Functional fat body proteomics and gene targeting reveal *in vivo* functions of *Drosophila melanogaster* α-Esterase-7

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ABSTRACT

Carboxylesterases constitute a large enzyme family in insects, which is involved in diverse functions such as xenobiotic detoxification, lipid metabolism and reproduction. Phylogenetically, many insect carboxylesterases are represented by multienzyme clades, which are encoded by evolutionarily ancient gene clusters such as the *α-Esterase* cluster. Much in contrast to the vital importance attributed to carboxylesterases in general, the *in vivo* function of individual *α-Esterase* genes is largely unknown. This study employs a functional proteomics approach to identify esterolytic enzymes of the vinegar fly *Drosophila melanogaster* fat body. One of the fat body carboxylesterases, *α-Esterase-7*, was selected for mutational analysis by gene targeting to generate a deletion mutant fly. Phenotypic characterization of *α-Esterase-7* null mutants and transgenic flies, which overexpress a chimeric *α-Esterase-7:EGFP* gene, reveals important functions of *α-Esterase-7* in insecticide tolerance, lipid metabolism and lifespan control. The presented first deletion mutant of any *α-Esterase* in the model insect *D. melanogaster* generated by gene targeting not only provides experimental evidence for the endogenous functions of this gene family. It also offers an entry point for *in vivo* structure-function analyses of *α-Esterase*-7, which is of central importance for naturally occurring insecticide resistance in wild populations of various dipteran insect species.

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1. Introduction

Carboxylesterases (CEs) play essential roles in lipid and xenobiotic metabolism throughout the animal kingdom. The broad substrate spectrum and their complex expression profiles reflect functional diversification among members of the CE multigene family, which promotes organismal diversity during Metazoan evolution (reviewed in Oakeshott et al., 1999). Insect genomes are particularly rich in CE genes and this complexity has been correlated with important organismic functions such as the insecticide resistance in the malaria vector *Anopheles gambiae* (Ranson et al., 2002). Overexpression has been described as one of two CE-mediated strategies for metabolic detoxification of organophosphorous (OP) insecticides found in insecticide-resistant populations of dipteran species (reviewed in Bass and Field, 2011; Oakeshott et al., 2005). This strategy makes use of OP sequestering to protect against the neurotoxic effect of the insecticides on their target, acetylcholinesterase. Alternatively, CE mutants with novel substrate spectrum are employed to detoxify OP insecticides by hydrolytic cleavage (see Section 4). Generally, much in contrast to the vital general importance of CEs, functional information about the *in vivo* role of particular CE family members is limited.

Electrophoretic zymograms of adult *Drosophila melanogaster* flies identified 22 soluble esterase isozymes, among them ten CEs (Healy et al., 1991). On the other hand computational annotation of the fly genome lists 35 genes, which encode enzymes with experimentally supported or predicted carboxylesterase activity (GO term 0004091; Tweedie et al., 2009; www.flybase.org). Among them are the ten genes (*α-Est1-10*) of the *α-Esterase* cluster located on the right arm of chromosome 3. Recently, the first two *α-Est* genes were assigned to esterase isozyme activities, i.e. *α-Est5* to EST9 and *α-Est7* to EST23 by peptide fingerprinting combined with recombinant protein expression (Campbell et al., 2003). This approach started to link the wealth of biochemical information on EST isozymes to the molecular identity of the corresponding genes. However, the *in vivo* roles of individual *α-Est* cluster genes remains obscure as none of them has been functionally characterized by mutant analysis.

Correlational analyses support diverse functions for *α-Est* genes. For example, *α-Est1*, *α-Est2* and *α-Est8* have a pronounced expression in adult fly heads (Campbell et al., 2003; Chintapalli et al., 2007). In heads of adult males *α-Est1* and *α-Est8* are transcriptionally up-regulated in fly lines selected for aggressive behavior (Dierick and Greenspan, 2006) and *α-Est2* behaves as a mating-responsive gene (Ellis and Carney, 2010). Moreover, the α-EST2 and α-EST7 proteins are members of the fat body lipid droplet proteome (Beller et al., 2006) suggesting a role in lipid metabolism. Notably, seven of the ten *α-Esterase* cluster genes are at least moderately expressed in the *Drosophila* fat body (Chintapalli et al., 2007). Multiple *α-Est* gene expression in the fat body highlights its central role of this organ as a metabolic hub in insects. Besides its most prominent role as major energy storage organ, the fat body is involved in vital organismal functions such as immunity, growth control and detoxification. The role of particular esterase activities in these processes awaits clarification.

This study employs a functional proteomics approach to molecularly identify esterases of the *Drosophila* fat body. Tissue profiling for esterase activities with subsequent enzyme identification via mass spectrometry has been successfully applied to characterize the lipolytic proteomes of mouse adipose tissue (Birner-Gruenberger et al., 2005) and liver (Birner-Gruenberger et al., 2008) as well as serine hydrolases up-regulated in invasive cancers (Nomura et al., 2010). Recognition of esterases is realized by activity-based probes, which contain *p*-nitrophenyl or fluoro phosphonates as reactive groups for the serine in the active site of the target enzymes as well as a fluorescent or biotin reporter tag for detection.

One of the major fat body esterases toward the activity-based probe NBD-HE-HP, which was identified in this study, *α-Est7*, was subjected to *in vivo* mutational analysis by homologous recombination. Initial phenotypic characterization of *α-Est7* mutant flies provides evidence that the gene is not essential for the *Drosophila* development but serves important functions for insecticide tolerance, lipid metabolism and lifespan control.

2. Material and methods

2.1. Functional proteomics

Fat body cells from young adult male flies (0−14 h of age) of the reference strain (RKF778; 6 h food deprived) were released into 25 mM Tris/HCl pH7.5, 0.25 M sucrose, 1 mM EDTA, 1 mM DTT, 20 μg/ml leupeptin, 2 μg/ml antipain, 1 μg/ml pepstatin by manual opening of the fly abdomina. The cells were lysed by repeated freeze/thaw cycles and intense vortexing followed by centrifugation of the homogenate at 5000*g* for 15 min. The protein content of the cytoplasmic layer between the fat layer and the pellet was

determined (Bio-Rad Protein Assay #500-0006) and adjusted to 0.5 μg/μl. Homogenates were incubated with 20 μM the fluorescent activity-based probe O-((6-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino)hexanoyl)aminoethyl-O-(n-hexyl)phosphonic acid *p*-nitrophenyl ester (NBD-HE-HP) (synthesized as published in Oskolkova et al., 2003; Schmidinger et al., 2005) and 1 mM Triton X-100 for 2 h at 28 °C with mild shaking (550 rpm), quick-frozen in liquid nitrogen and kept at −20 °C until further use. Unlabeled controls were also tested to exclude autofluorescent proteins. Proteins were precipitated with 10% trichloroacetic acid (w/v) and subjected to SDS-PAGE in Tris/glycine buffer (5% stacking gel, 10% resolving gel) or 2D-gel electrophoresis (pH 3−10 NL IPG strips; 10% SDS-PAGE) for detection of fluorescent NBD-HE-HP-labeled protein bands/ spots by laser scanning as described (Birner-Gruenberger et al., 2005). Relative fluorescence intensity spot volumes were determined using Delta2D 4.0.8 software for spot detection and quantitation after normalization on the total fluorescence intensity of all detected spots.

Protein spots were excised from gels and tryptically digested according to the method by Shevchenko et al. (1996). Peptide extracts were dissolved in 0.1% formic acid, separated by nano-HPLC and analyzed by LC-MS/MS with a Thermo-Finnigan LTQ linear iontrap mass spectrometer as described (Birner-Gruenberger et al., 2008). Proteins were identified by searching the *D. melanogaster* NCBI non-redundant public database with SpectrumMill Rev. 03.03.078 (Agilent). Acceptance parameters were two or more identified distinct peptides according to the parameters defined by Carr et al. (2004) except for the single high confident peptide of α-EST2 (see Fig. S1).

2.2. Esterase activity assay

Fat body cell homogenates were essentially prepared as described in Section 2.1. Specific total esterase activity was determined from the linear increase of absorbance at 405 nm of *p*-nitrophenolate released from 100 μM *p*-nitrophenyl butyrate in 20 mM Tris−HCl, pH8, 150 mM NaCl and 0.01%Triton X-100 (Shirai and Jackson, 1982).

2.3. Fly techniques

The *Act5C-Gal4* fly stock (BDSC #4414) is available from the Bloomington Drosophila Stock Center. The *FB-Gal4* inducer (RKF125; Grönke et al., 2003), the *α-Est7:EGFP* (formerly *CG1112:EGFP*) effector fly strain (RKF736; Beller et al., 2006) and the reference stock used for functional proteomics (RKF778; Grönke et al., 2007) have been described earlier. All flies were propagated at 25 °C on a complex malt-soy flour-corn flour-molasses food as described (Grönke et al., 2005).

The *α-Est7[1]* mutant allele lacks genomic sequences from 3R 3,345,260 to 3,347,419 and was generated by ends-out gene targeting as described (Gong and Golic, 2003). In brief, oligonucleotide primer pairs RKO535/536 (see Fig. 2A for primer positions and Supplemental Material and Methods for primer sequences) and RKO537/538 were used to amplify genomic regions flanking *α-Est7* exons 2−6 from the *w[1118]* fly stock (RKF972; called *α-Est7^{+}* control; Grönke et al., 2009), which were cloned via pBS-70w into the pP {EndsOut2} donor vector (both plasmids were a gift from Jeff Sekelsky; for details on the vectors see http://sekelsky.bio.unc.edu/ research/vectors/vectors.html). Transgenic lines carrying the pP {EndsOut2} *α-Est7 w[+]* donor were established in the RKF972 genetic background and crossed to *y[1] w*; P{ry[+t7.2] = 70FLP}11 P{v[+t1.8] = 70I-SceI}2B noc[Sco]/CyO, S[2]* (BDSC #6934), which provides heat shock-inducible *I-SceI* nuclease and FLP recombinase expression. Heat shock (1 h 38 °C) was performed at day 3 after egg laying and

mosaic w^+ F1 virgins were crosses to w^{1118}; P{ry$^{+t7.2}$ = 70FLP}10 (BDSC #6938), which provides a constitutive FLP source. From more than 1200 individual lines, which were established from F2 flies with homogenous w^+ eye color one mutant stock was singled out after a selection/characterization scheme detailed in Supplemental Material and Methods. Finally, the stock w^{1118}; α-Est7^1 (RKF973) was established by backcrossing to α-Est7$^+$ for four generations to achieve a genetically-adjusted mutant-control pair.

2.4. Body lipid content assay

Male flies were fed for six days after hatching at 25 °C under 12 h:12 h light/dark conditions on the propagation food and the body fat assayed on triplicate samples of eight males each using a commercial Triglyceride assay substrate as described (Grönke et al., 2003; Hildebrandt et al., 2011).

2.5. Longevity assay

Longevity assays have been performed as described (Grönke et al., 2005). In brief, $n = 180$ male flies (12 replicates of cohorts of 15 flies) for each genotype were set up and incubated at 25 °C with a 12 h:12 h light/dark cycle. The food was changed every 2–3 days and the surviving flies were scored at these points in time. Data plotted in Fig. 4 represent age-specific average survivorship rate means (in %) with 95% confidence intervals calculated from the replicates. A log-rank test was performed to assess statistical significance of the observed differences between the α-Est7^1 and the α-Est7$^+$ survivorship data.

2.6. Fecundity assay

Single virgin female flies of the respective genotypes were mated to 2-3 males of the same genotype. Flies were flipped to fresh food every 3–4 days and the F1 generation was allowed to develop until imaginal hatching. Pupal cases were scored to calculate the cumulative number of progeny per female fly.

2.7. Insecticide tolerance assay

Equal numbers of mutant and control embryos were mixed and raised in the same food vials. Male flies were fed for six days after hatching and subjected to a 4–6 h water-only starvation period. Cohorts of ten starved flies of each genotype were transferred to vials without fly food but equipped with a piece of Whatman paper soaked with 5% sucrose/5% Brilliant blue (www.omya-peralta.de) and the diazinon (Riedel-de-Haen 45842) insecticide parent molecule or solvent control (2% Acetonitrile for all conditions). Survival times were monitored and the survivor rates calculated on the basis of triplicate or duplicate (solvent controls) measurements. Qualitative food uptake was controlled by brilliant blue staining of the gut.

2.8. Fluctuating asymmetry analysis

Right-left distribution of large and small sternopleural bristle numbers were used to score for fluctuating asymmetry (FA) according to previously described parameters (Palmer and Strobeck, 1986; Parsons, 1962). There was no evidence of directional asymmetry or antisymmetry, and no size-dependent correlation for the analyzed asymmetry values, following a descriptive statistical analysis in male α-Est7$^+$ or α-Est7^1 flies raised at equal density in the same food vials at 25 °C. As both large and small sternopleural bristles showed an FA character they were used to estimate the developmental stability. The asymmetry index

calculated was the sum of the absolute differences between right and left side of an individual divided by the sample size (Polak et al., 2004):

$$FA = \frac{\sum(|R_i - L_i|)}{N}$$

A univariate analysis of variances was performed to examine differences between the α-Est7 mutant and α-Est7$^+$ control population. All analyses were performed using OriginPro v.8.0724.

2.9. Quantitative RT-PCR

Quantitative RT-PCR analysis was performed as described in Beller et al. (2010), using the QuantiTect Primer Assays QT00970193 for α-Est7, QT00970200 for α-Est6, QT00970186 for Ubc84D and QT00985677 for RpL32, respectively (www.quiagen.com).

2.10. Molecular biology

2.10.1. Inverse PCR

Genomic DNA equivalent to two flies of the mutant stock α-Est7^1 (RKF973) and the α-Est7$^+$ control (RKF972), respectively, were subjected to cleavage by 20U of restriction endonuclease Hin6I in a total volume of 25 μl. Endonucleolytic cleavage was terminated by a heat inactivation step (10 min at 65 °C) and 10 μl of the reaction was ligated overnight in a total volume of 400 μl with T4 DNA ligase at 8 °C. The ligation reaction was ethanol-precipitated, the pellet washed in 70% ethanol and subsequently resuspended in 150 μl Tris–EDTA. Of the ligation products 15 μl each were subjected to PCR reactions using the primer pairs RKO782/783 (gene targeting construct 5′ border) or RKO784/785 (gene targeting construct 3′ border), respectively (PCR program: 30sec 95 °C, 30sec 60 °C, 2 min 68 °C; 38 cycles). Resulting amplicons were size-separated on a 1% agarose gel and visualized by ethidium bromide staining. Note that approximate positions of primer binding sites are shown in Fig. 2A and primer sequences are presented in Supplemental Material and Methods. Calculated sizes: 5′ donor construct border on endogenous white gene of α-Est7^1 and the α-Est7$^+$: 594 bp. On α-Est7 of α-Est7^1 only: 902 bp. 3′ donor construct border on α-Est7 of α-Est7^1 only: 364 bp.

2.11. Statistical analysis

If not stated otherwise replicates were expressed as average values with corresponding standard deviation. Statistical significance was tested by unpaired Students' t-tests.

3. Results

3.1. Functional proteomics of Drosophila fat body esterases

A functional lipolytic proteome approach was used to identify esterases in the fat body cells of young adult _D. melanogaster_ flies. To this aim fat body cell homogenates were incubated with the fluorescent activity-based esterase probe NBD-HE-HP (for details see Section 2.1). Separation of the homogenate on 2D SDS-PAGE and subsequent detection of about 15 fluorescent spots (Fig. 1A) revealed about ten labeled proteins. Eight of these proteins were identified by LC-MS/MS analysis by multiple peptides and one by a single high confident peptide (Fig. 1B). Among these nine identifications are four proteins with the gene ontology annotated carboxylesterase activity according to gene ontology (GO term 0004091). These are three enzymes encoded by the _Drosophila_ α-_Esterase_ cluster i.e. α-Esterase-7, α-Esterase-5, α-Esterase-2 and
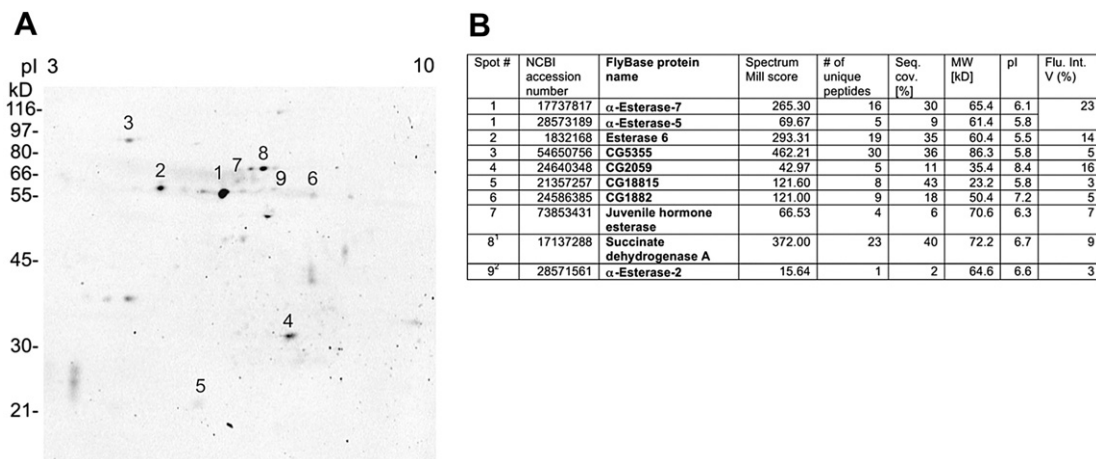
Fig. 1. Functional proteomics identifies α-Esterase-7 as a major *Drosophila* fat body esterase on NBD-HE-HP substrate. A. Fluorescence image of activity-based probe NBD-HE-HP-labeled fat body esterases after 2D electrophoretic separation. B. List of esterolytic proteins identified by LC-MS/MS corresponding to spot numbers in A. Shown are details of the protein identifications, predicted molecular weights, pI values of the precursor proteins and fluorescence intensity volumes in % of the spots. The identified proteins make up 85% of the total detected fluorescence intensity of all spots on the 2D-gel. [1]Note that the flavoprotein Succinate dehydrogenase A is a false positive identified due to protein auto-fluorescence. [2]Identification based on a single peptide. Fragment ions of the MS–MS spectrum of the single peptide used to identify α-Esterase-2 and peptide sequences of all identified unique peptides are shown in Fig. S1.

Esterase 6, which is encoded by the *Drosophila ß-esterase* gene cluster. Additionally, the proteomics approach identified Juvenile hormone esterase as well as four *in silico* predicted, functionally largely uncharacterized proteins. These proteins are members of the esterase/lipase superfamily (annotation cI12031) (Marchler-Bauer et al., 2011) called CG5355, CG2059, CG1882 and CG18815. *CG5355* encodes a predicted prolyl oligopeptidase domain (pfam0297) and *CG2059* encodes a predicted acyl-CoA esterase domain (region name PRK10673). CG1882 is a predicted αß fold family protein (region name PLN02894) and CG18815 encodes a predicted αß hydrolase 2 domain protein (pfam02230) with putative thioesterase activity. All of these functionally uncharacterized proteins will be discussed in more detail below (Section 4; for details on the LC-MS/MS-based protein identifications see Fig. S1).

The most intensely labeled protein using the NBD-HE-HP probe (spot 1 in Fig. 1A) corresponds to the gene product of the *α-Esterase-7 (α-Est7)* gene (also called *CG1112*; www.flybase.org). The protein product of *α-Est7*, α-Esterase-7 (α-EST7) is a membrane-associated α-Esterase with preference for substrates with shorter acid side chain length (Spackman et al., 1994; Parker et al., 1996). Inhibitor sensitivity studies classify α-EST7 as subclass I CE (Spackman et al., 1994). Intense labeling of α-EST7 by NBD-HE-HP (Fig. 1A) suggests tight binding of this suicide substrate and/or high protein abundance and is consistent with the pronounced transcriptional expression of the *α-Est7* gene in the fat body (Chintapalli et al., 2007; Campbell et al., 2003). Collectively these data predict *α-Est7* to encode one of the major *Drosophila* fat body esterases with hitherto unknown function *in vivo*.

3.2. Generation of Drosophila α-Esterase-7 mutant by gene targeting

Cytogenetically *α-Est7* is a central member of the esterase gene cluster on the right arm of *D. melanogaster* chromosome 3. The *α-Est7* gene is flanked by its paralogue *α-Esterase-6 (α-Est6)* and by the *Ubiquitin conjugating enzyme 84D (Ubc84D)* gene (Fig. 2A). To aid the characterization of *α-Est7* gene function *in vivo*, gene replacement by homologous recombination was used to generate the deletion mutant *α-Est7[1]*, which lacks all but the first exon of the *α-Est7* gene (Fig. 2A). The absence of *α-Est7* transcript in the *α-Est7[1]*

mutant and the lack of impairment of *α-Est6* and *Ubc84D* expression have been assessed by qRT-PCR (Fig. 2B). The successful deletion of *α-Est7*, has been genomically confirmed by PCR geno-typing (Fig. 2C) and sequencing (Fig. S3). The absence of additional donor construct integrations outside the *α-Est7* locus was verified by inverse PCR (Fig. 2D; Section 2.10; for details of all approaches to characterize the *α-Est7[1]* mutant see Supplemental Material and Methods). Consistent with the deletion of *α-Est7*, fat body homogenates of *α-Est7[1]* mutant flies lack the dominant esterase, which binds the NBD-HE-HP probe in genetically matched control homogenates (*α-Est7^{+}*) (Fig. 2E). The apparent molecular weight of the corresponding enzyme matches well with the predicted molecular weight of α-EST7 of 65.4 kDa as well as to the apparent molecular weight of recombinantly expressed α-EST7 (Campbell et al., 2003). Conversely, transgenic overexpression of a α-EST7:EGFP fusion protein (Beller et al., 2006) using the UAS/GAL4 technique (Brand and Perrimon, 1993) results in a strong esterase activity on *p*-nitrophenyl-butyrate conferred by a protein species, which matches the predicted fusion protein size of about 92 kDa (Fig. 2E). Lack of *α-Est7* causes a drastic reduction of total fat body esterase activity on *p*-nitrophenyl butyrate by 48% (Fig. 2F). Conversely, overexpression of a α-EST7:EGFP fusion protein increases total esterase activity on *p*-nitrophenyl-butyrate by 48−78% compared to controls (Fig. 2F). Collectively, these loss-of-function and gain-of-function studies demonstrate that *α-Est7* is a major contributor to total esterase activity on *p*-nitrophenyl-butyrate in the fly fat body.

3.3. Control of insecticide tolerance, lipid storage and lifespan by Drosophila α-Esterase-7

α-Esterase-7 has been proposed to act in xenobiotic metabolism based on its expression during *Drosophila* feeding stages (Campbell et al., 2003). Importantly, *α-Est7* orthologs of higher dipterans such as the blowfly *Lucilia cuprina* and the housefly *Musca domestica* have been implicated in OP insecticide detoxification in resistant insect strains (Newcomb et al., 1997; Claudianos et al., 1999). In these species metabolic OP detoxification is based on mutant *α-Est7* enzymes with modified substrate spectrum (Heidari et al., 2004). However, a dose/activity-dependent modulation of OP insecticide tolerance by wild type *α-Est7* has not been addressed *in vivo*. To

directly assess the impact of α-Est7 activity in Drosophila, α-Est7 loss-of-function and gain-of-function flies were exposed to varying doses of diazinon, an organophosphorothioate, which becomes bioactivated to the OP insecticide diazoxon (Fig. 3). Consistent with a protective role of α-Est7 toward the insecticide parent molecule, α-Est7[1] mutants are weakly diazinon-sensitive (Fig. 3A). Conversely, ubiquitous transgenic overexpression of α-Est7 confers moderate insecticide tolerance (Fig. 3B). Collectively, these data present in vivo evidence for a role of wild type α-Est7 in insecticide tolerance control in D. melanogaster.

Besides its role in detoxification of xenobiotics, α-Est7 has been hypothesized to act in dietary lipid metabolism based on the expression profile of the gene (Campbell et al., 2003). Accordingly, the body fat content has been quantified as a diagnostic marker of dysregulation of neutral lipid metabolism. Consistent with a lipid metabolism function of α-Est7, the lipid stores of α-Est7[1] mutant flies are significantly reduced by 24% (Fig. 4A). A body fat reduction of this magnitude is not attributable to genetic background effects in Drosophila strains, which are as closely related as the α-Est7[1] and α-Est7^{+} stocks used in this study. In contrast to the mutant phenotype, fat body-targeted α-EST7:EGFP overexpression has no significant impact on body fat storage (Fig. 4A) suggesting that the α-Est7 in vivo function on lipid metabolism is not restricted to the fat body. Currently it is unknown how the α-EST7 enzymatic function promotes storage fat accumulation (for a detailed discussion see Section 4).

The storage lipid metabolism function as well as pronounced expression of the α-Est7 in the female spermatheca (Chintapalli et al., 2007) suggests that the gene might influence reproductive fitness. However, offspring counts demonstrate no effect on female fecundity in α-Est7[1] mutant flies (Fig. 4B). But then, flies lacking α-Est7 function suffer from a median lifespan reduction between 6% and 14% (Fig. 4C and Fig. S2), which predicts a selective disadvantage of α-Est7 mutant flies in a natural environment. It should be noted that the genetic background of the α-Est7 mutant and corresponding control have been adjusted by a limited number of backcrosses. Accordingly, genetic background effects on the extent of the described physiological phenotypes cannot be ruled out. Collectively, these data demonstrate that α-Est7 is not essential for the development of the fly. However, the gene is necessary for selected physiological functions, which impact on genetic fitness parameters.

α-Esterase-7 has been proposed to provide a developmental function. This hypothesis is based on the finding of increased fluctuating asymmetry (FA), i.e. morphological left-right differences in specific bristle characters, in OP insecticide-resistant strains of Lucilia, which carry ali-esterase mutant alleles (Clarke et al., 2000; Davies et al., 1996; McKenzie and Clarke, 1988; McKenzie et al., 1982). Fluctuating asymmetry is indicative of developmental instability, which is believed to cause selective disadvantages and correspondingly a genetic fitness decline. Drosophila flies lacking α-Est7 show no gross developmental defects or impairment in fecundity. Small and large sternopleural bristle counts were used to test for an increase of FA in α-Est7 Drosophila mutants. Mean asymmetry values (±s.e.m.) for all sternopleural bristles of $n = 154$ flies per genotype were 2.00 ± 0.105 for α-Est7[1] mutants and 2.23 ± 0.123 for α-Est7^{+} control flies, respectively. Differences between the FA levels were not statistically different between the two genotypes (Levene's test $p = 0.86$). This result argues that the lack of α-Est7 function does not cause developmental instability in Drosophila.

Taken together this study molecularly identifies major esterases on the activity-based NBD-HE-HP probe in the Drosophila fat body based on a functional proteomics approach. α-Esterase-7, the most prominent fat body esterase toward the NBD-HE-HP suicide substrate, was selected to generate the first molecularly characterized Drosophila deletion mutant lacking a single α-Est gene by gene targeting. The initial mutant phenotype analysis provides evidence that α-Est7 confers moderate insecticide tolerance in wild type Drosophila flies. Moreover, although α-Est7 is not an essential gene it is implicated in important organismal functions in flies such as body fat storage and lifespan control. Accordingly, this study provides an entry point to a more comprehensive understanding of the biological functions of the esterase multigene family in insects.

4. Discussion

The fat body functional proteomics screen identified several enzymes with putative, yet uncharacterized roles in D. melanogaster metabolism. Among them are four predicted esterase/lipase superfamily (annotation cl12031) members: CG5355, CG2059, CG18815 and CG1882.

CG5355 is an ubiquitously expressed gene and encodes an 86.3 kDa protein with predicted prolyl oligopeptidase domain (pfam0297) (Chintapalli et al., 2007; Marchler-Bauer et al., 2011). The protein is well sequence-conserved among the Drosophilids (e.g. sequence identity to Drosophila virilis GJ21822 is 83.0%). Moreover, several Sophophoran Drosophilid species encode a CG5355 paralog of identical domain structure but unknown function, which is called CG2528 in D. melanogaster (69.9% sequence identity to CG5355). More distantly but still very closely related CG5355 homologs are encoded by the genomes of the body louse (54.5% identity) and the honeybee (60.5% identity; for more details see Fig. S4).

Another protein identified by the functional proteomics approach is CG2059, a functionally uncharacterized protein (calculated mass 35.4 kDa), which contains a predicted acyl-CoA esterase domain (region name PRK10673). Sequence alignments with related proteins show, that CG2059 is evolutionarily conserved among insect species (Fig. S4). Among the Drosophilids, which are distantly related to D. melanogaster, the Hawaiian Drosophila species Drosophila grimshawi encodes the putative protein GH24258, which has 48.2% sequence identity to D.m. CG2059 and among the Culicidae, A. gambiae encodes AGAP009289-PA, which is 39.6% identical to the D.m. protein (for more sequence similarity information see Fig. S4). The human protein with highest sequence similarity to D. melanogaster CG2059 is called αβ hydrolase domain containing 11 (ABHD11; also called WBSCR21 for Williams-Beuren syndrome chromosome region 21). Notably, mouse ABHD11 (sequence identity to D.m. CG2059 is 33.3%) is repressed in white adipose tissue (WAT) upon high fat diet feeding but induced by adding the PPARγ agonist rosiglitazone to the diet (Shen et al., 2007). In this tissue ABHD11 is co-regulated with the lysophosphatidic acid acyltransferase ABHD5 (also called the Chanarin-Dorfman syndrome gene or CGI-58) (Ghosh et al., 2008), which is the well-established activator of adipose triglyceride lipase (ATGL, also called PNPLA2) (Lass et al., 2006). ATGL is the vertebrate ortholog of the D. melanogaster Brummer lipase (Grönke et al., 2005). Interestingly, the presented functional proteomics approach also identified CG1882, which is encoded by the only Drosophila homolog of the vertebrate paralogous gene pair ABHD5/CGI-58 and ABHD4 (sequence identities 46.6% and 47.5%, respectively; see Fig. S4). Since CG1882 is expressed in the fat body and the protein localizes on the surface of lipid droplets (Cermelli et al., 2006) it is tempting to speculate that the gene executes a lipid metabolic function, either autonomously or in cooperation with the Brummer triglyceride lipase. The hypothesis of an evolutionarily ancient role of CG1882 in lipid metabolism gains further support by the finding that a mutation in the sequence-related Arabidopsis thaliana gene At4g24160 (amino acid sequence
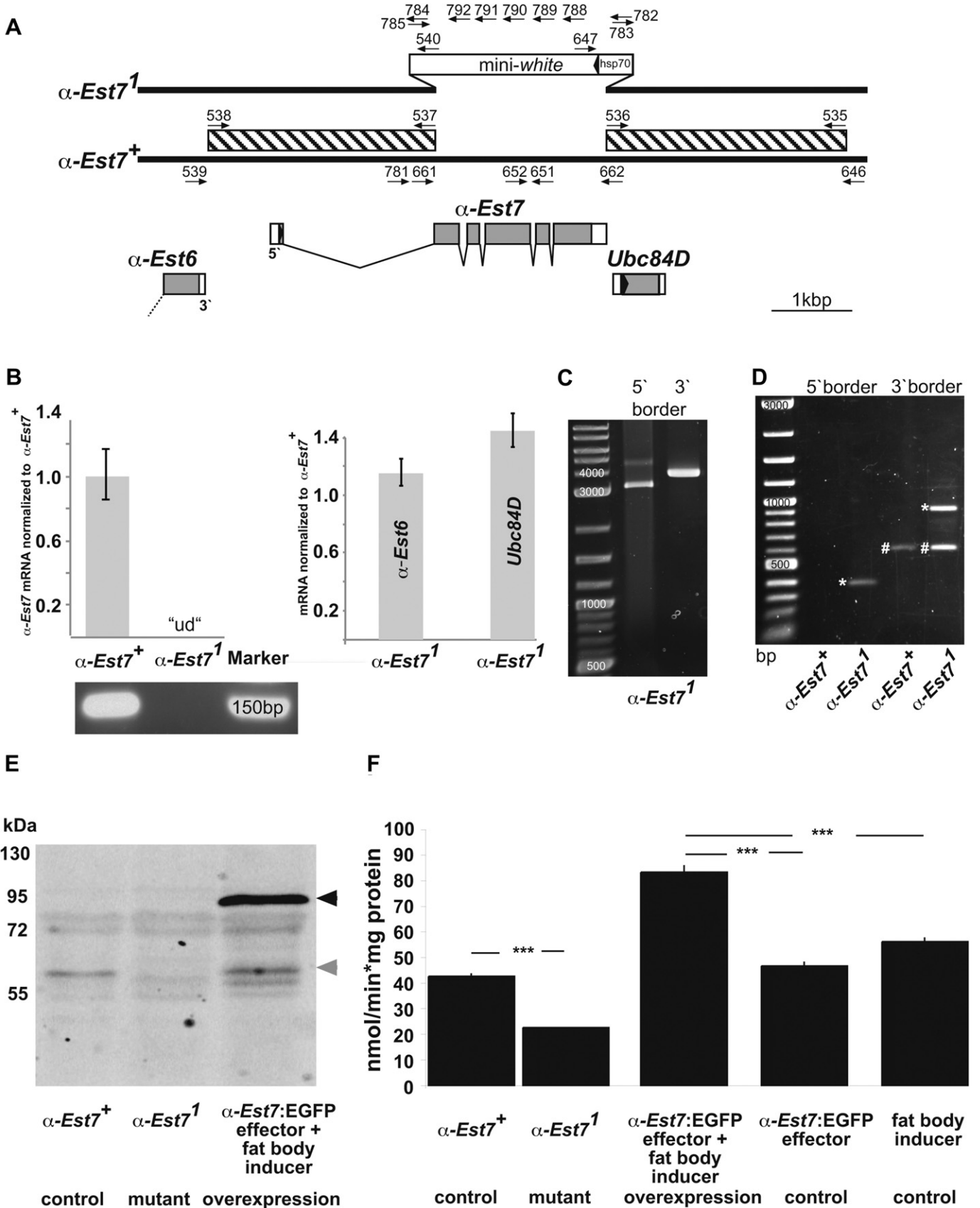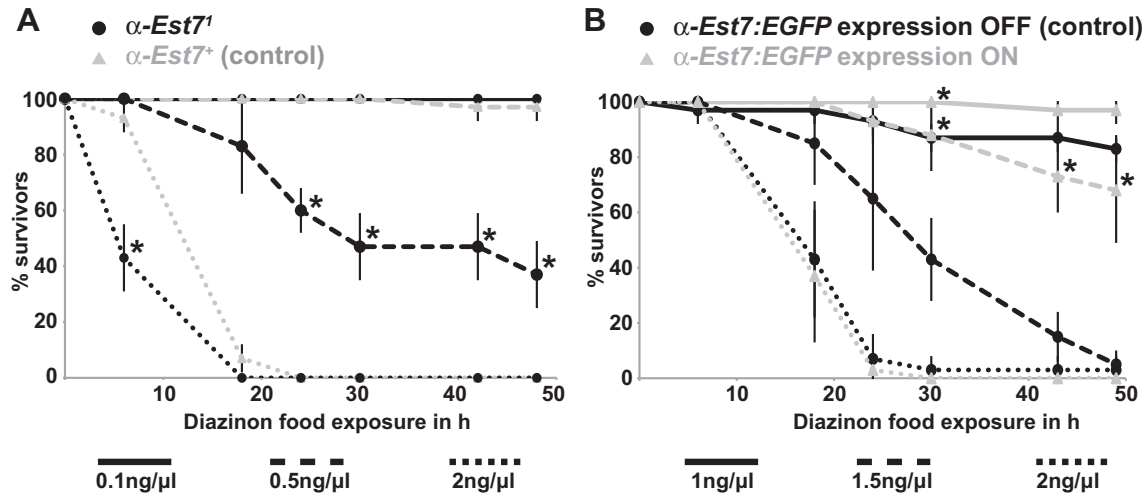
Fig. 3. α-Esterase-7 confers weak insecticide tolerance. α-Est7[1] mutants (A) are sensitive and α-Est7:EGFP overexpressing flies (B) moderately tolerant to the OP insecticide parent molecule diazinon in a dose-dependent manner. *$p \leq 0.05$. Note: Survivorship rates in % ($\pm$standard deviations) for the solvent control after 48 h food exposure were as follows: α-Est7[1] = 90($\pm$10), α-Est7[+] = 95($\pm$5) and α-Est7 overexpression OFF and ON = 100($\pm$0).

identity to CG1882 30.4%) causes neutral lipid accumulation in plants (James et al., 2010). It should be noted, however, that due to the comparably low sequence conservation between the discussed fly proteins and their vertebrate or plant relatives predictions on analogous protein functions deserve caution. CG1882 is also conserved in a wide range of insect species such as the Coleopteran *Tribolium castaneum* (55.2% sequence identity) and the pea aphid *Acyrthosiphon pisum* (47.8% identity).

Besides candidate enzymes of fly neutral lipid metabolism the presented screen also identified CG18815, the only *D. melanogaster* homolog of mammalian lysophospholipases I and II (LYPLA1 and LYPLA2; also called acyl-protein thioesterase 1 and 2 (APT1 and APT2); sequence identity to CG18815 is 54.2% see Fig. S4). CG18815 is an abundantly expressed putative thioesterase with largely cytoplasmic localization according to fusion protein expression data in *Drosophila* S2 cell culture cells (Bannan et al., 2008). Both mammalian LYPLAs regulate signaling molecules by deacylation. For example, LYPLA1 deacylates the orexigenic peptide ghrelin in serum (Satou et al., 2010) and down-regulates the Ras proto-oncogene by depalmitoylation (Dekker et al., 2010). While LYPLA2 deacylates the growth-associated protein-43 (Tomatis et al., 2010). CG18815 is well conserved among Drosophilids (e.g. 93.1% sequence identity to the *D. grimshawi* homolog) but also other insects such as the honeybee (60.2% sequence identity; see Fig. S4). The biological function of CG18815, however, awaits characterization.

The most prominent constituents of the molecularly identified fat body esterolytic proteome are CEs encoded by the α-*Esterase* and β-*Esterase* gene clusters. Among them is Esterase 6, a secreted esterase of the β-esterase cluster, which has an important though

evolutionarily derived role in *D. melanogaster* reproduction. In this and closely related Drosophilid species the gene is expressed in the male ejaculatory duct from where Esterase 6 is transferred as part of the seminal fluid to the female during mating (Richmond et al., 1980). In the female, male-derived Esterase 6 translocates to the hemolymph (Meikle et al., 1990) and stimulates egg laying whereas female receptivity for remating is delayed. Currently unknown, however, is the function of the evolutionary ancient Esterase 6 activity in the hemolymph. This Esterase 6 protein pool likely originates from the fat body and has been identified at larval stages by activity staining (Healy et al., 1991) and proteomics (Vierstraete, 2003).

α-*Esterase-7* is among the evolutionary ancient members of the α-*Esterase* cluster, which predicts important physiological gene functions (Oakeshott et al., 2005). To date, much of the current knowledge on *Drosophila* α-*Est7* function is derived from studies on α-*Est7* orthologs from insecticide-resistant strains of other dipteran species. In general, insecticide-resistant insect strains use one of two mechanisms to metabolically detoxify OP insecticides by CE activity modulation (reviewed in Feyereisen, 1995; Devonshire and Field, 1991; Ffrench-Constant et al., 2004; Oakeshott et al., 2005; Bass and Field, 2011). On one hand, overexpression of CEs by gene amplification or regulatory CE gene mutants confers OP tolerance by insecticide sequestration. This mechanism has been shown in resistant strains of aphids and lower dipteran insects (Field et al., 1999; Hemingway et al., 1998; Raymond et al., 1998) but also in the horn fly *Haematobia irritans* (Guerrero, 2000). On the other hand α-*Est7* can acquire insecticide tolerance by the mutant ali-esterase mechanism (Oppenoorth and van Asperen, 1960), i.e. increased OP hydrolase activity by structural mutation of the

Fig. 2. Esterase activity of fat body cells depends on α-*Esterase-7*. A. Schematic drawing of the α-*Est7* gene locus (based on www.flybase.org (Tweedie et al., 2009 and Robin et al., 1996)). Boxes represent the transcribed regions of α-*Est7* and parts of the neighboring genes α-*Est6* and *Ubc84D* with coding regions (gray boxes) and non-coding regions (open boxes). Hatched boxes indicate the genomic regions used for ends-out targeted gene replacement by a mini-*white* gene to generate the α-*Est7[1]* deletion mutant allele. Numbered arrows illustrate the approximate position of oligonucleotide primers (for details Sections 2.3, 2.10, 3.2 and Supplemental Material and Methods). B. qRT-PCR proves the absence of α-*Est7* transcripts but no impairment of α-*Est6* or *Ubc84D* in α-*Est7[1]*. Shown are the α-*Est7* (left upper panel) or α-*Est6* and *Ubc84D* transcript levels (right panel) normalized to α-*Est7^{+}* with corresponding 95% confidence intervals. Note that α-*Est7* transcripts are undetectable ("ud") in the α-*Est7[1]* mutant. Left lower panel: the corresponding α-Est7-specific qRT-PCR amplicons separated by agarose gel electrophoresis. C. Agarose gel size-separated PCR amplicons spanning the 5′ and the 3′ borders between the α-*Est7* genomic locus and the gene targeting donor sequences in the α-*Est7[1]* mutant. Expected amplicon sizes are 3 kbp (5′ border; RKO539/540) and 3.7 kbp (3′ border; RKO646/647), respectively. D. Absence of additional donor construct integrations outside the α-Est7 locus. Shown are inverse PCR amplicons separated by agarose gel electrophoresis. *denote amplicons derived from the α-Est7 gene locus of α-*Est7[1]* and # denote amplicons derived from the endogenous *white* gene locus (for more details and expected amplicon sizes see section 2.10). E. Fluorescence image of NBD-HE-HP-labeled fat body esterases after 1D SDS-PAGE separation. Positions of the endogenous α-EST7 (gray arrowhead) and the ectopic α-EST7:EGFP proteins (black arrowhead) are indicated. Note the band of unknown origin with an apparent molecular weight slightly smaller than α-EST7, which appears in α-*Est7:EGFP* (over)expressing flies. This band might reflect a partially degraded α-EST7(:EGFP) protein or an esterolytic activity, up-regulated in response to α-*Est7:EGFP* (over)expression. F. Total esterase activity on *p*-nitrophenyl butyrate of fat body cell homogenates is significantly reduced in α-*Est7[1]* mutants and increased upon α-*Est7:EGFP* overexpression in the fat body. ***$p \leq 0.001$.

A

B   **C**

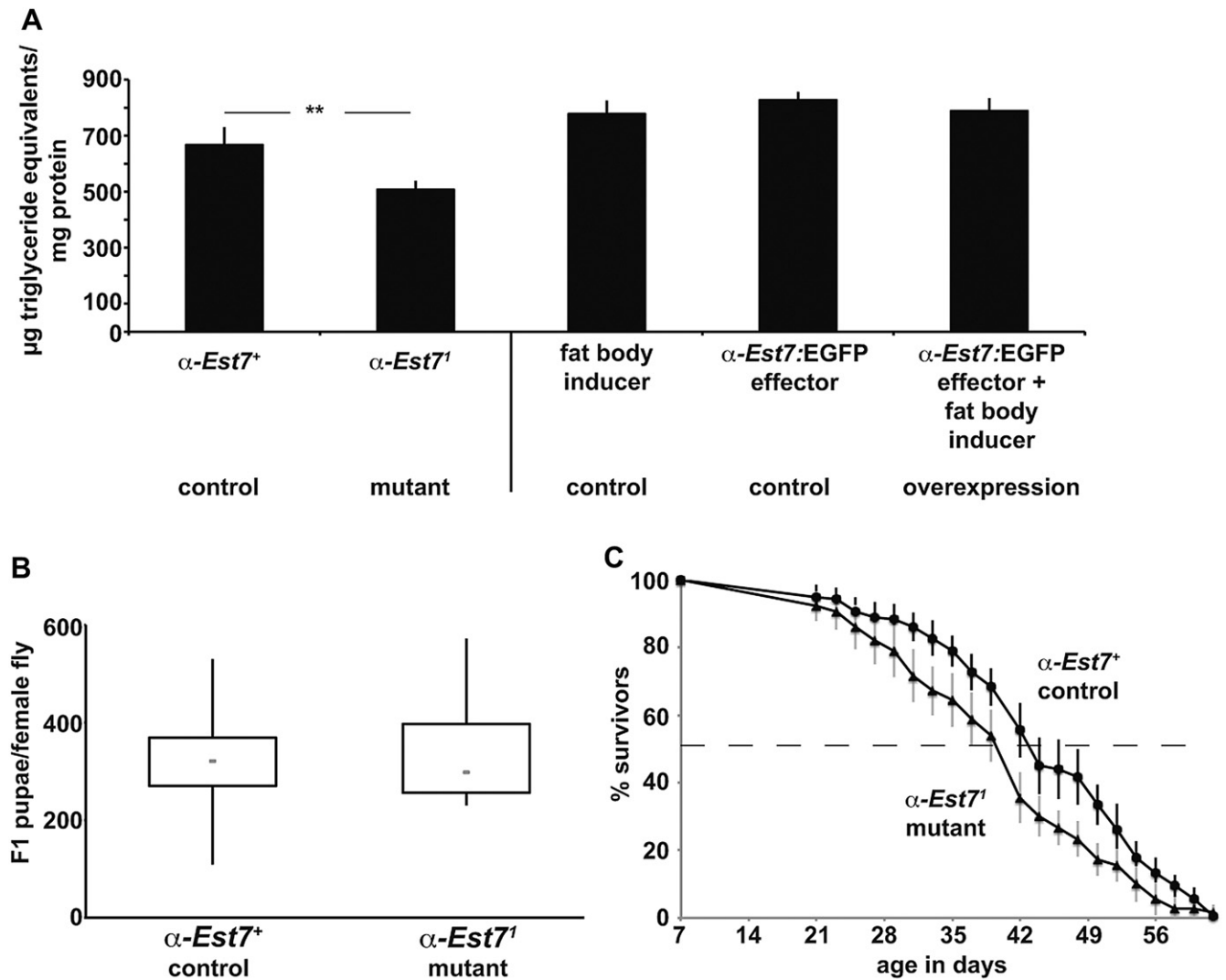**Fig. 4.** Metabolic and genetic fitness parameters of α-$Est7^1$ mutant flies. A. Leanness of α-$Est7^1$ mutant males but no impact of fat body-targeted α-$Est7{:}EGFP$ overexpression on body fat storage. $**p \leq 0.01$. B. Normal fecundity of α-$Est7^1$ females expressed by F1 pupae per female fly (depicted are medians (gray bar), upper and lower quartile (box) and maximum/minimum values of $n = 9$ (α-$Est7^+$) and $n = 11$ (α-$Est7^1$) female flies). C. Survival curves showing a median lifespan reduction of α-$Est7^1$ mutants (day 40.5) compared to α-$Est7^+$ control (day 43) male flies by 6% ($n = 180$ male flies for each genotype). Shown are mean age-specific survivorship data with 95% confidence intervals. Survivorship data of the two genotypes are significantly different (log-rank test $p < 0.00005$). For an independent longevity data set see Fig. S2. Note: As detailed in Section 2.3, α-$Est7^1$ has been backcrossed to α-$Est7^+$ for four generations prior to analysis.

enzymes oxyanion hole at the expense of reduced CE activity. Ali-esterase mutations in α-$Est7$ are causative for metabolic resistance toward OP insecticides in several higher dipteran species including the housefly *M. domestica* (Claudianos et al., 1999) and the blowfly *L. cuprina* (Newcomb et al., 1997). By contrast, α-EST7 mutants with mutant ali-esterase activity have not been found in OP-resistant *D. melanogaster* strains (Wilson, 2001). Instead, diazinon resistance of adult *D. melanogaster* from wild populations is low and of polygenic nature (Pyke et al., 2004). This seems paradoxical at first glance, because OP insecticide tolerance can be induced by transgenic expression of bacterial OP hydrolases in *Drosophila* (Phillips et al., 1990; Benedict et al., 1994), and because of the similar biochemical *in vitro* properties of the *D.m.α-Est7* and *L.c.α-Est7* isozymes (Parker et al., 1996). However, *D.m.α-Est7* shows low OP binding affinity compared to *L.c.α*-EST7, which has been proposed to grant less protection for the insecticide target acetyl-cholinesterase *in vivo* (Parker et al., 1996). Our data provide evidence for a moderate dose-dependent OP-tolerance function of wild type α-$Est7$, which is in line with these *in vitro* data. The

primary sequence mutations in α-EST7 found in OP-resistant insects have been demonstrated to confer mutant ali-esterase activity also to *Drosophila* α-EST7 (Devonshire et al., 2003) and a broad range of distantly related CEs *in vitro* (Cui et al., 2011). The presented α-$Est7$ null mutant fly opens the possibility to test the *in vivo* impact of α-EST7 ali-esterase mutants on OP insecticide tolerance in *Drosophila* by a gene replacement strategy. This approach will also allow the analysis of possible selective disadvantages connected with the shift in substrate specificity.

Drosophila α-$Est7$ mutants display a reduced body fat content, which invokes a role of the gene in lipid metabolism. In support of a lipid metabolic α-$Est7$ function, α-EST7 was identified on fat body lipid storage droplets (Beller et al., 2006) and the protein encodes an endoplasmic reticulum retention signal (Oakeshott et al., 2005), the organelle, where LDs are formed (Ohsaki et al., 2009). A number of LD-associated proteins in the fat body have been demonstrated to impact the body fat content in flies. Among them the triglyceride lipase Brummer (Grönke et al., 2005) and members of the Perilipin protein family, which are proposed to modulate lipase activity

(Grönke et al., 2003; Teixeira et al., 2003; Beller et al., 2010). However, *Drosophila* α-EST7 has low lipase activity (Campbell et al., 2003) and overexpression of the *α-Est7:EGFP* fusion gene in the fat body does not affect the body fat content, which suggests that *α-Est7* is not sufficient for storage fat control in the fat body. Alternatively the gene could act at least in part outside the fat body to adjust body fat storage. Notably, *α-Est7* is also expressed in parts of the gut, in particular in the crop (Chintapalli et al., 2007), which serves as a temporary food storage organ in flies. Accordingly, it is tempting to speculate that improper food storage or processing might contribute to the observed leanness of *α-Est7* mutant flies. However, a mechanistic understanding of how α-EST7 promotes neutral fat storage in flies requires the future identification of the *in vivo* substrates of the enzyme. It should be noted that differences in the body fat content might indirectly affect the toxicity of lipophilic OP insecticide parent molecules such as diazinon. Diazinon needs bioactivation (Pisani-Borg et al., 1996) typically by enzymes of the cytochrome P450 superfamily, some of which are expressed in the fat storage tissue (Chintapalli et al., 2007). Therefore it is tempting to speculate that a reduced fat content would cause decreased diazinon sequestration in the fat stores, which increases bioavailability of the parent molecule to activation to the OP insecticide diazoxon and accordingly would contribute to the moderately reduced insecticide tolerance level of the *α-Est7[1]* mutant flies.

In the light of the complex ontogenetic expression profile of *α-Est7* from embryos to aged adult flies of both sexes and in various fly organs, the observed *α-Est7* mutant phenotype is comparably mild. Notwithstanding the fact that the phenotypic analysis of *α-Est7* mutants is not comprehensive, this observation might also reflect functional redundancy among the *Drosophila* *α-Est* genes. Notably, EST9 encoded by the *α-Est5* gene is a fat body-expressed enzyme (Healy et al., 1991) and, consistently, *α-EST5* has been identified as fat body esterase by the presented functional proteomics approach (Fig. 1). In general, predictions regarding the *in vivo* functions of esterase genes deserve caution. Even a highly conserved gene such as *Est-P* (also called *ψEst-6*), a member of the *β-Esterase* cluster, is represented by functionally inactive alleles in many *D. melanogaster* wild populations suggesting no vital function of this gene, which might be in transition to a pseudogene in this species (Balakirev et al., 2003). This example emphasizes the need for a systematic generation and subsequent analysis of *Esterase* null mutants to assign functions to individual members of this important gene family. Accordingly, the presented initial phenotypic characterization of the *α-Est7* mutant pioneers the understanding of *α-Esterase in vivo* gene functions. Important questions regarding e.g. the full spectrum of the *α-Est7* gene functions, the tissue-specificity or the endogenous substrate spectrum of the enzyme deserve future attention. Moreover, the *Drosophila* *α-Est7* deletion mutant paves the way for *in vivo* structure-function analyses using a gene replacement strategy.

Author contributions

The study was designed by R.P.K. and R.B.-G. All fly experiments were done by I.B., J.L., P.H. and R.P.K. Functional proteomics was performed by R.B.-G., M.K., G.N.R. and A.H., R.P.K, P.H. and R.B.-G. analyzed the data and wrote the manuscript.

Conflict of interest

The authors declare no competing financial interests.

Appendix. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ibmb.2011.12.004.

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