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# Depletion of ribosomal protein L8 impairs *Drosophila* development and is associated with apoptosis

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Ribosomal protein L8 is a component of the 60S subunit of the ribosome and is involved in protein synthesis but its role in *Drosophila* development is not well understood. We depleted L8 through RNA interference (RNAi) to examine its effects on fly development both *in vivo* and *in vitro*. The results demonstrated that L8 RNAi caused embryonic or first-larval lethality, delay of larval development, defects in eye and wing morphology, and dramatically reduced the number of S2 cells. This indicated that L8 plays a crucial role in *Drosophila* development. Acridine orange staining of the wing discs showed that apoptosis occurred when L8 was depleted, indicating that depletion of L8 is tightly connected to apoptosis. RT-PCR analyses of the transcription level of genes that are known to be key factors in apoptosis (*p53*, *hid*, *reaper*, *dark*, *Dcp-1*) and cell cycle regulation (*cdc45*, *MCM3*, *cyclin B*, *incenp*) in L8-deficient S2 cells, were consistent with their role in apoptosis induction and cell cycle arrest. These results indicate that depletion of L8 strongly impairs *Drosophila* development, and that this depletion is associated with cell proliferation arrest and apoptosis, in which p53 may play a central role.

**ribosomal protein L8, *Drosophila*, RNAi, apoptosis, cell cycle, *p53***

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Ribosome proteins (RPs) are crucial components of the ribosomal subunits, with their functions involving ribosome assembly and protein synthesis. Recent studies have indicated that individual RPs have been implicated in a wide variety of biological functions, including cell cycle progression, apoptosis and DNA damage responses [1–4]. Their roles in these processes possibly arise independently of their role within the ribosome itself [5,6]. Abnormal expression of some RPs has been shown to be responsible for human afflictions such as Diamond-Blackfan anemia [7], Turner syndrome [8], hearing loss [9] and cancer [10].

Additionally, many Minute loci were originally identified from the phenotypes of flies heterozygous for a chromosomal deletion [11] and all Minute point mutations studied in depth have been found to be loss-of-function alleles. In *Drosophila*, accumulating data has shown that mutations in genes encoding some RPs cause the Minute phenotype, delayed larval development, short and thin bristles, reduced fertility and viability, and recessive lethality. This phenotype results in a slower rate of cell growth and proliferation [12,13], reflecting the reduced rate of protein synthesis due to impaired ribosome biogenesis [14].

L8 is ubiquitously and constitutively expressed at all stages of *Drosophila* development. Studies have found that a Minute locus (M(3)LS2) was mapped to 62E-63A [11] and the *Drosophila* L8 gene was mapped to position 62E6-7 on the third chromosome. It is unknown whether mutations in L8 generate Minute phenotypes [15]. In addition, it has been shown that *Drosophila* L8 has a role in the regulation of cell size in multiple tissues throughout development. It acts downstream of the insulin signaling pathway and might have additional functions affecting translational efficiency and output, and inter-

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acts with *Drosophila* Myc in regulating growth [16].

Although L8 has been studied in *Drosophila*, the mechanism by which L8 functions in this process remains unclear. In the present study, we successfully produced L8-depleted *Drosophila* and Schneider 2 (S2) cells by RNA interference (RNAi) and found that ablation of L8 strongly impairs *Drosophila* development, and is strongly associated with apoptosis and cell proliferation arrest.

## 1 Materials and methods

### 1.1 *Drosophila* strains and cultures

The cDNA fragments corresponding to the *Drosophila* RP L8 (nt 241–756) were amplified by PCR using primers with *Xho* I-*Eco*R I linkers. The forward primer used for amplification was 5′-CAACTCGAGAGGGAGCTGGTTCCGTG-TT-3′ and the reverse primer was 5′-CCGGAATTCCC-TTCAGGATGGGCTTGTC-3′. The resulting fragment was digested and cloned into the *Xho* I-*EcoR* I sites of the *SympUAST-w* vector. The resultant vector, *SympUAST-w*/L8 was sequenced and microinjected into $W^{1118}$ embryos to produce L8RNAi *Drosophila*. The other fly lines used in this study included *act5c*-Gal4, *Hsp70*-Gal4, *ey*-Gal4 and *vg*-Gal4. All the flies were maintained on the cornmeal sucrose-based medium and crosses were carried out at 18°C or 25°C unless otherwise stated.

### 1.2 Measurement of body length

To obtain viable flies, we crossed the L8RNAi males with *Hsp70*-Gal4 females and allowed them to lay on agar caps for 1 h at 25°C. Twenty-four hours after the eggs were laid, larvae were heat-shocked at 37°C for 30 min. Ten larvae in each group were randomly collected at 24-h intervals until the pupa appeared in the wild-type control. Pictures were captured by using an MZ16 stereomicroscope with a CCD camera (Leica). Images were analyzed using MetaMorph software (Molecular Devices) to determine the body length of each larva.

### 1.3 Staining of imaginal discs with acridine orange

Imaginal discs from third instar larvae were dissected in PBS (pH 7.2), and placed immediately into *Drosophila melanogaster* cell culture medium at 4°C. The dissected imaginal discs were transferred onto a slide and rinsed with 100 μL medium. They were then stained with 1 μg $mL^{-1}$ acridine orange (Sigma, St Louis, MO, USA) for 5 min at room temperature and observed immediately with an epifluorescence microscope.

### 1.4 RNAi in S2 cells

S2 cells were cultured at 25°C with the Schneider *Drosophila* basal medium (Gibco) supplemented with 10% fetal calf serum (Gibco), 160 μg $mL^{-1}$ penicillin, 250 μg $mL^{-1}$ streptomycin, and 4 mmol $L^{-1}$ L-glutamine (Sigma) in 75 $cm^2$ tissue culture flasks (Corning). Individual DNA fragments, containing the coding sequences for *Drosophila* L8 were amplified with primers containing the T7 promoter (forward primer: 5′-TAATACGACTCACTATAGGGAGAATGGG-TCGCGTTATTCGTGCA-3′; reverse primer: 5′-TAATAC-GACTCACTATAGGGAGATTACTTGTCCTTGCTGTC-GCC-3′). The PCR products were purified using the High Pure PCR Purification Kit (Roche Molecular Biochemicals). The purified PCR products were used as templates in the MEGASCRIPT T7 transcription kit (Ambion, Austin, TX, USA) to produce dsRNA. The dsRNA products were ethanol-precipitated and resuspended in RNase-free water. The dsRNAs were annealed by incubating at 65°C for 30 min followed by slow cooling to room temperature. The quality of dsRNAs was assessed by agarose gel electrophoresis and stored at −80°C until required.

RNAi in S2 cells was performed as described previously [17]. Briefly, S2 cells were diluted to a final concentration of $1\times10^6$ cells $mL^{-1}$ in a 35 mm culture dish (Corning) using the serum-free medium. L8 dsRNAs (5 μg $mL^{-1}$) were added directly to 1 mL culture medium and incubated for 60 min at room temperature. After that, 2 mL culture medium was supplemented with 10% fetal calf serum for routine culture. L8 RNAi-treated cells were harvested on day 4 or 8 for further analysis. Concurrently, dsRNAs for the GFP gene were used to produce control cells under the same conditions.

### 1.5 RT-PCR

Total RNA was extracted from L8 or GFP RNAi cells cultured for 8 d using an RNeasy Mini Kit (Qiagen) in accordance with the manufacturer's instructions. Prior to RT-PCR, the RNA was treated with DNase (TaKaRa Bio, Japan). The purified RNA was used for first-strand cDNA synthesis and reverse transcription was performed using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) with oligo-$dT_{18}$ primers in accordance with the manufacturer's instructions. Reactions lacking reverse transcriptase were also performed for each RNA sample tested to check for genomic contamination. For each 25 μL PCR, 1 μL of sample cDNA was used. Amplification was performed at 95°C for 5 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 45 s, and after the last cycle a final extension step at 72°C for 8 min was carried out. The primers used are shown in Table 1.

### 1.6 Statistical analysis

All experiments were repeated at least three times and statistical analyses were performed using Microsoft Excel software. All values were presented as mean±SD. Statistical

**Table 1** The primer sequences used in RT-PCR

| Gene name | Primer sequences |
|---|---|
| *L8* | Forward, 5′-TGCGTTCCCTGGACTTCG-3′<br>Reverse, 5′-GCTGGCACAGGTGCTCAA-3′ |
| *actin* | Forward, 5′-TGCCCATCTACGAGGGTTAT-3′<br>Reverse, 5′-AGTACTTGCGCTCTGGCGG-3′ |
| *MCM3* | Forward, 5′-GAGGGGGAGCAGTTCATCA-3′<br>Reverse, 5′-AAACCGTATTCGGTCTCCAG-3′ |
| *cdc45* | Forward, 5′-ATGTTTGTCCAGGATCTGCG-3′<br>Reverse, 5′-CTGAGCTTACGGCTCCTTG-3′ |
| *incenp* | Forward, 5′-ATGGAGGACATCTTGGGCG-3′<br>Reverse, 5′-CCGCATCTTACGGTTGAGTG-3′ |
| *cyclin B* | Forward, 5′-CACTGGAAGAAACAGCCACTGGGC-3′<br>Reverse, 5′-GGTGGTGGGCATCGTAGATGTGGT-3′ |
| *p53* | Forward, 5′-CATCGGTGGTTATTGCTTCA-3′<br>Reverse, 5′-GCTCAACGCTAAGGTGATTTT-3′ |
| *dcp-1* | Forward, 5′-AGGGTGATGAGAAGAAGATGG-3′<br>Reverse, 5′-TGAGATGCTTGCGTTGGTAT-3′ |
| *hid* | Forward, 5′-TGCTATACGCCCTCTACGAG-3′<br>Reverse, 5′-GATATGACGGATGTGGTTGC-3′ |
| *reaper* | Forward, 5′-ACAGAGCCACAACACCCACC-3′<br>Reverse, 5′-TAGCCAACTTCGACTCATCTTC-3′ |
| *dark* | Forward, 5′-GAGCATTCGGGATGGTCTGG-3′<br>Reverse, 5′-TATGTGGGCTGATGGAGGGA-3′ |

significance in body length was assessed by a Student's *t*-test. Values of $P<0.05$ were considered to be significant.

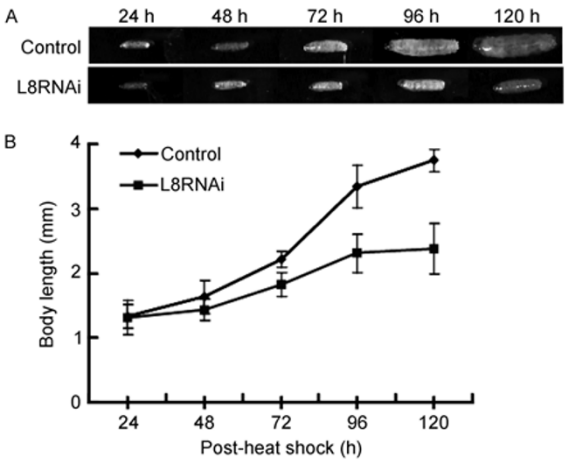


**Figure 1** Growth arrest in L8-depleted larvae. A, Photographs showing representative heat-shocked L8RNAi/*Hsp70*-Gal4 larvae compared with the control. B, The body length of L8RNAi/*Hsp70*-Gal4 larvae during the initial 48-h growth period was similar to the body length of controls. At 72 h, the L8RNAi/*Hsp70*-Gal4 larvae had significantly shorter body length. Data are expressed as mean±SD of body length calculated in at least three independent experiments, and 10 larvae were observed for each time point investigated. *$P<0.05$ compared with the corresponding time points in the L8-depleted group.

## 2 Results and discussion

### 2.1 RNAi of L8 delays larvae development

We crossed *act5c*-Gal4 drivers with L8RNAi flies to induce L8 gene silencing ubiquitously. All progeny of L8RNAi/*act5c*-Gal4 died at the embryo or early first-instar stages compared with control flies. These results are consistent with a previous report [16], suggesting that L8 plays a key role in embryonic or first instar development and its depletion leads to early death. To obtain viable L8-depleted flies, we used the *Hsp70*-Gal4 driver to induce L8 gene silencing ubiquitously in inducible expression systems. The larvae of L8RNAi/*Hsp70*-Gal4 showed an apparent growth delay and died in the larval stage at last. The control larvae grew normally into pupae and then adults (Figure 1A). During the initial 48 h, the growth of L8RNAi/*Hsp70*-Gal4 larvae had not slowed down compared with the control ($P>0.05$), their body lengths were significantly ($P<0.05$) shortened from 72 h (Figure 1B). This result showed that L8 plays an important role in *Drosophila* early development and depletion of L8 causes an arrest in cell proliferation.

### 2.2 RNAi of L8 causes defects in eye and wing development

We crossed L8RNAi male flies with *ey*-Gal4 female flies to exam the developing eye phenotype. The *ey*-Gal4 driver expresses Gal4 under the control of an enhancer element derived from the *eyeless* (*ey*) gene [18]. During early larval development, *ey* is expressed ubiquitously. In the third larval instar, its expression is restricted to the anterior of the morphogenetic furrow. Additionally, *ey* is expressed in the ventral nerve cord, optic lobes and other discrete domains of the brain [19].

The major phenotype (82%) consisted of headless adults lacking the entire head structures. These flies lost all eclosion ability and died in the pupa phase. The remaining 18% of flies had small eyes (Figures 2B and C) compared with the wild-type (Figure 2A). Eye size was highly variable (Figures 2B and C), indicating the differential influences of L8 depletion on fly eye development. The flies with smaller eyes were able to close normally. Similar phenotypes were observed previously in L14RNAi flies induced by *ey*-Gal4 [14]. *Ey* mutation was known to cause small eyes and extensive cell death was found in *ey* mutant eye discs [19]. Early ectopic expression of several transcription factors in the primordial eye-antennal disc interfered with the early function of *ey* and blocked cell division, thus generating headless flies, a phenotype much stronger than that caused by the *ey* mutation. Our results implied that L8 depletion caused apparent eye defects that were associated with cell proliferation and cell death.

Given that *Drosophila* L8 is ubiquitously expressed, we determined whether its depletion also caused a similar development blockade in other tissues. Consistent with the phenotype in eye structure, we saw a similar result in the wing, where *vg*-Gal4 was used to drive L8 silencing. These flies formed a very small and ruffled wing structure (Figure 2E) in comparison with the controls (Figure 2D). The L8RNAi-induced defect in wing development is consistent with defects in the eye structure, supporting that L8 affects

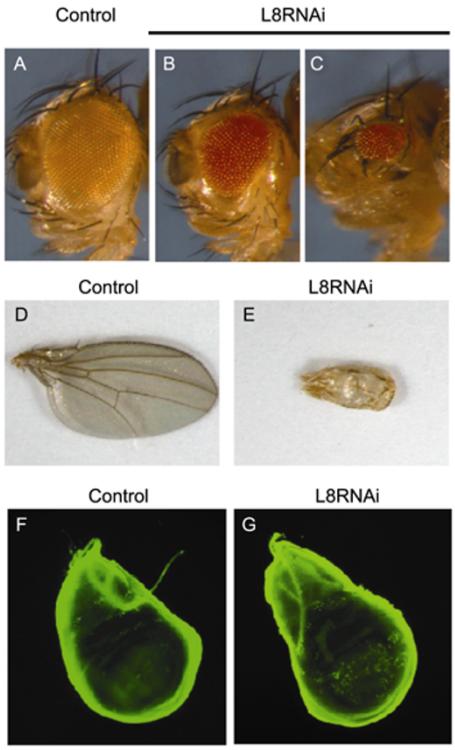


**Figure 2** Eye and wing defects in L8-depleted flies. In addition to the headless flies (82%), the remaining 18% of L8RNAi/*ey*-Gal4 progeny (B and C) showed eye defects in comparison with wild-type progeny (A). L8RNAi/*vg*-Gal4 progeny (E) showed wing defects in comparison with the wild-type progeny (D). Acridine orange staining (green) of wing discs from third-instar L8RNAi/*vg*-Gal4 larvae (G) showed apparent apoptosis of the wing disc (white arrow) compared with the wild type (F).

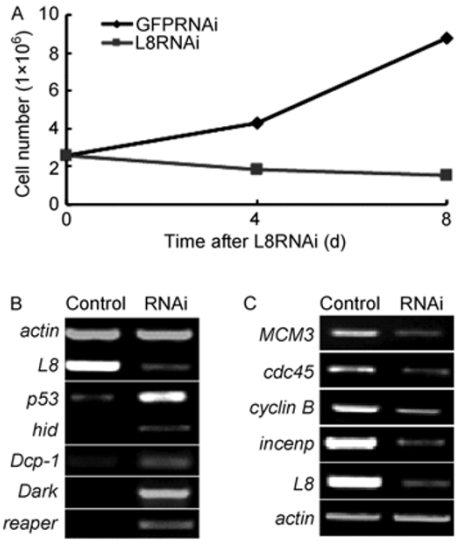


**Figure 3** Abnormal gene expression in L8-depleted S2 cells. A, Growth of L8-depleted cells was arrested immediately, and the cell numbers in cultures gradually reduced during continuous culture compared with the GFPRNAi control cells. RT-PCR analysis showed that depletion of L8 results in abnormal expression of *p53*, *hid*, *dcp-1*, *dark* and *reaper* corresponding to cell apoptosis (B) or abnormal expression of *MCM3*, *cdc45*, *cyclin B* and *incenp*, corresponding to cell cycle regulation (C), whereas the housekeeping gene *actin* is expressed normally in both GFP- and L8-depleted cells.

fly development in multiple tissues.

### 2.3 Apoptosis in the wing discs of L8RNAi flies

We stained the wing discs of the L8RNAi/*vg*-Gal4 flies with acridine orange (AO), which specifically labels dying cells with disrupted membrane integrity, and thus is a useful staining marker for apoptosis. The results clearly showed that the L8RNAi/*vg*-Gal4 wing discs (Figure 2G) exhibited severe cell death in the posterior region, where *vg*-Gal4 was expressed [20]. However, the wing discs of wild-type larvae had fewer dead cells (Figure 2F). This result strongly suggested that apoptosis occurred after RNAi was induced, and may be responsible for the growth defects in eye and wing development of *Drosophila*.

### 2.4 RNAi of L8 reduces S2 cell numbers

It is known that the cell number directly reflects the cell proliferation condition. The control of cell numbers is determined by an intricate balance of cell death and proliferation. Thus, to test the influence of L8RNAi *in vitro*, we transfected S2 cells with dsRNA derived from the L8 cDNA in the treatment group, and with dsRNA of GFP in the control group. Cell numbers were determined at different time points after transfection of dsRNA L8 and GFP. The number of S2 cells was dramatically reduced after transfection of L8 dsRNA compared with cells transfected with GFP dsRNA (Figure 3A) and continued to decrease as time went on. The results, in accordance with the eye and wing phenotypes, further confirmed that cell proliferation was blocked, possibly due to cell death. Previously, *Drosophila* L8 was shown to have a role in regulation of cell size in multiple tissues throughout development and L8RNAi caused a reduction in cell size rather than a decrease in cell number [16]. We also observed a reduction in S2 cell numbers following L8RNAi, implying a different mechanism by which L8 functions in *Drosophila* development.

### 2.5 Expression level of apoptotic inducers was enhanced in L8-depleted cells

Following L8RNAi, the occurrence of apoptosis was evident and we expected to see expression changes of related apoptotic factors. We investigated the expression of the well-known apoptosis inducer genes, *p53*, *hid*, *reaper*, *dark*, and *Dcp-1* by RT-PCR. The tumor protein, p53, plays a crucial role in inducing apoptosis [8] by caspase activation through mitochondrial cytochrome c release [21,22]. Hid and reaper are required for the induction of apoptosis in flies [23,24] by triggering p53 directly or indirectly [25]. In addition, it was found that the two proteins lead to apoptosis by activating a caspase pathway [26,27]. Dcp-1, one of the mem-

bers of the *Drosophila* caspase family of ICE/CED-3 proteases, also participates in apoptosis induction [28]. Dark, the *Drosophila* homologue of apaf1, results in apoptosis by releasing cytochrome c from mitochondria [29,30]. In our study, the transcription level of *hid*, *reaper*, *Dcp-1*, *dark* and *p53* increased as expected when expression levels of L8 were reduced in S2 cells (Figure 3B). The expression patterns of these genes were consistent with their role in apoptosis induction, supporting the occurrence of apoptosis after L8RNAi.

### 2.6 Expression alteration of cell cycle regulators in L8-depleted cells

It is known that apoptosis and proliferation are linked by cell-cycle regulators in proliferating cells, and apoptotic stimuli affect both cell proliferation and death [31]. We examined the expression level of some cell cycle regulators, such as *cdc45*, *MCM3*, *cyclin B* and *incenp* by RT-PCR. Cdc45 and MCM3 are the key regulators in promoting $G_1$/M transition, while cyclin B and incenp activation are required for $G_2$/M progression. It has been shown that the *cdc45* expression level is positively associated with cell proliferation [32]. MCM3 is cleaved early in several *in vitro* models of apoptosis [33]. The reduction of *cyclin B* levels causes $G_2$ arrest by p53-dependent transcriptional repression [34]. Incenp was shown to interact with Aurora B and survivin to form an Aurora B/incenp/survivin complex that is required for normal chromosome segregation [35–37]. It was reported that over-expressing survivin reduced p53 protein levels due to enhanced p53 degradation resulting from survivin-mediated inhibition of Mdm2 cleavage by caspases. Thus, incenp may affect cell cycle regulation in a manner similar to survivin. In our study, reduction of L8 resulted in the transcriptional decrease of *cdc45*, *MCM3*, *cyclin B* and *incenp* (Figure 3C). This result is in accordance with apoptosis related gene analysis and further indicates that L8RNAi leads to cell cycle arrest and apoptosis.

Recent studies have demonstrated that p53 plays a role in monitoring the status of ribosomal biogenesis [10]. Stresses on ribosomal biogenesis result in the arrest of cell growth or apoptosis to repair or remove the affected cells, probably via p53 activation. Previously, some studies found that depletion of S6 or L22 impairs T lymphocyte development in mice by activating a p53-dependant checkpoint response [38,39]. It is well known that p53 plays multiple roles in various aspects of cell metabolism, such as cell cycle regulation, apoptosis and DNA repair. It is likely that p53-dependant apoptosis is responsible for the stresses on ribosomal biogenesis. In our study, p53 increased remarkably following L8 depletion. As mentioned above, many genes related to apoptosis or regulation of the cell cycle were either directly or indirectly associated with p53 activity. Therefore, it is quite possible that ablation of L8 strongly blocks *Drosophila* development and is tightly associated with apoptosis, in which p53 seems to play a central role. In the future, the molecular interaction between L8 and p53, and detailed information regarding how p53 regulates cell cycle arrest and apoptosis in response to L8 depletion in *Drosophila* require further investigation.

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