**

dAtg1 is a suppressor of apoptosis-induced proliferation

AiP phenotypes of ey > hid-p35 animals vary from mild to moderate to severe overgrowth of head capsules characterized by pattern duplications of ocelli, bristles, and sometimes entire antennae (moderate) as well as deformed heads with amorphic tissue (severe) (Fig. 1a–d) [52]. To identify genes required for AiP, we are screening for suppressors of the ey > hid-p35-induced overgrowth phenotypes. For follow-up characterization of identified suppressors, we are using undead and regenerative (p35-independent) AiP models in eye and wing imaginal discs.
Fig. 1 Suppression of *ey > hid-p35* by loss of dAtg1. The hyperplastic overgrowth phenotype of *ey > hid-p35* can be grouped in three categories, weak (W, including wildtype-like), moderate (M) and severe (S), as previously described [52]. Moderate flies are characterized by overgrowth of head capsules with duplications of bristles (arrows) and ocelli (arrowhead), while severe flies have overgrown and deformed heads with amorphic tissue. Each screen analysis was repeated at least twice with scoring more than 50 *ey > hid-p35* (RNAi or mutant) adult flies. 

**a–h** Representative dorsal views of adult fly head capsules of the indicated genotypes. 

**a–d** Compared to the control *ey > p35*, which is similar to wildtype (a), percentages of *ey > hid-p35* flies display weak (b), moderate (c) and severe (d) phenotypes (9%, 46%, and 45%, respectively). Therefore, over 90% of *ey > hid-p35* flies show a clear hyperplastic overgrowth phenotype (either severe or moderate).

**e** Knockdown of dAtg1 by RNAi in *ey > p35* does not cause obvious defects on head capsules. 

**f–h** dAtg1 RNAi strongly reduces the percentage of *ey > hid-p35* flies showing severe (8%) and moderate (14%) overgrowth phenotype and largely extends the population of flies with a weak or wildtype-like appearance (78%).

**i** Summary of the suppression of the *ey > hid-p35* overgrowth phenotype by expressing dAtg1RNAi or dominant-negative dAtg1DN and the enhancement of the phenotype by expressing two constructs encoding dAtg1DN and dAtg1GS1079. Either 25 °C or room temperature (RT, 22 °C) was used for these analyses. The majority of *ey > hid-p35* flies (*ey > hid-p35/+*) display either severe or moderate overgrowth phenotypes at both 25 °C and RT. Blue indicates severe, orange indicates moderate, and green indicates weak or wildtype-like phenotypes.
Using this approach, we identified dAtg1 as a strong AiP suppressor of ey > hid-p35 by RNAi (Fig. 1f–h). The percentage of ey > hid-p35 animals with severe and moderate AiP phenotypes is strongly reduced upon dAtg1 knock-down (Fig. 1f–h; quantified in Fig. 1i). No effect was scored on control (ey > p35) animals (Fig. 1e). Although dAtg1 RNAi results in significant loss of dATG1 mRNA and protein levels (Additional file 1: Figure S1A–B') and no off-targets have been reported, we tested additional reagents for an involvement of dAtg1 in AiP. Expression of a dominant negative dAtg1 transgene also suppressed ey > hid-p35-induced overgrowth (Fig. 1i). Furthermore, increased expression of dAtg1, which does not alter apoptosis (Additional file 2: Figure S2), enhances the AiP phenotype and generates many animals with severe AiP phenotype (Fig. 1i). We therefore conclude that dAtg1 is required for tissue overgrowth in the undead AiP model.

dATG1 is required for regenerative apoptosis-induced proliferation

Encouraged by the identification of dAtg1 in the undead AiP model, we examined an involvement of dAtg1 in the regenerative (p35-independent) DEts > hid AiP model. When hid expression is induced for 12 h in the dorsal half of the eye imaginal disc, the dorsal half of the eye disc is severely ablated [52]. After 72 h recovery (R72h), the disc has recovered due to regenerative growth by AiP (Fig. 2b) [52]. The degree of the regenerative response can be easily assessed by visualization of the photoreceptor pattern using ELAV as a marker, because photoreceptor differentiation follows tissue growth of the disc [67]. DEts > hid control discs regenerate a normal ELAV pattern 72 h after hid-induced tissue ablation (Fig. 2b, b'). In contrast, DEts > hid imaginal discs expressing dAtg1RNAi are unable to fully regenerate the ablated tissue (Fig. 2c). The ELAV pattern in the dorsal half of the eye disc is incomplete (arrow in Fig. 2c'), suggesting that the regenerative response after hid-induced tissue ablation is partially blocked by dAtg1 RNAi. As additional control, DEts > dAtg1RNAi alone does not affect the ELAV pattern (Fig. 2a, a'). These findings are also confirmed by expression of a dominant negative dAtg1 transgene and quantified in Fig. 2d. In summary, these data illustrate that dAtg1 is an important gene required for AiP in both undead and regenerative models. We also considered examining the effect of overexpressed dAtg1 in regenerative AiP. However, expression of dAtg1 alone using the DE-Gal4 driver triggers strong apoptosis (Additional file 2: Figure S2C), consistent with a previous report [6], which may complicate the interpretation of the results. Therefore, we did not characterize the role of overexpressed dAtg1 in the regenerative AiP model.

dAtg1 is required for AiP downstream of Dronc in undead eye and wing imaginal discs

We further characterized the role of dAtg1 for AiP with molecular markers. Although dAtg1 is best characterized for a role in autophagy, it is theoretically possible that dAtg1 RNAi inhibits apoptosis and thus AiP indirectly. Therefore, we first tested how dAtg1 relates genetically to Dronc in the AiP pathway. As a marker for Dronc activity, we used the cleaved Caspase-3 (cCasp3) antibody. Although apoptosis is blocked by p35 expression, the cCasp3 antibody still labels undead cells (Fig. 3c, d), presumably because Dronc also has non-apoptotic substrates [52, 68]. dAtg1 RNAi suppresses the overgrowth and normalizes the morphology of the ey > hid-p35 eye disc as judged by ELAV labeling (Fig. 3e, f). However, cCasp3 labeling is not significantly altered by dAtg1 RNAi (Fig. 3e, f, j) despite the rescue of disc morphology suggesting that the loss of dAtg1 does not affect caspase activity in undead tissues.

We also characterized the involvement of dAtg1 in AiP in undead wing imaginal discs. Expression of hid and p35 under nub-Gal4 control (nub > hid-p35) causes strong overgrowth of the wing imaginal disc compared to nub > p35 control discs (Fig. 3g, h). dAtg1 RNAi suppresses the overgrowth of nub > hid-p35 wing discs, but leaves cCasp3 activity intact (Fig. 3i, k). To further confirm these data obtained by RNAi, we conducted mosaic analysis using dAtg1 null mutants in wing imaginal discs because homozygous dAtg1 mutants are early larval lethal in the ey > hid-p35 genetic background. Consistent with RNAi results, dAtg1 mutants do not alter cCasp3 labeling induced by co-expression of hid and p35 in MARCM clones (Additional file 3: Figure S3A–C). Similarly, dAtg1 mutant clones or RNAi do not suppress GMR-hid-induced apoptosis in the eye (Additional file 3: Figure S3D–F). Together, these data further confirm that loss of dAtg1 does not affect apoptosis and that dAtg1 controls AiP downstream of caspase (Dronc) activation.

dAtg1 is required for AiP downstream of JNK, but upstream of wingless in undead eye and wing imaginal discs

Next, because JNK is an important mediator of AiP [43, 52, 56, 57], we determined the position of dAtg1 relative to JNK in the AiP pathway. The JNK activity reporter puc-lacZ is strongly induced in AiP models compared to controls (Fig. 3a, c'; arrows) [52, 54, 56, 57]. The morphology of the discs is severely disrupted, which correlates with signal intensity of puc-lacZ, especially in overgrown areas. In response to dAtg1 RNAi, overgrowth and disc morphology, as judged by ELAV labeling, is restored to almost normal (Fig. 3e). Nevertheless, despite the rescue of disc morphology, puc-lacZ expression is not significantly reduced (Fig. 3e'; arrows). These data suggest that dAtg1
acts downstream of or in parallel to JNK activity in the AiP pathway.

Finally, we determined the position of dAtg1 relative to wingless (wg), another marker in the AiP pathway [54–56]. Wg and its orthologs are critical mediators of AiP in regenerative responses in many animals (reviewed by [43, 45]). In undead eye discs, inappropriate wg expression is induced compared to controls (Fig. 3b, b’, d, d’; arrows). dAtg1 knockdown normalizes wg expression in the disc (Fig. 3f, f’). In addition, in undead nub > hid-p35 wing imaginal discs, wg expression is strongly induced (arrows in Fig. 3h). However, similar to undead eye discs, co-expression of dAtg1 RNAi in nub > hid-p35 discs suppresses overgrowth (Fig. 3i) and normalizes the wg pattern (Fig. 3i’). Together, these analyses suggest that dAtg1 acts genetically downstream of Dronc and either downstream of or in parallel to JNK, but upstream of Wg, in the AiP network.

In addition to the RNAi analysis, we also co-expressed hid and p35 in either wildtype, dronc, or dAtg1 mutant clones (by MARCM) and examined for JNK activity (using MMP1 as JNK marker [69]) and Wg expression (Fig. 4a, a’, b, b’). Ectopic Wg expression is most frequently observed in the wing pouch area in close proximity to the dorsalventral boundary in the wing disc.
Fig. 3 dAtg1 acts genetically downstream of or in parallel to JNK and upstream of Wg expression. Late third instar eye (a-f) or wing (g-i) discs, anterior is to the left. The cleaved Caspase-3 (cCasp3) labeling (green in a-i) indicates activity of Dronc in p35-expressing tissues. White dotted lines in (a-f) indicate the anterior portion of the eye discs which expresses ey-Gal4. ELAV labels photoreceptor neurons (blue in a-f) and is used to mark the posterior differentiating eye field. (a-b') In ey > p35 control discs, puc-lacZ expression (β-Gal; red in a, grey in a') as a marker of JNK/Bsk activity is low (a', arrow) in the anterior portion of the eye discs. Expression of Wg (red in b, grey in b') is restricted to dorsal and ventral edges of the eye discs. Dronc activity indicated by cCasp3 labeling is low. (c-d') In ey > hid-p35 discs, Dronc activity (cCasp3 labeling) is strongly induced in undead anterior tissue (c, d). The anterior portion of the discs between the white dotted lines is significantly expanded and displaces the eye field into the posterior portion of the discs (ELAV). Compared to the ey > p35 control discs (a', b'), in the overgrown anterior eye portion, JNK activity (c', arrows) and expression of Wg (d', arrows) are strongly induced. (e-f') Expression of dAtg1RNAi suppresses hyperplastic overgrowth in about 80% of the ey > hid-p35-dAtg1RNAi discs (n > 60) indicated by the normalized ELAV pattern. This ratio corresponds to the suppression of the adult overgrowth phenotype (Fig. 1i). However, puc-lacZ expression and cCasp3 labeling are not suppressed by dAtg1RNAi (e', arrows) in contrast to ectopic Wg expression, which is blocked (f') in the anterior portion of the eye discs. (g-p') Compared to the control wing discs where p35 is expressed in the pouch area under the control of nub-Gal4 (nub > p35, g, g'), in nub > hid-p35 discs, co-expression of hid and p35 induces tissue overgrowth, increased cCasp3 labeling, and ectopic Wg expression (h, h', arrows). Similar to eye discs, expression of dAtg1RNAi largely blocks tissue overgrowth and ectopic Wg, but not the cCasp3 labeling (i, i'). A low level of ectopic Wg remains in nub > hid-p35-dAtg1RNAi discs (i', arrow). (j, k) Quantification of cCasp3 labeling intensity in eye and wing discs (mean ± SE). dAtg1 RNAi has no obvious effects on the cCasp3 labeling induced by expression of hid and p35 in both eye (j) and wing (k) discs.

Figures 4 and 5 illustrate the regulation of the caspase-dependent events induced by dAtg1. While expression of hid and p35 in dAtg1 mutant clones suppresses these AiP markers (Fig. 4c–d'), importantly, when hid and p35 were co-expressed in dAtg1 mutant clones, the expression of Wg was suppressed, while MMP1 expression was not affected (Fig. 4e–f') suggesting that dAtg1 acts downstream of or in parallel to JNK activity, but upstream of Wg. These data are consistent with the RNAi data (Fig. 3).

Because dAtg1 is required for wg expression in AiP, we tested if dAtg1 was also sufficient for expression of AiP markers including wg, dpp, and kekkon1 (kek), the latter being a marker of EGFR activity [51, 52, 54–56, 70]. However, while expression of hid in the DEx > hid model is sufficient to induce wg, dpp, and kek expression (Additional file 4: Figure S4A–B', D–E', G–H'), expression of dAtg1 alone under the same conditions (DEtx > Atg1) is not (Additional file 4: Figure S4C, C', F, F', I, I'). These observations suggest that, in addition to dAtg1 expression, additional caspase-dependent events have to occur in order to induce AiP.

dAtg1 is transcriptionally induced for AiP in a JNK-dependent manner

Next, we examined if protein and transcript levels of dAtg1 change in AiP. Indeed, using a dATG1-specific antibody (Additional file 1: Figure S1B, C) [71], we observed increased protein abundance of dATG1 in the undead compartment of wing discs compared to controls (Fig. 5a, b). To determine if this is a transcriptional or translational effect on dATG1 levels in undead cells,
we performed mRNA in situ hybridization assays on undead (hh > hid-p35) and regenerative (hh<sub>ts</sub> > hid) wing imaginal discs. In both AiP models, dAtg1 is transcriptionally induced (Fig. 5e – i). Additional file 5: Figure S5 demonstrates the specificity of the dAtg1 in situ probes. The hh<sub>ts</sub> > hid regenerative model allows determination of the timing of dAtg1 expression during AiP. dAtg1 expression is slow as a pulse of hid expression for 15 h only weakly induces it (Fig. 5h). Only after prolonged expression of hid (68 h), is a strong induction of dAtg1 expression detectable (Fig. 5i). These data suggest that dAtg1 expression occurs quite late in the AiP response.

Because dAtg1 acts genetically downstream of or in parallel to JNK (Figs. 3 and 4) and because JNK can induce dAtg1 expression under oxidative stress conditions and by ectopic activation of JNK [72], we tested if the transcriptional induction of dAtg1 in the AiP models is also dependent on JNK. The Drosophila JNK homolog is encoded by the gene basket (bsk) [73, 74]. Indeed, while bsk RNAi does not affect dAtg1 expression in control discs (Fig. 5c, c'), it suppresses the accumulation of dATG1 protein in undead and dAtg1 transcripts in regenerative wing discs (Fig. 5d, d', j). Consistent with a previous report [72], ectopic JNK activation by expression of a constitutively active JNKK transgene (hep<sub>CA</sub>) for a short pulse of 6 h with 6 h recovery at 18 °C (TS6hR6h) is sufficient to induce dAtg1 expression in wing imaginal discs (Fig. 5l). However, expression of the pro-apoptotic gene hid under the same conditions (TS6hR6h) cannot induce dAtg1 expression (Fig. 5k). Combined, these data suggest that dAtg1 expression is under direct control of JNK signaling, while it is far downstream of Hid expression.

Undead tissue produces autophagosome-like particles which do not contribute to apoptosis-induced proliferation

dAtg1 acts upstream in the autophagy pathway and its activation can induce autophagy [6, 10, 17]. Oxidative stress or ectopic activation of JNK has been previously reported to induce expression of multiple dAtg genes, including dAtg1, as well as autophagy in midgut and fat

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**Fig. 4** dAtg1 is required cell autonomously for Wg expression, but not JNK activation, in undead clones. Late third instar wing discs with mosaic clones positively marked by GFP, anterior is to the left. MMP1 labeling (red in a, c, e and grey in a', c', e') is used as marker of JNK activity. Wg (red in b, d, f and grey in b', d', f') is highly expressed at the dorsal/ventral (D/V) boundaries (arrowheads in b, d, f) of wing discs. (a–b) Simultaneous expression of hid and p35 in clones. MMP1 expression (arrows in a, a') is induced in all hid and p35 co-expressing clones. Ectopic expression of Wg (arrows in b, b') was observed in over 80% of clones (n = 66) generated in close proximity to the D/V boundaries in the wing discs. Genotype: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/FRT80B. (c–d') Simultaneous expression of hid and p35 in dronc mutant clones. Both MMP1 labeling and ectopic Wg expression, induced by co-expression of hid and p35, are completed blocked in dronc mutant clones (n > 30). Genotype: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/dronc<sup>I29</sup> FRT80B. (e–f') Simultaneous expression of hid and p35 in dAtg1 mutant clones. hid and p35-induced MMP1 expression persists in dAtg1 mutant clones (arrows in e, e'). In contrast, the ectopic Wg expression induced by hid and p35 is suppressed in over 70% of dAtg1 clones (n = 73) generated in close proximity to the D/V boundaries in the wing discs. Genotype: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/dAtg<sup>1Δ3D</sup> FRT80B.
body cells [72]. We therefore examined if autophagy is induced in undead disc tissue and whether it contributes to AiP. Because dATG8 is an essential part of autophagosomes, fusion proteins of dATG8 with fluorescent proteins such as GFP or mCherry are used as markers for formation of autophagosomes [7]. Moreover,
because GFP is stable in autophagosomes, but unstable in autolysosomes, whereas mCherry is stable in both compartments, the tandem fusion protein GFP-mCherry-dATG8a is used as marker for the maturation of autophagosomes into autolysosomes, indicating autophagic flux [75, 76]. Indeed, as shown in Additional file 6: Figure S6, undead ey > hid-p35-expressing tissue accumulates large quantities of GFP-mCherry-dATG8a-containing particles. However, it is unclear if these particles are classical autophagosomes. While the GFP signals are weaker compared to the mCherry signals, which may be an indicator of autophagic flux, there are clearly GFP-only particles which do not display mCherry fluorescence (compare Additional file 6: Figure S6b' and S6b”). This observation is inconsistent with the concept of autophagic flux [75]. Furthermore, even though dAtg1 RNAi suppresses AIP, it does not suppress the formation of the GFP-mCherry-dATG8a particles (Additional file 6: Figure S6C–C”). This result suggests that the ectopic expression of dAtg1 in undead tissue does not induce the formation of the GFP-mCherry-dATG8a-containing particles. Furthermore, and more importantly, these particles do not contribute to the overgrowth of undead tissue nor, thus, to AIP.

Other dAtg genes mediating autophagy and unc-76 are not required for apoptosis-induced proliferation

Because of this unexpected result, we tested other dAtg genes for an involvement in AIP. Surprisingly, RNAi targeting dAtg3, dAtg6, dAtg8a, dAtg8b, dAtg9, and dAtg17 as well as vps15 and vps34 had no effect on AIP (Fig. 6a). Most notable are dAtg3 and dAtg8 because they encode essential components for autophagosome maturation (see Background) [3, 19–21]. To ensure that the RNAi transgenes used to target these dAtg genes are functionally intact, we tested them in two functional assays. They suppressed starvation-induced autophagy in the fat body (Additional file 7: Figure S7A–E) demonstrating that dAtg3, dAtg8a, and dAtg8b are efficiently knocked down to induce an autophagy-deficient phenotype. In addition, the functionality of these RNAI stocks is further confirmed in that they all enhanced the eyeful phenotype (Additional file 4: Figure S4F–J) which is known to be enhanced by loss of autophagy [77]. The eyeful (ey-Gal4 L1AS-Dl,psq,lola) [78] condition uses the same Gal4 driver as in the ey > hid-p35 AIP model. Therefore, tissue-specific and/or Gal4-dependent differences do not account for the failure of these RNAI stocks to suppress AIP.

In addition to targeting essential autophagy components by RNAi, we also tested homozygous dAtg13 and dAtg7 mutants which can survive to pupal or adult stages, respectively, for suppression of AIP. dAtg13 encodes a component of the ATG1/ULK protein complex, while dAtg7 encodes the E1-conjugating enzyme for autophagosome maturation. However, dAtg13 and dAtg7 mutants fail to suppress the abnormal morphology of ey > hid-p35 discs as visualized by ELAV labeling and the ectopic Wg expression (Fig. 6b, c). These results suggest that the tested dAtg genes, except dAtg1, are not required for AIP. An involvement of dAtg1 in AIP is further confirmed by expression of a kinase dead form of TOR (TOR(TED)) [79], which activates dAtg1 [7], or RNAi knockdown of Raptor, an adaptor protein required for TOR activation [80], both of which enhance AIP (Fig. 6a).

Finally, we also examined the possibility that dAtg1 uses the same mechanism in AIP that it uses during neuronal development. However, RNAi targeting unc-76, which is an important mediator of the function of dAtg1 during neuronal development [27], does not suppress the overgrowth phenotype of the undead ey > hid-p35 AIP model (Fig. 6a). Three independent RNAi lines gave consistent results. Therefore, in addition to autophagy and neuronal development, our data define a third function of dAtg1 for AIP.

Discussion

In this paper, we show that the sole ULK ortholog in Drosophila, dAtg1, is required for AIP both in undead and regenerative models. We demonstrated that dAtg1 acts downstream of JNK activity in AIP and is transcriptionally induced by JNK, consistent with a previous study on oxidation response [72]. Furthermore, dAtg1 is required for the expression of Wg, a mitogen associated with AIP [51, 52, 54–56, 81]. Finally, our data provide evidence that the role of dAtg1 in AIP is independent on its role in canonical autophagy.

It is generally assumed that the secreted mitogens Wg, Dpp, and Spitz promote the proliferation of surviving cells during AIP [43, 45]. The expression of these genes is under control of JNK activity. Until recently, it was unknown how JNK signaling promotes expression of these genes. However, very recently, it was reported that an enhancer element in the wg gene that drives expression of wg under regenerative conditions contains three AP-1 binding sites required for regeneration [81]. AP-1 is composed of the transcription factors Jun and Fos (Kayak in Drosophila), which are controlled by JNK activity. This observation suggests a direct way of wg expression by JNK-dependent AP-1.

How does dATG1 fit into the AIP network? Our genetic data suggest that dAtg1 acts downstream of or in parallel to JNK. Furthermore, we placed dAtg1 genetically upstream of wg expression. Therefore, dAtg1 may act in at least two different ways in the AIP network. It may directly modulate the activity of the AP-1 transcriptional complex. An indirect mode of action is also
possible in which dATG1 provides a permissive environment for AP-1 activity. However, dAtg1 does not control all AP-1 activities. Expression of puc-lacZ and MMP-1 are not affected by dAtg1 RNAi and dAtg1 mutants, respectively (Figs. 3 and 4). In contrast, wg expression is suppressed under these conditions. Therefore, of the known transcriptional targets of JNK and AP-1 during AiP (puc-lacZ, MMP-1, dAtg1, and wg), dAtg1 affects only wg expression. Future work will address the mechanistic role of dATG1 for the control of AiP.

Although dAtg1 is required for AiP, it is not sufficient. Overexpression of dAtg1 using DE4-Gal4 for 12 h followed by 24 h recovery does not trigger AiP markers such as wg-lacZ, dpp-lacZ, or kek-lacZ (Additional file 4: Figure S4). Expression of hid under the same conditions is able to induce these markers ectopically. These observations suggest that, in addition to dAtg1 expression, apoptotic signaling triggers an additional activity required for wg expression and AiP.

The best characterized function of dATG1 and of ULKs in general is the initiation of autophagy under starvation or stress conditions [1, 2, 5, 10, 72]. Autophagy requires a total of 36 Atg genes [3]. Although we did not test all 36 Atg genes for a role in AiP, we tested several genes which are critical for autophagy, including dAtg3, dAtg6, dAtg8a, dAtg8b, dAtg9, dAtg17, vps15, and vps34. dAtg13 and dAtg17 (aka Fip200) encode subunits of the ATG1/ULK complex [10–12]. ATG6 and VPS34 are subunits of the ATG6/Beclin complex, which is activated by ATG1 during autophagy. Phosphorylation of
ATG9, the mammalian ortholog of dATG9, by ULK1 is required for autophagy [16, 17]. Finally, lipidation of ATG8, which is essential for formation of autophagosomes requires the function of ATG3 and ATG7 [3, 20, 21]. In contrast to dAtg1, inactivation of any of these genes does not suppress the overgrowth phenotype of ey > hid-p35 animals. Furthermore, although we detect the formation of ATG8a-containing particles in undead eye imaginal discs, these particles are not dependent on dAtg1 and do not contribute to AiP and overgrowth (Additional file 6: Figure S6). Combined, these data suggest that dATG1 does not trigger canonical autophagy in an AiP context.

In addition to autophagy, ULK proteins have also been implicated in neuronal development, most notably axon guidance and axonal growth [27, 30]. However, we also exclude a neuronal function of dAtg1 in AiP because inactivation of unc-76, a mediator of dAtg1 for neuronal development [27], does not suppress overgrowth induced by ey > hid-p35.

Conclusions

We revealed a third function of dAtg1 in Drosophila for the control of regenerative proliferation after massive apoptotic cell loss. Future work will address if this role of dAtg1 in regenerative proliferation is also conserved in other organisms, the molecular mechanism of this function, and whether it is potentially misregulated in pathological conditions such as cancer.

Methods

Fly strains and the ey > hid-p35 assay

UAS-dAtg1(1KR6SB) or UAS-dAtg1(1K86Q) were used to express a dAtg1 kinase-dead mutant that functions as a dominant negative [6]. Either UAS-dAtg168 or UAS-dAtg1G10797 were used to express wild-type dAtg1 [6]. Both constructs gave similar results under the control of Gal4 lines tested in this study. Dorsal Eye-Gal4 (DE-Gal4) [66], dromED9 [82], dAtg1ASSD [7], dAtg1ASS7 [10], dAtg7d14, dAtg7d77 [83], dAtg8a::GFP-mCherry-dAtg8a [76], and eyefid (ey-Gal4 > UAS-delta, GS88A8 UAS-lola and UAS-pipsqueak) [78] were as described. puc-lacZ665, wg-lacZ, dpp-lacZ, kek-lacZ, ey-Gal4, hh-Gal4, tub-Gal4, GMR-Gal4, tub-Gal80d, UAS-p35, UAS-hid, UAS-hepA, UAS-GFP, and UAS-TORED were obtained from the Bloomington Stock Center. UAS-based RNAi stocks of the following genes were obtained from Bloomington, VDRC or NIG-FLY stock centers: bsk (BL 32977, V34138), dAtg1 (BL26731), dAtg3 (BL34359, V101364), dAtg6 (V22122, V10197), dAtg8a (V43076, V43097), dAtg8b (V17097), dAtg9 (V10045), dAtg17 (V106176), Vps15 (V110706, NIG9746R-2), Vps34 (V100296), raptor (BL34814, BL41912), and unc-76 (V20721, V20722, V40495). Comparable results were obtained from multiple RNAi lines targeting the same gene. Functionality of BL26731, V101364, V43097 and V17097 was tested on inhibition of starvation-induced autophagy [7] (Additional file 7: Figure S7). The exact genotype of ey > hid-p35 is either UAS-hid; ey-Gal4 UAS-p35 (UAS-hid on X; ey-Gal4 UAS-p35 on second chromosome) or UAS-hid; ey-Gal4 UAS-p35 (UAS-hid on X; ey-Gal4 UAS-p35 on third chromosome; only used in Fig. 6c, c'). For analysis of ey > hid-p35 adult hyperplastic phenotype, three categories, weak (W), moderate (M) and severe (S), were used as previously described [52]. Each screen analysis was repeated at least twice at 25 °C, or at room temperature (RT, 22 °C) if strong lethality was caused by expressing RNAi or dominant-negative mutant constructs at 25 °C in the background of ey > hid-p35, with scoring more than 50 ey > hid-p35(RNAi or mutant) adult flies.

Temperature-sensitive regenerative assays and statistical analysis

Larvae of the following genotypes (1) DEts > hid (UAS-hid/+; UAS-GFP/+; DE-Gal4 tub-Gal80f/+) (2) DEts > dAtg1RNAI (UAS-GFP/+; DE-Gal4 tub-Gal80f/ UAS-dAtg1RNAI); (3) DEts > hid-dAtg1RNAI (UAS-hid/+; UAS-GFP/+; DE-Gal4 tub-Gal80f/ UAS-dAtg1RNAI); (4) DEts > dAtg1DN (UAS-GFP/+; DE-Gal4 tub-Gal80f/ UAS-dAtg1DN); (5) DEts > hid-dAtg1DN (UAS-hid/+; UAS-GFP/+; DE-Gal4 tub-Gal80f/ UAS-dAtg1DN) were raised at 18 °C. Expression of UAS-constructs (GFP, hid, dAtg1RNAI, dAtg1DN) was induced by a temporal temperature shift to 29 °C for 12 h. After a 72 h recovery period at 18 °C, late third instar eye discs were dissected and analyzed as indicated in the panels (Fig. 2). Full details of the DEts > hid assay have been described previously [52]. At least three independent experimental repeats were done for each genotype and the results were consistent. For statistical analysis shown in Fig. 2d, at least 10 eye discs from each of the following genotypes, DEts > hid; DEts > dAtg1RNAI; DEts > hid-dAtg1RNAI; DEts > dAtg1DN; and DEts > hid-dAtg1DN, were measured for their sizes of dorsal versus ventral half of discs using the “histogram” function in Adobe Photoshop CS6. For such measurement, location of the optic stalk at the center of the posterior edge of eye disc was used as a landmark to horizontally divide eye discs into dorsal versus ventral halves. The dorsal/ventral size ratio was then calculated for each genotype. The statistical significance was evaluated through a one-way ANOVA with Bonferroni multiple comparison test (at least P < 0.01). For the developing wing tissue (Fig. 5), hh-Gal4 tub-Gal80f (hhf) was used to temporally control expression of UAS-constructs in the posterior compartment of wing discs.
Mosaic analysis
For mosaic analysis with “undead” cell clones in larval discs (Fig. 4), the 3-L-MARCM assay was used [84]. Mid second instar (32–40 h post-hatching) larvae of the following genotypes were heat shocked for 1 h at 37 °C, raised at 25 °C, and analyzed at the late third instar larval stage. (1) Generation of hid and p35 co-expressing “undead” clones: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/FRT80B. (2) Generation of hid and p35 co-expressing dronc mutant clones: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/dronc29 FRT80B. (3) Generation of hid and p35 co-expressing dAtg1 mutant clones: hs-FLP tub-GAL4 UAS-GFP/UAS-hid; UAS-p35/+; tub-GAL80 FRT80B/dAtg1^{ASSD} FRT80B. (4) Generation of dAtg1 mutant clones in GMR-hid eye discs: ey-FLP/Δ; GMR-hid/Δ; dAtg1^{ASSD} FRT80B/ubi-GFP FRT80B. The mosaic assay in starving fat body (Additional file 7: Figure S7) was done according to Neufeld [85]. UAS-RNAi lines targeting dAtg1, dAtg3, dAtg8a, and dAtg8b were crossed to yw hs-FLP; r4-mCherry-Agt8a Act > CD2 > Gal4 UAS-GFPnls [86] and incubated at 25 °C. Offspring were starved for 3 h on 20 % sucrose solution before dissection.

Immunohistochemistry and quantification of cCasp3 labeling intensity
Imaginal discs were dissected from late third instar larvae and stained using standard protocols [87]. Antibodies to the following primary antigens were used: anti-cleaved Caspase-3 (Cell Signaling), β-GAL, ELAV, MMP1 (388D12 and 5H7B1 used as a 1:1 cocktail), and Wg (all DHSB). dATG1 antibodies were kindly provided by Jun Hee Lee [71]. Secondary antibodies were donkey Fab fragments conjugated to FITC, Cy3 or Cy5 from Jackson ImmunoResearch. For the dATG1 labeling, HRP-labeled secondary antibodies were used and amplified with Tyramide Signal Amplification (TSA, PerkinElmer). Fluorescent images were taken with a Zeiss confocal microscope. Adult fly images were taken using a Zeiss stereomicroscope equipped with an AxioCam ICC1 camera.

For quantification of cCasp3 labeling intensity in eye or wing discs (Fig. 3, k and Additional file 3: Figure S3C), the average cCasp3 signal intensities in certain disc areas were acquired through Adobe Photoshop CS6 and normalized to the corresponding background level of cCasp3 labeling in the same disc. The background cCasp3 labeling intensity was obtained from the antenna discs for measurement in eye discs (Fig. 3j), the notum regions for measurement in wing discs (Fig. 3k), and the non-clonal areas for the Additional file 3: Figure S3C. At least five representative discs of each genotype were used for such quantification. The statistical significance was evaluated through either a one-way ANOVA with Bonferroni multiple comparison test (at least \( P < 0.01 \), Fig. 3j, k) or a two-tailed, unpaired Student’s \( t \) test (Additional file 3: Figure S3C).

In situ hybridization
For in situ hybridization to detect dAtg1 transcripts, Drosophila cDNA clone LD18893 (Berkeley Drosophila Genome Project expressed sequence tags, Drosophila Genomic Resource Center) was used as a template to generate digoxigenin-labeled sense and antisense RNA probes (Roche). Labeled probes were detected with a TSA Cy3 kit (PerkinElmer) as previously described [88].

Quantitative real-time PCR (qPCR)
Total RNA was isolated from 100 eye discs collected from either the control ey-GAL4 or ey-GAL4 UAS-Atg1RNAi (ey > dAtg1^{RNAi}) third instar larvae using the TRIzol Reagent (Thermo Fisher Scientific). cDNA was then generated from 1 μg of total RNA with the GoScript Reverse Transcription System (Promega). This is followed by the real-time PCR using the SensiFAST SYBR Hi-Rox kit (BIOLINE) with a ABI Prism7000 system (Life technologies). dAtg1 mRNA levels were normalized to the reference gene ribosomal protein L32 (RPL32) by using the ΔΔCt analysis. Three independent biological repeats were analyzed. The following primers suggested by the FlyPrimeBank [89] were used: dAtg1 Fw, CGTCAGCCTGGT CATGGAGTA; dAtg1 Rv, TAACGTTATCCTCGCTGAG; RPL32 Fw, AGCATACAGGCCCCAGATCG; RPL32 Rv, TGTGGTCGATACCCCTTGGGC.

Additional files

**Additional file 1: Figure S1.** Specificity of dAtg1 antibodies and dAtg1^{RNAi}. (A) dAtg1 transcript levels were determined by qPCR from total RNA extracted from eye discs without (control) or with expression of dAtg1^{RNAi} driven by ey-GAL4. dAtg1 RNAi suppresses dAtg1 transcript levels to less than 30 %. Error bars represent SD of three biological repeats. (B, C) A hh > dAtg1^{RNAi} wing disc labeled with dAtg1 antibodies (red in B, grey in C) by expression of dAtg1^{RNAi}. Expression of dAtg1 is strongly reduced in the posterior compartment (GFP*) where dAtg1^{RNAi} is expressed. C A GMR > dAtg1 eye disc labeled with dAtg1 antibodies (red in C, grey in A). C Atg1 antibodies specifically recognize dAtg1 proteins expressed in the GMR domain (GFP†). (TIF 5165 kb)

**Additional file 2: Figure S2.** Expression of dAtg1 enhances caspase activity and apoptosis. Late third instar larval eye discs labeled with the cleaved Caspase-3 antibodies (cCasp3, green in A, B, grey in A; B, blue in C, and grey in C), anterior is to the left. (A–B*) Compared to ey > hid-p35 discs (A, A), cCasp3 labeling indicating activity of Dronc is not affected by expression of dAtg1 which enhances ey > hid-p35-induced overgrowth phenotype (B, B). C Expression of dAtg1 under control of DE-Gal4 and tub-Gal80ts (DEt) and indicated by GFP. Expression of dAtg1 by a temperature shift (ts) to 29 °C for 48 h induces apoptosis as indicated by cCasp3 labeling (C, arrow) and developmental defects in the eye disc indicated by the affected pattern of ELAV labeling (C, arrow). (TIF 4775 kb)
Additional file 3: Figure S3. Loss of dAtg1 does not suppress apoptosis. (A–B) Mosaic late third instar wing discs with hid-p35-expressing clones positively marked by GFP. Simultaneous expression of hid and p35 in clones induces strong cCap3 labeling (A, arrow), similar cCap3 labeling persists in dAtg1 mutant clones (B, arrow). (C) Quantification of cCap3 labeling intensity in hid-p35-expressing clones and hid-p35-expressing dAtg1 mutant clones (mean ± SE). No significant difference of cCap3 labeling was observed. (D–F) A representative late third instar GMR-hid eye disc with dAtg1 mutant clones negatively marked by GFP (highlighted by yellow dotted lines). The wave of apoptosis (arrow) induced by GMR-hid persists in dAtg1 mutant clones. (E, F) Representative adult eyes of the indicated genotypes. GMR-hid-induced eye ablation phenotype (E) is not altered by RNAi knockdown of dAtg1 (F). (TIF 6416 kb)

Additional file 4: Figure S4. Expression of dAtg1 is not sufficient to induce growth arrest for AIP. Late third instar eye disc labeled by yellow dotted lines expressing RNAi constructs are also observed. (A) dAtg1 is expressed at low level in wildtype eye discs. (B, C) Labeling of GMR > dAtg1 discs using sense probes (B) and antisense dAtg1 probes (C). The dAtg1 antisense probes recognize high levels of dAtg1 transcripts driven by GMR-Gal4 (C, the GMR domain expressing dAtg1 is highlighted). (TIF 1140 kb)

Additional file 5: Figure S5. Specificity of in situ probes to detect dAtg1 transcripts. In situ hybridization of late third instar larval eye discs with DIG-labeled probes detected with Tyramide Signal Amplification. (A) Endogenous dAtg1 is expressed at low level in wildtype eye discs. (B, C) Labeling of GMR > dAtg1 discs using sense probes (B) and anti-sense dAtg1 probes (C). The dAtg1 antisense probes recognize high levels of dAtg1 transcripts driven by GMR-Gal4 (C, the GMR domain expressing dAtg1 is highlighted). (TIF 1140 kb)

Additional file 6: Figure S6. (A–C) Autophagy flux reporter expression in ey > hid-p35 eye discs. Late third instar larval eye discs expressing the autophagy flux reporter GFP::mCherry-dAtg8a under control of the dAtg8e promoter [76]. The yellow dotted lines indicate the anterior portions of the eye discs which expresses ey::G4a. Note the overgrowth of the anterior eye disc portion in ey > hid-p35 imaginal discs (B–B”). Expression of GFP and mCherry is low in the control ey > p35 discs (A–A”). In contrast, the numbers of GFP and mCherry positive particles are strongly increased in the overgrown ey::G4a expressing area of ey::hid-p35 discs (B–B”). Although the overgrowth of ey > hid-p35 eye discs is strongly suppressed by dAtg1 RNAi, the GFP and mCherry signals are not significantly reduced (C–C’). (TIF 7345 kb)

Additional file 7: Figure S7. Functional tests of the RNAi lines targeting dAtg1, dAtg3, dAtg8a, and dAtg8b. (A–E) Stavasson assay of fat bodies from third instar larvae. Formation of autophagosomes was visualized by mCherry-Atg8 (red in A, E; green in A–E’). Cells expressing RNAi constructs are labeled by GFP and outlined by yellow dotted lines. (A) Wildtype fat body displaying mCherry-Atg8 puncta both in clone cells and surrounding cells. (B–E) Cells expressing dAtg1, dAtg3, dAtg8a, and dAtg8b RNAi (GFP”) fail to form mCherry-Atg8 marked autophagosomes. (TIF 8240 kb)
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