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A microarray analysis of genes involved in relating egg production to nutritional intake in *Drosophila melanogaster*

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Abstract

Egg chambers of *Drosophila* are reabsorbed under conditions of nutritional shortage by inducing apoptosis at stages 8 and 9, midway through oogenesis. Nutritional shortage leads to an increase in ecdysone concentration in flies. Apoptosis at stage 8/9 is also induced by 20-hydroxyecdysone injection into the females maintained with adequate nutrition. The expression pattern in the ovary of some ecdysone response genes, *E75A, BR-C*, is different according to the nutritional environment and the overexpression of these genes induces apoptosis. Apoptosis is suppressed by Juvenile hormone analog treatment of females under nutritional shortage. We predict nutritional and stress response genes control hormone levels and the increase in ecdysone concentration in the flies following starvation induces the ovarian apoptosis. We therefore used a microarray approach to identify the genes involved in receiving the nutritional signal from the environment and translating it in the ovary, thus initiating and executing apoptosis.

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Abbreviations: 20E, 20-hydroxyecdysone; JHA, juvenile hormone analogue

Introduction

Egg laying in *Drosophila* is closely linked to food availability. With adequate availability of nutrients and appropriate laying substrates, mated females of *Drosophila melanogaster* can lay up to half of their body weight a day as eggs. However, following nutrient withdrawal, the female responds rapidly and egg laying almost ceases. Increased cyst death in region 2a/b of the germarium occurs very early oogenesis in response to nutritional shortage.[1] In addition, mature eggs are frequently retained by the female, young previtellogenic egg chambers do not enter vitellogenesis and some egg chambers in early vitellogenesis undergo apoptosis.[2,3] Those egg chambers that have completed vitellogenesis, complete chorion synthesis but are not laid. Subsequently, the female reduces production of yolk proteins in the fat body, but many of the early responses to the change in food conditions are in the ovary itself.[3,4] Under adequate nutrition, the nurse cell nuclei in the egg chamber show DNA fragmentation from stage 12 of oogenesis, following the completion of cytoplasmic transfer from the nurse cells to the oocyte.[5] Some *reaper*, *hid* and *grim* including genes crucial for the apoptosis in *Drosophila* have been identified,[6–8] but they are not involved in the apoptosis of the nurse cells of egg chamber which commences at stage 12 during normal development.[5]

There seems to be a checkpoint or control point at stage 8/9 of oogenesis,[9] just as vitellogenesis begins, when individual egg chambers make the decision to either develop or undergo apoptosis. The link between nutrients and the ovary seems to be mediated by the balance of the two key hormones, ecdysone and juvenile hormone (JH).[2,10] We have recently shown that both hormones are crucial in the decision-making process in the egg chamber and they do this by modulating early ecdysone response genes including *Broad-Complex* (*BR-C*).[3] *BR-C* has four isoforms, *Z1, Z2, Z3* and *Z4,* encoding a family of zinc-finger transcription factors[11] and control oogenesis either by inducing apoptosis or controlling *yolk protein* gene expression and hence key developmental choices.[3] The *BR-C* isoform expression pattern is different in the ovary of females kept under nutritional shortage and those kept with adequate nutrition. *BR-C Z2* and *Z3* are not expressed in egg chambers at the stage 8 developmental checkpoint under adequate nutrition, but under nutritional shortage, both isoforms are expressed in the egg chamber at this stage.[3] These expression patterns are possibly controlled by ecdysone concentration that is higher in the females under nutritional shortage than in the females under adequate nutrition.[10] It seems likely that the expression of some genes, which can modulate hormone levels, are affected by nutritional status and consequently change and control the ecdysone concentration in the females. In addition, to actually induce apoptosis at stages 8 and 9, genes that control and select the apoptosis pathway should be induced. Some of these may be known stress response genes, since lack of nutrients will be a stress to the physiology of the female. Diverse stimuli, such as nutritional shortage and cytotoxic chemicals, can induce the apoptosis at mid-oogenesis (reviewed by McCall[12]). The nurse cells are the first cells to show signs of apoptosis.[3,12]

To investigate this process further and understand more of the genes involved in this switch between development and apoptosis, we undertook a microarray analysis. A model for the relationship between nutritional conditions and egg development is shown in Figure 1 (based on Terashima and

Bownes[3]). Our experiments aim to identify some of the genes in the ovary needed to execute the decision as to whether an individual egg chamber should undergo development into a mature egg or die by apoptosis. When the females are maintained under adequate nutrition, the flies produce many eggs. However, when the flies are maintained under starvation or when 20-hydroxyecdysone (20E) is injected into the abdomen of the females under adequate nutrition, an increase in apoptosis in egg chambers at stages 8 and 9 of oogenesis is observed (Figure 1, Table 1[3]). The apoptosis induced by starvation is suppressed by But juvenile hormone analog (JHA) treatment of the abdomen of the females.[2,3] For the microarray analysis, we used 3-day-old flies. This is because hormone levels fluctuate during the first days following eclosion as metamorphosis is completed. We therefore selected those flies that were mature and already producing viable eggs.

Our first comparison was therefore between the ovaries of starved and fed females. JH can partially rescue the apoptosis induced by starvation,[2,3] so we compared the ovaries of starved females with and without JH analogue (JHA, methoprene) treatment. Finally, since 20E can mimic to some degree the effect of starvation by inducing apoptosis of egg chambers,[2,3] we next compared the ovaries of fed females with ovaries from fed females treated with 20E.

In this paper, we describe some of the groups of genes that showed altered expression in the ovary and are likely to be involved in the execution of this survival/death developmental decision. We selected from the results genes in several categories including nutrient-sensing genes, stress response genes, genes capable of modulating hormone levels and apoptosis-related genes that may be involved in this process. In addition, we carried out *in situ* hybridization and RT-PCR for some genes to check their expression in the ovary and investigate if it matched the microarray data. In this way, we compared gene expression patterns under two sets of apoptotic conditions and two sets of conditions that promote development.

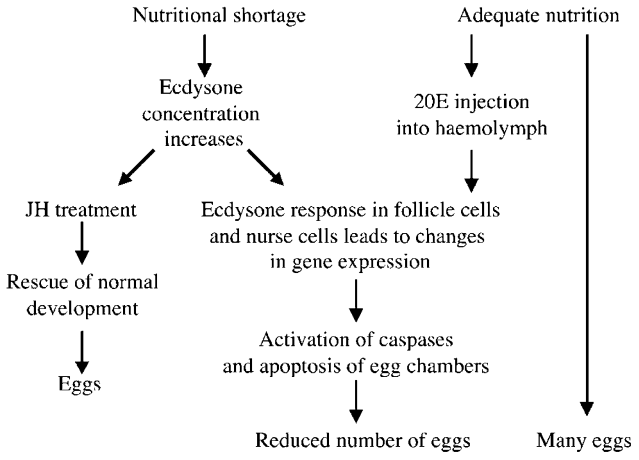


Figure 1 A model for the apoptosis pathway activated by nutritional shortage. The scheme proposes a model for the interaction of nutrition, ecdysone and apoptosis. Apoptosis is induced in the egg chambers at stages 8 and 9 under nutritional shortage and in 20E-treated flies

Results

Table 2 shows a summary of the results for the set of cDNAs used in the microarray and ovarian tissues. We carried out the microarray on a chip with 5364 genes (the list of genes on the chip is presented on http://www.ebi.ac.uk/arrayexpress. We carried out comparisons as follows: the ovary of starved flies and fed flies (S/F), JHA (methoprene)-treated starved flies and starved flies (JH/S) and 20E-treated fed flies and fed flies (E/F). The aim was to determine which genes are up- or

Table 2 Comparison of the numbers of identified genes and their relative expression levels between various apoptotic conditions

Experimental conditions compared	Upregulated	No change	Downregulated
Starved/fed (S/F)	2604	1385	1375
JHA-treated/starved (JH/S)	1900	973	2491
20E-treated/fed (E/F)	2469	953	1942

The table indicates the numbers of genes that upregulated, downregulated or remain unchanged in each combination of starved / fed (S/F), JHA-treated/starved (JH/S) and 20E-injected/fed (E/F). Upregulated (red column) shows that gene expression increases under those conditions. Upregulation means that gene expression is activated by starvation (S/F) and 20E injection (E/F) and JHA application (JH/S). Nochange (yellow column) indicates that gene expression is not changed with different experimental conditions. Downregulated (green column) indicates that gene expression is lower.

Table 1 Number of egg chamber at each stage and percentage of apoptosis under various condition

	Number of egg chambers (S.D.)						% Apoptosis	
	Stage 5	Stage 6	Stage 7	Stage 8	Stage 9	Stage 10	Stage 8	Stage 9
Fed	31.4 (3.4)	20.8 (4.3)	27.6 (2.8)	16.4 (2.9)	21.1 (1.8)	19.9 (1.8)	1.5	5.5
Starved	*23.8 (2.7)	19.3 (2.9)	22.1 (3.5)	*11.3 (1.2)	*10.9 (2.8)	*3.4 (1.8)	*15.5	35.2
20E-treated	32.9 (2.2)	21.3 (1.9)	22.2 (2.7)	*2.9 (2.1)	*3.9 (1.9)	*0.5 (0.9)	*82.9	*90.3
Ringer-treated	31.5 (4.2)	22.6 (4.3)	26.5 (5.2)	17.3 (2.5)	20.9 (2.3)	21.2 (2.5)	1.5	4.6
JHA-treated	*32.5 (4.1)	20.5 (4.2)	22.0 (3.1)	*16.3 (1.9)	*17.9 (2.1)	*8.7 (4.2)	*8.2	16.5
Acetone-treated	24.6 (4.9)	21.3 (2.8)	24.3 (4.6)	10.1 (1.1)	9.8 (1.5)	3.6 (2.5)	15.2	35.6

Significant difference (at 5% level, comparing fed and starved, 20E-treated and Ringers-treated, and JHA-treated and Acetone-treated). % of apoptosis is calculated as follows: % = (mean of egg chamber in which apoptosis is induced/mean of total number of egg chambers) × 100 ($n = 12$). The table shows the number of egg chambers at each of the stages from 5 to 10 and the percentage of apoptosis at stages 8 and 9 in ovaries of fed, starved and 20E treated, Ringer-treated (control for 20E-treated), JHA-treated and acetone-treated (control for JHA-treated) flies. Nuclear condensation and fragmentation were detected by Hoechst staining and observed under a fluorescent microscope. *indicates significant differences within 5% ($n = 12$ flies).

downregulated by starvation, JH and 20E, respectively. We predicted that genes involved in apoptosis should be upregulated by starvation and 20E treatment and downregulated by JHA treatment, because 20E injection into fed flies and JHA treatment of starved flies induces and suppresses, respectively, the apoptosis of egg chambers at stages 8 and 9.[2,3] Further, the 20E concentration in starved flies is higher than the concentration in fed flies[10] (Terashima J, Takaki K, Sakurai S, and Bownes M, unpublished data).

The increase of 20E concentration during starvation and the induction of the apoptosis by *BR-C Z2, Z3* and *E75A* (Terashima and Bownes[3] and unpublished data), which are ecdysone response genes, suggest that the higher levels of apoptosis observed during starvation are likely to be controlled indirectly by 20E concentration. Any genes in the ovary that control or modulate hormone titre should be up- or downregulated by starvation. We did not know whether any of these genes would be altered in their expression levels in the ovary itself as other tissues of the fly could equally well modulate hormone levels under different nutritional conditions. However, it is also possible that some genes in the ovary receive the starvation signals via other mechanisms and not through hormone regulation. Therefore, we analysed 2604 genes that are upregulated by starvation and 1375 genes that are downregulated by starvation. We expect these to include nutritional and stress response genes that regulate hormone synthesis or metabolism. In addition, we carried out RT-PCR and *in situ* hybridization to confirm expression levels and observe where and when the genes are expressed in the ovary. Some RT-PCR results did not reflect the results of the microarray analysis. This is likely to be because we used the whole ovary for the microarray. However, for the RT-PCR, we wanted to see what was happening prior to and at the checkpoint in oogenesis, thus we extracted total RNA from all the egg chambers up to stage 9 or from just stage 8 and 9 egg chambers. The different results between RT-PCR and microarray analysis is therefore likely to be caused by removing the egg chamber after stage 10. This removes them in the next stage of analysis as we move from the microarray to the more detailed analysis of gene expression in genes which are simply upregulated as late development proceeds.

Rather than report on long lists of random genes, we are making the full data set available on http://www.ebi.ac.uk/arrayexpress. We have grouped some of the affected genes, which are known to be involved in pathways whose expression we expect to be altered by nutrition and which alter hormone levels. We will concentrate in this manuscript on those genes which may enable us to better understand the role of pathways that are at least partially known and which we propose are involved in relating environmental cues to reproduction.

Nutrition- and stress-related genes

One of the aspects of the decision to undergo development or apoptosis is that cells need to be induced to undergo cell proliferation or proliferation needs to be inhibited, respectively. We predict that there are nutritional response and stress response pathways upstream of the pathways that modulate hormone levels. However, we have no idea how many of the enzymes that can lead to ecdysone concentration increases will operate in the ovary itself as opposed to other tissues of the fly. We did not know if the ovary itself was capable of modifying hormone levels either in the ovary itself or affect haemolymph levels. *takeout* (*to*) and *basket* (*bsk*) are starvation and stress response genes, respectively.[13,14] *bsk* encodes c-jun N terminal kinase (JNK),[14] which induces apoptosis in the *Drosophila* wing[15] and neural tissues through activation of *Plenty of SH3s* (*POSH*).[16] Higher expression of *to, bsk* and *POSH* in the ovary were induced by starvation (Figure 2a). *bsk* and *POSH* expression were downregulated in the JH/S combination. Higher *POSH* expression was induced by 20E application, but *bsk* was not affected by 20E. *to* expression was upregulated by JHA treatment (JH/S) and downregulated by 20E treatment (E/F). RT-PCR also detected higher expression in starved (compared with fed), but differences in expression levels between starvation and JHA treatment was not detected by RT-PCR (Figure 2b). We propose that *to* expression was enhanced after stage 10 in oocyte development and suppressed in stage 10 egg chambers under starvation. We thought that *bsk* and *POSH* could induce the apoptosis observed in stage 8 and 9 egg chambers. Therefore, we checked expression levels in the egg chambers at stages 8 and 9 (Figure 2b). *bsk* and *POSH* expression reflected the results of the microarray analysis; starvation induced higher expression of *bsk* and *POSH* in the ovary prior to stage 10 and in the egg chambers at stages 8 and 9. 20E induced higher expression of *POSH* but not *bsk,* and JHA treatment suppressed the expression of both genes in the ovary prior to stage 10 and in the egg chambers at stages 8 and 9 (Figure 2b). Starvation and stress must be sensed in the ovary and the first step in responding to starvation may well involve alterations in the expression of these genes. We expect that expression of these genes will be clearly upregulated by starvation, and are upregulated or remain unchanged following 20E treatment Therefore, *to, bsk* and *POSH* are candidates for sensing starvation and subsequently affecting the progress of oocyte development.

The mechanism of body size control in *Drosophila* is described by Potter and Xu[17] and Oldham and Hafen.[18] The scheme in Figure 2c shows the relationship between some of the genes involved in cell growth in response to nutrition and their relationship to the insulin signalling pathway (based on Potter and Xu,[17] and Oldham and Hafen[18]). Target of rapamysin (Tor) responds to nutrition and induces cell growth through activation of RPS60-p70-protein kinase (S6k). *S6k* mutants have a smaller body size than wild-type flies.[17] Simultaneous activation of S6k and TOR suppresses the *Drosophila* homologue of 4E-BP[19] and Thor negatively regulates body size in *Drosophila*.[20] TOR and S6k are inactivated by Tuberous Sclerosis Complex 1 (TSC1)/TSC2, which induces cell proliferation.[17,18] To induce cell growth, TSC1/TSC2 is inhibited by Protein kinase B (*Akt*), which is activated by the insulin receptor through activation of *Dstpk61* (the *Drosophila* homologue of PDK1).[21] If insulin binds to the insulin receptor, the receptor activates AKT through *Dstpk61* and AKT inhibits the TSC1/TSC2 complex. As a result, the TOR and S6k pathway is not suppressed and cell growth is activated in *Drosophila*.

a

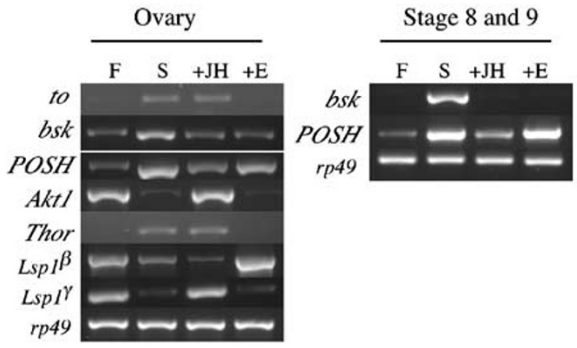
Up-regulated
No-change
Down-regulated

Expression levels of genes thought to be involved in response to stress, nutrients and starvation

S/F	JH/S	E/F	Name	Biological process affected
1.11 (0.036)	0.17 (0.042)	-0.77 (0.043)	*to (takeout)*	Response to starvation
0.85 (0.038)	-1.35 (0.003)	-0.15 (0.710)	*bsk (basket)*	Response to stress, JNK, MAPK cascade
1.57 (0.025)	-1.00 (0.007)	1.04 (0.020)	*POSH (Plenty of SH3s)*	Receptor signaling complex, scaffold activity
0.04 (0.678)	-0.16 (0.027)	-0.55 (0.038)	*Tor (Target of rapamycin)*	Positive regulation of cell size and cell growth
-0.95 (0.004)	1.00 (0.007)	-1.45 (0.008)	*Akt1 (Protein kinase B)*	Protein serine/threonine kinase activity, anti-apoptosis
0.54 (0.044)	-0.58 (0.042)	-0.07 (0.797)	*Tsc1 (Triple sex combs 1)*	Negative regulation of cell size and cell growth
-0.33 (0.002)	0.01 (0.423)	0.07 (0.094)	*Dstpk61**	Positive regulation of cell size
0.90 (0.013)	0.75 (0.043)	-0.14 (0.607)	*Thor (Thor)*	Negative regulation of cell size
-0.68 (0.021)	-1.18 (0.033)	1.14 (0.004)	*Lsp1$^{\beta}$ (Larval serum protein 1$^{\beta}$)*	Nutrient reservoir
-1.09 (0.008)	1.07 (0.033)	-0.38 (0.038)	*Lsp1$^{\gamma}$ (Larval serum protein 1$^{\gamma}$)*	Nutrient reservoir

* = (*Drosophila* serine/threonine protein kinase at position 61)

b RT-PCR analysis of transcripts from the genes identified by the microarray analysis.



c Gene hierarchy involved in *Drosophila* body size control.

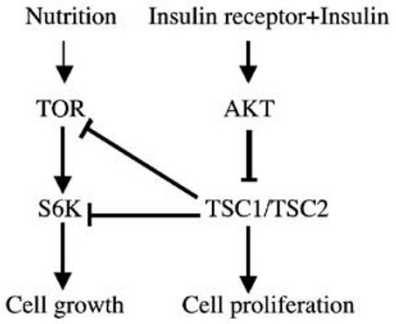


Figure 2 The expression of nutrition- and stress-related genes in the ovary. (**a**) The nutrition and stress response gene expression levels comparing apoptotic and nonapoptotic conditions is shown. Red – gene expression was upregulated. Yellow – gene expression was not changed. Green – gene expression was downregulated under apoptotic conditions. Starved females were compared with fed females (S/F) and JH-treated females (S/JH). 3nbh 20E-injected females were compared with fed (E/F) females. The gene names are shown as abbreviations with full names in parentheses. Index = $\log_2$ (detected signal ratio) and ( ) = *P*-value. (**b**) The RT-PCR analysis. Total RNA was extracted from the ovary containing stages prior to 10 (Ovary) and the egg chambers at stages 8 and 9 (Stages 8 and 9). F, S, +JH and +E represent fed, starved, JHA treatment and 20E treatment, respectively. (**c**) Cell growth and proliferation pathway that is known to be controlled by nutrition and insulin. This scheme is modified from Potter and Xu[17]

We observed that *Akt1, Tsc1, Tor, Thor* and *Dstpk61* are expressed in the ovary (Figure 2a). The expression of positive cell size regulators, *Akt1* and *Dstpk61*, was downregulated and negative cell size regulators, *Tsc1* and *Thor* were upregulated by starvation (Figure 2a). However, expression of the positive cell size regulator, *Tor* did not change under starvation. JHA treatment suppressed the expression of *Tor* and *Tsc1,* and enhanced the expression of *Akt1* and *Thor* and did not affect *Dstpk61* expression. 20E treatment suppressed the expression of *Tor* and *Akt1,* but did not affect the expression of *Tsc1, Dstpk61* and *Thor* (Figure 2a). RT-PCR showed the same results for *Akt1* and *Thor* expression (Figure 2b). Expression of the positive cell size regulator, *Akt1* was suppressed by starvation and that of the negative cell size regulator, *Thor* was enhanced by starvation (Figure 2b).

The genes that positively regulate cell and body size namely *Akt1* and *Dstpk61* were downregulated following starvation. On the other hand, the genes that negatively regulate cell and body size *Tsc1* and *Thor* were upregulated by starvation. These results suggest that cell size in the egg chamber may be downregulated under starvation and upregulated under adequate nutritional conditions.

The cells of the fat body of *Drosophila* larvae produce large amounts of storage proteins, generally referred to as hexamerins.[22] Two immunologically distinct hexamerins have been identified as larval serum proteins (LSP).[23,24] LSP contains LSP1 and LSP2, and LSP1 is composed of three subunits, α, β and γ, each encoded by different genes scattered throughout the genome.[24,25] *Larval serum protein 1* β (*Lsp1*β) and γ (*Lsp1*γ) expression in the ovary were downregulated by starvation. *Lsp1*γ expression was lower under all apoptotic conditions, 20E injection suppressed and JHA treatment enhanced expression. However, *Lsp1*β expression was suppressed by JHA treatment and 20E injection induced higher expression in fed flies (Figure 2a and b).

Thus, we see changes in gene expression within the ovary of genes known to be involved in the stress response or known to be related to the nutritional status of the organism in other tissues or at other times in development. It was not previously known for several of these genes that they were expressed in the ovary.

a

Up-regulated
No-change
Down-regulated

Expression of hormone related genes

S/F	JH/S	E/F	Name	Biological process affected
0.79 (0.009)	0.31 (0.017)	-0.58 (0.025)	*dib (disembodied)*	Ecdysone biosynthesis, Ecdysteroid 22-dehydrolxyase activity
1.10 (0.023)	-1.00 (0.014)	0.92 (0.023)	*Dare (defective in the avoidance of repellents)*	Cholesterol catabolism
-0.03 (0.441)	0.77 (0.047)	-0.75 (0.036)	*EcR (Ecdysone receptor)*	Ecdysone receptor activity
-1.29 (0.012)	-0.53 (0.048)	0.62 (0.009)	*Vha44 (Vacuolar H⁺ATPase 44kD subunit)*	Regulation of JH biosynthesis
0.38 (0.042)	-0.05 (0.184)	-0.11 (0.509)	*Jheh2 (Juvenile hormone epoxide hydrolase2)*	JH catabolism
0.52 (0.009)	-0.39 (0.004)	0.71 (0.003)	*Jheh3 (Juvenile hormone epoxide hydrolase3)*	JH catabolism

b

RT-PCR analysis of the transcripts for genes identified by the microarray analysis.

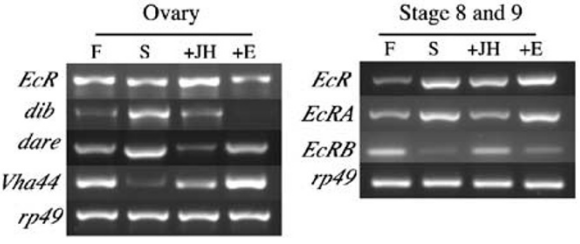


Figure 3 The expression of genes related to hormone biosynthesis and metabolism. (**a**) The expression of genes that could affect the titre of JH and ecdysone is shown. Red – gene expression upregulated. Yellow – gene expression was not changed. Green – gene expression was downregulated under apoptotic conditions. Starved flies were compared with fed females (S/F) and JH-treated females (S/JH). 20E-injected females were compared with fed females (E/F). Index = $\log_2$ (detected signal ratio) and ( ) = *P*-value. (**b**) The RT-PCR analysis. Total RNA was extracted from the ovary containing egg chambers up to stage 10 only and the egg chambers at stages 8 and 9 (Stages 8 and 9). F, S, + JH and + E indicate fed, starved, JHA treatment and 20E treatment, respectively

Genes affecting hormone metabolism

Although it is well documented that JH and ecdysone modulate the progress of oogenesis, it is not clear how some egg chambers can make a decision to develop while others undergo apoptosis when the haemolymph levels of hormone will be the same for all egg chambers in a given female. Our results show changes in expression of some genes that could well affect metabolism of the JH and ecdysone *within* the egg chambers (Figure 3a and b).

Cholesterol metabolism, essential to produce 20E, is mediated by 22-hydroxylase, 2-hydroxylase and 20-hydroxylase, which are encoded by *disembodied* (*dib*), *shadow* (*sad*) and *shade,* respectively.[26,27] In addition, it is possible that *defective in the avoidance of repellents* (*Dare*) interacts to synthesize ecdysteroids.[28] In our microarray and RT-PCR, *dib* expression in the ovary of starved flies showed a higher expression than in the ovary of fed flies. JHA treatment induced higher expression and 20E treatment suppressed it (Figure 3a and b). *Dare* expression also changed between starvation and feeding conditions. In addition, JHA treatment suppressed its expression (Figure 3a and b). The microarray analysis showed that 20E treatment induced *dare* expression, but the results of RT-PCR did not show much difference in *dare* expression between fed and 20E treated females (Figure 3b). These results suggest that the ecdysone synthesis pathway is activated in the ovary itself by starvation and includes the expression of *dib* and *Dare*.

We have shown that some ecdysone response genes induce apoptosis of egg chambers at stages 8 and 9 of oogenesis in *Drosophila*.[3] Under apoptotic conditions, *E75A, BR-C Z2* and *Z3* are expressed in egg chambers at stages 8 and 9 (Terashima and Bownes,[3] and unpublished data); this results in the induction of apoptosis in egg chambers. In addition, ecdysone response genes and ecdysone receptor (EcR) need to be expressed for normal oogenesis to proceed. In clones of cells mutant for *E75* and *EcR*, oogenesis is arrested at mid-oogenesis stages and egg chambers degenerate.[29,30] *EcR* expression showed no difference between fed and starved ovaries in the microarray analysis and RT-PCR, but JHA treatment induced and 20E treatment suppressed expression (Figure 3a and b). We thought that the *EcR* expression in stage 8 and 9 egg chambers could be important for inducing apoptosis. Therefore, we checked the expression of *EcR* isoforms, *EcRA* and *EcRB,* in egg chambers at stages 8 and 9 by RT-PCR (Figure 3b). *EcR* expression at stages 8 and 9 was enhanced by starvation and 20E treatment, and JHA treatment suppressed this expression (Figure 3b). This result was mirrored by the expression of *EcRA* at stage 8 and 9, *EcRA* expression was enhanced by starvation and 20E treatment but suppressed by JHA treatment. However, *EcRB* expression at stages 8 and 9 showed the opposite expression pattern being suppressed by starvation and 20E treatment and enhanced by JHA treatment (Figure 3b). These results suggested that *EcRA* and *B* may have opposite affects in relation to inducing apoptosis of stage 8 and 9 egg chambers. *EcRA* may induce expression of *E75A, BR-C Z2* and *Z3,* all apoptosis inducers by binding ecdysone and ultraspiracle.

JH epoxide hydrolase (JHEH) may play a pivotal role in regulating insect juvenile hormone (JH) titers along with JH esterase.[31] *Vacuolar H^+ ATPase 44 kDa subunit* (*Vha44*) may also be involved in JH synthesis.[32] Under starvation conditions, *Juvenile hormone epoxide hydrolase 2* (*Jheh2*) and *3* gene expression were upregulated and *Vha44* expression was downregulated (Figure 3a).

Further investigations of the functions of these genes in the ovary and how titers of hormones may change within the ovary itself would be a good way forward to investigate exactly how the regulation of hormone titers may affect and be affected by the ovary. It also may explain how different egg chambers in the same haemolymph can respond differently to the environmental conditions present.

Apoptosis-related genes

Since the end result of nutritional deprivation is apoptosis of many egg chambers, it is not surprising that expression of many genes known to be involved in apoptosis at other stages of the life cycle are expressed at higher levels in the ovaries of starved flies than fed flies.

DP transcription factor (*Dp*) is a positive regulator of nurse cell apoptosis and interacts with *E2F, a transcription factor* (*E2f*).[33] The *Drosophila E2F* includes *E2F* and *E2F transcription factor 2* (*E2f2*).[33] *Dp* was upregulated in starved flies and was suppressed by JHA treatment in the ovary containing egg chambers up to stage 9 and the egg chambers at stages 8 and 9 (Figure 4a and b). 20E treatment did not affect *Dp* expression in the ovary excluding all egg chambers beyond stage 10 (Figure 4a), but in the egg chambers at stages 8 and 9, *Dp* expression was enhanced by 20E treatment (Figure 4b). *Dp* expression was detected in the nurse cells and oocytes of egg chamber at stage 9 under all conditions. Expression at stage 8 was weaker than at stage 8 following feeding and JHA treatment. The expression at stage 8 during starvation and 20E treatment was stronger than during feeding and JHA treatment (Figure 5a). *E2f2* was upregulated in the ovary of starved flies in a similar way to *Dp* expression. However, JHA enhanced *E2f2* expression in starved flies and 20E suppressed expression in the fed flies. *licorne* (*lic*) is a MAPK kinase, targeting the MAPK known as *p38*.[34] It is known that *p38* signalling has a role in apoptosis and is activated by many environmental stress signals.[35] *lic* expression was enhanced by starvation and 20E treatment, and JHA did not affect its expression (Figure 4a).

The caspase pathway is one of the important pathways for inducing apoptosis in *Drosophila* (reviewed in Meier *et al*.[36]). *Nedd 2-like caspase* (*Nc, Dronc*) and *dream* have caspase activity.[37,38] *Nc* (*DRONC*) was upregulated by starvation and 20E treatment of fed flies, but there are no differences in the expression levels between the ovary of starved flies and JHA-treated flies in the ovary containing stages up to stage 10 (Figure 4a). *Nc* expression in the egg chambers at stages 8 and 9 was also enhanced by starvation and 20E treatment; moreover, JHA treatment suppressed *Nc* expression under starvation (Figure 4b). *Nc* expression was detected in the nurse cells of stage 8 and 9 egg chambers during starvation and 20E treatment (Figure 5a), but expression was not

a

Expression of apoptosis related genes

Up-regulated
No-change
Down-regulated

S/F	JH/S	E/F	Name	Biological process affected
0.99 (0.027)	-0.87 (0.012)	-0.07 (0.670)	*Dp (DP transcription factor)*	Positive regulation of nurse cell apoptosis
0.65 (0.045)	0.52 (0.021)	-0.91 (0.021)	*E2f2 (E2F transcription factor 2)*	Interaction with E2F (Positive regulation of nurse cell apoptosis)
0.59 (0.034)	-0.05 (0.857)	1.27 (0.048)	*Nc (Nedd 2-like caspase)*	Caspase activity
1.35 (0.038)	-1.04 (0.0002)	1.24 (0.047)	*dream (dream)*	Apoptosis inducer activity
0.96 (0.001)	-2.00 (0.024)	0.64 (0.002)	*p53 (p53)*	Apoptosis inducer activity, response to radiation
0.99 (0.034)	-0.58 (0.025)	0.67 (0.017)	*lok (loki)*	DNA damage response, resulting in induction apoptosis
0.80 (0.043)	-0.04 (0.888)	0.70 (0.038)	*lic (licorne)*	MAPKK activity
-0.03 (0.675)	1.04 (0.024)	-1.00 (0.036)	*th (thread)*	IAP1 activity, apoptosis inhibitor activity
-0.60 (0.045)	0.05 (0.891)	-0.05 (0.734)	*Iap2 (inhibitor protein of apoptosis 2)*	Apoptosis inhibitor activity
-1.86 (0.040)	0.50 (0.026)	-0.80 (0.041)	*Aac11 (Aac11)*	Apoptosis inhibitor activity

b

RT-PCR analysis of the transcripts for genes identified by the microarray analysis.

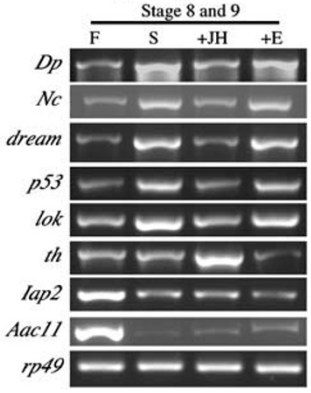


Figure 4 The expression of apoptosis-related genes. (**a**) Apoptosis-related gene expression levels. Red – gene expression was upregulated. Yellow – gene expression was not changed. Green – expression was downregulated under apoptotic conditions. Starved flies were compared with fed females (S/F) and JH-treated females (S/JH). 20E-injected females were compared with fed females (E/F). Index = $\log_2$ (detected signal ratio) and ( ) = *P*-value. (**b**) The RT-PCR analysis. Total RNA was extracted from the egg chambers at stages 8 and 9 (Stages 8 and 9). F, S, + JH and + E indicate fed, starved, JHA treatment and 20E treatment, respectively

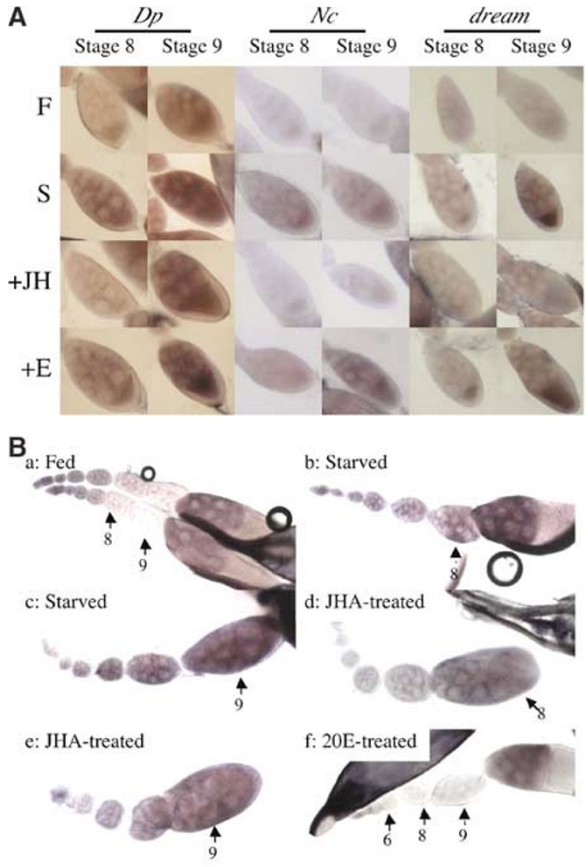


Figure 5 Temporal and spatial expression of apoptosis-related genes in oogenesis. (**A**) The expression patterns of *Dp, Nc* and *dream* at stage 8 and 9 egg chamber, as detected by *in situ* hybridization. F, S, +JH and +E indicate fed, starved, JHA treatment and 20E treatment, respectively. (**B**) *th* expression in the ovary: a, the ovary from fed female, b and c, starved females; d and e, JHA-treated females and f, 20E-treated females. The numbers on the panels indicate stages

detected in the ovaries of fed and JHA-treated females. *dream* was upregulated under starvation and following 20E treatment and suppressed by JHA treatment. *p53,* crucial for inducing apoptosis, responds to DNA damage and *reaper* is a direct transcriptional target of *Drosophila p53* following DNA damage;[39] however, *p53* has not reported as an inducer of developmental apoptosis in *Drosophila* and its function in oogenesis is unknown. *loki* (*lok*) is the *Drosophila* chk-homolog and *lok* regulates irradiation-induced, *p53*-mediated apoptosis in *Drosophila.*[40] *p53* and *lok* were upregulated under all apoptotic conditions (starvation and 20E treatment) in the ovary containing only egg chambers prior to stage 10 and the egg chambers at stages 8 and 9 (Figure 4a and b). This means that *p53* and *lok* were induced by starvation and 20E treatment and suppressed by JHA application.

The *Drosophila* homologue of *inhibitor of apoptosis 1* (*Iap1*) is *thread* (*th*) and *Drosophila* also have the *inhibitor of apoptosis 2* (*Iap2*).[41] Starvation did not suppress *th* expression, but 20E treatment suppressed and JHA treatment enhanced it. *Iap2* expression was suppressed by starvation and 20E treatment, but there was no change comparing starvation and JHA treatment (Figure 4a). *th* and *Iap2* expression in the egg chamber at stages 8 and 9 showed same results as the microarray (Figure 4b).

Aac11 has apoptosis inhibitor activity[42] and its expression was suppressed by starvation and 20E treatment in the ovary containing egg chambers up to stage 10 and the stage 8 and 9 egg chambers; and JHA treatment induced expression in the ovary excluding stages beyond stage 10 (Figure 4a and b). However, in the egg chambers at stages 8 and 9, JHA treatment had no effect *Aac11* expression (Figure 4b).

Figure 5b shows *th* expression in the ovary. *th* expression was not detected in the stage 8 and 9 egg chambers and 20E treatment suppressed *th* expression almost completely, except in the nurse cells at stage 10 (Figure 5Ba) Under starvation and JHA treatment, *th* was expressed in many egg chambers at stages 8 and 9 (Figure 5Bb–d).

Some apoptosis inducer gene expression showed different expression patterns in the egg chambers at stages 8 and 9 when comparing adequate nutrition, JHA treatment and starvation and 20E treatment. We propose that these different expression patterns are important for inducing apoptosis in the egg chamber at stages 8 and 9. One of the apoptosis inhibitors, *th*, is expressed in many egg chambers at stages 8 and 9 under starvation, but is not expressed under adequate nutrition. We suggest that *th* is expressed in those egg chambers at stages 8 and 9 that survive and develop to mature eggs under starvation and that it acts as an apoptosis inhibitor.

Discussion

Nutrient and stress response pathways in the ovary

The genes we see with changing expression levels in the microarray experiment support the model shown in Figure 6, suggesting how the ovary responds to environmental change. Red and Green indicate the genes that are down and upregulated by nutritional shortage and/or 20E treatment. Nutrient shortage is one of several environmental stresses for *Drosophila*. Probably, many tissues and organs respond to nutritional shortage; however, until these experiments were undertaken we did not know how the ovary responded. The ovary is affected by nutritional shortage by ultimately reducing yolk protein synthesis, by fewer eggs being laid and we know there is an altered expression pattern of some ecdysone response genes.[2,3] We propose that the transmission of the nutritional response signal includes stress responses. It is likely that the nutrient and stress response pathways in other tissues, such as the gut and fat body, respond to nutrient shortage, and that signals from these tissues activate a subsequent pathway in the ovary. It seems, however, that regulation of hormone concentration occurs within the ovary itself and this, one might have expected, would be controlled entirely by other tissues.

The gut is one of the candidates for releasing the first signal to respond to nutritional status. It is possible that the gut monitors the intake of the fly and releases signals into the haemolymph. A midgut hormone in liver-fed *Phormia regina* adult females leads to the completion of oogenesis.[43]

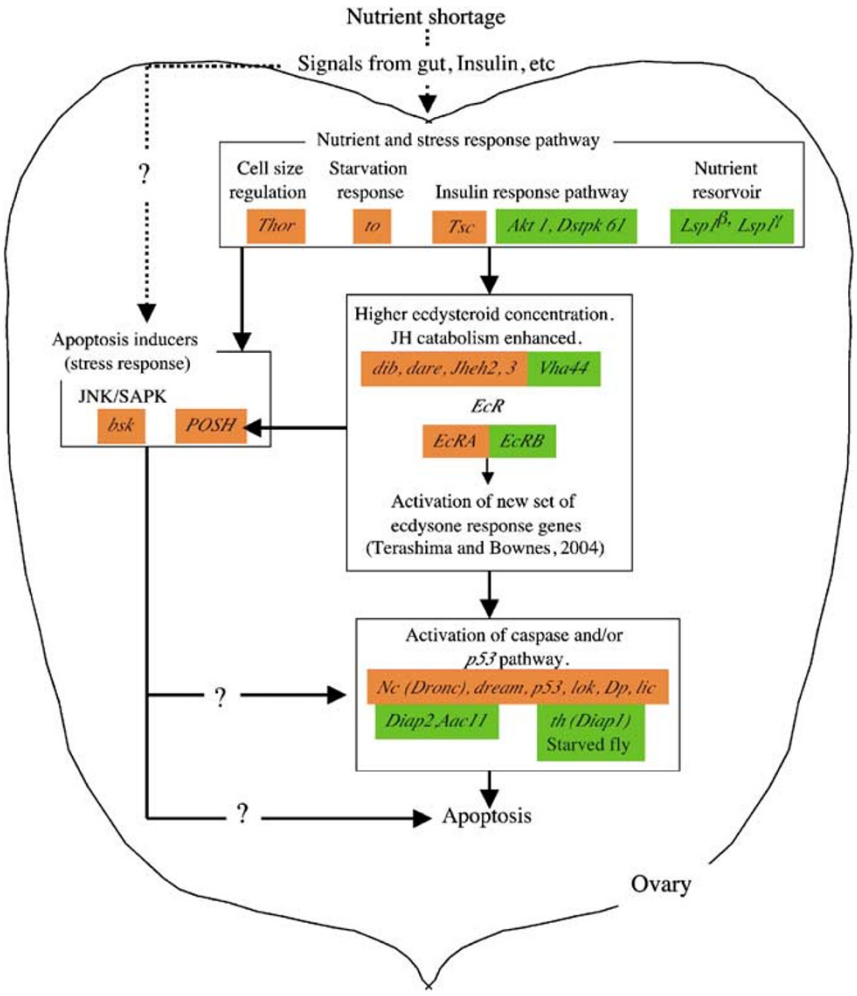


Figure 6 Model linking nutritional status and apoptosis in the egg chamber. Nutrient shortage activates or inactivates the nutrient and stress response pathway directly or indirectly. It is possible that genes that modulate ecdysone and JH levels are downstream of the nutrient and stress response pathway. Ecdysone activates a new set of ecdysone response genes, and these genes activate the caspase pathway or *p53* pathway to induce apoptosis. It is possible that there is JNK/SAPK pathway downstream of stress response pathway in the ovary. In this case, the JNK/SAPK pathway would need to be affected by ecdysone and ecdysone response genes, because to induce the apoptosis of egg chamber at stages 8 and 9 needs higher ecdysone concentration and ecdysone response gene expression is enhanced. The apoptosis of egg chambers at stages 8 and 9 of oogenesis has three possible apoptosis pathways, the JNK/SAPK pathway, the caspase pathway and the *p53* pathway. It is likely that these three pathways interact each other and induce the apoptosis observed at stages 8 and 9

Subsequently, the nutrient and stress response pathways in the ovary respond themselves to nutritional shortage, since the expression of some nutrient and stress response genes are up- or downregulated within the ovary (Figure 2a and b). The nutrient and stress response genes are categorized into subgroups (Figure 6). We expect that insulin has an important role for sensing nutritional status, and insulin release from the gut should be reduced under nutritional shortage. The ovary of insulin receptor mutants are immature and arrest at mid-oogenesis,[44] and *chico* mediates insulin receptor signalling in *Drosophila*, and it has a significant effect on egg chamber progression into vitellogenesis.[1] We propose that the insulin response pathway is inactivated by lack of insulin from the gut and suppresses cell growth (Figures 2c and 6). In the fed fly, the insulin receptor in the ovary activates AKT and induces cell growth. On the other hand, in starved flies, the insulin receptor is inactivated resulting in inactivation of AKT. This inactivation could lead to the activation of the TSC1/TSC2 complex and induce cell proliferation. In this case, gut or other tissues would release signals like insulin to activate or inactivate the nutrition and stress response pathway in the ovary. We suggest that the ovary then uses the genes which show altered expression levels to control hormone concentration and ultimately, therefore, the apoptosis pathway. The insulin receptor interacts with AKT and AKT interacts with DSTPK61.[21] The *Akt1* product has a role in antiapoptosis,[45] and the *Dsptk61* product is a positive regulator of cell size.[21,46] The expression of both these genes in the ovary were downregulated by starvation. In addition, *Tsc1* and *Thor*, which are negative regulators of cell size, were upregulated by starvation (Figure 2a and b). Nutritional shortage signals should modulate cell size and nutrient storage suppression and prepare cells to induce apoptosis in the egg chamber at stages 8 and 9.

How might apoptosis be induced?

It seems likely that nutrition and stress response pathways regulate ecdysone concentration in the ovary. The ovary is one of several tissues in the adult that is capable of ecdysone synthesis,[47,48] and it is possible that secretion of ecdysone from ovary itself can affect ecdysone concentration in the haemolymph. Simultaneously, other tissues that synthesize ecdysone may also affect the concentration in the fly, and it has been shown that the total ecdysone concentration in the fly is increased in starved flies.[10] In the ovary, *dib* and/or *Dare* expression could affect the synthesis of ecdysone or 20E. Higher ecdysone concentration in the fly induces apoptosis of the egg chamber at stages 8 and 9,[2,3] and overexpression of some ecdysone response genes induce apoptosis of egg chambers at stages 8 and 9.[3] Therefore, we expect that to induce the apoptosis at stages 8 and 9 requires an increase in ecdysone concentration and either a decrease in JH concentration or the suppression of JH action. *Vha44,* a JH synthesis-related gene is suppressed and JH catabolism genes, *Jheh2* and *3* are enhanced by starvation. This hormone-related gene expressions is probably controlled by the nutrient and stress response pathways, and as discussed in the previous section, the strongest candidate is the insulin pathway. Insulin receptor mutant females require continuous exposure to JHA to induce any vitellogenesis.[45]

The EcR with ecdysone bound to it and Ultraspiracle act as a transcription factor. Oogenesis in *EcR* mutant is arrested at mid-oogenesis and the egg chambers are degenerated.[29] This means that the EcR is essential to progress normal oogenesis. However, the results of RT-PCR suggest EcR may also act in the egg chamber at stages 8 and 9 to induce the apoptosis of the nurse cells. *EcRA* expression in the egg chamber at stages 8 and 9 was enhanced by starvation and 20E treatment (Figure 3b) but *EcRB* expression in the egg chamber at stages 8 and 9 was suppressed by starvation and 20E treatment. These results suggest that *EcRA* acts as transcription factor for the genes involved in inducing apoptosis such as *BR-C Z2* and *Z3*.[3] We predict that EcR should have affects not only on normal oogenesis but also on the induction of apoptosis, possibly different isoform of *EcR* have different roles in normal oogenesis and in the induction of apoptosis during *Drosophila* oogenesis.

There are some candidate genes for the pathway that leads to apoptosis. *Dp, lic, p53,* JNK/SAPK and the caspase family were identified by the microarray, RT-PCR and *in situ* hybridization. *Dp* normally induces the apoptosis of nurse cells that commences at stage 10 during normal oogenesis and is known to interact with *E2f* and *E2f2*.[34] Most apoptosis in *Drosophila* is induced by the caspase, including *Nc* (*Dronc*) and *dream,* pathway that is activated by ecdysone response genes.[37] The germ line of nutrient-deprived flies carrying either a loss of function mutation in *Death caspase-1* (*Dcp-1*) or a transgene overexpressing the caspase inhibitor *Diap1* fail to die, and large numbers of abnormal egg chambers accumulate in the ovaries.[49,50] In our microarray analysis, *Dcp-1* expression was increased by nutrient shortage and 20E treatment and suppressed by JHA treatment (Index, $S/F = 1.00$, $JH/S = -0.95$, $E/F = 0.89$), but all the *P*-values were over 0.05 (Data not shown) and therefore was not significant and could not be included in our list of genes altered by these experimental conditions. Nonetheless, we expect that *Dcp-1* is likely to interact with the apoptosis process during mid-oogenesis. The apoptosis activity of *p53* is regulated by *lok* in *Drosophila*.[41] *lic* is the homologue of *p38* in *Drosophila* and is required for oogenesis.[35] *POSH* belongs to the JNK/SAPK pathway downstream of the stress response pathway and induces apoptosis in the *Drosophila* wing and neural tissues.[14–16] These genes are regulated by 20E, probably mediated by *BR-C Z2*, *Z3* and *E75A. bsk* also belongs to JNK/SAPK pathway although starvation induced *bsk* expression, 20E treatment did not induce its expression (Figure 2a and b). This suggests that there is one ecdysone-dependent pathway and another ecdysone-independent pathway that can induce apoptosis. 20E treatment of fed females induces ovarian apoptosis[2,3] and this requires changes in expression of response genes. It seems likely that an increase in ecdysone concentration in the fly is essential for inducing the apoptosis in the egg chamber at stages 8 and 9. We propose that *bsk* may support the main apoptosis pathway. In addition, there are some candidates for apoptosis inhibitors, including *thread, Iap2* and *Aac11*. The homologues of the apoptosis inhibitors, *Iap1* and *2* have been identified as *thread* (*th, Drosophila Iap1, Diap1*) and *Drosophila Iap2* (*Diap2*).[41] *Aac11* has apoptosis inhibitor activity in mammalian cells,[42] but the mechanism of action is unknown. *th* expression levels did not change between the ovary of starved flies and fed flies. However, we have observed that *th* expression at stages 8 and 9 differed spatially and temporally between the ovaries of starved flies and fed flies (Terashima and Bownes, unpublished data). *th* was not expressed in the egg chambers at stages 8 and 9 in fed flies, but was expressed in some egg chambers in starved flies. This expression pattern has been reported by Foley and Cooley.[5] They have shown the *Diap1* expression is detected beginning at region 2B in the germarium and downregulated at mid-oogenesis stages.[5] In addition, as we have mentioned, flies overexpressing the caspase inhibitor *Diap1* as a transgene fail to die and large numbers of abnormal egg chambers accumulate in the ovaries.[50] We expect that *th* is expressed in the small number of egg chambers of starved flies that survive and develop into mature eggs. In starved flies, some egg chambers always show normal development[3] and we suggest that *th* has a role inhibiting the apoptosis at stages 8 and 9 of these egg chambers. *Diap 2* expression is detected in the nurse cells of these egg chambers.[5] Although *Iap2* expression was suppressed by starvation, JHA treatment did not affect its expression in the ovary of starved flies. Perhaps *th* and *Iap2* inhibit apoptosis in starved flies and fed flies, respectively. Probably, starvation signals switch the affects of the apoptosis inhibitors *th* and *Iap2* in early oogenesis.

We propose a model for inducing apoptosis in egg chambers at stages 8 and 9 by starvation. The genes that are candidates for inducing the apoptosis are grouped as nutrient and stress response, hormone-related and apoptosis-related genes. The nutrient and stress response group includes the genes that are related to JNK/SAPK, and the insulin pathway that control cell size regulation and nutrient storage. Some of these genes should regulate hormone synthesis or catabolism to increase ecdysone concentration.

The increase in ecdysone levels must activate the apoptosis executing pathway, caspase, *p53* and/or JNK/SAPK. One of the JNK/SAPK genes *bsk* may act as an apoptosis inducer that is independent of ecdysone concentration or has other unknown affects in oogenesis. We predict that lack of an insulin signal marks beginning of activation of the apoptosis pathway in the mid-oogenesis, and probably induces ecdysteroid synthesis. Tu *et al*.[51] reported *insulinlike receptor* mutant *virgin* females show reduced *production* of ecdysteroids in the ovary. In our measurement, ecdysteroid secretion from the ovaries was decreased, but the *ecdysteroid concentration* in the ovaries was increased in *Drosophila* after mating (Terashima J, Takaki K, Sakurai S, and Bownes M, unpublished data). We therefore predict that the higher ecdysteroid concentration may be induced by lack of an insulin signal. In this paper, we have identified some of the candidate genes in the ovary for sensing nutrient changes and stress, modulating hormone titre and initiating and executing apoptosis.

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* was used throughout. Flies (3-day old) were transferred from a standard diet to one of sugar and water (starved: 1% agar medium that contains 5% sucrose and 0.005% Nipagen in 95% ethanol) or one of yeast (fed: 2 g bakers yeast on approximately 50 ml 1% agar medium that contains 2.5% cornflour, 5% sucrose, 1.75% lyophilized 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), or injected with 20E and maintained on yeast for 1 day (F3EF1).

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.[52] The concentration of 20E was determined according to Bownes;[10] with a haemolymph volume of approximately 1 μl/female,[52] injecting 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 undertaken by injecting Ringer's only and treating flies with acetone.

Microarray and data analysis

The comparisons between experimental conditions in the microarray experiments were starved *versus* fed, starved *versus* JHA-treated and 20E-injected *versus* fed (each three sets). The ovaries were dissected in Insect Ringer's solution and homogenized in 500 μl TRIZOL. There are 27–55 pairs of ovaries in each sample (40–55 mg). The samples were stored at −80°C until undertaking the microarray analysis. When the microarray was carried out, the same volume RNA was used from each sample. There were 5364 genes on the chip and the microarray processes are described on http://www.flychip.org.uk/Project/Protocols.

We prepared three experimental sets for each combination and carried out the microarray. Cy3 (red) and Cy5 (yellow) fluorescence intensities were normalized and nonbackground subtracted. The mean ratios (Cy3 and Cy5) are calculated and sorted by the absolute log ratio. Upregulation or downregulation is determined by the average log ratio (ALR). Upregulation is ALR >0 and downregulation is ALR <0. The validity of the results is provided by the *P*-value of ALR, which is shown as a percentage. The genes whose *P*-value of the log ratio are over 95% are categorized as 'no-change' in gene expression and the genes with expression levels that have a significant difference between the test sample and control sample ($P<0.05$) are either 'up or downregulated'. The expression index was calculated as follows: Log_2 (the ratio of detected signals, S/F, JH/S and E/F), as the signals detected by a factor of 2 change have a signal ratio of 2, whereas the signals detected by the same factor have a signal ratio of −0.5. The most widely used alternative transformation of the ratio is the logarithm base 2, which has the advantage of producing a continuous spectrum of values and treating up- and downregulated genes in a similar fashion.[53] It is important to remember that logarithms treat numbers and their reciprocals symmetrically: $\log_2(1)=0$, $\log_2(2)=1$ $\log_2(1/2)=-1$, $\log_2(4)=2$, $\log_2(1/4)=-2$, and so on.

In situ hybridization and RT-PCR

The protocol is based on the procedure previously described[54] 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 hybrix (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 hybrix containing digoxigenin-labelled probe (DIG-DNA labelling 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 (Boehringer Mannheim). Anti-DIG-AP conjugate was preabsorbed with postfixed wild-type (Oregon R) ovaries at 4°C overnight. The ovaries were mounted in a mixture of PBS/glycerol (1 : 4) for microscopy. Egg chambers were staged according to their size and morphology.[55]

The levels of the gene transcripts in ovaries were detected by reverse transcriptase (RT)-PCR as described previously.[56] The egg chambers after stage 10 were removed from the ovaries and the egg chambers at stages 8 and 9 were isolated from the ovaries in ice-cold Ringer's solution. The details of total RNA extraction, reverse transcription reactions and the PCR reaction are described in Tzolovsky *et al*.[57]

We used two types of starved flies, S3 (as a control for comparison with F3) and F3S1 (as a control for comparison with F3JHS1) in the microarray analysis. The gene expression levels and the spatial and temporal expression patterns resulting from *in situ* hybridization were similar between S3 and F3S1 females. Therefore, we show the results of the ovaries in S3 flies as the example for starved flies in the RT-PCR and *in situ* hybridization analysis.

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