**Drosophila acinus** encodes a novel regulator of endocytic and autophagic trafficking

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**SUMMARY**
Endosomal trafficking affects many cellular pathways from cell signaling to metabolism, but little is known about how these effects are coordinated. In a genetic screen for mutants affecting endosomal trafficking, we identified *Drosophila acinus* (*dacn*; hook-like). Its mammalian homolog Acinus has been implicated in RNA processing and chromatin fragmentation during apoptosis. Loss-of-function analysis of *dacn* revealed two distinct functions. First, *dacn* is required for stabilization of early endosomes, thus modulating levels of Notch and Egfr signaling. Second, loss of *dacn* interferes with cellular starvation responses by inhibiting autophagosome maturation. By contrast, overexpression of *dacn* causes lethality due to enhanced autophagy. We show that this enhanced autophagy is independent of the Tor pathway. Taken together, our data show that *dacn* encodes a regulator of endosomal and autophagosomal dynamics, modulating developmental signaling and the cellular response to starvation.

**KEY WORDS:** Endocytic trafficking, Autophagosomes, Acinus, Egfr, Notch, *Drosophila*

**INTRODUCTION**
Cells must regulate the transport of proteins and lipids between numerous organelles in order to maintain basic cellular functions. Regulation of this trafficking is complex. In yeast, for example, more than fifty genes are required for the transport of endocytosed material from early endosomes to multivesicular bodies (MVBs) and finally the vacuole (Mullins and Bonifacino, 2001). The regulation of lysosomal trafficking in multicellular organisms is even more complex, as cells must also regulate their interactions with neighboring cells and their environment. For example, the strength of developmental signaling is controlled by the endocytosis and trafficking of activated receptors (Zwang and Yarden, 2009). Once internalized, many receptors continue to signal from early endosomes until they are segregated from the cytoplasm inside MVBs. Mutations that block transport from early endosomes into MVBs result in increased levels of receptor signaling (Lloyd et al., 2002; Vaccari et al., 2008). Thus, cells may regulate the trafficking of activated receptors to ensure proper levels of signaling and to modulate signal strength.

In order to identify novel components that mediate endosomal trafficking, we undertook a genetic screen in *Drosophila* for abnormal eye pigmentation, a sensitive readout of endocytic trafficking (Lloyd et al., 1998). We identified mutations in the *Drosophila* (d) homolog of Acinus (*dacn*; hook-like – FlyBase). Mammalian Acinus [apoptotic chromatin condensation inducer 1 (Acin1)] is a primarily nuclear protein that has been implicated in apoptotic chromatin destruction (Joselin et al., 2006; Sahara et al., 1999) and that physically interacts with RNA-binding proteins (Schwer et al., 2003; Tange et al., 2005). We find that DAcn is also primarily nuclear, but that it is not required for DNA condensation or fragmentation during apoptosis. Instead, *dacn* mutants exhibit reduced levels of early endosomes resulting in a reduction of Notch and Egfr signaling. Furthermore, *dacn* mutants also exhibit reduced maturation of autophagosomes into autolysosomes. Strikingly, overexpression of *dacn* is lethal due to an overabundance of autophagy. Thus, *dacn* appears to be a nuclear regulator of endosomal transport and autophagosomal maturation.

**MATERIALS AND METHODS**

**Genetic screen and fly genotypes**
To find new regulators of endocytic trafficking, we performed a two-tiered screening of ~190,000 mutagenized male flies (25 mM EMS or 3000 rads of β-irradiation). F1 flies carried whole-eye clones of a single mutated chromosome arm (Stowers and Schwarz, 1999) and were screened for defects in eye color. Flies carrying FRT insertions at 40A, 42B, 80D and 82B were screened without prior isogenization. First, eye color mutants were identified in adult whole-eye clones (Stowers and Schwarz, 1999). Approximately 500 eye color mutants were subsequently examined for defects in endosomal trafficking by staining for Boss in third instar eye discs. Forty mutant lines showed defects in both eye pigmentation and Boss trafficking.

Additional fly strains used were: l(2)37Ba¹, Df(2L)TW130 (Stathakis et al., 1995), N¹¹⁶¹¹-¹¹ (Slizynska, 1938), Egfr¹ (Baker and Rubin, 1989), UAS-Rhee (Scott et al., 2004), UAS-Tor¹ (Scott et al., 2004), UAS-Atg5-RNAi (Scott et al., 2004), UAS-Atg10-RNAi (Scott et al., 2004), UAS-p110 (Scott et al., 2004) and Lsp2-Gal4, ey>FLP, UAS-Atg8-GFP (Rusten et al., 2007).

**Molecular biology**
A 4.2 kb *dacn* genomic rescue fragment was amplified using primers 5'-GGGGATCCAAAGCGCGGTAAAGACG-3' and 5'-GGGGCGCGGCTCGCGCTGATGACGTTAT-3' and cloned into pCaeSpeR4. For a second set of transgenes a 2×Myo epitope was inserted between codon 1 and 2 of *dacn* in the context of the 4.2 kb genomic rescue fragment. Both transgenes restored viability to *dacn*⁻⁻/¹⁺ dacn⁻⁻/¹⁺ transheterozygotes and rescued endocytosis and autophagy defects of *dacn*⁻⁻/¹⁺ larvae and *dacn*⁻⁻/¹⁺ clonal. However, they did not restore viability to the individual mutant lines, most likely because of second-site mutations.

To make pUAS-2×Myo-dAcn, *dacn* was amplified from cDNA LD46360 using primers 5'-GAATTCAATGACGATGCACGCTCGAG-3' and 5'-GTCGACACGTCGACGCTCTCCGTCCT-3'. The PCR product was
cloned into the EcoRI and Xhol sites of pMT-2×Myc (Akbars et al., 2009). A 2×Myc-dAcn fragment was excised with KpnI and SalI and cloned into pUAST. Embryo injections were performed by Best Gene.

**Antibodies**

A GST fusion protein containing amino acids 423-599 of dAcn was expressed in bacteria, purified and injected into guinea pigs. Specific antibodies were affinity purified using a His-tagged version of the same dAcn fragment. For detection of endogenous proteins by immunofluorescence, we used anti-dAcn (1:1000), anti-Avl (1:1000), anti-Vps16A (1:1000) (Pulipparachuruvil et al., 2005), anti-Boss1 (1:300) (Krämer et al., 1991), anti-Dir (1:400) (Klueg et al., 1998), anti-Hrs (1:300) (Lloyd et al., 2002), anti-Rab5 (1:50) (Wucherpfennig et al., 2003) and anti-Rab7 (1:250) (Chinchore et al., 2009). Cell-surface DI was labeled with antibodies against Tubulin (1:5000; DM1a, Sigma-Aldrich), Protein (10 μg/ml) (DC Protein Assay, Bio-Rad) were run in each lane and measured with ImageJ (NIH). Fluorescence microscopy of whole-mount tissues, and light and electron microscopy of plastic sections of Epon-embedded tissues, were described as (Akbars et al., 2009) and adjusted for brightness and contrast using Photoshop (Adobe). For PAS staining, sections of Epon-embedded eyes were dehydrated for 10 minutes in 50% periodic acid (Sigma-Aldrich), rinsed with water, incubated for 20 minutes in Schiff’s Reagent (Sigma-Aldrich), rinsed in water and mounted in Permount. Scanning electron microscopy of adult eyes was performed on an FEI XL30 environmental scanning electron microscope. Transmission electron microscopy was performed on an FEI Tecnai G2 Spirit BioTwin. Measurements of organellar size from transmission electron micrographs were performed using ImageJ. Organellar peripheries were traced and the area of the outlined organellae was calculated. The perimeter of tissue in micrographs was traced and measured, and the percentage of tissue composed of organellae was calculated as the sum of organellar areas divided by tissue area.

**Histology**

Micrographs of eyes and wings were obtained on a SteREO Discovery V12 microscope. Images of eyes are a composite of pictures taken at multiple z positions and compressed using C2Focus software. Wing notches were measured with ImageJ (NIH). Fluorescence microscopy of whole-mount tissues, and light and electron microscopy of plastic sections of Epon-embedded tissues, were described as (Akbars et al., 2009) and adjusted for brightness and contrast using Photoshop (Adobe). For PAS staining, sections of Epon-embedded eyes were dehydrated for 10 minutes in 50% periodic acid (Sigma-Aldrich), rinsed with water, incubated for 20 minutes in Schiff’s Reagent (Sigma-Aldrich), rinsed in water and mounted in Permount. Scanning electron microscopy of adult eyes was performed on an FEI XL30 environmental scanning electron microscope. Transmission electron microscopy was performed on an FEI Tecnai G2 Spirit BioTwin. Measurements of organellar size from transmission electron micrographs were performed using ImageJ. Organellar peripheries were traced and the area of the outlined organellae was calculated. The perimeter of tissue in micrographs was traced and measured, and the percentage of tissue composed of organellae was calculated as the sum of organellar areas divided by tissue area.

**Immunofluorescence quantitation** was performed using Amira software. Mutant and wild-type regions were identified based on nuclear GFP fluorescence. Signals were thresholded to remove background fluorescence, and the average signal intensity per area in the regions was measured. Fluorescence in mutant patches was compared with that of wild-type patches from the same tissue to determine the percentage of intensity in the mutant. Percentages from three different tissue samples were averaged.

**Hemocyte culture**

The lethal phase for dacn1/dacn7 transheterozygotes is distributed, with most dying during the third larval instar and only a few surviving to early pupal stages. dacn1/dacn7 transheterozygote third instar larvae were used to isolate dAcn hemocytes and dAcn fat bodies. Third instar larval hemocytes were cultured (Chew et al., 2004) and apoptosis was induced by an 8-hour incubation in 100 μM cycloheximide or 1-hour incubation in 50 μM Smac mimetic (Li et al., 2004). TUNEL staining was performed using the ApopTag Red In Situ Apoptosis Detection Kit (Chemicon). A 10-minute incubation in Hoechst was performed just prior to mounting.

**Biochemistry**

For western blots, ten third instar larvae were crushed in 300 μl of lysis buffer (10% SDS, 6 M urea, 50 mM Tris-HCl pH 6.8) at 95°C, boiled for 3 minutes, and spun for 10 minutes at 20,000 g to remove larval cuticle. Protein (10 μg; DC Protein Assay, Bio-Rad) were run in each lane and detected with antibodies against Tubulin (1:5000; DM1a, Sigma-Aldrich), dAcn (1:4000), Avl (1:3000), Vps16 (1:3000) (Pulipparachuruvil et al., 2005), Hrs (1:3000) (Lloyd et al., 2002), Rab5 (1:2000) (Wucherpfennig et al., 2003) and Vps28 (1:1000) (Sevrioukov et al., 2005). Western blots were quantified using the Odyssey System as recommended by the manufacturer (LI-COR Biosciences).

**Statistical methods**

All data sets were compared by two-tailed Student’s t-test using GraphPad Prism 5. Data are plotted as mean ± s.e.

**RESULTS**

**dacn regulates pigment granule and lysosome function**

To identify novel regulators of endosomal trafficking, we took advantage of the observation that mutations in genes that interfere with the transport of cargo to lysosomes and lysosome-related organelles affect pigment granules in the fly eye (Lloyd et al., 1998). We screened ~190,000 mutant flies for pigment defects in FLP/FRT-induced whole-eye clones (Stowers and Schwarz, 1999). Approximately 500 mutants with altered eye color were further tested for defects in the trafficking of internalized ligands. Here, we describe one of these mutants with abnormal eye pigmentation and defects in ligand trafficking (Fig. 1). This line carried a nonsense mutation in the Drosophila (d) homolog of Acinus (dacn; also known as CG10473 and hook-like); we named this allele dacn7. Flies with whole-eye clones mutant for dacn7 exhibited rust-colored eyes with no pseudopupil (Fig. 1A,B).

Complementation tests identified a second allele dacn1, previously called I(2)37Ba’ (Stathakis et al., 1995). When crossed to each other or to Df(2L)WT130, which removes the dacn gene region (Stathakis et al., 1995), dacn1 and dacn7 are lethal. The lethal phase for dacn1/dacn7 transheterozygotes is distributed, with most larvae dying during third instar and a few after pupariation. Viability of dacn1/dacn7 transheterozygote flies was restored by transgenes containing a 4.2 kb genomic rescue fragment. These transgenes also rescued all other phenotypes that we tested, including the defects in eye color and the external roughness of dacn7 mutant eyes (Fig. 1C). Acinus proteins share the highly conserved central P17 region, which functions in chromatin destruction during apoptosis in vertebrates (Joselin et al., 2006; Sahara et al., 1999). A conserved C-terminal domain contains two consensus Akt1 phosphorylation sites. Importantly, dacn1 and dacn7 truncate dAcn after amino acids 242 and 302, respectively, deleting all of these conserved sequences (Fig. 1J). Together, these data indicate that we have identified two strong loss-of-function or null alleles of dacn.

To examine lysosomal delivery, we analyzed the localization of two ligands, Boss and Delta (DI). Boss is expressed on the apical surface of R8 photoreceptor cells in the developing eye disc, and upon binding to the Sevenless receptor is endocytosed into R7 precursors (Fig. 1D, arrowheads) located in a stereotypical position next to R8 (Krämer et al., 1991). In dacn27/dacn1 eye disc, Boss protein was present on R8, but was not detected in R7 (Fig. 1E). Boss staining in R7 was restored upon addition of a transgene carrying the dacn genomic region (Fig. 1F).

To further investigate this defect, we generated random mitotic clones in eye discs, so that dacn27 cells were present next to wild-type cells. Whereas 87% (n=179) of wild-type R7 cells exhibited Boss staining, only 2% (n=58 from nine eye discs) of dacn27 R7 cells showed staining for Boss (Fig. 1G). Importantly, wild-type R8 cells did not rescue defects in neighboring mutant R7 cells (Fig. 1G, yellow arrows) indicating that this is a defect in endocytosis rather than secretion.

A second ligand, DI, is present in endosomal puncta in many developing photoreceptors (Klueg et al., 1998) and is visible by its partial colocalization with the early endosomal marker Avalanche (Avl; SxY7 - FlyBase) (Fig. 1H, inset; see Fig. S1 in the supplementary material). DI puncta were reduced in dacn27 clones.
(Fig. 1H), and normal levels were restored by a dacn transgene (data not shown). Thus, endosomal levels of both ligands were reduced in dacn cells, showing that loss of dacn alters endocytic trafficking.

We generated an antibody to the C-terminus of dAcn, including the P17 domain, to determine its subcellular localization. On western blots of third instar larval lysates, the antibody recognized a protein present in wild-type but not dacn1/dacn27 lysates. The protein was restored to wild-type levels in lysates from dacn larvae carrying a genomic dacn transgene (Fig. 2A). Anti-dAcn staining in eye discs was abolished in dacn1/dacn27 mutant clones (see Fig. S2 in the supplementary material). Interestingly, dAcn was diffusely distributed in the cytoplasm and accumulated in nuclei in a subset of cells. When compared with markers for various endocytic organelles, we did not detect any significant colocalization (see Fig. S2 in the supplementary material). In the developing eye disc, dAcn was present in the nuclei of cone cells and photoreceptors in a dynamic and developmentally regulated pattern (Fig. 2B-F).

dacn is not required for apoptotic chromatin destruction

Mammalian Acinus has been implicated in chromatin condensation (Sahara et al., 1999) and fragmentation (Joselin et al., 2006) during apoptosis. Both dacn alleles remove the region that is similar to the P17 domain of mammalian Acinus, which is sufficient to condense chromatin in vitro (Sahara et al., 1999). We examined whether dAcn has a similar function. In hemocytes isolated from wild-type third instar larvae, a 1-hour treatment with Smac mimetic induces apoptosis (Chew et al., 2004). This caused nuclear condensation, as assayed by Hoechst staining, and chromatin fragmentation, as assayed by TUNEL labeling (Fig. 3A-F). By both criteria, wild-type hemocytes were indistinguishable from those isolated from dacn1/dacn27 third instar larvae (Fig. 3G-I). Of 60 wild-type and dacn127 cells scored each, all showed the phenotypes depicted in Fig. 3. Although these experiments cannot completely rule out a role of dacn in apoptosis, they indicate that it is not strictly required for the destruction of chromatin during apoptosis in Drosophila.

Early endosomes are reduced in dacn cells

The reduced endosomal levels we observed for Boss and Dl could be due to a failure to endocytose these ligands or to their enhanced degradation after internalization. We performed two experiments to distinguish between these possibilities.

First, we incubated eye discs containing dacn clones with chloroquine for 2 hours to neutralize lysosomal pH and inhibit lysosomal degradation. After chloroquine treatment, Dl staining was detected in all dacn cells (Fig. 3J; see Fig. S1 in the supplementary material) and levels of DI in dacn clones were similar to those of surrounding wild-type cells (Fig. 3I). Therefore, the loss of staining for endosomal ligands in dacn is largely due to enhanced lysosomal degradation of endocytosed proteins.

To further investigate the trafficking of endocytosed proteins, we used anti-DI antibodies as a ligand to follow its trafficking in live eye discs containing dacn clones. DI exposed on the cell surface of eye discs was tagged with the antibody and its trafficking compared in dacn and neighboring wild-type cells. Immediately after labeling, the level of DI antibodies on apical membranes appeared indistinguishable between wild-type and mutant cells, indicating that there is no difference in the steady-
state levels of DI at the cell surface (Fig. 4A). After a 20-minute chase, the level of DI antibodies present in apical punctae appeared lower in dacn cells (Fig. 4B; see Fig. S1A,B in the supplementary material). The difference was not due to the basal displacement of the staining (Fig. 4B) and also did not reflect a change in total uptake as there was no difference in the amount of DI antibodies that remained on the cell surface; after a 60-minute chase, the levels of remaining DI antibody were again indistinguishable between the wild type and dacn (Fig. 4C). Fluid-phase endocytosis was not altered as dextran particles were endocytosed efficiently into dacn cells and did not show significant differences in fluorescence intensity compared with wild-type cells (Fig. 4D). These data indicate that dacn cells have no change in the internalization of cargo but may exhibit a change in endosome distribution or stability.

To further analyze possible endosomal defects, we stained third instar eye discs containing dacn clones for markers of different endosome populations. The early endosome and MVB proteins Avl, Rab5, and Hrs all showed reduced apical staining in dacn mutant clones (Fig. 5A-C,F; see Fig. S1 in the supplementary material), compared with only minor changes for the late endosome protein Rab7 (Fig. 5D) and the recycling endosome marker Rab11 (Fig. 5E). These changes in endosomal markers did not reflect a basal displacement of endosomes in dacn cells (see Fig. S1 in the supplementary material) and thus might indicate that these normally apically enriched proteins are dispersed throughout mutant cells, making their detection above background difficult. Alternatively, the reduced staining might reflect changes in steady-state levels. Levels of Hrs, Rab5, and Avl appeared unchanged on western blots of lysates of dacn/dacn cells third instar larvae (Fig. 2A; see Fig. S3 in the supplementary material); however, it is possible that tissue-specific differences in expression might obscure reduced expression in some cell types. When taken together with the altered ligand trafficking, the change in endosomal staining suggests that early endosomes in dacn cells are less stable than those in wild-type cells, resulting in enhanced delivery of endocytosed proteins to lysosomes.

dacn regulates developmental signaling molecules

As many receptors signal from early endosomes (Fortini and Bilder, 2009), altered endosomal stability may result in developmental defects. Multiple signaling pathways contribute to the stereotypical trapezoidal array of photoreceptors in wild-type Drosophila eyes (Fig. 6A). In sections of dacn27 eyes, some ommatidia were patterned correctly, but 41% had missing or extra photoreceptors or were disorganized (Fig. 6B). Because such
defects point to possible defects in Egfr or Notch signaling, we performed genetic interaction studies to determine whether either signaling pathway was affected in dacn cells.

To investigate interactions with Egfr signaling, we used Egfr\textsuperscript{F1}, a dominant gain-of-function allele that causes excessive signaling during development (Baker and Rubin, 1989) resulting in rough eyes (Fig. 6C,D). Removal of one functional copy of dacn rescued the Egfr\textsuperscript{F1}/+ rough eye phenotype (Fig. 6E). This was specific for dacn as Egfr\textsuperscript{F1} roughness was restored by a dacn genomic transgene (Fig. 6F). By contrast, removing one copy of dacn did not rescue the rough eye phenotype caused by overexpression of activated Ras\textsuperscript{12} (see Fig. S4 in the supplementary material), which activates the Egfr signaling pathway downstream of the receptor (Fortini et al., 1992). Although different sensitized systems might show differential sensitivity to a reduction in the dacn gene dosage, the most straightforward explanation for this observation is that reduced Egfr signaling in dacn reflects a defect in the trafficking of the Egfr protein, rather than an event downstream of the receptor.

To investigate interactions between dacn and Notch (N), we used N\textsuperscript{264-39/+} flies, which have notched wings due to reduced Notch signaling during wing patterning (Slizynska, 1938) (Fig. 6G). Reducing the dose of dacn by one copy doubled the size of the notches (Fig. 6H,I). The addition of a genomic dacn transgene abrogated the dacn effect. Together, these data are consistent with a requirement of dacn in stabilizing early endosomes that serve as signaling platforms for multiple receptors.

dacn mutations inhibit the maturation of autophagosomes

To visualize ultrastructural defects in dacn mutant cells, we analyzed sections of adult whole-eye clones by transmission electron microscopy (TEM). In pigment cells, we noticed abundant 40- to 50-nm granules that were reminiscent of glycogen granules (Fig. 7A,B). Because glycogen degradation occurs to a significant extent via autophagy (Kotoulas et al., 2006), inhibition of autophagy was caused by changes in autophagic signaling. Expression of Pten or a dominant-negative Target of
rapamycin (Tor) kinase induced autophagy in wild-type fat bodies (Rusten et al., 2004; Scott et al., 2004), but failed to so in dacn fat bodies (Fig. 7I-L). Thus, dacn is required downstream of, or parallel to, the Tor signaling pathway in autophagy. The localization of dacn to a subset of fat body nuclei was not altered by starvation (see Fig. S5 in the supplementary material).

To determine the step in autophagy at which dacn is required, we analyzed dacn fat bodies by TEM. Autophagosomes in TEM appear as round structures containing cytoplasm and organelles surrounded by an electronlucent region between the two membranes, whereas autolysosomes are filled with degradative electron-dense material (Eskelinen, 2008). After starvation, autophagosomes were larger and more abundant in dacn than in

Fig. 6. Egfr and Notch signaling are reduced in dacn mutants. (A,B) Sections of adult Drosophila eyes showed stereotypical patterning in a wild-type whole-eye clone (A), but 41% of ommatidia in dacn27 whole-eye clones had patterning defects (B). (C-F) Scanning electron micrographs of adult eyes showing regular patterning in wild-type (C) and roughness in Egfr1/4 (D) eyes. Removing one copy of dacn from Egfr1/4 flies restored eye patterning to close to that of the wild type (E), and a dacn genomic rescue construct abrogated the effect of the dacn1 (F). (G,H) N263-39/+ wings had small wing notches (G) due to reduced Notch signaling. Removal of a copy of dacn (H) enhanced the wing notch phenotype. (I) Measurement of wing notch area showed that removal of one copy of dacn doubled the size of the notches. This effect is suppressed by a genomic rescue construct. Notch area: wild type, 1870±151.2 μm², n=105; dacn1, 3391±417.9 μm², n=82, P<0.002; dacn27, 3637±233.9 μm², n=150, P<0.0001; dacn1 plus rescue, 2306±233.1 μm², n=72, P<0.0306 versus dacn1 only; dacn27 plus rescue, 1990±151.6 μm², n=126, P<0.0001 versus dacn27 only. Genotypes: (A,C) Oregon R; (B,D) dacn27 nGFP38 FRT40A/FRT40A; (E,F) dacn27 rescued; (G,H) N263-39/+; dacn1; (I,J) N263-39/+; dacn27 nGFP38 FRT40A/FRT40A; (K,L) N263-39/+; dacn1; (M,N) N263-39/+; dacn27 nGFP38 FRT40A/FRT40A. N263-39/+ was used as a control.

Fig. 7. Impaired autophagosome maturation in dacn mutants. (A) TEM of adult Drosophila eyes. Compared with wild-type eyes (A), dacn27 eyes (B) contained high levels of glycogen-like particles in pigment cells (those not pseudo-colored yellow). Inset shows a higher magnification image of the glycogen-like particles. (C,D) Sections of dacn27 whole-eye clones (D), but not wild-type clones (C), tested positive for glycogen by PAS staining. (E-L) Micrographs of fat body cells stained with Lysotracker (red) and Hoechst (blue). Four hours of starvation induced Lysotracker-positive autolysosomes in the wild-type fat body (E,F). Autolysosome formation induced by expression of torTEX (J,K,L) was also blocked in dacn1/dacn27 fat bodies (F,J). Autolysosome formation induced by expression of torTEX (J,K,L) was also blocked in dacn1/dacn27 fat bodies (J). (M,N) TEM of fat bodies revealed autophagosomes (AP) and autolysosomes (AL) in wild-type (M) and dacn1/dacn27 (N) starved fat bodies. (O) Quantification of organelle size and quantity in electron micrographs. Autophagosome area as a percentage of total tissue increased in dacn, whereas autolysosome area was reduced. All phenotypes were restored to wild-type by the addition of a genomic dacn rescue construct. *P<0.0001 compared with wild type or dacn plus rescue. Genotypes: (A,C) Oregon R; (B,D) dacn27 nGFP38 FRT40A/FRT40A; (E,F) dacn27 rescued; (G,H) dacn27 nGFP38 FRT40A/FRT40A; (I,J) dacn27 rescued; (K,L) Lsp2-Gal4; UAS-TorTEX; (Lsp2-Gal4; UAS-TorTEX; (I,J) Lsp2-Gal4; UAS-TorTEX; (Lsp2-Gal4; UAS-TorTEX; (K) Lsp2-Gal4; UAS-Pten; (L) Lsp2-Gal4; dacn27 nGFP38 FRT40A/FRT40A; (J) Lsp2-Gal4; UAS-Pten; (L) Lsp2-Gal4; dacn27 nGFP38 FRT40A/FRT40A; UAS-TorTEX; (K) Lsp2-Gal4; UAS-Pten; scale bars: 25 μm in E-L; 1 μm in N for M,N.
wild-type fat bodies or in those of dacn larvae carrying a genomic dacn transgene. By contrast, autolysosomes were less abundant (P<0.0001 for all autolysosome comparisons; P=0.0001 for all autophagosome comparisons; n=47 sections for wild type, n=46 for dacn1/27 and n=35 for dacn1/27 plus rescue transgene) (Fig. 7M-O). Autolysosomes were also significantly smaller in dacn than in wild-type fat bodies or in those of dacn larvae with the rescue transgene (n=100 autolysosomes each; P<0.0001 for dacn1/27 compared with the wild type or dacn1/27 plus rescue transgene). Together, these data indicate that dacn is not required for the formation of autophagosomes but for their maturation into autolysosomes.

**dAcn overexpression induces autophagy**

To further define the role of dAcn in autophagosome maturation, we overexpressed dAcn using the fat body-specific driver Lsp2-Gal4. Overexpression of dAcn induced autolysosome formation in the absence of starvation (Fig. 8A). To determine whether dAcn induces autophagy via activation of the Akt/Tor pathway, we co-expressed repressors of the Akt/Tor pathway along with dAcn. Co-expression of p110 or Rheb (Scott et al., 2004) did not interfere with enhanced Lysotracker staining in dAcn-overexpressing cells (Fig. 8B,C), indicating that dAcn induces autophagy downstream of, or in parallel to, Tor. Consistent with Lysotracker staining in dAcn-overexpressing cells, TEM detected an increased number of small multi-lamellar organelles, which may represent late endosomes or autolysosomes (Fig. 8H). To distinguish between these possibilities, we used RNAi to knock down Atg5, an essential autophagy component (Scott et al., 2004). In cells expressing an inverted repeat targeting Atg5 RNA (Atg5IR), dAcn expression no longer induced Lysotracker staining (Fig. 8D). Furthermore, Atg8-GFP, which labels autophagosomes and autolysosomes (Rusten et al., 2007), localized to punctae that accumulated in response to dAcn overexpression (Fig. 8E-G). Together, these results indicate that overexpression of dacn induces autolysosome formation.

Expression of dAcn throughout the developing fly with the Tubulin-Gal4 driver is lethal, as no adult flies emerged. Control flies lacking the UAS-dAcn transgene survived to adulthood (n=503 from several independent crosses). To determine whether this lethality is due to induction of autophagy, we analyzed the survival of flies in which autophagy had been blocked by knock down of Atg5 (Fig. 8I). Expression of Atg5IR with Tubulin-Gal4 caused some loss of viability, as only 40.3% of the expected number of flies surviving (controls lacking UAS-dAcn transgene, n=258). Co-expression of Atg5IR and dAcn resulted in a similar degree of viability, with 37.9% of the expected number of flies surviving (controls lacking UAS-dAcn transgene, n=286). By contrast, the presence of an unrelated UAS transgene (UAS-CD8-GFP) did not ameliorate the survival of dAcn-expressing flies (0%, n=111). Thus, blocking autophagy rescued the lethality of dacn overexpression to the level of an otherwise wild-type fly in which autophagy is blocked. The resulting flies had no obvious developmental defects. This indicates that upregulation of autophagy is the principal cause of dacn-induced lethality.

**DISCUSSION**

Cells modulate the rate of autophagy in response to metabolic changes, cellular stress and developmental signals. Thus, it is not surprising that multiple inputs regulate distinct steps along the autophagic pathway. Much progress has been made in understanding the initial stages of autophagy and their regulation by the canonical Tor pathway (Cecconi and Levine, 2008; Mizushima et al., 2008; Nakatogawa et al., 2009; Neufeld and Baehrecke,
Less is known, however, about the mechanisms that regulate the maturation of autophagosomes and their interaction with endosomes and lysosomes. Here, we describe dAcn as a novel element in the regulation of endocytic trafficking and autophagy.

**dAcn is not required for apoptosis in Drosophila**

Mammalian Acinus was initially described for its role in the destruction of chromatin during apoptosis (Joselin et al., 2006; Sahara et al., 1999). Although the fragment of Acinus that functions during apoptosis is highly conserved with dAcn, we found no evidence for a role of dacin in chromatin destruction during apoptosis in third instar larval hemocytes. Nevertheless, we cannot rule out the possibility that dacin functions in apoptosis but is not absolutely required, and recent studies have shown that dAcn physically interacts with the anti-apoptotic protein Aac11 (Rigou et al., 2009). Furthermore, although dAcn overexpression is lethal, this is unlikely to be due to a direct induction of chromatin condensation or fragmentation as the lethal effect of dAcn in Drosophila is suppressed by inhibiting autophagy.

**dAcn regulates early endosome function**

Cells that are mutant for dacn exhibit two distinct phenotypes. First, early endosomes appear destabilized. This was evident by the reduced level of staining for multiple early endosomal markers in the absence of an overall reduction in their levels. The defect in early endosome function was reflected in reduced signaling by Egr and Notch. Such defects in signaling are also observed in Avl or Rab5 loss-of-function mutants (Vaccari et al., 2008). This similarity is striking as the effect of these two mutants on the trafficking of cell-surface proteins is opposite to that of dacn. In Avl or Rab5 mutant cells, endosomal fusion is blocked and ligands and receptors are inhibited from reaching early endosomes and progressing to MVBs and eventually lysosomes (Lu and Bilder, 2005). By contrast, dacn cells internalize ligands at a rate that is indistinguishable from wild-type cells and hasten lysosomal degradation. Thus, the only common effect of these two classes of mutant with regard to trafficking is the reduced time that activated receptors spend in early endosomes. The observation that a shortened presence of activated receptors in the early endosome results in reduced signaling, regardless of the underlying mechanism, further supports the notion that endosomes serve as crucial signaling platforms for activated receptors including Egr and Notch (Vaccari et al., 2008).

It is important to note that endocytosis of the Dl ligand is also crucial for Notch signaling (Fortini and Bilder, 2009). Therefore, the effect of dacn on Notch signaling might be due to an effect on the endocytosis of Notch or Dl, or both. An analysis of this by cell-autonomy studies is hindered, however, by the mild effect of dAcn on Notch signaling. Unlike shibire2, which blocks endocytosis at the non-permissive temperature and completely inhibits Notch signaling (Fortini and Bilder, 2009), the effect of dacn on Notch or Dl trafficking is less severe and, as judged by the mild phenotypes observed in dacn mutant eyes, causes an accordingly modest reduction in Notch signaling.

Interestingly, dAcn itself appears to be regulated because the degree of its nuclear localization dynamically changes in the developing eye at stages at which active Notch and Egr signaling occur (Fig. 2). In mammalian cells, Akt-mediated phosphorylation alters the binding of Acinus to zyxin and, thereby, its nuclear access (Chan et al., 2007; Hu et al., 2005). As Akt activity is regulated by receptor tyrosine kinases, the regulated localization of Acinus might be part of a feedback loop to fine-tune signaling in development.

**dAcn regulates autophagosome maturation**

A second distinct phenotype of cells lacking dAcn is a block in the autophagic pathway. When flies are starved, fat body cells respond with an increased formation of autophagosomes, which mature to acidified autolysosomes upon fusion with lysosomes (Rusten et al., 2004; Scott et al., 2004). By contrast, dacn cells accumulate autophagosomes as they fail to mature to autolysosomes. A straightforward explanation for such a phenotype is a role of dAcn in regulating the fusion of autophagosomes with endosomes or lysosomes. Alternatively, dAcn might play a role in the fusion of autophagosomes to endosomes that is known to be important for autophagosome maturation in mammalian cells (Razi et al., 2009; Rusten and Simonsen, 2008). This requirement for functional endosomes during the maturation of autophagosomes generates a complex interplay between these two distinct pathways to lysosomes (Filimonenko et al., 2007; Lee et al., 2007; Razi et al., 2009; Rusten et al., 2007), making it difficult to pinpoint the precise mechanisms by which ESCRT proteins participate in autophagy (Rusten and Stenmark, 2009).

A function of dAcn in a late step in autophagy is consistent with our epistasis experiments using mutants affecting the Tor pathway. This pathway is crucial in regulating the initial formation of autophagosomes (Neufeld, 2003; Noda and Ohsumi, 1998). Expression of Pten or a dominant-negative Tor kinase induces autophagy in wild-type cells, but not in dacn cells. Furthermore, dAcn expression causes increased autophagy, which is not suppressed by inhibitory elements of the Tor pathway such as PI3 kinase (p110) or Rheb (Scott et al., 2004). Thus, dAcn acts downstream of, or in parallel to, the canonical Tor pathway at a step that is required for autophagosomes to mature into autolysosomes.

**Other Drosophila mutants** that interfere with such late steps in autophagy include deep orange (dor) and carnation (car), which both encode subunits of the HOPS complex (Sevrioukov et al., 1999). This multi-protein complex is necessary for the fusion of lysosomes to late endosomes and autophagosomes (Akbar et al., 2009; Klionsky, 2005; Lindmo et al., 2006; Pulipparacharuvil et al., 2005). Regulation of HOPS activity in tethering or fusion might explain the function of protein complexes containing beclin 1, Uvrag and rubicon proteins in mammalian cells (Liang et al., 2008; Matsunaga et al., 2009; Zhong et al., 2009). Overexpression of Uvrag enhances both autophagosome maturation and endosomal fusion (Liang et al., 2008). Loss of rubicon, which binds beclin 1, also enhances both autophagosome and endosome maturation (Matsunaga et al., 2009; Zhong et al., 2009). Interference with any of those interactions might explain the consequences of dAcn in modulating autophagy and endocytic trafficking.

It is important to note, however, that mutants inactivating the HOPS subunits Dor and Car have effects that are opposite to those of dacn with regard to lysosomal delivery of endocytosed cargo. In dor and car cells, internalized ligands are inhibited from reaching lysosomes and instead accumulate in Rab7-positive late endosomes (Akbar et al., 2009; Sevrioukov et al., 1999; Sriram et al., 2003). This sharply contrasts with the enhanced degradation of ligands we observe in dacn cells. The observation that dAcn and HOPS components appear to act synergistically in autophagic trafficking, yet antagonistically in endosomal trafficking, is not easily reconciled with a straightforward role of dAcn in modulating the HOPS-mediated lysosomal tethering and fusion reactions in these two pathways.

As dAcn is found in the nucleus in many cell types, it might affect autophagy by regulating gene expression. Mammalian Acinus physically interacts both with spliceosomes (Chen et al.,
dAcn regulates autophagosome-lysosome fusion

2007; Zhou et al., 2002) and the exon junction complex, which regulates mRNA export and stability (Tang et al., 2005). These observations raise the possibility that in Drosophila, dAcn might modulate autophagy through the regulation of post-transcriptional RNA processing, but we do not know yet which targets might be affected by its activity. If dAcn regulates RNA processing, it might affect just one of the RNAs that encode these key regulatory proteins or, alternatively, a large number of RNAs. A recent example for the concerted regulation of a broad collection of functionally related targets comes from the gene network that regulates lysosomal biogenesis and function, in which transcription factor EB regulates the expression of more than twenty lysosomal genes (Sardiello et al., 2009). Although this network operates at the transcriptional level, dAcn might participate in a similar coordinated regulation at the level of RNA processing.

Alternatively, nuclear localization of dAcn might reflect a function that is distinct from its role in membrane trafficking. Many cytoplasmic regulators of endocytic trafficking have distinct nuclear functions. For example, Epsin, which functions in Clathrin-mediated endocytosis and autophagy (Csikos et al., 2009), and the ESCRT III protein Snf7, which functions in the cytoplasm to sort proteins into MVBs, also function in the nucleus to regulate gene expression or chromatin remodeling (Borlido et al., 2009; Pilecka et al., 2007). These proteins shuttle between the nucleus and cytoplasm, and many appear to be exclusively cytoplasmic until nuclear export is blocked. Current studies aim to dissect which of the activities of dAcn depend on its nuclear or cytoplasmic localization.

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