FEBS Letters 543 (2003) 154–158 FEBS 27248

The *Drosophila* homolog of Aut1 is essential for autophagy and development

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Received 10 February 2003; revised 4 April 2003; accepted 5 April 2003

First published online 1 May 2003

Edited by Jesus Avila

Abstract The *Drosophila* homolog of yeast Aut1, CG6877/Draut1, is a ubiquitously expressed cytosolic protein. Draut1 loss of function was achieved by expression of an inverted repeat transgene inducing RNA interference. The effect is temperature-dependent and resembles an allelic series as described by Fortier, E. and Belote, J.M. (Genesis 26 (2000) 240–244). Draut1 loss of function larvae are unable to induce autophagy and heterophagy in fat body cells before pupariation and die during metamorphosis. To our knowledge, this is the first report of a multicellular animal lacking the function of a gene participating in the protein conjugation systems of autophagy.

Key words: *Drosophila*; Autophagy; Fat body; Aut1/Apg3; CG6877/Draut1; RNA interference

1. Introduction

Autophagy is a bulk cytoplasm degradation process, resulting in the digestion of self-material by lysosomal hydrolases. The main and best-known pathway of autophagy is macroautophagy (hereafter: autophagy), when an isolation membrane of yet uncertain origin engulfes portions of the cytoplasm, including organelles [2,3]. The emerging autophagosome fuses with a lysosome, forming an autophagic vacuole, in which the sequestered material is degraded [4,5].

Autophagy is highly induced during starvation to ensure the survival of the cell/organism [5,6], and during autophagy-type cell death, when usually large, post-mitotic cells end up containing an enormous number of autophagic vacuoles and die without showing classic apoptotic features like caspase activation and cellular shrinkage [7].

In holometabolous insects, autophagy is induced in larval tissues before and during metamorphosis, when the majority of these cells die. Autophagy in larval cells ensures the breakdown of most of the cytoplasm before cell death. The timing and hormonal control of this process are well-known, making these animals (including *Drosophila*) a remarkable model system to study autophagy [8–12].

Recent studies in yeast characterized many genes essential for autophagy [6]. Seven of them are involved in two, ubiquitin-like protein conjugation systems essential for autophagy, the Apg12 and Apg8 systems, named after the small ubiquitin-like proteins involved [4]. The homologs of these genes can be identified in other eukaryotes as well, suggesting that the core machinery of autophagy is very conserved during evolution [6,13].

Aut1/Apg3 is the conjugating enzyme for activated Aut7/Apg8, which is a crucial step during autophagy in yeast [14]. Yeast strains lacking wild-type Aut1 are defective for autophagy [15]. We have identified, characterized and created a loss of function for the *Drosophila* homolog of Aut1 to show its essential role during the fruit fly development.

2. Materials and methods

2.1. Molecular cloning of Draut1 constructs

For all cloning steps standard procedures were employed. Briefly, for the bacterial expression of Draut1, the expressed sequence tag (EST) clone GH28859 (1226 bp, coding sequence: 111–1103) was polymerase chain reaction-amplified using primers CATATGCAGAGTGTTCTCAACACCG and CTCGAGAGACATGTTGAAGTTTTGCGTA, TA cloned into pBLTA (pBL KS digested with Eco*RV* and a single T overhang added by Taq polymerase) creating pBLTADraut1, digested with Nde*I* and Xho*I* and cloned into the Nde*I*–Xho*I* site of pET21a creating pET21Draut1. For the inverted repeat construct, an Eco*RI*–Xho*I* fragment of GH28859 (223–1226, lacking translation start codon) was cloned into the Eco*RI*–Xho*I* site of pUAST, creating pUASDraut1-start. Insert orientation of pBLTA-Draut1 clones was analyzed by restriction digestion, and an Xho*I*–Xba*I* insert was cut out and cloned into Xho*I*–Xba*I*-digested pUAS-Draut1-start, downstream and in reverse orientation of the first insert, thereby creating pUASDraut1IR. DL709 cells were used for the molecular cloning of the last construct to reduce palindrome instability [16]. 3′ non-translated sequences (1103–1226) of GH28859 served as a spacer. pUASDraut1-start was digested with Eco*RI*, dephosphorylated and a small Eco*RI* fragment of GH28859 (1–223) was ligated. Appropriate recombinant clones were identified by sequencing with a pUAST promoter-specific primer.

2.2. Generation of transgenic flies

Germ-line transformation was carried out as described in [17]. Nine independent lines of UASDraut1IR were established as stocks. As all transgenic lines exhibited the same temperature-sensitive lethality when crossed to Act5CGal4, only experiments on a second chromosomal homozygous viable line are presented. In case of UASDraut1, four independent lines were recovered.

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Abbreviations: EST, expressed sequence tag; PAGE, polyacrylamide gel electrophoresis; Draut1p, Draut1 protein; 20E, 20-hydroxy-ecdysone; RNAi, RNA interference; UAS, upstream activating sequence; EIR, expression of an inverted repeat

0014-5793/03/$22.00 © 2003 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.
doi:10.1016/S0014-5793(03)00431-9

2.3. Antibody production

Recombinant Draut1 protein (Draut1p) was expressed in BL21 cells, inclusion bodies were separated by ultracentrifugation, further purified by polyacrylamide gel electrophoresis (PAGE) followed by elution of the appropriate band and subsequent immunization of C57Black mice.

2.4. Western blots

Pooled samples were collected and homogenized in 20 μl SDS sample buffer (Sigma)/1 mg wet tissue. Dissected organs were homogenized in phosphate-buffered saline (Sigma) supplemented with Complete Mini protease inhibitor cocktail (Roche), and the protein content of samples was determined by a protein assay kit (Bio-Rad). Usually 10 μg of sample per lane was subjected to SDS–PAGE and immunoblotting followed by development with alkaline phosphatase-conjugated rabbit anti-mouse antibody (1:2000, Sigma). Molecular masses were estimated using prestained molecular weight markers (Bio-Rad, Fermentas).

2.5. Light and electron microscopy

Dissected larvae were processed for electron microscopy as described in [9]. Semithin sections for light microscopy were stained with toluidine blue-Azur II mixture.

3. Results

3.1. The Drosophila homolog of Aut1/Apg3, Draut1, is expressed in all stages of development

The *Drosophila* homolog of Aut1, Draut1, shares 32% identity and 48% similarity with the yeast protein, and 62% identity and 76% similarity with the human homolog. Draut1 can be found in the annotated *Drosophila* genome as CG6877, and it localizes on the left arm of the third chromosome, in 75D4. The longest available EST, GH28859, was fully sequenced and found to perfectly match with the GadFly data (www.fruitfly.org/annot), both in respect of nucleotide sequence and intron–exon boundaries.

We raised polyclonal antisera against the purified recombinant protein in mice. Six sera (M1–M6) were tested, and all reacted with the same 46 kDa protein (Fig. 1).

With the help of the best-reacting polyclonal antiserum M1 (1:500), we analyzed the expression pattern of Draut1p in *Drosophila*. Draut1p was expressed in all stages of development, as revealed by Western blots (Fig. 1a) with a slightly elevated level during metamorphosis. It was also present in all organs of L3 stage wandering larvae tested, most abundant in brain (Fig. 1b). Cell fractionation experiments revealed that Draut1p localizes exclusively in the cytosol (not shown), just like its yeast counterpart [4].

3.2. Draut1p is involved in autophagy in Drosophila

The last larval stage of holometabolous insects can be divided into two parts: feeding and wandering period. During the feeding period, autophagy is rarely observed in larval tissues like the fat body (Fig. 2a,e). After reaching a critical mass required for successful metamorphosis, the start of the wandering period is triggered by the elevated level of molting hormone 20-hydroxy-ecdysone (20E) [8]. As a consequence of the 20E peak, autophagic and heterophagic vacuoles appear en mass in the cytoplasm of late wandering larval fat body cells [10,18], which can be easily assayed by light (Fig. 2b) and electron microscopic (Fig. 2f) methods.

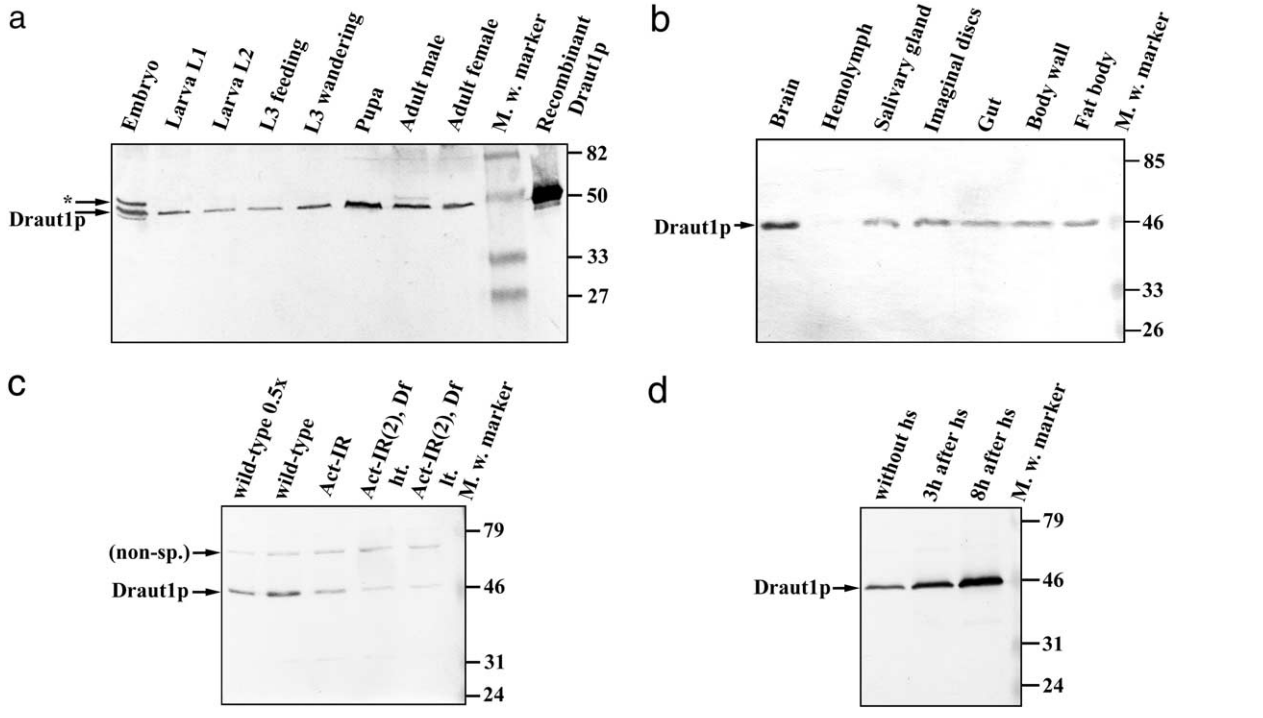

Fig. 1. a: Draut1p is expressed in all stages of development, peaking in pupal stage (*indicates another band present in embryos and adult males, which can represent either a covalent modification or a non-specific cross-reaction). b: Draut1p is present in all organs of L3 stage wandering larvae tested here, with elevated level in brain (lane 1). The very faint band in hemolymph (lane 2) probably originates from hemocytes. Body wall consists of the cuticle, epidermis and attached muscles. c: EIR Draut1 transgene greatly reduces protein level. Act-IR: *UASDraut1-IR/Act5CGal4*, Act-IR(2), Df: *UASDraut1IR/UASDraut1IR*; *Act5CGal4/Df(3L)Cat* at 30 (ht.) or 18°C (lt.). (non-sp.) refers to a non-specific band which also appears in the normal serum of C57Black mice and here serves as a loading control (this blot was slightly overdeveloped). d: Draut1p level is greatly increased by a heat shock driven transgene in *UASDraut1/+*; *Hsp70Gal4/+* L3 stage feeding larvae (hs: 1 h heat shock at 37°C).

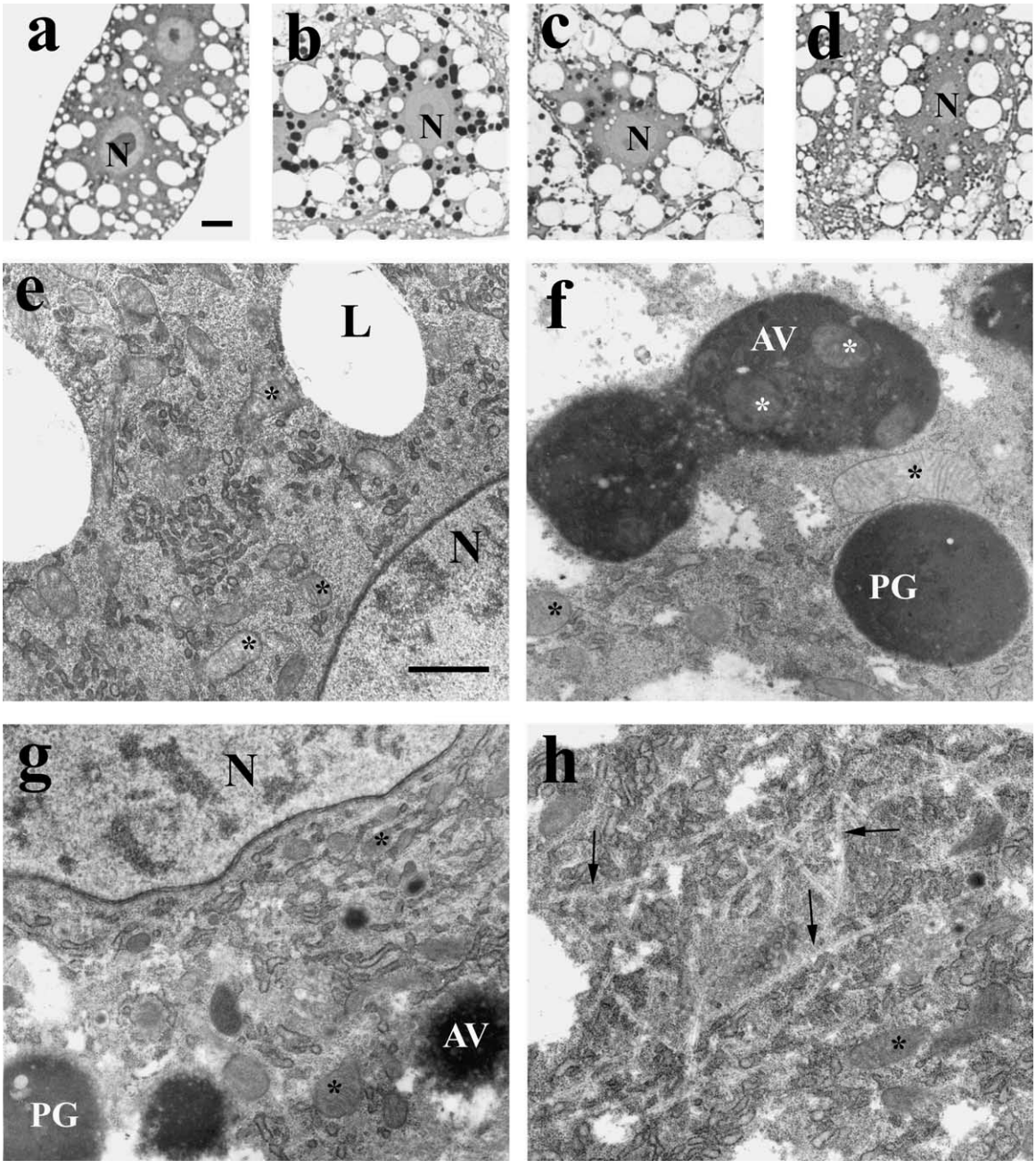

Fig. 2. a–d: Light microscopic images of L3 stage larval fat bodies. a: No granules can be seen during the feeding period in wild-type animals. b: Many granules appear in the cytoplasm during the wandering period (small dark dots, mainly around the nucleus (N)). c,d: In Draut1 loss of function (*ActGal4/UASDraut1IR* at 30°C) animals at the wandering period there are less, smaller granules: a representative intermediate ('hypomorph', c), and 'null' phenotype (d) is shown here. e–h: Electron microscopic images of fat bodies shown in a–d. e: No sign of autophagy can be observed during the feeding period. f: Large autophagic vacuoles (AV) containing mitochondria (*) and protein granules (PG) accumulate in the cytoplasm during the wandering stage. g: Draut1 loss of function intermediate phenotype: smaller AVs and PGs. h: AVs and PGs are absent from this cell; however, the number of microtubules is increased (arrows), which might represent an overcompensation reaction. Bars equal 10 μm for light and 1 μm for electron microscopic images.

Injection of double-stranded RNA into embryos produced only a weak effect on autophagy (not shown), possibly due to the fact that our analysis followed injections by 5 days, and the effect of RNA interference (RNAi) is lost by that time in *Drosophila*.

A palindromic transgene containing Draut1 cDNA sequences in inverted orientation was cloned in our lab, and transformed into *Drosophila*. If the transgene, Draut1IR (for inverted repeat) is transcribed, the emerging palindromic RNA is capable of forming double-stranded RNA in the self-complementary regions, thereby triggering RNAi and silencing of the endogenous Draut1 gene [1,17]. The transgene is normally silent, and only expresses upon crossing the stock to another transgenic line containing an appropriate Gal4 driver. The Gal4 transcription factor activates the transgene, which is under the control of a Gal4-responsive promoter (UAS, upstream activating sequence). Thus, the promoter of the Gal4 transcription factor determines the expression pattern of not

only Gal4, but of UASDraut1IR also [1]. The expression of an inverted repeat (EIR) method is temperature-sensitive: no effect is seen at low temperature (18°C) and strongest effect is seen at high temperature (30°C) [1].

We examined flies expressing Draut1IR under the influence of the Actin5CGal4 constitutive driver, which had the most prominent effect in our hands. Western blot experiments demonstrated a strong decrease in Draut1p level of L3 stage wandering larvae (Fig. 1c, lane 3), and of adult flies also (not shown). No difference was seen between individual loss of function larvae on Western blots (not shown).

Morphological analysis of larvae expressing the palindromic transgene at 30°C revealed a considerable variance in the individual responsiveness of the animals to decreased Draut1p level. Three main categories could be set up: in the first category, autophagy and heterophagy are normally induced in fat body cells at the onset of wandering (nine of 20 larvae examined, not shown, see Fig. 2b,f). In the second category, the number and size of the granules in larval fat body cells are reduced (7/20 larvae examined, Fig. 2c,g), which resembles a typical weak hypomorph phenotype. Animals from the third category gave the best phenocopy: no autophagic vacuoles or protein granules were observed in cells (4/20 larvae examined, Fig. 2d,h), thus proving the essential function of Draut1 in autophagy and in the receptor-mediated endocytosis of larval serum proteins and protein granule formation.

3.3. *Draut1 function is essential in* Drosophila

We carried out lethality assays to determine the lethal phase of Draut1 loss of function flies. Animals containing the palindromic transgene in one copy under the influence of the continuous Gal4 driver Actin5CGal4 showed 64% lethality at the restrictive temperature (167 adults hatched at 30°C of *Actin5CGal4/UASDraut1IR* versus 461 of *UASDraut1IR/BcGla* from the same vial), however, the viability is similar to balancer chromosome carrying siblings at a lower temperature, at which the EIR technique works poor [1] (387 adults hatched at 18°C of *Actin5CGal4/UASDraut1IR* versus 353 of *UASDraut1IR/BcGla*).

The effectiveness of the EIR technique can be further increased using multiple copies of the palindromic transgene and a deletion that uncovers the target gene [1]. In our case, animals containing two copies of UASDraut1IR and a deletion that uncovers Draut1 besides the constitutive Gal4 driver Actin5CGal4 (*UASDraut1IR/UASDraut1IR*; *Actin5CGal4/Df(3L)Cat*) showed an even decreased Draut1p level compared to the one-copy transgenes without the deletion (Fig. 1c, lane 4, compare to lane 3). Interestingly, Draut1p level was also further decreased at lower temperature (Fig. 1c, lane 5, compare to lane 3; see Section 4 for possible reasons). These animals showed 100% lethality at the restrictive temperature, dying as late L3 larvae, prepupae or pupae, while controls of the genotype *Actin5CGal4/Df(3L)Cat* develop normally at 30°C.

3.4. *Increased expression of Draut1 has no effect on the level of autophagy*

At the start of the wandering period in L3 stage, autophagy is induced in the fat body [10] and midgut [11] by a 20E peak. Six to eight hours before reaching wandering stage, L3 larvae are already competent to respond to autophagy-inducing stimuli like 20E treatment (unpublished). However, overexpression of Draut1 by a heat shock (UASDraut1 driven by hsGal4) did not induce autophagy significantly in the fat body and midgut (not shown), in spite of the large overall increase in Draut1p level (Fig. 1d). Constitutive expression of Draut1 (UASDraut1 driven by Actin5CGal4) did not increase the level of autophagy either, nor did it interfere with proper development (not shown).

4. Discussion

The biogenesis and consumption of autophagosomes by lysosomes are widely investigated on the molecular level in the last decade. In case of single cells (e.g. *Saccharomyces cerevisiae*), autophagy is not required for viability, although a defect in this pathway is proposed to be deleterious in the natural environment, where infinite amounts of nutrients are not available [4]. In plants, this process is also dispensable, although the lack of autophagy accelerates leaf senescence and starvation-induced chlorosis [19].

One study reported a *Drosophila* null mutant for one of the homologs of yeast Aut2/Apg4, CG6194 [20]. The fact that CG6194 and CG4428 both can rescue the autophagy-defective phenotype of Aut2-deleted yeast strains argues that they are both capable of processing Aut7-like proteins. Flies mutant for CG6194 are viable and fertile, and autophagy is induced on time in larval fat body cells of these mutants (unpublished).

Aut1 is a conjugating enzyme for Aut7/Apg8, which is an essential step for autophagy in yeast [14]. Mammalian cells have three homologs of Aut7: MAP-LC3 (microtubule-associated protein light chain 3), also involved in autophagy, GATE-16 (Golgi-associated ATPase enhancer of 16 kDa), an intra-Golgi transport modulator, and GABARAP ($GABA_A$ receptor-associated protein), responsible for the targeting and/or degradation of the receptor, with Aut1 being the conjugating enzyme for all three [21]. However, as in *Drosophila* there are only two Aut7 homologs (CG12334 and CG1534), probably one or both of them are involved in autophagy. During the induction of autophagy in the fat body, both homologs are strongly induced (manuscript in preparation).

The EIR technique was effectively used to create 'mutant' phenotypes [17], however, it mimics an allelic series [1], which might be responsible for the different phenotypic categories of Draut1 loss of function animals. On the basis of the phenotype of these animals, we conclude that Draut1 is also involved in autophagy. In animals belonging to the most severe phenotype category, the induction of autophagy was completely blocked in fat body cells of wandering larvae. The formation of protein granules was also blocked, in accordance with the fact that protein granules always appear later than autophagic vacuoles and may represent a special endolysosomal hybrid organelle [22], where degradation of storage proteins is delayed during the course of metamorphosis [23]. Alternatively, Draut1p may also be involved in cellular trafficking processes other than autophagy, just like its human counterpart. Further studies are needed to reveal the possible role of Draut1 in the endocytosis of larval serum proteins and sequestration into protein granules by the fat body.

Surprisingly, the Draut1p level of EIR flies was strongly decreased at both high and low temperatures (Fig. 1c, lanes 4 and 5). Probably a low level of Draut1p becomes rate-limit-

ing in a cellular process at higher temperature, but not at lower temperature (e.g. fly development takes only 7 days at 30 but 3 weeks at 18°C).

Draut1p is quite abundant in spite of its low mRNA level (manuscript in preparation), and slightly induced before/during metamorphosis. Draut1p is contributed maternally as revealed by Western blots of 0–1 h old embryos (not shown), enabling animals to proceed through embryonic development even if the mRNA level is reduced by RNAi. The main lethality peak is associated with the large body reorganization phase of *Drosophila*, the metamorphosis. Starving Draut1 loss of function larvae do not increase lethality, however, in adult flies it significantly reduces lifespan as compared to wild-type flies (not shown).

The overexpression of human Aut1 was reported to facilitate the formation of Apg12–Apg5 conjugate [21], but morphologically was not evaluated. Here we showed that the overexpression of Draut1 has no effect on the level of autophagy in *Drosophila* larval tissues, and it does not interfere with proper development either.

Further studies are required to define the exact role of Draut1 in *Drosophila*, as well as the causes and foci of lethality caused by the loss of Draut1 function. In this paper, we have proven its involvement in autophagy and heterophagy in larval fat body and its essential role during metamorphosis.

Acknowledgements: We thank the following people for stocks, reagents, discussions and allowing G.J. to work in their labs: Csaba Ádori, John Belote, László Farkas, Erzsébet Fellinger, Tatsuhiko Kadowaki, Orbán Komonyi, János Kovács, Zoltán Kristóf, Lajos László, David Leach, Péter Maróy, Attila Nagy, László Szilágyi. We thank the skillful assistance of Károlyné Válóczy, Mariann Saródy, Sarolta Sipos. This work was supported by the Hungarian Ministry of Education under Grant MEDICHEM 1/047 NKFP provided to M.S.

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