Identification of a novel *Drosophila* gene, *beltless*, using injectable embryonic and adult RNA interference (RNAi)

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Abstract

**Background:** RNA interference (RNAi) is a process triggered by a double-stranded RNA that leads to targeted down-regulation/silencing of gene expression and can be used for functional genomics; i.e. loss-of-function studies. Here we report on the use of RNAi in the identification of a developmentally important novel *Drosophila* (fruit fly) gene (corresponding to a putative gene CG5652/GM06434), that we named *beltless* based on an embryonic loss-of-function phenotype.

**Results:** *Beltless* mRNA is expressed in all developmental stages except in 0–6 h embryos. In situ RT-PCR localized *beltless* mRNA in the ventral cord and brain of late stage embryos and in the nervous system, ovaries, and the accessory glands of adult flies. RNAi was induced by injection of short (22 bp) *beltless* double-stranded RNAs into embryos or into adult flies. Embryonic RNAi altered cuticular phenotypes ranging from partially-formed to missing denticle belts (thus *beltless*) of the abdominal segments A2–A4. Embryonic *beltless* RNAi was lethal. Adult RNAi resulted in the shrinkage of the ovaries by half and reduced the number of eggs laid. We also examined Df(1)RK4 flies in which deletion removes 16 genes, including *beltless*. In some embryos, we observed cuticular abnormalities similar to our findings with *beltless* RNAi. After differentiating Df(1)RK4 embryos into those with visible denticle belts and those missing denticle belts, we assayed the presence of *beltless* mRNA; no *beltless* mRNA was detectable in embryos with missing denticle belts.

**Conclusions:** We have identified a developmentally important novel *Drosophila* gene, *beltless*, which has been characterized in loss-of-function studies using RNA interference. The putative *beltless* protein shares homologies with the *C. elegans* nose resistant to fluoxetine (NRF) NRF-6 gene, as well as with several uncharacterized *C. elegans* and *Drosophila melanogaster* genes, some with prominent acyltransferase domains. Future studies should elucidate the role and mechanism of action of *beltless* during *Drosophila* development and in adults, including in the adult nervous system.

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**Background**

RNA interference (RNAi) is a process that leads to targeted down-regulation/silencing of gene expression [1,2]. Typically, RNAi is triggered by double-stranded RNAs (dsRNAs) that are cleaved into small 21–23 bp small interfering RNAs by a dsRNA-specific enzyme, and thereafter, cellular machinery that causes degradation of dsRNA-complementary endogenous mRNA is set in motion. The physiological role of RNAi, particularly in the central nervous system (CNS), remains speculative [3],
but the applicability of experimentally induced RNAi for functional genomics, i.e. loss-of-function studies, is becoming more apparent [4,5].

Recently, we developed a method of dsRNA-injectable RNAi as a tool for loss-of-function studies in adult Drosophila (fruit fly) [6,7] that combines loss-of-function phenotypes with neuropharmacological techniques [8]. Injectable RNAi has been successfully applied to adult honeybees [9], snails [10], and also to mice [11] and rats [12]. This method can be used both during various developmental stages of Drosophila, e.g., embryonic RNAi, as well as selectively in adult flies. In Drosophila, RNAi appears to be cell- and isoform-specific [13]. Whereas the GAL4-driven hairpin-induced RNAi can be cell autonomous [14], injecting adult Drosophila intra-abdominally with either long or short dsRNAs resulted in cell-nonautonomous silencing of the complementary endogenous mRNA and produced an altered phenotype [7]. Thus, these two methods of RNAi induction in Drosophila could be used as complementary experimental approaches; the injectable cell-nonautonomous RNAi appears preferable for studies of genes in which cell- or tissue-specificity of expression is unknown.

Previously, we demonstrated that injection into adult flies of dsRNA corresponding to a putative gene CG5652 (GM06434) destroys its cognate mRNA [6]. A homologue of this putative Drosophila gene has been characterized in C. elegans. Thus, Choy and Thomas [15] carried out a genetic screen for mutants that were resistant to a certain behavior, i.e., nose twitches, induced by antidepressant drugs such as fluoxetine (Prozac) (hence, "nose resistant to fluoxetine", NRF). However, in our work we did not observe any major behavioral effects of the fluoxetine in fruit flies, which precluded us from attempting to find the Drosophila correlate of fluoxetine-triggered nose twitches observed in C. elegans. This could be attributed in part to the failure of fluoxetine, whose mechanism of action involves binding to and inhibition of the serotonin transporter, to bind and inhibit the Drosophila serotonin transporter [16]. Moreover, these findings suggested to us that the CG5652 gene in Drosophila may have a different role from its putative homologue in C. elegans, and that this gene does not mediate behavioral actions of fluoxetine in fruit flies. Nevertheless, in pilot experiments with CG5652 gene silencing using injectable RNAi in adult flies, we observed an effect of RNAi on the spontaneous egg laying behavior of adult females [17].

In our present work, we used both embryonic and adult RNAi to further characterize this putative Drosophila gene. Our results indicate a developmental role for a novel gene that we named beltless based on the embryonic loss-of-function phenotype.
Localization of blt mRNA determined by in situ RT-PCR (A, B) and in situ hybridization with a digoxigenin-labeled blt riboprobe (C-H). In stage 8–11 embryos, specific staining was observed in the neuroectoderm (nec) and in the invaginating pole cells (pc) (A). In stage 15–16 embryos, specific staining appears in the developing central nervous system (CNS) (B). In the third instar larvae, blt mRNA is localized in neuromuscular junctions (C and D; arrows), and in the ring gland (RG) (E). (F-H) In adult flies, panel F shows blt mRNA localization in the interneurons of the optic lobe (arrowheads) and in the large cells (arrows), panel G shows specific staining in the corpus allatum (CA) and panel H, in the ovary.
Beltless phenotype triggered by embryonic RNAi

In this set of experiments, we injected syncytial blastoderm embryos with blt dsRNA and staged the samples under mineral oil at 25°C. Compared to control embryos injected with human 5-lipoxygenase 23 nt dsRNA (there is no 5-lipoxygenase homologous gene in Drosophila), blt RNAi embryos appeared smaller and displayed a number of altered cuticular phenotypes ranging from partially differentiated cuticle to misdirected and only partially-formed denticle belts, and to missing denticle belts (beltless) of the abdominal segment A2–A4 (Fig. 3). The blt RNAi embryos developed and moved around in the eggshell, but failed to hatch, indicating that the embryonic blt “knockdown” is lethal.

Short 22 bp blt dsRNA injections into adult flies destroy endogenous blt mRNA

The injection of short (22 bp) blt dsRNAs into adult Drosophila is effective in inducing blt-specific silencing; i.e., this type of RNAi led to the destruction of the blt mRNA assayed with RT-PCR (Fig. 4).

Phenotype triggered by the adult blt RNAi

In pilot studies, we observed a marked reduction of the number of progeny from females injected with either a long [6] or a short 22-nucleotide blt dsRNA. This observation was interesting because we observed in situ localization of blt mRNA expression in the ovaries (Fig. 2), leading us to a further characterization of ovarian morphology following blt dsRNA injection into adult female flies. In Drosophila, each ovary consists of a compact group of parallel ovarioles held together by a peritoneal sheath; each ovariole is surrounded by an epithelial sheath, which is a thin nucleated membrane. Seventy-two hours after injection, the ovaries of blt dsRNA-injected flies had shrunk to half the size of control dsRNA-injected flies, and the connections between the ovarioles were disorganized and loose (Fig. 5). Although ovarioles of blt dsRNA-injected flies contained the proper number of egg chambers, their appearance was altered, suggesting that the maturation rate of the eggs might have slowed down (Fig. 5).

As a measure of ovarian function, we tested the effects of blt RNAi on oviposition (egg laying behavior). Table 2 shows that the injection of control dsRNA into adult females did not alter oviposition, whereas injection of blt dsRNA, which altered ovarian morphology (Fig. 5), significantly reduced the number of eggs laid.

Blt mutants

The Blt gene is localized in region 13B1 of the X chromosome (see below). Insertion/deletion mutagenesis screens of the Drosophila X chromosome revealed several key developmental genes associated with embryonic/first instar larval cuticle development [18]. These genes are also recessive-lethal and have been assigned to loci 11A-13B1. To test whether a deletion of blt results in an embryonic phenotype similar to that produced by embryonic blt RNAi, we examined flies carrying a deficiency on the X chromosome region 13A9-B1; i.e., Df(1)RK4 flies. In this strain, deletion removes 16 genes, including blt. In some embryos (about 3%) of these flies, we observed cuticular abnormalities similar to our findings with blt RNAi (Fig. 6). For example, we observed that the denticle belt is typically either interrupted or missing from the abdominal A4 segment. In a large number of embryos, the phenotypical changes are more severe; for example, denticle belts have not been formed at all. However, even in these severe cases, the mouth hook formation is not affected (Fig. 6). In this mutation, we observed extensive embryonic lethality.

After differentiating the Df(1)RK4 embryos based on their blt phenotype into two groups; i.e., those with visible denticle belts and those missing denticle belts, we assayed the presence of blt mRNA. No blt mRNA was detectable in embryos with missing denticle belts (Fig. 7).

The predicted structures of the blt gene and protein

According to a computer search and analysis of the available sequence data bases of the Drosophila genome (Berkeley Drosophila Genome Project; BDGM), the blt gene is located on the X chromosome and has been mapped cytologically to region 13B1 (FlyBase report for CG5652; http://flybase.bio.indiana.edu). The mRNA transcript from CG5652-RA is 2069 nt; the genomic sequence encompasses about 3.7 kb and comprises 7 exons (Fig. 8A). Our Northern blot hybridization analysis of blt mRNA from total RNA extracted from adult Drosophila identified a single band of about 2 kb (Fig. 8B).

The predicted blt gene mRNA encodes 659 amino acids (Q9VXX9, NM_167430). The PROSITE [19,20] motif search for Blt predicts potential phosphorylation sites for cAMP- and cGMP-dependent protein kinases, protein kinase C, and tyrosine kinase (Fig. 9A). In addition, the blt gene contains possible amidation, N-glycosylation, and three N-myristoylation sites. The primary sequence and hydrophobicity profile of the Blt protein reveals 7 transmembrane domains and regions with low complexity composition. A BLAST homology search aligned the protein with several uncharacterized C. elegans and Drosophila genes with acyltransferase domains (Fig. 9B).

Discussion

Using embryonic and adult RNAi, we have identified a loss-of-function phenotype for a novel Drosophila gene. Both embryonic RNAi and a mutation/deletion of this gene, e.g., Df(1)RK4 flies, are embryonic lethal and result
Embryonic blt RNAi induces a prominent cuticular phenotype.

Figure 3
Embryonic blt RNAi induces a prominent cuticular phenotype. (A) A control embryo, injected with a human 5-lipoxygenase 22 bp dsRNA; (B-E) embryos injected with blt dsRNA. RNAi induced phenotype: Panels B, D and E show missing or interrupted denticle belts from the abdominal segments A2, A3 and A4 (arrows). Panel C shows an example of a more severe cuticular phenotype.
in visible cuticular abnormalities, mainly the disappear-
ance of abdominal A2, A3 or A4 denticle belts, hence the
name beltless. In addition to this evidence for a
developmentally crucial role for blt, a selective silencing of
blt in adult female flies resulted in prominent anomali-
ies of the ovaries and eggs.

The lack of some but not all denticle belts suggest that blt
could participate in the functioning of cis-regulatory ele-
ments in the denticle pattern formation cascade. Possibly
this function of blt could take place downstream of Hox
transcription factors, which are the prime Drosophila seg-
mental morphology identifiers. Thus, secreted signaling
proteins Wingless (Wg) and Hedgehog (Hh) are essential
for establishing the denticle pattern in the epidermis of
Drosophila embryos. In the embryonic epidermis, these
proteins regulate the expression of Ser and rho, which are
critical for the final steps of denticle formation [21,22].

In this process, secondary modifications of signaling pro-
teins may be crucial for the biological activity of these pro-
teins. For example, the Hh protein undergoes
autocatalytic cleavage by its own C-terminal domain,
which produces the Hh signaling peptide. The cleavage of
Hh is followed by the addition of cholesterol to the C-ter-
minal end of its signaling protein [23,24]. On the other
hand, the N-terminal of this protein is modified by an
acyltransferase named skinny hedgehog (Ski). Ski, which
is a member of a group of segment polarity genes, is
required for Hh activation by palmitoylation of the N-ter-
minal [25]. Alternatively, the N-terminal of the Hh signal-
ning protein can be modified by the Rasp protein, another
member of the segment polarity genes that is catalytically
active as an acyltransferase [26].

Interestingly, the primary sequence and hydrophobicity
profile of the Blt protein suggest that this is a membrane-
associated protein with 7 transmembrane domains,
whereas its homology with several uncharacterized C. ele-
gans and Drosophila genes with acyltransferase domains
suggests that Blt could possess acyltransferase activity.
These characteristics point to putative similarities between
blt and other segment polarity genes. This possibility is
further supported by our findings of the blt loss-of-func-
tion embryonic phenotype, and we postulate that the
putative acyltransferase activity of blt may be needed for
Hh activation in anterior abdominal segments A2–A4.
Namely, after blt RNAi, we did not observe embryos with
missing A1 or A5–A8 denticle belts.

Based on our characterization of the localization of
embryonic blt mRNA, i.e. in the neuroectoderm, CNS, and
the invaginating pole cells, an additional developmental
role for blt should be considered. Developmental blt
expression also includes the ring gland, which degenerates
during metamorphosis and produces hormones that par-
ticipate in molting and metamorphosis. The embryonic
lethality of blt RNAi and blt deletion also point to a puta-
tive crucial developmental function of this gene.

The selective use of blt RNAi in adult flies enabled us to
investigate blt loss-of-function phenotype in adult Dros-
ophila. Thus, in addition to the embryonic beltless pheno-
type, we observed that blt silencing produces a major
disruption in egg development and maturation in adult
females. Whether the putative acyltransferase activity of
blt participates in the adult loss-of-function phenotype
should be investigated.

Localization of blt mRNA in the nervous system of devel-
oping and adult Drosophila indicates a possible role for Blt
in neuronal development and functioning. As indicated
above, the primary sequence and hydrophobicity profile
of the Blt protein suggests 7 transmembrane domains and
regions with low complexity composition. There was no
evidence for an N-terminal signal sequence; this would
classify Blt as a type III membrane protein typically found
in endoplasmic reticulum Golgi bodies. Furthermore, the
predicted amino acid composition of Blt suggests similar-
ities to the peroxisome microbody. Peroxisomes belong to
the microbody family of organelles and peroxisomal pro-
teins are crucial for neuronal development; defects of per-
oxosomal assembly factors lead to severe neurological
diseases known as peroxisome biogenesis disorders [27].
The peroxisomal signal motif N/T/K/KL (PTS1; the perox-
osome-targeting signal 1) [28] was found at the C-terminal
end of Blt protein.

Found in neural cell peroxisomes [29], acyltransferase
activity is important during insect development,
particularly in the larval synthesis of triacylglycerols and
phosphoglycerides [30]. Acyltransferase activity also
appears to be important for Drosophila synaptic vesicle

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Adult *blt* RNAi-induced alterations of ovarian morphology. (A) Control ovary isolated from flies injected with a human 5-lipoxygenase 22 bp dsRNA. (B) Confocal microscopy of a control developing egg. (C) Ovary isolated from flies injected with *blt* 22 bp dsRNA. (D) Confocal microscopy of a *blt* RNAi developing egg. Note the smaller size and presence of deformities in the ovaries and eggs in C and D, respectively.
budding at the neuromuscular junction [31] and in general, is involved in synaptic vesicle biogenesis [32]. Interestingly, we found prominent blt mRNA presence in the neurite-like structures at the neuromuscular junctions of the larvae. It has recently been proposed that the dendritic localization of mRNAs underlies certain aspects of synaptic plasticity [33].

Finally, acyltransferase activity was found in insect ovaries [34] where we also observed significant blt expression. Based on our RNAi experiments in adult flies, the presence of Blt in the ovaries appears to be functionally important and the loss-of-function phenotype in adult flies included both morphological and functional (e.g., oviposition) alterations and deficits. It is possible that these phenotypes are a reflection of an essential function of Blt in the corpus allatum, which releases the juvenile hormone that regulates egg maturation, as well as of Blt in the surrounding epithelial sheath in the ovariole.

Conclusions
In conclusion, we identified a novel Drosophila gene that we named beltless. Our results demonstrated that blt is crucial during oogenesis and embryogenesis. The expression of blt in the adult nervous system suggests that besides its developmental role blt might be important for neuronal functioning. Further studies are needed to fully characterize this novel gene.

Methods
Fly stocks and injections
Wild type Drosophila melanogaster (CS/S) flies and Df(1)RK4 mutant flies (Bloomington Drosophila Stock Center, Donors: Robert Kreber and Rachel Drysdale) carrying a deficiency of the X chromosome region 13A9-B1 were cultured at 25°C, 50–60% humidity, 12 h/12 h light/dark cycle, on yeast, dark corn syrup, and agar food. For the injections, flies were anesthetized with CO2 (at a maximum of 5 min) [6,7]. Using custom-beveled glass pipettes (20 × 40 µm tip diameter) coupled to a cell injector (Narashige IM-200) and a micromanipulator under a stereo microscope (Leica GZ6), we injected a volume of 0.2 µl/fly by a pulse pressure of 300 kPa [6,7]. Injection of embryos was performed using the Nanoliter 2000 injector (World Precision Instruments).

dsRNA synthesis
For RNAi studies, we targeted two 22-nucleotide long regions of the blt gene; i.e., regions 2–23 and 685–708. Eight 39-mer DNA oligonucleotides with an attached T7 RNA polymerase promoter sequence were synthesized (Integrated DNA Technology, Inc.); these oligonucleotides represented both sense and antisense strands. The sequences of the sense and antisense DNA oligonucleotides corresponding to the blt gene used for the in vitro transcription reaction are shown in Table 1; only one match was found in the genome database pattern search analyses for each chosen targeted sequence.

One set of oligonucleotides produced dsRNA probes with a 3’ overhanging 2 UUUn, and a second set was used for the production of blunt-end dsRNAs with GG..CC flanking nucleotides. Equal amounts of oligonucleotides were annealed to form a double-stranded template by heating at 80–85°C for 5 min and cooling down on ice. The in vitro transcription reaction (30 µl volume) for the synthesis of the 22 nt RNA run of transcripts contained 0.1 µg of a template, 500 µM each CTP, GTP, ATP, and UTP, 1 × transcription buffer (Tris-HCl, pH 7.5, 10 mM dithiothreitol, 1% bovine serum albumin), 20 U of

| Table 1: Oligonucleotides used for the synthesis of blt dsRNAs |
|---------------------------------------------------------------|
| **T7 promoter (bold letters)** | **Targeted cDNA sequence (22 nt)** |
| Sense | 5’-TAATACGACTCACAATAATGCAGGATGCCTGGTATT-3’ |
| Antisense | 5’-AAACACGCGGATGCGCATATAGTGACGAGCT-3’ |
| Sense | 5’-TAATACGACTCACAATAATGCAGGATGCCTGGTATT-3’ |
| Antisense | 5’-TGTGGTCCGCTAGCCGCTTTTATAGTGACGAGCT-3’ |
| Sense | 5’-TAATACGACTCACAATAATGCAGGATGCCTGGTATT-3’ |
| Antisense | 5’-GGAGCGCACTGCTGCAATGAGCTATAGTGACGAGCT-3’ |
| Sense | 5’-TAATACGACTCACAATAATGCAGGATGCCTGGTATT-3’ |
| Antisense | 5’-CCCTCGGTCAGCAGGTTACTGATAGTGACGAGCT-3’ |

| Table 2: Inhibitory effect of adult blt RNAi on oviposition 72 h after injection |
|----------------------------------------|-----------------|
| Treatment of adult females | Number of eggs laid/fly/24 h (mean ± s.e.m.) |
| Control 1 (buffer injection) | 8.7 ± 0.7 |
| Control 2 (dsRNA injection) | 8.3 ± 0.7 |
| blt dsRNA injection | 4.8 ± 0.6* |

*P < 0.001 (Dunnett’s test; n = 21)
Figure 6
**Df(1)RK4 embryonic cuticular phenotype.** In this mutation, the denticle belt is missing from the abdominal A4 segment (A), or is interrupted (B) (arrows). (C, D) In a large number of embryos, the phenotypical changes are more severe; for example, no denticle belts are formed; panel C: dorsal view. However, even in these severe cases, the mouth hook formation is not affected (panel D, ventral view).
RNA was extracted from samples collected at different stages of *Drosophila* development. For adult flies, the total RNA was extracted separately from the heads and bodies of male and female flies; two flies were used for each experiment. Two embryos displaying cuticular defects and two normal embryos from Df(1)RK4 flies were used for RT-PCR. RNAs were DNAase treated prior to RT reaction. The rp49 gene was used as an internal control. The following are the primers used in the RT-PCR assay: for *blt* – direct 5'-atcgatcaggaacttcttggt-3'; reverse 5'-atttcgctgggatcataac-3'; for rp49 – direct 5'-atgaccatccgcccagcat-aca-3'; reverse 5'-tggtcttgccgagcaggtta-3'. The reaction conditions were as follows: first strand synthesis, 1 h at 37°C in 20 µl of reaction mix with a 0.2 mM of each dNTPs, 10 pmol of a hexanucleotide primers, 20 U RNAse inhibitor, and 100 U reverse transcriptase. We performed 30 cycles of PCR in 20 µl of reaction mix at the following conditions: 92°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec. Agarose gel electrophoresis was used to resolve and visualize the PCR reaction products.

**Northern blot assay**

Total RNA was extracted from the heads and bodies of male and female flies with a Trizol reagent (Gibco BRL). Ten micrograms of total RNA per sample were analyzed according to a standard protocol for the Northern blot analysis [35]. A digoxigenin-labeled 524 bp PCR fragment of *blt* was used as the hybridization probe. The signal was detected via chemiluminescence with the CSPD reagent (a substrate for alkaline phosphatase; Roche, catalog number 1655884); the blots were exposed for 12 h to Kodak BioMax film.

**In situ hybridization assay of *blt* mRNA**

The heads of 20–30 male and female flies were collected 72 h after injection with dsRNA oligonucleotides or ringer solution, and heads from naïve flies were also collected. The heads were sliced on dry ice with a sterile surgical blade (#11) and transferred into the tubes with a fixation buffer (15 % formaldehyde in phosphate-buffered saline; PBS; pH 7.4) for 20 min. Ovarian, corpus allatum, and ring gland hybridization was done on cryostat sections of female flies and 3rd instar larvae. For the 3rd instar larval muscle preparation, larvae were dissected from the ventral side with a surgical blade (#11), the cuticle was stretched and pinned down, and the preparations were fixed. The ovaries also were collected from anesthetized flies for confocal microscopy (Leica TCS-NT 1.6.587) without the in situ hybridization. They were manually dissected in PBS and fixed in a 1% glutaraldehyde in PBS buffer for 20 min, rinsed in PBS, and mounted in a NaCl/glycerol solution.

The in situ hybridization procedure was applied as described elsewhere [36]. Briefly, the samples were incubated with proteinase K (10 µg/ml) for 5 min at room temperature.
Figure 8
The exon/intron structure of the \textit{blt} gene and Northern blot analysis. (A) Exons: filled squares, introns: lines. (B) Northern blotting revealed a single band (the arrow indicates the 2.37 kb marker size), visualized with digoxigenin-labeled probe, using the total RNA extracted from heads and bodies of male and female flies. Lane 1: female body, lane 2: male body, lanes 3 and 4: their respective heads. The lower panel shows the corresponding ribosomal \textit{rp46} mRNA.
The putative beltless protein; analysis and multiple alignment.

(A) The putative beltless (Blt) protein shares homologies with the *C. elegans* nose resistant to fluoxetine (NRF) NRF-6 gene, including the NRF domain and the acyltransferase domain (Acyl_transf_3). Using the PROSITE motif search engine, we found a high probability for multiple potential phosphorylation sites in the putative Blt (shaded rectangles). (B) The BLAST homology search and the CLASTLOW multiple alignment tools aligned the Blt protein (Q9VXX9) with several uncharacterized *C. elegans* and *Drosophila melanogaster* genes, some with prominent acyltransferase domains.

Figure 9
The putative beltless protein; analysis and multiple alignment. (A) The putative beltless (Blt) protein shares homologies with the *C. elegans* nose resistant to fluoxetine (NRF) NRF-6 gene, including the NRF domain and the acyltransferase domain (Acyl_transf_3). Using the PROSITE motif search engine, we found a high probability for multiple potential phosphorylation sites in the putative Blt (shaded rectangles). (B) The BLAST homology search and the CLASTLOW multiple alignment tools aligned the Blt protein (Q9VXX9) with several uncharacterized *C. elegans* and *Drosophila melanogaster* genes, some with prominent acyltransferase domains.
temperature, rinsed with a glycine (2 mg/ml) in PBS, and prehybridized (6x SSC, i.e., 3 M NaCl, 0.3 M sodium citrate, 10 μg/ml sonicated salmon sperm DNA, 10 μg/ml yeast tRNA, 50 % formamide, 0.01 % Tween-20) at 55°C for 1 h. The samples were hybridized overnight at 55°C with digoxigenin-UTP-labeled (Roche) sense and antisense strands of a 524 bp fragment of blt gene (sequence 128–652; AF 173374). They were washed three times in PBS, 1 h each at 55°C, and incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:1000 in PBS at room temperature for 1 h). Thereafter, sections were washed 4 times 30 min each in PBS at room temperature, and rinsed twice in a blue color development buffer for alkaline phosphatase (100 mM NaCl, 100 mM Tris-HCl, pH 9.5, 50 mM MgCl₂, 0.1% Tween-20). The color was developed with NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate; Roche) and the reaction was stopped with 2 mg/ml glycine in PBS, pH 2.2. Samples were mounted in a mounting solution (80% glycerol, 0.25 mM NaCl) and images were taken under the microscope.

Since we were not able to detect mRNA staining in embryos with conventional in situ hybridization (probably because the signal is below detection level), we used an in situ RT-PCR assay with digoxigenin-labeled dUTP to localize blt mRNA in the embryos. Briefly, the 0–20 h embryos were collected on agar plates and fixed and processed as described elsewhere [36]. The RT step was carried out with at least 200 embryos per tube and in 20 μl of digoxigenin-labeling PCR mix (Roche); the samples were processed for 10 cycles (92°C 15 sec, 57°C 30 sec, 72°C 30 sec). Thereafter, they were washed several times in PBT (PBS/Tween-20), incubated with anti-digoxigenin antibody conjugated with alkaline phosphatase (1:1000 in PBS) for 30 min, and washed in PBS for 2 h. Specific primers used in this PCR reaction were as follows: direct-5′-tagtggctgtagaggattgcc-3′, reverse-5′-acagttggttagttcaccctagg-3′. As a negative control, either one or both primers were omitted from the above-described procedure.

Cuticle preparation
Embryos were manually dechorionated, covered with mineral oil on a glass slide, and flattened with a cover slip. They were steam-fixed on top of boiling water for 1 min, the cover slips were slowly removed and the mineral oil was replaced with a mounting solution (glycerol/NaCl). The vitelline membrane and all debris were manually removed and covered with a cover slip. The preparations were examined under a microscope and photographed.

Oviposition
Mated female flies were injected with 10 ng/μl of blt dsRNA, control dsRNA, or with buffer and individually placed for 24 h into small glass tubes with food. Flies were transferred to new tubes every 24 hours. The tubes were inspected under the microscope and the number of eggs laid was counted.

Author’s contribution
SD carried out the molecular studies, analyzed the predicted structures of the blt gene and protein, and drafted the manuscript. ND carried out the adult injectable RNAi and functional assays. HM conceived of the study, and participated in its design and coordination. All authors read and approved the final manuscript.

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