A gain-of-function screen identifies \textit{wdb} and \textit{lkb1} as lifespan-extending genes in \textit{Drosophila}

Masabumi Funakoshi, Manabu Tsuda, Keigo Muramatsu, Hiroshi Hatsuda, Shinichi Morishita, Toshiro Aigaki

1. Introduction

The insulin/insulin-like growth factor (IGF) signaling pathway plays an important role in controlling metabolism, organ growth and lifespan [1,2]. In \textit{Drosophila}, mutations in the insulin receptor substrate \textit{chico} or overexpression of phosphatase and tensin homolog (PTEN) reduced body size and extended the lifespan [3,4]. The insulin/IGF pathway activates Akt, followed by the target of rapamycin (TOR), which promotes protein synthesis by activating S6K [2]. Mutations in \textit{dTOR} or \textit{dS6K} were also reported to reduce body size [5] and increase lifespan [6,7]. TOR is also regulated by AMP-activated protein kinase (AMPK) signaling pathways. AMPK is a cellular energy sensor that is activated in response to low levels of nutrition [8]. The activation of AMPK leads to downregulation of TOR signaling by stimulating the GTPase-activating protein (GAP) activity of tuberous sclerosis protein-2 [9,10] and phosphorylation of raptor, a component of the TOR complex [11]. In \textit{Caenorhabditis elegans}, a mutation in \textit{oak-2}, a homolog of AMPK, reduced the lifespan while the expression of the constitutive active form of AMPK extended the lifespan [12]. Therefore, AMPK could be involved in the regulation of cell growth and lifespan.

A number of genes could modify insulin/IGF and TOR signaling. However, relatively few genes have been investigated regarding their effects on lifespan. Since the lifespan is one of the most complex biological traits, experimental studies are necessary to understand the mechanisms involved in the regulation of lifespan.

2. Materials and methods

2.1. \textit{Drosophila} stocks and culture

Flies were raised on a standard corn meal, yeast, glucose agar medium at 25 °C. The (UAS) expression vector containing an upstream activating sequence (UAS) was inserted into the genome at random, allowing for \textit{Gal4}-dependent forced expression of the vector-flanking genes [13]. Since the lifespan is often inversely correlated with body size, we first screened for insertions that reduce wing size when misexpression was induced in the developing wing discs. We also examined these insertions to determine whether they also reduced eye size when misexpressed in eye discs. Among the insertions that reduced the sizes of wings and eyes, we found two genes, \textit{wdb} and \textit{lkb1}, that extended lifespan when overexpressed ubiquitously. We provide biochemical evidence showing that overexpression of \textit{wdb} reduces the level of phosphorylated AKT, while overexpression of \textit{lkb1} increases the level of phosphorylated AMPK and reduces the level of phosphorylated S6K. Our results suggested that \textit{wdb}- and \textit{lkb1}-dependent lifespan extension is mediated by downregulation of S6K, a downstream component of the insulin/IGF and TOR signaling pathways.
Wings were removed from flies with forceps and mounted on a microscopic slide with a drop of Fly Line Dressing (TIEMCO, Tokyo, Japan). Wing images were captured using a stereoscopic microscope (Leica MZ16F, Leica Microsystems, Tokyo, Japan) equipped with a DP-70 digital camera system (Olympus, Tokyo, Japan). Wing size was measured using Image J software (version 1.38, http://rsb.info.nih.gov/ij/download.html).

2.4. Real-time PCR analysis

Total RNA was extracted from 3 to 5-day-old adult flies using Trizol reagent (Invitrogen, Carlsbad, CA), treated with DNAse I, and then reverse-transcribed using a SuperScript VILO cDNA Synthesis kit (Invitrogen). Real-time PCR was carried out using SYBR Premix Ex Taq (TaKaRa Bio, Shiga, Japan) and a Chromo 4 Detector (MJ Research, Hercules, CA). The results were normalized by the level of Actin 5C mRNA. The relative expression levels of wdb and ikb1 mRNA in flies as hs > wdb and hs > ikb1, respectively, were calculated against that of a control (hs > GFP), and means ± standard error were calculated from triplicate assays. The primer sequences used for real-time PCR were as follows: wdb_F, 5'-CAAGGTTCTCCTGCCCTACAGAA-3'; wdb_R, 5'-ACACGCAAACACTGGCCACCTT-3'; ikb1_F, 5'-GCACACTGTTCCTGCGCATGC-3'; ikb1_R, 5'-TGCGATACGGTGCCGGTCTT-3'; Actin5C_F, 5'-GGGATGCTTTGATCTCGG-3'; Actin5C_R, 5'-AATCTCCACCACTCGCACTTG-3'.

2.5. Lifespan measurement

The lifespan of adult flies was measured at 25 °C, as previously described [14]. Briefly, newly eclosed males and females of the appropriate genotypes were collected and transferred to new vials containing standard food every 2-3 days until all of the flies had died. One hundred flies (20 flies/vial) were used for each genotype, and the number of dead flies was counted at the time of transfer. The logrank test was used to evaluate the difference between the two genotypes for each sex.

2.6. Western blot analysis

Adult heads were cut from 5-day-old flies, homogenized in SDS-sample buffer (12.5 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 2% 2-mercaptoethanol and 0.001% bromophenol blue) and boiled for 10 min at 95 °C. The samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). After blocking with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO), the membranes were incubated with a primary antibody in Tris-buffered saline containing Tween (TBST) overnight at 4 °C and then with a secondary antibody in TBST for 1 h at 25 °C. The signals were detected with an ECL-plus kit (GE Healthcare). As primary antibodies, rabbit anti-phospho-Akt (Cell Signaling Technology, Danvers, MA), rabbit anti-phospho-p70 S6 kinase (Cell Signaling Technology) and mouse anti-a-tubulin (Sigma-Aldrich) were used at dilutions of 1:1000, 1:1000 and 1:8000, respectively. HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated anti-mouse IgG (GE Healthcare) were used as secondary antibodies at dilutions of 1:2000 and 1:1000, respectively.

2.7. Immunohistochemistry

Wing imaginal discs were dissected from third instar larvae, immersed in TBS, and fixed for 20 min in 4% paraformaldehyde in TBS.
The tissues were washed in TBST, blocked in 10% goat serum in TBST for 2 h, and incubated with rabbit anti-phospho-AMPK antibody (Cell signaling Technology) at a dilution of 1:100 in TBST overnight at 4 °C. After washing in TBST, the samples were incubated with Alexa Fluor 488-conjugated anti-rabbit IgG (1:500, Invitrogen), washed in TBST, mounted with GEL/MOUNT (Cosmo Bio, Tokyo, Japan), and then observed under a Nikon C1 laser scanning confocal microscope (Nikon, Tokyo, Japan).

3. Results and discussion

3.1. Generation, mapping and a gain-of-function screening of P(GS) vector insertion lines

To perform the gain-of-function screen, we first mobilized the P(GS)-element vector containing a UAS, and established 763 independent insertion lines (GS lines). We next determined the insertion sites of the P(GS) element in these lines by LM-PCR. Using this technique, 716 lines (94%) were successfully mapped to single locations of the Drosophila genome. The numbers of P(GS) inserts on chromosomes X, 2 and 3 were 57, 324 and 335, respectively. More than 60% of insertions were mapped within 0.5 kb from the transcription start site (5'-end of mRNA) of nearby genes, most frequently between 100 and 1000 bp from the transcription start site (Fig. 1A). A total of 340 insertions were found within or near annotated genes. Of these, the numbers of insertions in the UTRs of genes were 94 (Fig. 1B). The insertion preference of the P(GS)-element near to the 5'-end of genes was consistent with that observed for other P-element vectors [15].

We next screened for the insertions whose misexpression affected organ size. The GS lines were crossed with vg-GAL4, which drives misexpression of vector-flanking genes in the developing wing imaginal discs. Approximately 1.3% (10 lines) of the insertions were lethal, and 13% (99 lines) induced visible phenotypes, which were classified into six categories: notched wings, missing veins, extra veins, blistered wings, increased wing size, and reduced wing size (Fig. 2 and Supplementary Table 1). The most fre-
quently observed phenotypes were notched wing (45 lines, 45%) and reduced wing size (22 lines, 22%). In the subsequent studies, we focused on the insertions that reduced wing size because they might extend the lifespan of flies.

We examined whether the insertions only reduced the size of wing or whether they affected other organs as well. A total of 22 P(GS) lines that reduced wing size were crossed with GMR-GAL4 to induce misexpression in the developing eye imaginal discs. Six insertions (GS2157, GS10079, GS9756, GS7058, GS11006, and GS9548) reduced the size of the wings and eyes. Interestingly, among the genes flanked by these insertions (Supplementary Table 1), wdb and lkb1 are implicated in insulin/IGF or TOR signaling, while the remaining four genes (dpld, CG8963, CG13577 and CG13894) are poorly characterized. Therefore, we characterized the phenotypes associated with overexpression of wdb and lkb1 in more detail.

3.2. Overexpression of wdb reduces organ size and extends lifespan by inhibiting Akt activity

The GS9548 line had a P(GS) vector insertion located 29-bp upstream of the transcription start site of the wdb gene, which encodes a regulatory subunit of protein phosphatase 2A (PP2A) (Fig. 3A). We performed real-time PCR to determine whether wdb is overexpressed in a GAL4-dependent manner. To induce GAL4 expression, hs-GAL4 was used as a driver, and heat induced at

![Image of figures](https://example.com/figure4.jpg)

Fig. 4. Overexpression of lkb1 reduces organ size and extends lifespan by activating AMPK signaling. (A) Genomic organization of the lkb1 locus with the P(GS) vector insertion in the line GS11006. Black and open boxes represent the protein-coding and untranslated regions, respectively. (B) Real-time PCR analysis of lkb1 mRNA expression level in hs > lkb1 and hs > GFP (control) flies. Mean (±SE) values were calculated from three experiments (t-test: *P < 0.01). (C) Superimposed images of the wings of vg > Rbb1 (magenta) and vg > GFP (green) flies. (D) Overexpression of lkb1 reduced the size of the compound eyes. (E) Immunohistochemical staining of wing imaginal disc with an anti-phospho-AMPK antibody. The ptc-GAL4 driver was used to overexpress lkb1 on the anterior side of the anterior/posterior border of imaginal discs (right panel, ptc > lkb1). An ectopic phospho-AMPK signal was detected in the wing disc corresponding to lkb1 overexpression (arrow head), whereas no signal was detected in the control flies (left panel, ptc-GAL4). (F) Western blot analysis of phosphorylated AMPK and Akt in GMR > lkb1 and GMR > GFP (control) flies. (G) Survival curves of males (left panel) and females (right panel). The mean lifespan of hs > lkb1 flies (black) was significantly longer than that of hs > GFP flies (gray) in females (logrank test: *P < 0.01) but not in males. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

37 °C for 30 min. The mRNA expression of wdb in flies carrying both the G59548 and hs-GAL4 insertion was approximately 3 times higher than that control flies (hs > GFP) (Fig. 3B). Thus, we used this insertion as a transgene to characterize the wdb overexpression phenotype.

The mean wing area of flies overexpressing wdb in wing imaginal discs (vg > wdb) was reduced by 15.5% compared with control flies (vg > GFP), with a marked reduction in the posterior half of the wing (Fig. 3C). Overexpression of wdb in eye imaginal discs using the GMR-GAL4 driver also reduced the size of the compound eyes (Fig. 3D, left panels). In Drosophila, wdb has been characterized as a gene that protects against apoptosis in cultured S2 cells [16] and regulates planer cell polarity in vivo [17]. In addition, it has been shown that Wdb binds to Akt, and genetically interacts with the PI3K/PTEN/Akt signal [18]. We found that overexpression of wdb suppressed the increase in eye size caused by overexpression of akt in imaginal eye discs using GMR-GAL4 (Fig. 3D, right panels), indicating that wdb negatively regulates the role of akt in controlling eye size. Furthermore, overexpression of wdb reduced the level of phosphorylated Akt in GMR > wdb flies as compared with that in control flies (GMR > GFP) (Fig. 3E). These results suggest that the wdb-dependent reduction of organ size is mediated by inhibition of Akt signaling.

We next determined the effects of wdb overexpression on lifespan using hs-GAL4 as a ubiquitous driver. At 25 °C, the expression level of wdb in hs > wdb flies was approximately 1.8-fold higher than that in control flies (hs > GFP). The mean lifespan of hs > wdb flies was 68.2 ± 1.1 days in males and 73.5 ± 0.4 days in females, representing increases of 17% and 13%, respectively, versus control flies (hs > GFP) (Fig. 3F). These results suggest that overexpression of wdb extends the lifespan by inhibiting Akt, a downstream component of the insulin/IGF signaling pathway.

3.3. Overexpression of lkbl reduces organ size and extends lifespan by activating AMPK signaling

The P(GS) vector insertion site in the GS11006 line was mapped to the first exon of the lkbl gene (Fig. 4A), which encodes a serine/threonine kinase implicated in Peutz-Jeghers syndrome and tumor suppression [19,20]. Since the insertion site is within the 5'-UTR, lkbl is likely overexpressed upon GAL4 activation. Overexpression of lkbl was confirmed by real-time PCR of hs > lkbl flies (Fig. 4B). Thus, we used this insertion as a transgene to overexpress lkbl. When lkbl was overexpressed in the developing wing imaginal discs (vg > lkbl), the mean wing area was reduced by 7.4%, compared with that in control flies (vg > GFP) (Fig. 4C). Overexpression of lkbl in imaginal eye discs reduced the size of compound eyes (Fig. 4D). Lkbl plays crucial roles in the regulation of energy sensing pathways. In response to low energy availability, Lkbl inhibits the TOR signaling pathway by activating AMPK [9,10]. Therefore, we investigated whether overexpression of lkbl activates AMPK in Drosophila. Overexpression of lkbl in imaginal wing discs using the ptc-GAL4 driver induced ectopic activation of AMPK along the border of the anterior/posterior compartments (Fig. 4E). Furthermore, the level of phosphorylated S6K was greatly reduced, while that of phosphorylated Akt remained unchanged (Fig. 4F). These results suggest that the lkbl-dependent wing size reduction was mediated by the activation of AMPK and inhibition of the TOR signal.

We subsequently examined the effects of lkbl overexpression on adult lifespan. The expression level of lkbl in hs > lkbl flies at 25 °C was 1.7 times higher than that of control flies. The mean lifespan of hs > lkbl females was 69.6 ± 1.3 days, representing an increase of 6.5% relative to control females (hs > GFP) (65.3 ± 1.6 days) (Fig. 4G). No significant increase in lifespan was observed in males, being 60.3 ± 1.3 days in males overexpressing lkbl and 58.3 ± 1.6 days in control males. The female-specific extension of lifespan could be due to metabolic differences between the two sexes. Genetic and environmental methods to manipulate the lifespan have often resulted in sex differences [21]. Furthermore, several studies have reported sex-dependent effects of manipulating the insulin and TOR signaling pathways on lifespan [22,23]. In general, female flies show greater response than male flies to the manipulation of the insulin and the TOR signals. Although the mechanism underlying the sex difference in the effects of lkbl is unclear, our results suggest that overexpression of lkbl extended the lifespan of females by activating AMPK and, in turn, inhibiting TOR signaling.

In conclusion, we have identified two lifespan-extending genes, wdb and lkbl, in Drosophila. We provide molecular evidence that overexpression of wdb inhibits Akt and that of lkbl activates AMPK and reduces S6K activity. Thus, overexpression of wdb and lkbl mimics the downregulation of the insulin/IGF and TOR signaling pathways in Drosophila.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.01.090.

References


