Cell Death and Differentiation (2006) 13, 454–464

www.nature.com/cdd

E75A and *E75B* have opposite effects on the apoptosis/development choice of the *Drosophila* egg chamber

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Received 15.2.05; revised 06.7.05; accepted 13.7.05; published online 07.10.05
Edited by J Abrams

Abstract

The number of *Drosophila* egg chambers is controlled by the nutritional status of the female. There is a developmental checkpoint at stage 8, which is controlled by *BR-C* in the follicle cells along with ecdysteroid. During this period, developmental decision is made in each egg chamber to determine if it will develop or die. During nutritional shortage, inducing apoptosis in the nurse cells of stages 8 and 9 egg chambers reduces the number of egg chambers. We show that ecdysone response genes *E75A* and *E75B* are involved in inducing or suppressing apoptosis. It is thus possible that the *E75* isoforms *A* and *B* are involved in the decision to develop or die in oogenesis. We have established part of the pathway by which ecdysone response genes control apoptosis of the nurse cells and hence select between degeneration or development of individual egg chambers at stages 8 and 9.
Cell Death and Differentiation (2006) **13,** 454–464.
doi:10.1038/sj.cdd.4401745; published online 7 October 2005

Keywords: *Drosophila*; oogenesis; apoptosis; *E75*; ecdysone

Abbreviations: *BR-C*, *Broad-Complex*; JHA, juvenile hormone analog; 20E, 20-hydroxyecdysone

Introduction

The *Drosophila* egg chamber consists of a cluster of 16 cells interconnected by ring canals, which are surrounded by a monolayer of somatic follicle cells. One cell in the cluster develops into the oocyte and the other 15 cells become nurse cells that support the oocyte in its development.[1] The 15 nurse cells degenerate by apoptosis when the oocyte is mature.[2,3] Normally, apoptosis of the nurse cells is completed at stage 14 of oogenesis when they have transferred their contents to the oocyte.[3,4] Apoptosis of the nurse cells commences at stage 10B, when nurse cells undergo cytoskeletal rearrangement.[5,6] However, in flies that are maintained under starvation conditions or have 20-hydroxyecdysone (20E) at physiological concentrations injected into the abdomen, apoptosis is observed in the nurse cells much earlier, at stages 8 and 9.[7,8] Simultaneous application of the juvenile hormone analogue (JHA), Methoprene, inhibits this apoptosis.[7,8] The ecdysteroid concentration in starved females is higher than in females that are maintained on normal food.[9] Thus it seems likely that the monitoring of nutrition and the response of the fly in terms of oogenesis will be controlled in part by ecdysone and juvenile hormone (JH).

Ecdysone functions through the Ecdysone/Ultraspiracle nuclear receptor complex (EcR/USP).[10,11] The complex directly regulates the early ecdysone response genes such as the *Broad-Complex (BR-C), E74* and *E75*. The *BR-C* encodes a family of zinc finger transcription factors.[12] *E75* encodes three orphan members of the nuclear receptor superfamily, designated *E75A, E75B* and *E75C*.[13] Early ecdysone response genes are essential for oogenesis. Transcription of *E75* and *E74* appeared to be upregulated during stage 8 in both the nurse cells and somatic follicle cells.[14] Germline clones of *E75* mutant cells result in degeneration of egg chambers after stage 8, with a phenotype similar to that of the temperature-sensitive mutant *ecdysoneless[1]*.[14] The ecdysone receptor, *EcR,* which is crucial for regulation of expression of the early response genes, has also been shown to be crucial in the germline, as mutant germline clones arrest development during mid-oogenesis.[14] In addition, we have shown that *EcR* isoforms are differentially suppressed, with *EcRA* showing higher expression and *EcRB* lower expression under nutritional shortage.[15] *BR-C* expression in the somatic follicle cells regulates the establishment of dorso-ventral polarity of the eggshell later in oogenesis[16] and leads to prolonged endoreplication and to additional amplification of selected genes.[17] In addition, *BR-C* expression in the follicle cells controls the cell fate of the egg chamber, determining if it will progress to develop into an egg or induce apoptosis.[8] *BR-C* isoforms, *Z1* and *Z3,* affect yolk protein gene expression, while *Z2* and *Z3* expression in the follicle cells induce apoptosis of the stages 8 and 9 egg chambers.[8] Thus ecdysone, ecdysone receptor and ecdysone response genes are clearly crucial for a number of developmental decisions in normal oogenesis.

Apoptosis in many tissues and glands in insects is induced by 20E and is regulated by the same ecdysone response genes that are crucial in oogenesis.[18] *Dronc* is one of the apoptosis inducers in *Drosophila*,[19] and is upregulated by *reaper*,[19,20] which also induce apoptosis in *Drosophila*.[21,22] *Reaper* expression is regulated by *BR-C* and *hid* expression is regulated by *BR-C* and *E74A* in *Drosophila* salivary glands.[23] But Foley and Cooley[4] have established that *reaper, grim* and *hid* do not affect the apoptosis of nurse cells that commences during normal development of an egg at stage 12 of oogenesis.[4] *E75* is required for the suppression of *diap2*, which inhibits the progression of apoptosis in the larval salivary glands during *Drosophila* metamorphosis.[23,24] The relationship between *BR-C, E75A* and *E75B* is described by Woodard *et al*.[25] and White *et al*.[26] during early metamorphosis in

Drosophila. The ecdysone/EcR/USP complex activates a small set of genes (early and early-late genes), some of which encode transcription factors. These gene products in turn negatively autoregulate, and turn on a large set of effecter genes (late genes), and control the expression of the mid-prepupal factor β*FTZ-F1*. This factor then specifies the appropriate early gene response to the subsequent prepupal pulse of ecdysone.[25,26]

In this paper, we describe the network of ecdysone response genes used in the control of apoptosis of nurse cells in the stages 8 and 9 egg chamber of *Drosophila*. *BR-C Z2* and *Z3*, along with *E75A* and *E75B* in the follicle cells, control the premature apoptosis of the nurse cells. *BR-C* responds to the signal initiated by nutritional or hormonal conditions, and controls the expression of *E75A* and *B* in the egg chamber. *E75A* and *B* have opposite effects on apoptosis, *E75A* acts as an apoptosis inducer and *E75B* acts as an apoptosis inhibitor.

Results

E75A expression in the ovary

Ecdysone response genes have been shown to be involved in developmental decisions in many insect tissues and glands.[18] We investigated the early ecdysone response genes, *E75A* and *E75B*, to establish whether or not they affect apoptosis in the nurse cells of the egg chamber at stages 8 and 9. Transgenic flies of *E75A* and *E75B* were heat shocked to induce overexpression in the egg chamber, both in the nurse cells and follicle cells. *E75A* overexpression induced 12- and 15-fold higher expression than in *OrR* under fed and starved conditions, respectively, and *E75B* overexpression induced 19- and 17-fold higher expression than in *OrR* under fed and starved conditions, respectively. *E75A* overexpression in the egg chamber induced apoptosis of the nurse cells at stages 8 and 9 in fed flies (F3) and the percentage of egg chambers undergoing apoptosis increased in starved flies (F3S1). *E75B* overexpression in the egg chamber suppressed the apoptosis of nurse cells at stages 8 and 9 in starved flies (Table 1). *E75A* and *E75B* were then investigated to see if they showed a different temporal or spatial expression pattern in the ovary when comparing apoptotic (starved and 20E injected) and nonapoptotic (fed and JH-treated) conditions.

E75A expression was observed in the germarium, and follicle cells and nurse cells at early stages, then in the nurse cells at stage 10 of oogenesis in fed flies. However, in the egg chambers at stages 8 and 9, *E75A* expression in the follicle cells was not observed and expression in the nurse cells was weak (Figure 1A a and f). On the other hand, *E75A* expression at stages 8 and 9 was observed in follicle cells of starved (S3 and F3S1) flies (Figure 1A b and g) and was also present in the follicle cells at stages 8 and 9 when 20E was injected into the abdomen of the fed flies (Figure 1A d and e). There was no obvious difference in *E75A* comparing levels in nurse cells between apoptotic and nonapoptotic conditions, but in the follicle cells *E75A* expression was significantly different (Figure 1A f and g). The expression of *E75A* at stages 8 and 9 in starved flies was suppressed by JHA, Methoprene-treatment of the abdomen (Figure 1A c). We carried out RT-PCR to compare *E75A* expression levels between apoptotic and nonapoptotic conditions, using total RNA from dissected egg chambers at stages 8 and 9 only (Figure 1B). In starved flies (S3 and F3S1), *E75A* expression levels at stages 8 and 9 were higher than in fed flies (F3), and JHA-application suppressed the higher levels in starved flies (F3JHS1). 20E inject ion of fed flies induced higher *E75A* expression at stages 8 and 9 (F3EF1).

Apoptosis of nurse cells following starvation and 20E injection was observed at late stage 8 or stage 9. *E75A*

Table 1 Percentage of apoptosis at stage 8 and 9 in the ovary of various transgenic flies

		Fed females (%) of apoptosis)				Starved females (% of apoptosis)			
***OrR* Transgenic flies**		**+hs stage 8 2.2**	**−hs stage 8 1.7**	**+hs stage 9 5.0**	**−hs stage 9 4.7**	**+hs stage 8 16.2**	**−hs stage 8 14.9**	**+hs stage 9 28.0**	**−hs stage 9 29.2**
Overexpression	**Line**	**+hs stage 8**	**−hs stage 8**	**+hs stage 9**	**−hs stage 9**	**+hs stage 8**	**−hs stage 8**	**+hs stage 9**	**−hs stage 9**
E75A	283 w; +/ SM5; P[hs-E75A]/TM3	5.9	4.1	*13.0	5.9	17.3	17.7	*45.6	28.5
	286 w; P[hs-E75A-H4]; Dr/TM6	*13.1	5.1	*44.9	26.6	*29.8	16.3	*41.8	23.9
E75B	267 $_{w}$; hs-hsE75B^{34-22X}	5.6	6.4	5.5	5.1	*16.2	26.9	*19.0	47.6
	269 hs-E75B 7-4	5.3	4.6	5.8	4.3	17.2	26.1	*19.9	37.5

*Significant difference (at 5% level); +hs = with heat shock, −hs = without heat shock; % of apoptosis is calculated as follows: % = (mean of egg chamber in which apoptosis is induced/mean of total number of egg chambers) × 100, (n = 12). Percentage of apoptosis in the nurse cells at stage 8 and 9 in ovaries of transgenic for *E75A* and *E75B* with or without heat shock. Flies were heat shocked at 39°C for 30 min and were maintained at 25°C for 6 h with or without yeast prior to dissection. Nuclear condensation and fragmentation in the nurse cells were detected by Hoechst staining and observed under a fluorescent microscope. The fed flies were maintained for 3 days with yeast, and starved flies were maintained for 1 day of starvation after being maintained for 3 days with yeast

expression, however, was observed at stages 6 and 7 in all egg chambers of the females under all experimental conditions. If *E75A* expression induces apoptosis of the nurse cells at stages 8 and 9, the expression level in the follicle cells at stage 8 was likely to be important in determining whether or not apoptosis was induced. As shown in Figure 1C, there were no significant differences between the percentage of egg chambers at stage 8 in which *E75A* expression in the follicle cells was observed and the percentage of the nurse cells at stage 9 in which apoptosis was induced. This was true for all experimental conditions. But when compared with the controls, fed *versus* starved, starved *versus* JHA treated and fed *versus* 20E injected, these percentages changed dramatically (Figure 1C). The proportion of egg chambers expressing *E75A* at stage 8 and undergoing apoptosis at stage 9 was higher in starved flies (S3 and F3S1) than in fed flies (F3); and this higher level in starved flies was suppressed by JHA treatment. 20E treatment increased the proportions in fed flies.

We therefore propose that *E75A* expression in the follicle cells at stages 8 and 9 has a role in the induction of apoptosis in the nurse cells at stages 8 and 9. In flies, under nonapoptotic conditions, it seems that *E75A* expression level in the follicle cells is suppressed to prevent induction of apoptosis in the nurse cells at stages 8 and 9. *E75A* expression levels in the egg chamber are increased under apoptotic conditions, which leads to apoptosis in the nurse cells at stages 8 and 9.

E75B expression in the ovary

E75B expression was seen in region 2B of the germarium and the expression was faint in egg chambers at early stages. *E75B* expression was seen in the follicle cells and nurse cells at stages 4 or 5 again and expression continued until stage 9 in the ovary of fed flies (Figure 2A a and e). The *E75B* expression pattern was similar under all experimental conditions. However, expression levels appeared to be different by observation under the microscope when comparing non-apoptotic, fed and JHA treated with apoptotic, starved and 20E treated, flies. *E75B* overexpression in the egg chamber (in both nurse cells and follicle cells) suppressed apoptosis at stages 8 and 9 in starved flies (Table 1). *E75B* expression at stage 8 was observed in follicle cells and nurse cells (Figure 2A e, f, g and h), but expression levels were different in the egg chamber at stages 8 and 9 when comparing fed flies (F3) and

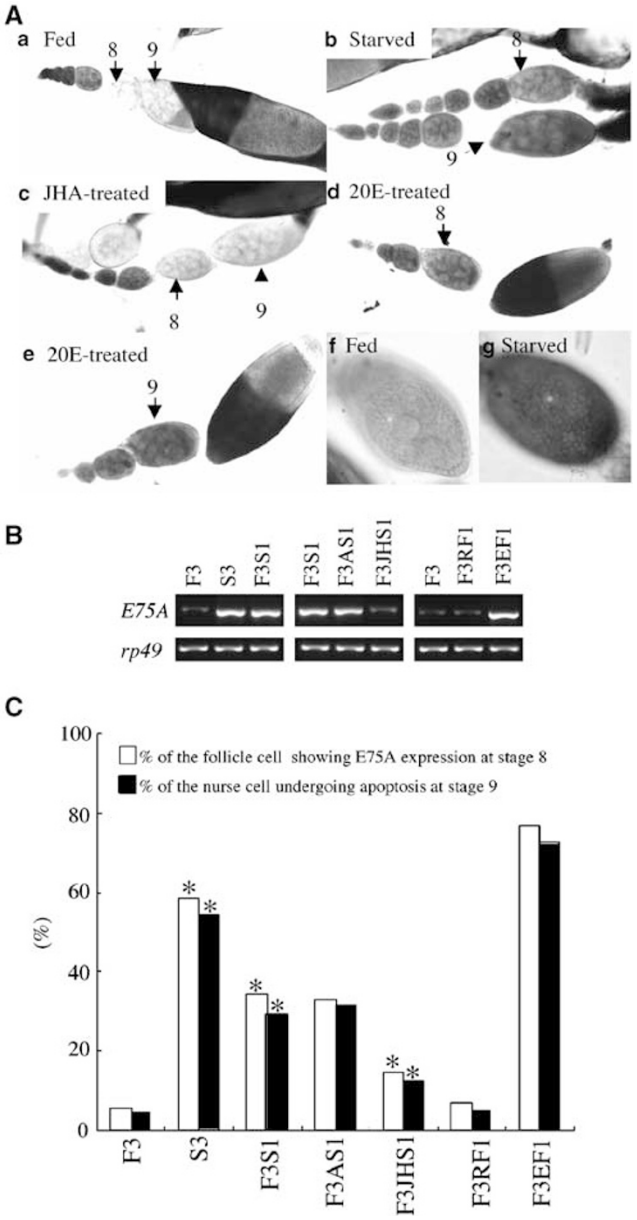

Figure 1 *E75A* expression in ovaries of *Drosophila* under various nutritional and hormonal conditions and expression levels in stage 8 and 9 egg chambers. (**A**) Results of *in situ* hybridization to the ovaries of females under various hormonal or nutritional conditions are shown. (a) Fed: maintained with yeast for 3 days (F3), (b) starved: starved for 1 day after maintaining with yeast for 3 days (F3S1), (c: JHA treated: starved for 1 day with JHA application to the abodomen (1 μg/100 nl acetone) after maintaining with yeast for 3 days (F3JHS1), (d) and (e) 20E treated: fed for 1 day with 20E injection into the abdomen (100 pg/50 nl Ringer's solution) after maintaining with yeast for 3 days. (f) Fed and (g) starved are enlarged to show the egg chamber at stage 8 and focus on the follicle cells of fed (F3) and starved (F3S1) flies, respectively. The number on the panel indicates the stage of oogenesis. (**B**)Results of RT-PCR using total RNA from the egg chamber ($n=150$ flies) at stages 8 and 9 only are shown. F3: with yeast for 3 days, S3: without yeast for 3 days, F3S1: starved for 1 day after maintaining with yeast for 3 days, F3AS1: starved for 1 day with acetone application (100 nl) after maintaining with yeast for 3 days, F3JHS1: starved for 1 day with JHA application (1 μg/100 nl) after maintaining with yeast for 3 days, F3RF1: fed for 1 day with Ringer's injection (50 nl) after maintaining with yeast for 3 days, F3EF1: fed for 1 day with 20E injection (100 pg/50 nl) after maintaining with yeast for 3 days. *rp49* is used as a control. (**C**) Percentage of the egg chambers at stage 8 in which *E75A* expression is induced and at stage 9 in which apoptosis is induced in the nurse cells. Percentage of egg chamber expression was calculated as follows: {(number of egg chambers at stage 8 showing *E75A* expression in the follicle cells)/(total number of the egg chambers at stage 8)$-$(number of stage 8 egg chambers showing nuclear condensation or fragmentation in the nurse cells)} $\times$ 100. The percentage of egg chambers showing apoptosis in nurse cells at stage 9 was calculated as follows: {(number of egg chambers at stage 9, showing nuclear condensation and fragmentation in nurse cells)/(total number of egg chambers at stage 9)} $\times$ 100. Nuclear condensation and fragmentation were detected by staining with Hoechst. There were no significant differences between the percentage of egg chambers showing *E75A* expression in the follicle cells at stage 8 and the percentage showing apoptosis in the nurse cells at stage 9. *Indicates that there are significant differences (within 5%), between F3 *versus* S3 and F3S1, F3AS1 *versus* F3JHS1 and F3RF1 *versus* F3EF1. $n=12$ flies

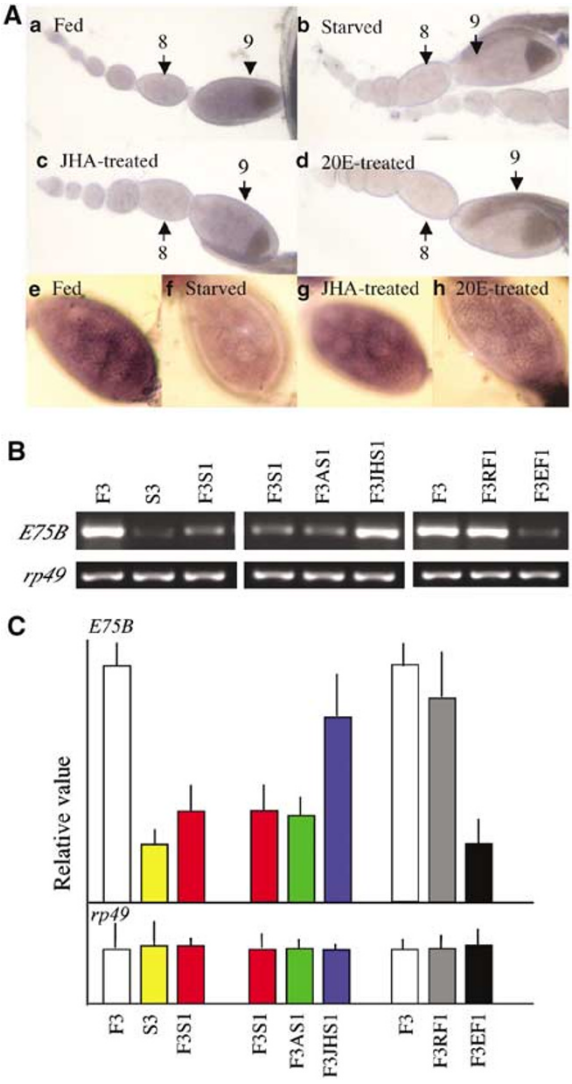

Figure 2 *E75B* expression in ovaries of *Drosophila* under various nutritional and hormonal conditions. (**A**)Results of *in situ* hybridization to the ovaries of females under various hormonal or nutritional conditions are shown. (a) Fed: maintained with yeast for 3 days (F3), (b) starved: starved for 1 day after maintaining with yeast 3 days (F3S1), (c) JHA treated: starved for 1 day with JHA application to the abdomen (1 μg/100 nl acetone) after maintaining with yeast for 3 days (F3JHS1), (d) 20E treated: fed for 1 day with 20E injection (100 pg/50 nl Ringer's solution) after maintaining with yeast 3 days. (e) Fed, (f) starved, (g) JHA treated and (h) 20E treated are enlarged the egg chamber at stage 8 to focus on the follicle cells of fed (F3), starved (F3S1), JHA treated (F3JHS1) and 20E treated (F3EF1) flies, respectively. The number on the panel indicates the stage of oogenesis. (**B**) Results of RT-PCR, using total RNA extracted from egg chambers ($n=150$ flies) at stage 8 and 9 only are shown. F3: with yeast for 3 days, S3: without yeast for 3 days, F3S1: starved for 1 day after maintaining with yeast for 3 days, F3AS1: starved for 1 day with acetone application (100 nl) after maintaining with yeast for 3 days, F3JHS1: starved for 1 day with JHA application (1 μg/100 nl) after maintaining with yeast for 3 days, F3RF1: fed for 1 day with Ringer's injection (50 nl) after maintaining with yeast for 3 days, F3EF1: fed for 1 day with 20E injection (100 pg/50 nl) after maintaining with yeast for 3 days. *rp49* is used as a control. (**C**) The graph indicates the expression level using RT-PCR results that are shown in (**B**) using relative value $\pm$S.D. (measured by NIH image). *Indicates that there are significant differences between fed (F3) and starved (S3 and F3S1), starved (F3S1 and F3AS1) and JHA treated (F3JHS1) and fed (F3 and F3RF1) and 20E treated (F3EF1) flies

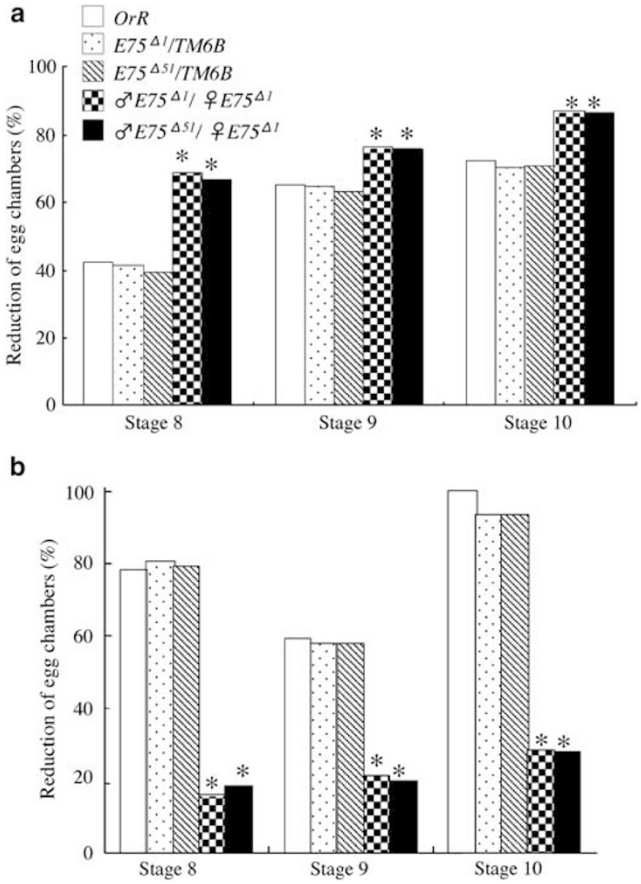

Figure 3 *E75B* mutant is sensitive to starvation. We made *E75B* mutants ($E75^{\Delta1}/E75^{\Delta51}$) by crossing $E75^{\Delta1}/TM6B$, which is an $\sim$3 kb deletion, most of the first exon of *E75B* and $E75^{\Delta51}/TM6B$, which is an $\sim$30 kb deletion that removes the first exon of *E75B*, as well as the adjacent exon, shared by all three *E75* isoforms, that encodes the second zinc finger of the DNA binding domain (Bialecki *et al*., 2002). (**a**) Percentage reduction of stages 8, 9 and 10 egg chambers comparing fed (F3) and starved (F3S1) flies is shown. $n=12$ flies. The egg chambers that indicated nurse cell apoptosis were not included in these graphs. (**b**) The percentage of recovery at stages 8, 9 and 10 egg chambers following JHA treatment of the starved flies (F3JHS1) is shown. $n=12$ flies. The egg chambers that showed nurse cell apoptosis were not included in these graphs

starved flies (S3 and F3S1, Figure 2B and C). *E75B* expression levels in the egg chamber at stages 8 and 9 of starved flies were rescued by JHA treatment of starved flies (F3JHS1, Figure 2B and C). *E75B* is one of the ecdysone response genes in *Drosophila,* but *E75B* expression in the egg chamber at stages 8 and 9 was suppressed by 20E injection of fed flies (F3EF1, Figure 2B and C).

E75A and *C* mutants show similar lethal phases, but *E75B* mutants are viable and fertile, with no detectable phenotype.[27] We thought that perhaps *E75B* mutants might be sensitive to nutritional stress even though under normal conditions they are fertile. Therefore, we examined *E75B* mutant flies that were maintained under nutritional shortage and checked how many stages 8, 9 and 10 egg chambers were present (Figure 3). The egg chambers of wild-type starved females were reduced by approximately 40, 65 and 70% of stages for 8, 9 and 10 egg chambers, respectively, compared to

wild-type fed females, but the egg chambers of *E75B*-mutant starved flies was reduced approximately 65, 80% and over 80% for stages 8, 9 and 10 egg chambers, respectively (Figure 3a). JHA treatment of the starved wild-type flies suppresses apoptosis in stages 8 and 9 egg chambers[7,8] and led to recovery of the number of stages 8, 9 and 10 egg chambers (Figure 3b). However, when JHA was applied to the starved *E75B* mutant flies, the recovery levels were lower than in starved wild-type flies (Figure 3b). These results indicate that the *E75B* mutant is particularly sensitive to nutritional stress that *E75B* overexpression and JHA treatment suppressed this apoptosis at stages 8 and 9 (Table 1) and that JHA suppresses the apoptosis through *E75B*. From these results, we propose that *E75A* and *E75B* have opposite roles in determining if apoptosis of nurse cells at stages 8 and 9 is induced or not in *Drosophila*. Further, although E75B mutants are fertile and visible under normal conditions, this mutation is detrimental to fertility under conditions of nutritional stress.

BR-C Z2 and *Z3* control *E75A* and *E75B* expression in egg chambers

BR-C Z2 and *Z3* are not expressed in the egg chambers at stages 8 and 9 of fed females, but they are expressed in the follicle cells under apoptotic conditions. In addition, overexpression in the egg chamber induces apoptosis of that egg chamber at stages 8 and 9 in fed flies.[8] We have suggested that *BR-C* controls the developmental checkpoint at stages 8 and 9 of oogenesis in *Drosophila*,[28] inducing either yolk protein synthesis or inducing apoptosis in the nurse cells at stages 8 and 9.[8] On the basis of these results, we investigated whether or not *Z2* and *Z3* overexpression in the follicle cells affects *E75A* and *E75B* expression in egg chambers at stages 8 and 9.

Figure 4A shows the *E75A* expression pattern in *hsZ2* and *Z3* transgenic flies under fed conditions (F3) with or without heat shock. *E75A* expression in the follicle cells at stages 8 and 9 was not seen or was very faint in *hsZ2* and *hsZ3* transgenic flies under fed conditions without heat shock (Figure 4A c, f and g). When *Z2* and *Z3* overexpression in the egg chamber was induced by heat shock, *E75A* was expressed in the egg chamber, in nurse cells and follicle cells at stages 8 and 9 under fed conditions (Figure 4A a, b, d and e). The *E75A* expression pattern in egg chambers at stages 8 and 9 thus reflects the results of *in situ* hybridization, as shown in Figure 3a (Figure 4B). *E75A* expression level at stages 8 and 9 following *Z2* and *Z3* overexpression was higher than the expression levels without heat-shock induction in not only fed flies but also starved flies (F3S1, Figure 4B). The percentage of egg chambers at stage 8 in which *E75A* expression was seen corresponded to the percentage of egg chambers at stage 9 in which apoptosis was induced in fed flies (Figure 4C). *Z2* and *Z3* overexpression increased the percentage of egg chambers at stage 8 in which *E75A* expression was observed and the percentage of nurse cells at stage 9 in which apoptosis was induced.

E75B expression patterns in the ovaries of *hsZ2* and *hsZ3* transgenic flies under fed conditions were the same as the

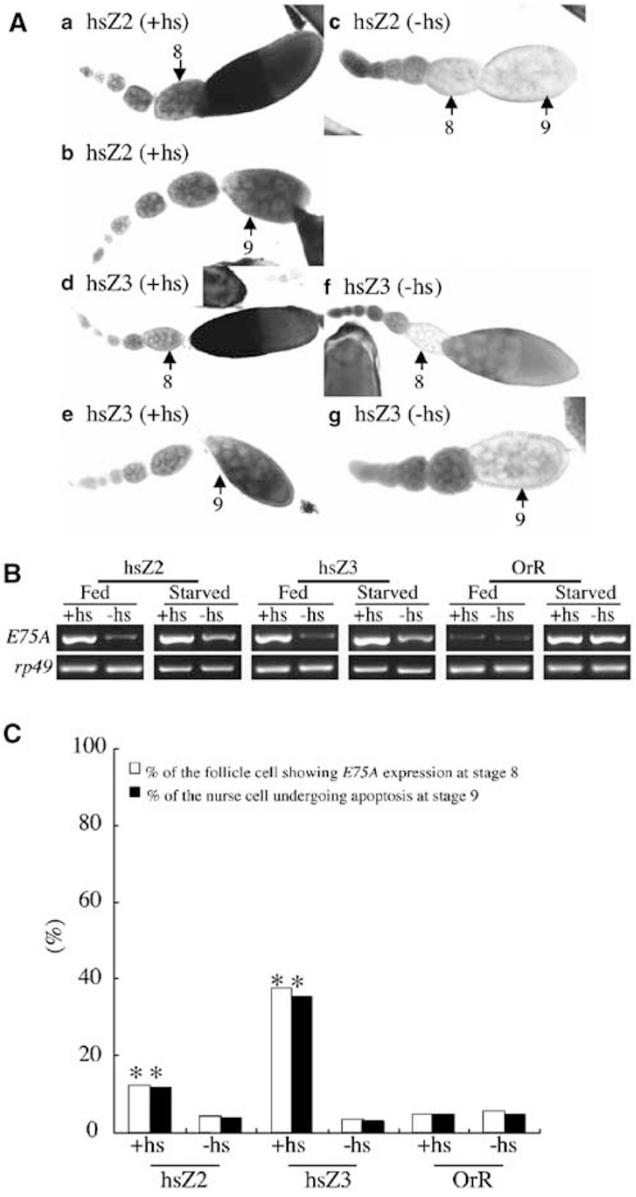

Figure 4 *E75A* expression in the follicle cell under inducing *Z2* and *Z3* overexpression. (**A**) Results of *in situ* hybridization to the ovaries of *hsZ2* and *Z3* transgenic flies with adequate nutrition, with or without heat shock. (a, b) *E75A* expression in the adequate nutrition (F3) with heat shock (+hs). (c)*E75A* expression in the ovary of F3 female without heat shock (−hs). Number indicates the stage of oogenesis. (**B**) Results of RT-PCR, using total RNA, extracted from egg chambers ($n=150$ flies) at stages 8 and 9 only of fed (F3) and starved (F3S1) *hsZ2* and *hsZ3* transgenic flies with or without heat shock (39°C 30 min). (**C**) Percentage of stage 8 egg chambers in which *E75A* expression was induced in follicle cells and at stage 9 in which apoptosis was induced in the nurse cells of *BR-C Z2* and *Z3* transgenic flies (fed flies). The percentage was calculated as follows: {(number of egg chambers at stage 8, *E75A* expression in the follicle cell)/(total number of egg chambers at stage 8)−(number of stage 8 egg chambers showing nuclear condensation and fragmentation)} × 100. The percentage of the egg chambers (apoptosis in the nurse cells at stage 9) is calculated as follows: {(number of egg chambers at stage 9 indicate nuclear condensation and fragmentation in the nurse cells)/(total number of egg chambers at stage 9)} × 100. There were no significant differences between the percentage of egg chambers showing *E75A* expression in the follicle cells and the percentage of egg chambers showing apoptosis in nurse cells at stage 9 in each condition. *Indicates that there are significant differences (within 5%) comparing results with and without heat shock. $n=12$ flies

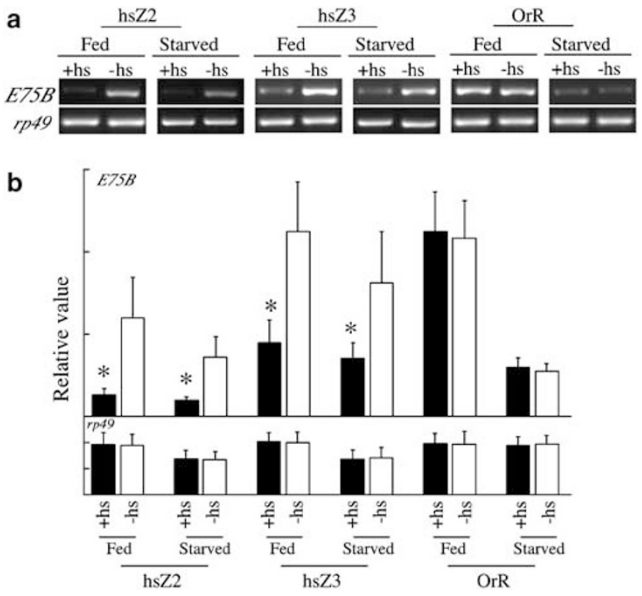

Figure 5 *E75B* expression in the ovary following *Z2* and *Z3* overexpression. (**a**) Results of RT-PCR, demonstrating *E75B* expression, using total RNA from the stages 8 and 9 egg chambers of fed (F3) and starved (F3S1) *hsZ2* and *Z3* transgenic flies with or without heat shock (39°C 30 min). $n = 150$ flies. (**b**) The graph indicates the expression levels with $\pm$S.D. (measured by NIH image). *Indicates that there is a significant difference (within 5%) comparing results with and without heat shock

ovaries of *OrR* flies under fed conditions, and when these transgenic flies underwent heat shock, the expression patterns did not differ from the flies without heat shock (data not shown). However, *E75B* expression levels were different with and without the heat shock inducing *Z2* and *Z3* overexpression (Figure 5) and decreased in the egg chambers at stages 8 and 9 under fed and starved conditions (Figure 5). Heat shock did not affect *E75B* expression at stages 8 and 9 in wild-type control flies.

Z2 and *Z3* overexpression induced *E75A* expression and suppressed *E75B* expression in the egg chambers at stages 8 and 9. *BR-C* controls the developmental checkpoint in oogenesis at stages 8 and 9, by inducing apoptosis and regulating yolk protein gene expression.[8] Probably, *E75A* and *E75B* expression in the follicle cells and nurse cells at stages 8 and 9 are regulated also by *BR-C*, in this case *Z2* and *Z3*, and thus *E75A* and *B* expression in the follicle cells may ultimately induce the apoptosis of the nurse cells at stages 8 and 9, which leads to degeneration of the egg chamber. Apoptosis must therefore involve communication between the nurse cells, oocyte and follicle cells.

E75B suppresses *E75A* expression

We investigated whether or not *E75A* and *E75B* affected each other in the egg chambers at stages 8 and 9. *E75A* overexpression did not affect the *E75B* expression pattern in the ovary (data not shown), but *E75B* overexpression affected *E75A* (Figure 6A). In starved flies, *E75A* was expressed in the egg chambers at stages 8 and 9 of *hsE75B* transgenic flies under starvation in the same pattern as wild-type flies under starvation (Figure 6A b and c); but when the transgenic flies were heat shocked under starvation, *E75A* expression at stages 8 and 9 was not seen or was very faint (Figure 6A a). *E75A* overexpression did not affect *E75B* expression at stages 8 and 9 and *E75B* overexpression suppressed *E75A* expression in the egg chamber at stages 8 and 9 (Figure 6B, C and E). In starved wild-type flies, the percentage of egg chambers in which *E75A* expression was induced at stage 8 and in which apoptosis was induced at stage 9 was approximately 40%, but when *E75B* transgenic flies underwent heat shock to induce overexpression, this percentage was decreased to approximately 20% (Figure 6D). This is a significant drop in the number of egg chambers undergoing apoptosis.

Therefore, in the egg chamber at stages 8 and 9, *E75B* suppressed *E75A* expression in the follicle cells and nurse cells under fed and starved conditions. We propose that *E75A* expression in the follicle cells at stages 8 and 9 induces apoptosis in the nurse cells at these stages, but if *E75B* expression in the follicle cells at stages 8 and 9 is above threshold level, *E75B* suppresses *E75A* in the follicle cells, which in turn suppresses apoptosis in the nurse cells at stages 8 and 9.

Discussion

Early ecdysone response genes control apoptosis in nurse cells at stages 8 and 9

Apoptosis of many insect tissues and glands are affected by 20E and JH. 20E induces apoptosis and JH III treatment inhibits apoptosis in a *Drosophila* cell line, *(l) 2 man*.[29] In *Maduca sexta,* 20E induces and JH suppresses the apoptosis of the prothoracic gland.[30,31] It is known that the early ecdysone response genes, *BR-C, E74* and *E75* are essential for the regulation of apoptosis in insect tissues and glands.[18] *E74A* and *BR-C* activate the apoptosis inducer, *hid*, in the *Drosophila* salivary gland and *BR-C* activates *reaper,* also an apoptosis inducer.[23] *E75A* and *B* induce apoptosis of salivary glands through suppression of *diap2,* an apoptosis suppressor.[23]

We have recently established that the *BR-C* controls the fate of the egg chamber, by progressing either development or apoptosis.[8] The number of *Drosophila* egg chambers is reduced under nutritional shortage[8] and in response to increasing ecdysone concentration.[7] Apoptosis of the nurse cells at stages 8 and 9 causes a reduction in the number of egg chambers. Degenerating stages 8 and 9 egg chambers often appear elongated and the surface of the egg chamber has a rough appearance.[7] In degenerating stages 8 and 9 egg chambers, follicle cells and their nuclei increase in size.[7] Follicle cells are likely to phagocytose the dying nurse cells.[3] After that the follicle cells die.[7] Further, the apoptosis seems to be induced by *BR-C Z2* and *Z3* expression at stage 8. Nutritional shortage induced increased ecdysone concentration in flies;[9] thus, it seemed likely that other ecdysone response genes may also affect apoptosis in the nurse cells at stages 8 and 9.

As shown in Table 1, *E75A* induces apoptosis in nurse cells at stages 8 and 9 of oogenesis. Stronger *E75A* expression in

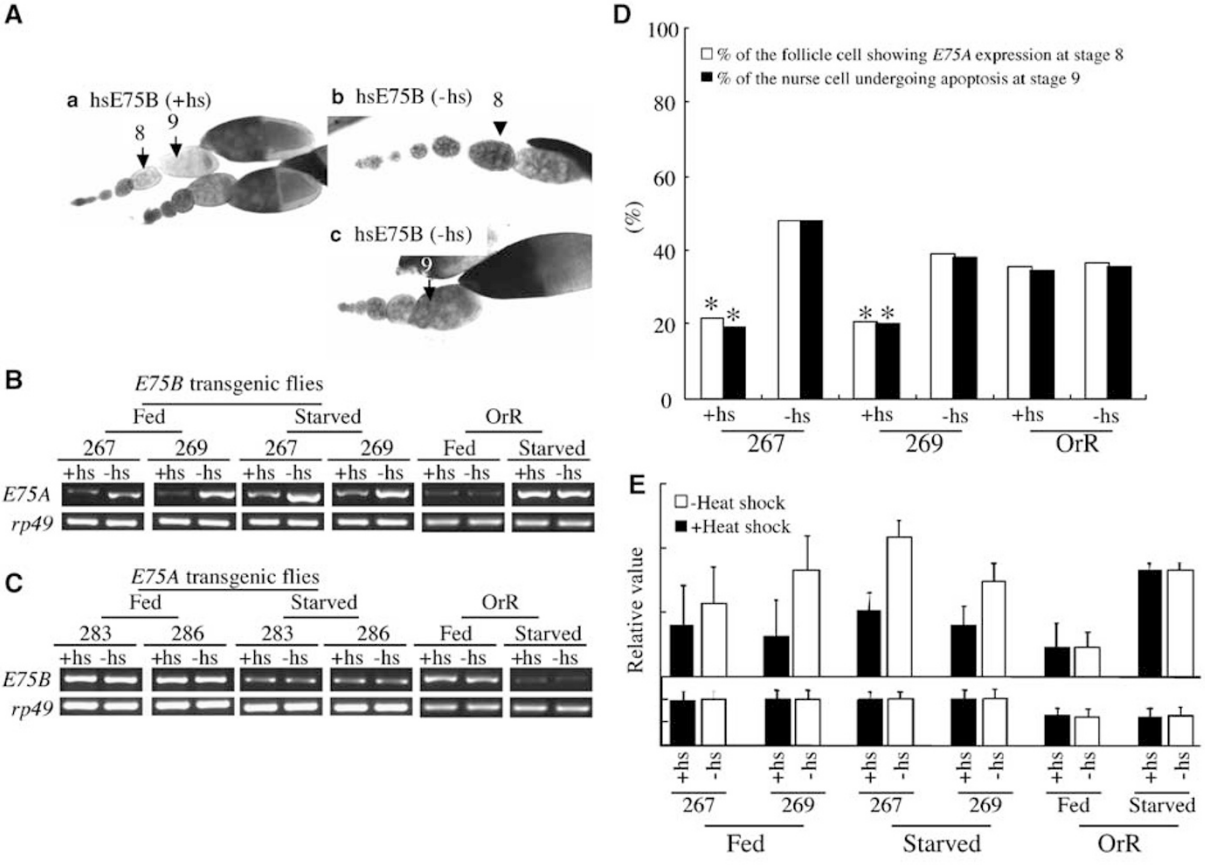

Figure 6 *E75A* expression in *Drosophila* is inhibited by *E75B* overexpression. (**A**) Results of *in situ* hybridization to the RNA of ovaries of *hsE75B* transgenic flies (269) under nutritional shortage with or without heat shock. (a) *E75A* expression in the ovary under nutritional shortage (F3S1) with heat shock (+hs), resulting in *E75B* being expressed in nurse cells and follicle cells. (b and c) *E75A* expression in the ovary of S3 females without heat shock (−hs). The number on the panel indicates the stage of oogenesis. (**B**)Results of RT-PCR (*E75A* expression) using total RNA extracted from the egg chambers ($n = 150$ flies) at stages 8 and 9 in *E75B* transgenic flies (fed, F3 and starved, F3S1 flies). (**C**) tResults of RT-PCR (*E75B* expression), using total RNA, extracted from the egg chambers ($n = 150$ flies) at stages 8 and 9 in *E75A* transgenic flies (fed, F3 and starved, F3S1 flies). (**D**) Percentage of stage 8 egg chambers in which *E75A* expression was induced in the follicle cells and at stage 9 in which apoptosis was induced in the nurse cells of *E75B* transgenic flies (starved flies, line 267 and 269). There were no significant differences between the percentage of the egg chambers showing *E75A* expression in the follicle cells at stage 8 and the percentage of egg chambers showing *E75A* expression in the nurse cells at stage 9. *Means that there are significant differences (within 5%) comparing with and without heat shock. $n = 12$ flies. (**E**) The graph indicates the *E75B* expression levels using RT-PCR, and total RNA from stages 8 and 9 egg chambers of *E75B* transgenic flies. The results are summarized in (**C**) showing relative value ± S.D. (measured by NIH image). *Mean that there are significant differences between experiments with and without heat shock

the follicle cells at stages 8 and 9 is observed in the egg chambers of females that are starved or injected with 20E, namely under apoptotic conditions (Figure 1A). In addition, *BR-C, Z2* and *Z3* (which induce apoptosis at stages 8 and 9) overexpression induced *E75A* expression at stages 8 and 9 (Figure 4). However, *E75B* does not act as an apoptosis inducer in the egg chamber. *E75B* overexpression suppresses apoptosis in the egg chambers of starved flies (Table 1). *E75B* expression levels at stages 8 and 9 reflect these results, *E75B* expression at stages 8 and 9 in fed and JHA- treated females (namely nonapoptotic conditions) is stronger than under apoptotic conditions (Figure 2B). In addition, *E75B* mutant flies were sensitive to nutritional shortage (Figure 3a). *E75B* mutant flies do not show abnormal development,[14] but the percentage of egg chamber reduction in *E75B* mutants was higher during starvation than in wild-type females (Figure 3a). JHA treatment of starved flies suppresses the apoptosis of stage 8 and 9 egg chambers.[7,8] This suppression induced a recovery of the numbers of stage 8, 9 and 10 egg chambers (Figure 3b). JHA treatment induced higher *E75B* expression in stage 8 and 9 egg chambers (Figure 2B and C). On the other hand, starvation, 20E treatment and *BR-C Z2* and *Z3* overexpression in the follicle cells suppressed *E75B* expression in stage 8 and 9 egg chambers (Figures 2B, C and 5). Starvation induces high ecdysteroid concentrations in the haemolymph and ovary,[32] which in turn may induce *BR-C Z2* and *Z3* expression in the follicle cells and thus induces apoptosis at stages 8 and 9.[8] These results indicate that the two isoforms, *E75A* and *E75B*, have opposite effects on apoptosis of nurse cells. *E75A* acts as an apoptosis inducer and *E75B* acts as an apoptosis suppressor.

In summary, during apoptosis of the nurse cells at stages 8 and 9, *BR-C Z2* and *Z3* and *E75A* act as apoptosis inducers and *E75B* acts as an apoptosis suppressor as their expression is controlled in the follicle cells.

Interactions between ecdysone response genes

There is a developmental checkpoint at stages 8/9 in oogenesis.[28] At this checkpoint, the egg chambers undergo a developmental selection, becoming committed to produce a mature egg or to undergo apoptosis. *BR-C* is a key gene at this developmental checkpoint. The progression of development or the induction of apoptosis depends on which *BR-C* isoforms are expressed in the follicle cells at stage 8.[8] When flies are maintained under starvation conditions or 20E is injected into the abdomen, *BR-C Z2* and *Z3* are expressed in the follicle cells of the egg chamber at stage 8.[8] In addition, *BR-C Z1* and *Z3* regulate yolk protein gene expression that begins at stage 8 in the ovary.[8]

BR-C Z2 and *Z3* affect *E75A* and *E75B* expression at stages 8 and 9 (Figures 4 and 5). *BR-C Z2* and *Z3* overexpression induces stronger *E75A* expression in the egg chamber, both in nurse cells and follicle cells, at stages 8 and 9 under fed and starvation conditions (Figure 4A and B). In contrast, *Z2* and *Z3* overexpression suppresses *E75B* expression under fed and starved conditions (Figure 5). *E75A* and *E75B* are therefore downstream of the *BR-C* isoforms *Z2* and *Z3*. Ecdysteroid concentration in starved flies is higher than in fed flies.[9] We suggest that increasing ecdysteroid concentration induces *BR-C Z2* and *Z3* expression in the follicle cells at stages 8 and 9 and that *Z2* and *Z3* control the fate of egg chambers by regulating *E75A* and *B* expression in the follicle cells. Buszczak *et al*.[14] show that *BR-C, E75* and *E74* are required for egg chamber development during mid-oogenesis. These genes are needed to permit the mature egg to develop, yet we find they are also needed to induce apoptosis. Expression of early ecdysone response genes is sensitive to changes in the ovarian ecdysone titre.[14] Probably, there is a threshold of ecdysteroid titre in the ovary. Carney and Bender[33] established that *EcR* is required for normal oogenesis in *Drosophila.* This means ecdysteroid is required for normal oogenesis in *Drosophila.* In contrast, ecdysteroids induce apoptosis at mid-oogenesis in *Drosophila*.[8] We propose that these two opposite effects of ecdysteroid depend upon a threshold level of ecdysteroid titre. If levels are below the threshold, ecdysteroid induces the appropriate gene expression profile for normal oogenesis, but if it exceeds the threshold, ecdysteroids induce an alternative gene set that executes premature apoptosis at mid-oogenesis. Starvation would therefore cause the ecdysteroid concentration to exceed the threshold. We suggest that the ovarian ecdysone titre controls the apoptosis/development decision of individual egg chambers by regulation of the patterns of *BR-C* isoform expression.

A model for the network of ecdysone response genes, which control apoptosis in the nurse cells of the stages 8 and 9 egg chambers

Figure 7 proposes the first steps in a model for the ecdysone response gene network regulating apoptosis in the nurse cells of the egg chamber. In fed flies, only *BR-C Z1* expression is observed in the follicle cells at stages 8 and 9;[8] therefore, *E75B* expression in the follicle cells at stages 8 and 9 is not suppressed. *E75B* suppresses *E75A* expression in the follicle cells at stages 8 and 9, which in turn results in apoptosis of the nurse cells at these stages being suppressed and thus leads to the production of many mature eggs. When 20E is injected

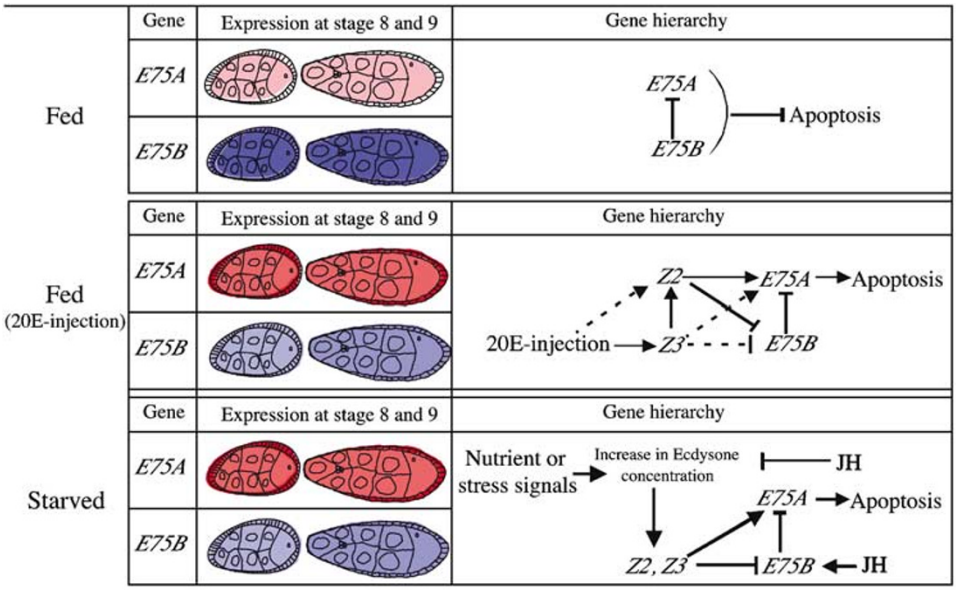

Figure 7 Model to explain the hierarchy of ecdysone response genes regulating apoptosis of stages 8 and 9 egg chambers. Complete nutrition induces normal development of the mature egg during oogenesis. In this case, just *BR-C Z1* is expressed in the follicle cells at stage 8. *E75B* suppresses *E75A* expression in the follicle cells to prevent apoptosis. However, if 20E is injected into the abdomen of fed flies, *Z2* and *Z3* are expressed in the follicle cells at stage 8. In this case, *Z2* expression in the follicle cells was induced by *Z3* overexpression in the egg chambers. This results in a decrease in *E75B* and *E75A* induces apoptosis of the nurse cells at stages 8 and 9. Under starved conditions, *Z2* and *Z3* are also expressed in the follicle cells at stage 8 and suppress *E75B* and activate *E75A* expression in the follicle cells. As a result, the *E75B* expression level is not enough to suppress *E75A* expression, so *E75A* induces apoptosis in the nurse cells of the stages 8 and 9 egg chambers

into the abdomen of fed flies, *BR-C Z2* and *Z3* are expressed in the follicle cells at stages 8 and 9. In fed flies, *Z2* expression is induced by *Z3* expression[8] and it is possible that 20E activates *Z2* expression directly. *Z2* expression activates *E75A* expression in the follicle cells and suppresses *E75B* expression at stages 8 and 9. *Z3* overexpression also activates *E75A* expression and suppresses *E75B* expression in the egg chamber at stages 8 and 9. However, we do not know if *Z3* activates *E75A* and suppresses *E75B* by inducing *Z2* expression or acts directly. *E75A* expression is increased in the follicle cells of the egg chamber at stages 8 and 9 by *Z2,* and *E75A* may activate the apoptosis pathway in the nurse cells and/or follicle cells at stages 8 and 9.

In starved flies, nutritional shortage induces an increase in ecdysone concentration in flies[9]; ecdysone activates *Z2* and *Z3* expression in the egg chamber at stages 8 and 9.[8] In this case, *Z2* and *Z3* expression do not affect each other; this suggests that ecdysone activates *Z2* and *Z3* expression in the follicle cells independently. *Z2* and *Z3* expression in the follicle cells activate *E75A* expression in the follicle cells at stages 8 and 9 and suppress *E75B* expression. As a result, *E75A* expression in the follicle cells should activate the apoptosis pathway in the nurse cells and/or follicle cells of the egg chamber and apoptosis commences at stages 8 and 9. Follicle cell development is partly independent of germ-line cell differentiation in *Drosophila* oogenesis,[34] but induction of apoptosis at stages 8 and 9 under nutritional stress needs interactions between follicle cells and nurse cells.

The pattern of *BR-C* isoform expression in the follicle cells controls the checkpoint by interacting in each egg chamber to control a developmental switch leading to the development of a mature egg or to undergo apoptosis. *BR-C* expression is controlled by ecdysteroid concentration, which is increased in females under nutritional shortage.[32] Ecdysteroids are crucial for normal oogenesis in *Drosophila*.[14,33,35] On the other hand, our results suggest that ecdysteroids are also needed to induce apoptosis of the egg chamber at stages 8 and 9. We propose that there is a threshold ecdysteroid concentration in the fly and if ecdysteroid levels are below the threshold, Normal oogenesis is induced, but if levels exceed the threshold, ecdysteroids induce apoptosis of egg chambers at stages 8 and 9. When apoptosis of a nurse cell at stages 8 and 9 is induced, the genetic pathway that is activated differs between the fed and starved conditions. However, both pathways result in the activation of *E75A* expression in the follicle cells at stages 8 and 9 and the suppression of *E75B* expression at stages 8 and 9. *E75A* may activate the apoptosis pathway, for example the caspase pathway, which executes apoptosis. We do not know how the apoptosis that we observe in nurse cells is affected by what is happening to gene expression in the nurse cells. It is possible that *E75A* activates an apoptosis inducer in the follicle cells and the inducer activates the apoptosis pathway in the adjacent nurse cells or is transported to nurse cells so that both cell types die. We have identified some candidate genes for inducing or suppressing apoptosis by a microarray analysis.[15] Our results show that *E75A* is an apoptosis inducer and *E75B* is the inhibitor. *E75A* may control expression of apoptosis inducers, which we identified by the microarray analysis, including *Dp, p53* or the caspase family. On the other hand, *E75B* controls expression of an apoptosis inhibitor, and we therefore suggest this may be *Diap1* and *Diap2*. The expression of *E75A* and *E75B* are regulated by alternative splicing in the yellow fever mosquito *Aedes aegypti*.[36] We propose that the regulation of alternative splicing of *E75* in the *Drosophila* ovary is controlled by *BR-C Z2* and *Z3* expression in the follicle cells of stages 8 and 9. The network of genes involved in controlling this developmental decision, which regulates how many eggs a female will produce, is complex and further investigations on how events are coordinated in the nurse cells, oocyte and follicle cells remain to be undertaken.

Materials and Methods

Drosophila maintenance

Flies were maintained on standard yeast, maize meal, sugar and agar medium at 25°C. The wild-type strain, *Oregon R (OrR)* was used throughout. All the flies for each experiment were 3 days old. Flies (3 days old) were transferred from a standard diet to one of sugar and water (starved, 1% agar medium, which contains 5% sucrose and 0.005% 10% Nipagen in 95% ethanol) or one of yeast (fed, 2 g bakers yeast on approximately 50 ml 1% agar medium, which contains 2.5% cornflour, 5% sucrose, 1.75% lypophilized yeast and 0.005% Nipagen in 95% ethanol). After 3 days on sugar or yeast, flies were dissected (sugar: S3, yeast: F3), transferred to sugar for 1 day after 3 days on yeast (F3S1), or topically treated with Methoprene and maintained on sugar and water for 1 day (F3JHS1), injected with 20E and maintained on yeast for 1 day (F3EF1). We used *BR-C* transgenic flies (*TN-Q^1-Q^2-Z1*, *Z2*, *Z3* and *Z4*, kindly provided by C Bayer), *E75A* transgenic flies (line 283, 286), *E75B* transgenic flies and *E75$^{\Delta 1}$* and *E75$^{\Delta 51}$* to make *E75B$^-$/Df* (line 267 and 269, *E75A, E75B* transgenic and *E75$^{\Delta 1}$* and *E75$^{\Delta 51}$* flies were kindly provided by CS Thummel). Flies carrying the *E75$^{\Delta 51}$* allele were crossed to *E75$^{\Delta 1}$* mutants. The flies were maintained at 25°C for 3 days with yeast (Fed, F3) or starved for 1 day after maintaining with yeast for 3 days (Starved, F3S1), then underwent heat shock at 39°C for 30 min and were maintained at 25°C for 6 h.

Injection of 20E and application of JHA

20E (Sigma) was dissolved in Insect Ringer's solution (130 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) and 50 nl was injected at a concentration of 2 μg/ml.[37] The concentration of 20E was determined according to Bownes.[9] With a haemolymph volume of approximately 1 μl/female,[37] 100 pg 20E/female leads to a concentration of 2×10^{-7} M. Methoprene (ZR515, Zoecon) was applied topically to the ventral abdomen in 100 nl acetone. Methoprene diluted 1 : 100 in acetone corresponds to a concentration of about 1 μg/100 nl. Controls were also undertaken injecting Ringer's only and treating flies with acetone.

Hoechst staining

Ovaries were dissected in Insect Ringer's solution and fixed in 4% paraformaldehyde. After fixation and permeabilization in 1% Triton X-100 in PBS, ovaries were stained in 1 μM Hoechst in PBS for 5 min, then washed twice in PBS for 2 h and mounted in FISH medium (220 mM 1, 4-diazabicyclo [2.2.2] octane, 90% glycerol, 100 mM Tris-HCl pH 8.5) and examined immediately using fluorescent filters.

RNA *in situ* hybridization

The protocol is based on the procedure previously described[38] and modified as follows. The ovaries were dissected in Ringer's solution and fixed for 20 min in 4% *p*-formaldehyde in PBS. After rinsing the tissue in PBT, it was treated for 10 min in methanol/0.5 M EGTA, pH 8 (9 : 1). The ovaries can then be stored in methanol at -20°C for several months. The stored ovaries were rehydrated in PBT. The prehybridization was carried out for 1 h at 45°C in DNA hybrid (50% deionized formamide, 5 × SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml Heparin, 0.1% Tween-20). The ovaries were hybridized overnight at 45°C in DNA hybrid containing digoxigenin-labeled probe (DIG-DNA labeling and detection kit, Boehringer Mannheim). For detection, a 1 : 1000 dilution of anti-DIG-AP-conjugated Ab was used. The staining reaction was performed in 100 mM Tris pH 9.5, 50 mM MgCl, 10 mM NaCl, 0.2% Tween-20, 8 mM levamisole, 4.5 μl/ml NBT and 3.5 μl/ml X-phosphate (Boeringer Mannheim) for 5 h. Anti-DIG-AP conjugate was preabsorbed with postfixed wild-type (Oregon R) ovaries at overnight 4°C. The ovaries were mounted in a mixture of PBS/glycerol (1 : 4) for microscopy. After the ovaries had been double stained using Hoechst and *in situ* hybridization to RNA, they were washed in PBS and stained in 1 μM Hoechst in PBS for 5 min, then washed twice in PBS for 2 h in the dark and mounted in FISH medium.

RNA extraction and RT-PCR

Transcript levels in ovaries were detected by reverse transcriptase (RT)-PCR as described previously.[39] Total RNA was extracted from egg chambers at stages 8 and 9. The egg chambers at stages 8 and 9 were isolated from the ovary after dissection. The primer sequences are as below: *E75A*, forward 5′-TCAAGTGTCATTTCGAAGCCA-3′ and reverse 5′-AGATTGGCGATTTCCTTGTG-3′, *E75B,* forward 5′-GCTCTAGACAC CAAAGCCATGTGCCGATCT-3′ and reverse 5′-GGCGCAGGAGATTGG CGATT-3′. The expression levels (relative value and standard deviation) were quantified by NIH image (downloaded from http://rsb.info.nih.gov/nih-image/download.html).

Acknowledgements

This project was supported by the BBSRC and a University of Edinburgh Wellcome VIP award. We are grateful to Hilary Anderson for assistance with preparation of the manuscript.

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