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Master thesis

Role of Jun and Fos in oogenesis of the beetle *Tribolium castaneum*

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Annotation

The aim of this work was to examine the function of the nuclear effectors of JNK signaling, Jun and Fos, in insect telotrophic ovaries, and compare it to the polytrophic ovary of *Drosophila*. RNA interference knockdown of Jun and Fos in the beetle *Tribolium castaneum* blocked oogenesis by causing death of follicle cells during growth of the vitellogenic oocytes through caspase-dependent apoptosis. Jun and Fos are therefore required for follicle cell maintenance, which is not the case in *Drosophila*. Since knockdown of the Jun N-terminal kinase (JNK) had no such effect, Jun and Fos likely act downstream of another kinase during *Tribolium* oogenesis. We propose that Jun and Fos ensure follicle cell survival upon physical stretching of the follicle epithelium resulting from the oocyte growth.

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Vlastimil Smýkal

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1. Introduction

The tiny red flour beetle, *Tribolium castaneum* (Coleoptera, Polyphaga, Tenebrionidae; Bonneton, 2008), is a rising star among model organisms. A general type of insect development, systemic RNAi and the sequenced genome (*Tribolium* Genome Sequencing Consortium, 2008) make the beetle *Tribolium* a perfect model for comparative studies with the highly advanced fly *Drosophila melanogaster*.

As a holometabolous species, *Tribolium* develops through embryonic, larval, pupal and adult stages (Fig. 1). The lengths of these developmental stages are temperature-dependent, and the number of larval instars varies between 7 and 8 (Tab. 1).

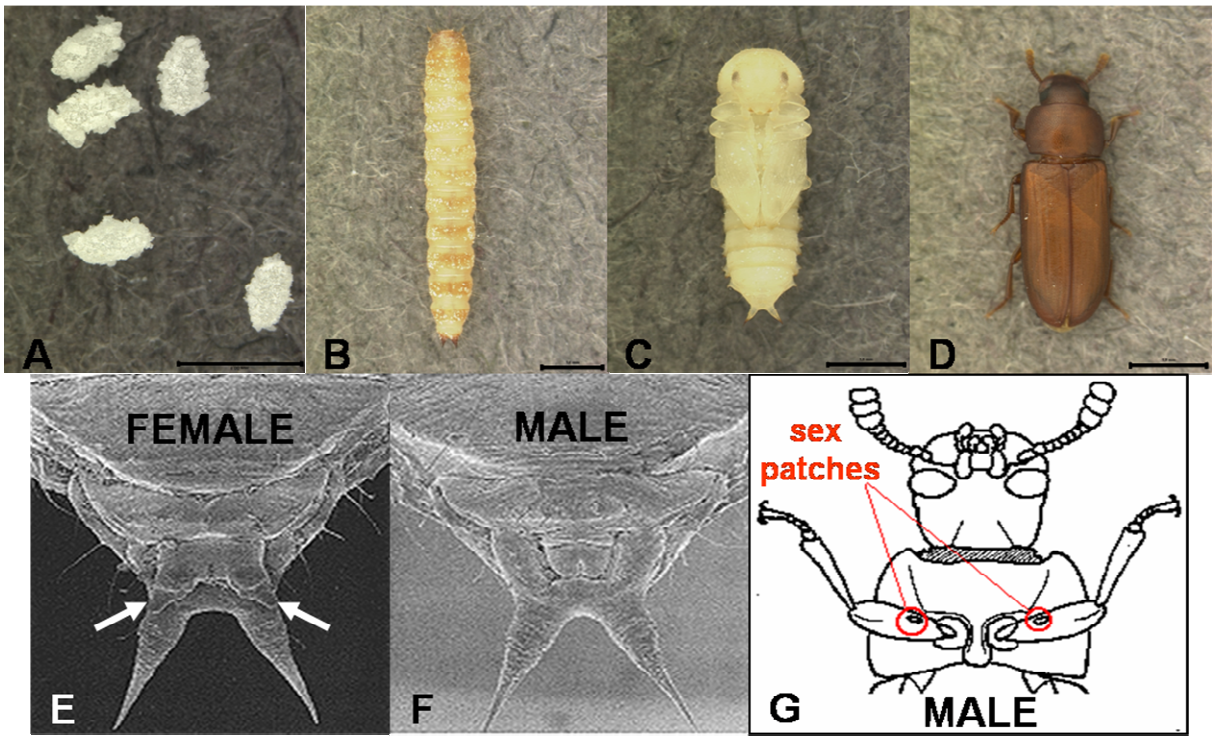

Fig. 1. ***Tribolium* life cycle and sex specific characters**. Eggs (**A**), last-instar larva (**B**), pupa (**C**) and adult beetle (**D**) of *Tribolium castaneum*. Male (**E**, **G**) and female (**F**) specific characters. Female pupae have two pointed genital papillae (**E,** arrows). Adult males have a small patch of short bristles on the femur of the first pair of legs (**G**). Scale bar in A, B, C and D = 1 mm. (Panels E-G are modified from Dr. Beeman's web page "BEETLE WRANGLING TIPS" , http://bru.gmprc.ksu.edu/proj/tribolium/wrangle.asp)

Table 1. **Time-course of *Tribolium* development at 32°C.** First-instar larvae hatch after 3 days of embryonic development. Larvae go through 7 or 8 instars, demarcated by molting and ecdysis from the old cuticle. The last instar takes about 2.5 days plus 1 day of prepupal development (prepupa is a pharate pupa before ecdysis). Adults emerge after 4.5 days of pupal development and live for approximately 200 days (single males up to 3 years).

embryo	larva (instar)								prepupa	pupa	adult
	I.	II.	III.	IV.	V.	VI.	VII.	VIII.			
3	1 - 2	2 - 3	2 - 3	2 - 3	2 - 3	2 - 3	2 - 3	2 - 3	1	4.5	ca 200

1.1 Why study *Tribolium castaneum*?

Although genetic research in *Drosophila melanogaster* is highly productive, some aspects of insect development and reproduction need to be studied in species that are less derived or possess different features than the evolutionarily advanced fly. For example, the short-germ band segmentation, the primitive type of metamorphosis, or the telotrophic ovaries make *Tribolium* a perfect tool for comparative studies.

Insect embryogenesis can go through two basic modes: short (plus intermediate) and long germ band embryogenesis. In short germ band embryos, such as *Tribolium*, only a small number of anterior segments are created at the beginning of embryogenesis, and the other segments are sequencially added at the posterior growth zone (Schröder et al., 2008). In long germ band embryos, such as *Drosophila*, all body segments form at the same time. Contrary to *Drosophila* with extremely reduced head and no appendages, *Tribolium* embryos develop head with antennae and mouthparts (Bucher & Wimmer, 2005), and their legs develop directly (and not later from imaginal discs) (Davis & Patel, 2002).

As a basal holometabolan species, *Tribolium castaneum* represents a „typical" holometaboly. Unlike in *Drosophila*, entry to metamorphosis in *Tribolium* is controlled by juvenile hormone (Konopova & Jindra, 2007, 2008). As in most insects the larval epidermis of *Tribolium* is reprogrammed to make pupal and then adult structures, which are not built from early-forming imaginal discs as in *Drosophila*. Finally, *Tribolium* and *Drosophila* also differ in the type of oogenesis. Although both possess meroistic ovaries, *Tribolium* has telotrophic instead of polytrophic ovaries (see Section 1.3 below).

1.2 Genetic tools for study of *T. castaneum*

Success of model organisms depends on methods that are available for their study. For *Tribolium*, many molecular tools have been established within the last several years. Two most important ones are hereditary genetic transformation with transposable elements and systemic RNA interference.

1.2.1 Genetic transformation with transposable elements

Stable germline transformation was achieved in *Tribolium* by insertion of vectors based on the *piggyBac*, *Hermes* and *Minos* transposable elements that have a broad host range (Berghammer et al., 1999; Lorenzen et al., 2002, 2003; Pavlopoulos et al., 2004). These vectors contain color-specific fluorescent markers (Berghammer et al., 1999). Remobilization of *piggyBac* by crossing transgenic beetles with a helper strain that carries a *Minos* element

with integrated *piggyBac* transposase gene has enabled mutagenesis and enhancer-trap screening. The GEKU (Goettingen-Erlangen-Kansas State University-USDA) insertional mutagenesis screen has yielded many lines whose specific tissues are marked with EGFP, and uncovered new gene-specific mutants (e.g. Lorenzen et al., 2003, 2007).

1.2.2 Systemic RNAi

RNA interference (RNAi) was discovered ten years ago, and has immediately become a commonly used method in biology (Fire et al., 1998; Montgomery, 2006). RNAi is a conserved cellular mechanism in eukaryotes, in which presence of a double-stranded RNA (dsRNA) in the cell leads to post-transcriptional gene silencing through degradation of homologous mRNA. A related endogenous micro-RNA (miRNA) mechanism regulates gene expression via translational repression (Fig. 2; Meister & Tuschl, 2004). In both pathways, dsRNAs are processed by the Dicer RNase III (Carmell & Hannon, 2004) into short interfering RNAs (siRNAs, 20-25 nucleotides long). In the RNAi pathway, siRNAs enter the RNA-induced silencing complex (RISC) (Filipowitz, 2005). RISC uses these small RNAs to find and cleave the complementary mRNA (Meister & Tuschl, 2004) (Fig. 2).

In some species dsRNA can be delivered by injection or feeding; then it is uptaken by the animal's cells where siRNAs are generated. siRNAs can spread through the body and affect all or most other tissues of the organism. This effect is known as systemic RNAi, and in *C. elegans* it depends on a transmembrane protein SID-1 (Winston et al., 2002). RNAi clearly has a systemic nature also in *Tribolium* but not in some other insect species including *Drosophila* (Tomoyasu et al., 2008). Although the molecular mechanism of systemic RNAi in *Tribolium* seems to differ from that in *C. elegans* (Tomoyasu et al., 2008), the systemic response to dsRNA gives *Tribolium* a great advantage for reverse-genetic studies.

All developmental stages of *Tribolium* are susceptible to systemic RNAi upon dsRNA injection. Methods for embryonic RNAi (Brown et al., 1999), larval, pupal and adult RNAi (Tomoyasu & Denell, 2004; Bucher et al., 2005), and parental RNAi in which the knockdown effect is transmitted to the next generation (Bucher et al., 2002) have been established. We have used the advantage of systemic RNAi in this work to study gene function in the adult *Tribolium* ovary.

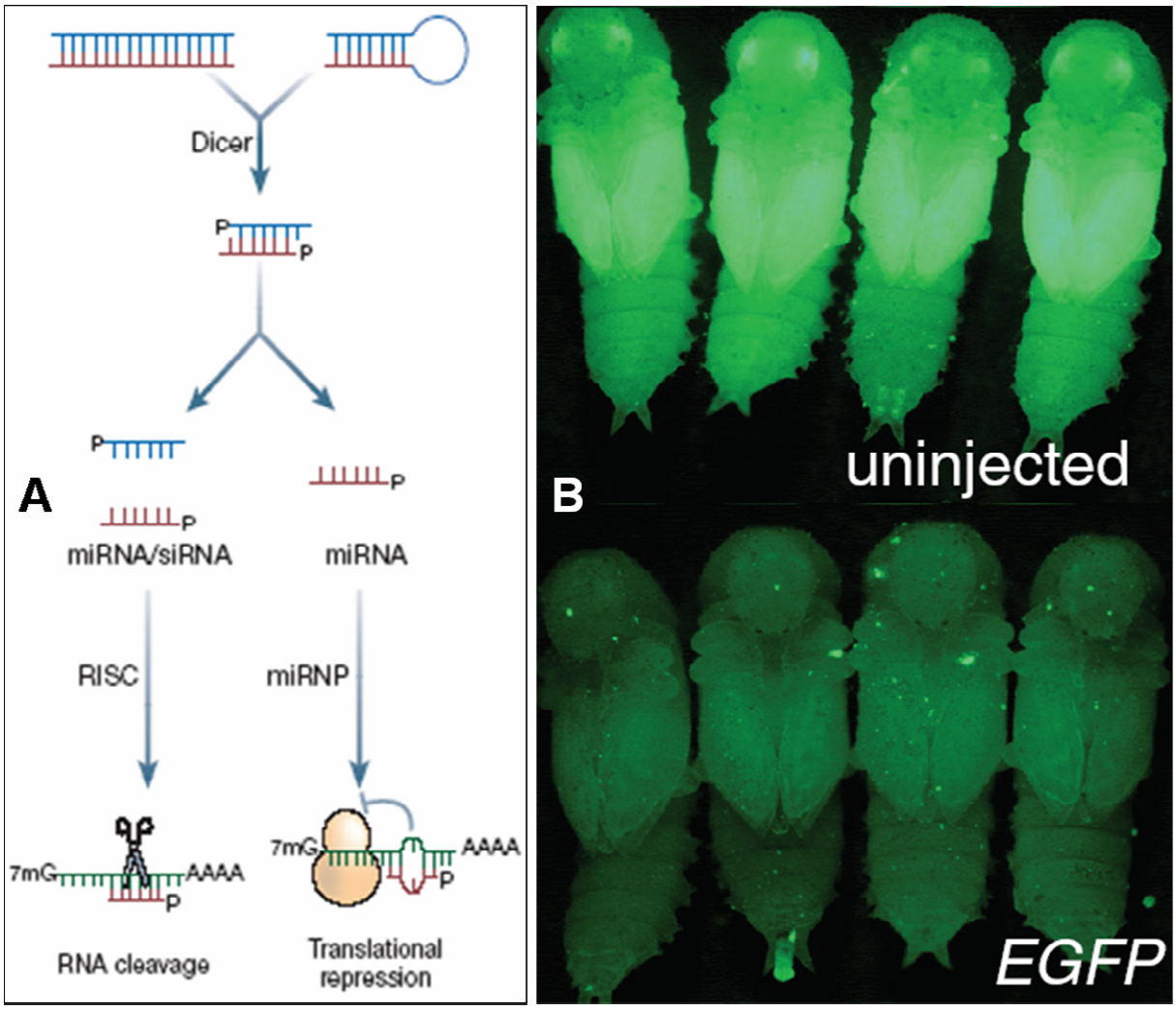

Fig. 2. **RNA interference.** (**A**) A simplified mechanism of RNAi. Exogenous or endogenous dsRNA is cleaved by Dicer. Resulting small interfering RNAs or microRNAs are loaded into the RISC or miRNP complexes and used for cleavage or for translational repression of mRNA targets, respectively (from Meister & Tuschl, 2004). (**B**) Example of systemic RNAi in *Tribolium*. Transgenic EGFP expression in wings and eyes of pupae becomes totally silenced upon EGFP dsRNA injection during the previous larval stage (from Tomoyasu et al., 2008).

1.3 Oogenesis in the telotrophic ovaries of *T. castaneum* and the polytrophic ovaries of *D. melanogaster*

Insect ovaries consist of ovarioles where oogenesis takes place. The ovariole has two main functional parts: (1) the germarium where germ cells originate, and (2) the vitellarium where oocytes are enclosed with the somatic follicle cells (FC) and where the growth of follicles takes place. Each ovariole is connected to the oviduct via the pedicel and is covered with a somatic-cell sheath. We recognize two basic types of ovaries – panoistic, which are not equipped with nutritive (nurse) cells, and meroistic, in which the oocyte is supplied with maternal components by the germline nurse cells (NC) (Fig. 3).

Meroistic ovaries divide in two types – polytrophic and telotrophic – and these differ in the location of the NC. In polytrophic ovarioles, well known from *Drosophila*, each oocyte

and its sibling NC are enclosed within the same follicle and pass through the ovariole together (Fig. 3C). In the telotrophic ovary of *Tribolium*, NC (trophocytes) remain in the posterior part of the germarium (here termed tropharium) and do not accompany the oocyte to the vitellarium (Fig. 3B,D). Each oocyte is covered by the somatic FC and maintains connection with NC via cytoplasmic bridges called nutritive cords (Büning, 1994).

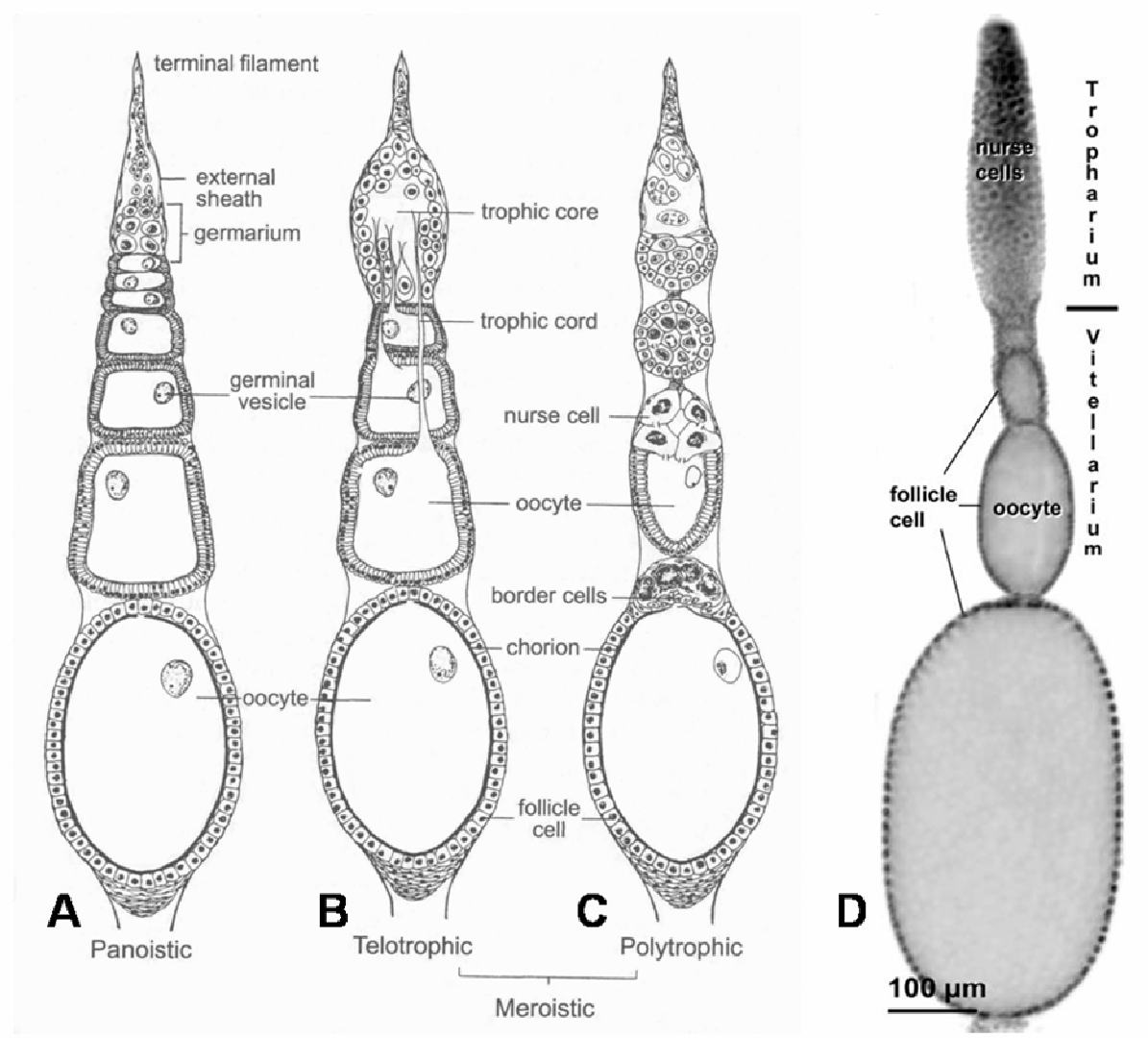

Fig. 3. **The basic types of ovarioles.** (A) Panoistic, (B) telotrophic meroistic and (C) polytrophic meroistic ovarioles. Germinal vesicle is the nucleus of the oocyte (2n) before meiosis. (**D**) Ovariole of *Tribolium* female. (Figure A-C is from Schwalm (1988); D is from Trauner & Büning, 2007).

NC are sister cells of the oocyte that are formed by asymmetric divisions of the oogonia (Huynh & St. Johnston, 2004). They become highly polyploid (4096n in *Drosophila*) and these hugely metabolically active cells pass RNAs, proteins, mitochondria, centrioles, ribosomes and other materials into the oocyte (during stages 8-14 in *Drosophila*). After the *Drosophila* NC complete oocyte dumping, they undergo apoptosis (McCall, 2004).

1.3.1 Role of follicle cells in oogenesis

In the follicle epithelium of *Drosophila*, which derives from the gonadal mesoderm, at least seven sub-populations of FC with different roles during oogenesis can be distinguished. These subgroups co-operate with the germline cells – the oocyte and NC – to establish both anterior-posterior and dorsal-ventral axes of the follicle and the future embryo. To do that, FC sub-groups undergo several tightly orchestrated movements that are necessary to form a fully developed egg (Dorman et al., 2004; Horne-Badovinac & Bilder, 2005). In contrast, *Tribolium* follicles are composed of only two types of FC – the main body FC and stalk cells, which are located on both ends of the follicle and separate follicles from each other (Trauner & Büning, 2007).

Once the *Drosophila* oocyte is released from the germarium, it enters the apex of the vitellarium where it is surrounded by the somatic pre-follicular cells, which start to proliferate and then enclose the oocyte in a single epithelial layer (Dobens & Raftery, 2000). At the onset of vitellogenesis, the FC switch from proliferation to three cycles of endoreplication and thus become polyploid. After polyploidization, FC become columnar and have bigger and lobed nuclei. Columnar FC separate from each other during vitellogenesis and create intercellular channels necessary for the receptor-mediated endocytosis of vitellogenins (or yolk polypeptides in *Drosophila*; Sappington, 2002) from the hemolymph into the oocyte (Sappington & Raikhel, 1998; Schonbaum et al., 2000). FC also synthesize some yolk proteins (Lossky & Wensink, 1995). After vitellogenin uptake is complete, FC synthesize vitelline membrane and deposit chorion proteins on the surface of the oocyte (Cernilogar et al., 2001). The complete chorion has an operculum (the entry point for the sperm) and in *Drosophila* it also includes structures with special functions such as the respiratory dorsal appendages. Formation of these structures depends on the proper specification of the FC populations primarily by the EGFR signaling, and on their epithelial movements that require several signaling pathways including the Jun N-terminal kinase (JNK) pathway (Berg, 2005).

A recent study of Trauner & Büning (2007) shows that the FC in the telotrophic ovary of *Tribolium* behave similarly to the FC in *Drosophila* in that they also encapsulate the early oocyte and proliferate only during early oogenesis. The morphology of the FC nuclei suggests that they also become polyploid and then finally degenerate when the egg is released into the oviduct. However, genetic regulation of FC functioning in the telotrophic ovary has yet to be studied, and *Tribolium* provides an excellent model for this research.

1.4 Mitogen-activated protein kinases (MAPKs)

Mitogen-activated protein kinases (MAPKs) transmit extracellular signals and regulate both transcriptional and non-transcriptional responses to them. MAPKs are involved in many cellular processes in eukaryotes ranging from development (Stronach & Perrimon, 1999; Harden, 2002), cell cycle control (Zhang & Liu, 2002) and apoptosis (Feuerstein & Young, 2000) to stress signaling and immunity (Dong et al., 2000; Kyriakis & Avruch, 2001).

The canonical MAPK pathway is a phosphorylation cascade, in which an activated MAP kinase kinase kinase (MAPKKK) activates a downstream kinase, finally culminating by phosphorylation of target transcription factors such as Jun and Fos (Pearson et al., 2001; Imajo et al., 2006) (Fig. 4A).

Positive or negative stimuli from the extracellular space are detected by receptors on the cell membrane and MAPKKKs are activated via G-proteins. MAPKKKs contain a serine/threonine protein kinase domain on their C-terminus and phosphorylate two serines or threonines of dual-specific MAPKKs, which phosphorylate both serine/threonine and tyrosine residues of the active site S/T-X-Y motif of MAPKs.

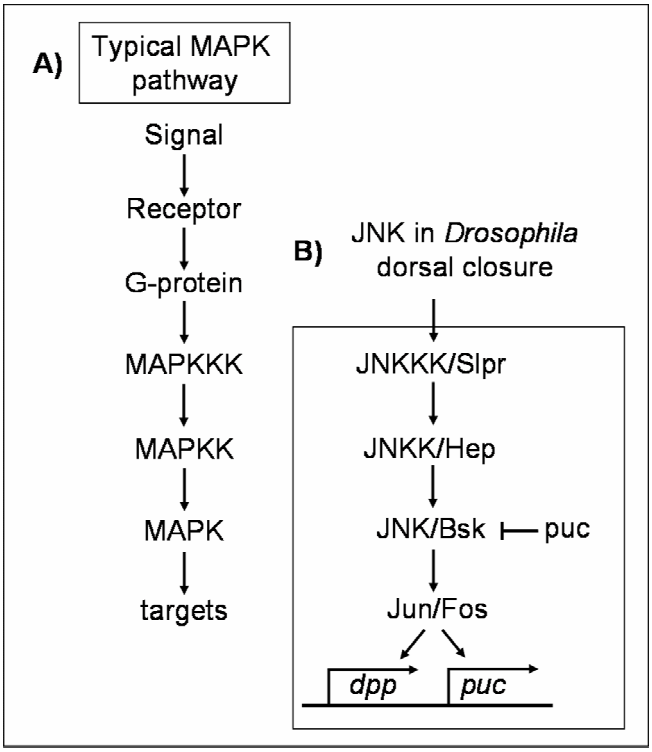

Fig. 4. **General MAP kinase pathway signaling.**
(**A**) Signal transduction through a canonical MAPK cascade. (**B**) JNK pathway components active during *Drosophila* embryonic dorsal closure. The homologous kinases are named Slipper (Slpr), Hemipterous (Hep) and Basket (Bsk). They lead to activation of genes including *dpp* (a TGFβ) and *puckered* (*puc*), which encodes a JNK/Bsk phosphatase and thus provides a negative feedback loop.

Three MAPK cascades are known in *Drosophila* – the Jun N-terminal Kinase (JNK) pathway (in mammals also known as Stress-Activated Protein Kinase, SAPK), Extracellular signal-regulated protein kinase (Erk), and p38 kinase cascade. These are conserved with MAPK pathways known in mammals, but display less complexity and lower degree of redundancy. MAP kinases differ in stimuli that activate them, in the targets they phosphorylate, and consequently in processes they regulate.

JNK as well as other MAPK pathways can activate the Activator protein-1 (AP-1) via phosphorylation or via transcriptional induction of AP-1 molecules. AP-1 is a heterodimer comprised of basic region leucine zipper (bZIP) transcription factors, typically Jun, Fos or ATF (Fig. 4B). The bZIP transcription factors contain a leucine zipper that enables them to dimerize, and a region of basic amino acids that mediates DNA binding. Jun and Fos bear MAPK-specific phosphorylation sites and upon activation, the AP-1 complex can bind DNA and activate or repress target genes (van Dam & Castellazzi, 2001).

1.5 Developmental roles of Jun N-terminal kinase pathway in *Drosophila*

Similar to other eukaryotes, *Drosophila* JNK signaling plays important roles from embryogenesis to adulthood. JNK-dependent embryonic dorsal closure, thorax closure during metamorphosis, planar cell polarity, and regulation of apoptosis are all necessary for proper development of *Drosophila* (Kockel et al., 2001). JNK is also involved in wound healing, stress and immune response, growth and aging (Liu, 2006). Finally, JNK signaling is also active during *Drosophila* oogenesis.

1.5.1 JNK in embryonic dorsal closure and pupal thorax closure of *Drosophila*

During *Drosophila* embryogenesis, germband retraction leaves the embryo with the extraembryonic amnioserosa but no epidermis on the dorsal side. In the process of dorsal closure (DC), lateral sheets of epidermis move dorsally across the amnioserosa to close the embryo (Kockel et al., 2001; Homsy et al., 2006). The closure has three stages: initiation, spreading and suturing. During the initiation, dorsal-most row of epidermal cells (called leading edge, LE) start to elongate along dorso-ventral axis and to accumulate myosin and F-actin in actin-nucleating centers (ANCs), which organize the LE cytoskeleton. *JNK/bsk*, *jun* and *fos* are expressed in the LE cells, but down-regulated in the amnioserosa (via *puckered* expression, Fig. 4). These genes seem to affect the maturation and stabilization of the ANCs and also stimulate the formation of filopodia and lamellipodia. After an actin cabel is created in the LE, the cells of the LE as well as the rest of the epithelium start to spread over the amnioserosa. *jun* and *fos* are expressed in the spreading epithelial sheets. Finally, LEs from the opposite sides fuse at the dorsal midline and form stable adhesion junctions (Martin & Wood, 2002). Mutations in *JNK/bsk*, *jun* and *fos* disrupt DC and produce embryos with a dorsal open phenotype (Sluss et al., 1996; Kockel et al., 1997; Zeitlinger et al., 1997).

Thorax closure (TC) occurs during metamorphosis, when the larval epidermis of the thorax must be replaced by the imaginal epithelium. The notum of the adult fly originates

from the bases of wing imaginal discs that, several hours after puparium formation, span across the thorax until they meet and fuse at the dorsal midline (Usui and Simpson, 2000). The process is similar to dorsal closure, but differs in some aspects. Rather than being driven by cell shape changes, TC depends on cell migration (Zeitlinger & Bohmann, 1999). The leading edge of the wing imaginal discs is not one but several rows of cells wide, and *dpp* expression is not regulated by JNK in the thorax (Agnes et al., 1999). Overexpression of *puckered* or disrupted *hep*, *bsk*, *fos* or *jun* function all lead to incomplete TC, manifest as cleft of the imaginal epidermis and cuticle along the dorsal midline. Therefore, JNK signaling is important for epithelial movements during both embryonic and adult tissue closure processes.

1.5.2 JNK function in *Drosophila* apoptosis

In *Drosophila*, apoptosis can be activated by both intrinsic and extrinsic death signals. Extrinsic (extracellular) death pathway starts at the cell membrane where Eiger, a tumor necrosis factor (TNF) homolog, binds its receptor Wengen. Signal is then transmitted via the JNK pathway and culminates in apoptosis (Igaki et al., 2002; Moreno et al., 2002b). Intrinsic cell death pathway can be triggered through expression of the effectors of apoptosis – Reaper, Hid and Grim. In response to gamma irradiation, JNK pathway activates the cell death effector genes, which elicit caspase-mediated apoptosis (McEwen & Peifer, 2005). JNK-mediated cell death is necessary for normal morphogenesis of the wing (Adachi-Yamada et al., 1999; Moreno et al., 2002a), and ectopic JNK activation in the eye imaginal disc induces cell death and leads to an eye ablation phenotype (Takatsu et al., 2000). JNK signaling can also serve as a relay of compensatory proliferation induced by apoptotic cells in imaginal discs (Ryoo et al., 2004).

1.5.3 JNK, Jun and Fos in *Drosophila* oogenesis

During *Drosophila* oogenesis, formation of the egg respiratory appendages requires the shaping of anterior and dorsal follicle cells (FC). This is achieved by cooperation of several signaling pathways including JNK (Fig. 5). Fos is expressed in the main body FC during their posterior migration. Follicles containing FC clones homozygous for an amorphic *fos* allele (kay^1) degenerate during stage 9 due to failed FC migration (Dequier et al., 2001). Fos is also required for the dumping of the maternal components from the NC into the oocyte. JNK, Jun and Fos are expressed in the anterior dorsal floor cells, where they regulate the morphogenetic reorganization of the FC that will secrete the dorsal appendages. In FC clones mutant for *JNK*, *jun* or *fos*, the dorsal appendages are abnormally short (Dequier et al., 2001;

Dobens et al., 2001; Suzanne et al., 2001). Likewise, altered activity of Puckered, the JNK phosphatase, leads to abberrant dorsal appendages and incomplete NCs dumping (Dobens et al., 2001). Delayed disappearance of NC nuclei in follicles lacking Fos suggest that Fos is required for timely apoptosis of the NC after dumping of their material into the oocyte (Dequier et al., 2001). Recently, JNK has also been shown to stimulate mitoses in FC prior to stage 6, when the mitotic-to-endocycle transition occurs (Jordan et al., 2006).

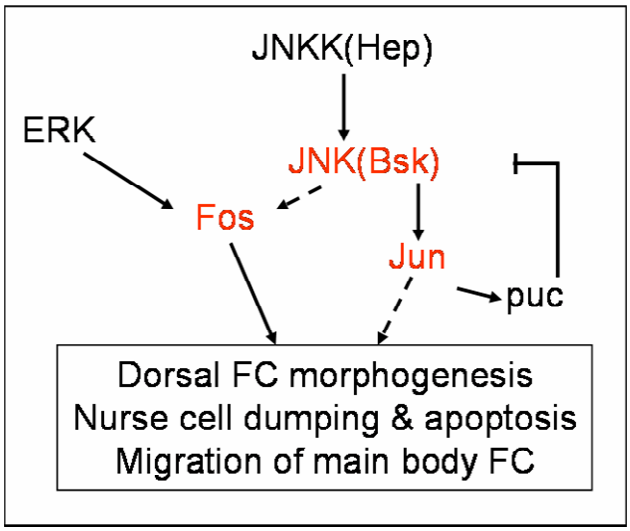

Fig. 5. **Role of JNK, Jun and Fos in *Drosophila* oogenesis**. At least Fos may act downstream of both JNK and ERK, possibly in different processes during oogenesis.

In summary, although Jun, Fos and JNK apparently play some roles in *Drosophila* oogenesis, mainly in the FC epithelium, their loss-of-function phenotypes are not very robust (particularly not in the case of Jun) and therefore do not directly uncover the requirement of these genes for oogenesis. Roles of Jun, Fos and JNK in insect oogenesis have not been addressed outside the *Drosophila* model, and nothing is known about them in the case of the telotrophic ovary. Since Jun, Fos and JNK are structurally conserved in *Tribolium*, this species can now be used for such a comparative study.

2. Goals of the work

1. To clone *Tribolium* genes encoding the Jun N-terminal kinase (JNK) and its targets Tc-Jun and Tc-Fos

2. To knockdown *Tc-JNK*, *Tc-jun* and *Tc-fos* genes by using systemic RNAi and determine their requirement for *Tribolium* development

3. To analyze the role of *Tc-JNK*, *Tc-jun* and *Tc-fos* in *Tribolium* oogenesis

3. Results

3.1 Sequence conservation of *Tribolium jun, fos* and *JNK* orthologs

We have cloned single orthologs of *Drosophila jun, fos* and *JNK* genes from the sequenced *Tribolium* genome. Based on homology, mainly of the functional domains and phosphorylation sites, with *Drosophila* (Fig. 6) and humans, we will refer to these genes as *Tc-jun, Tc-fos* and *Tc-JNK*, respectively. We will use designations, *Dm-jun, Dm-fos* and *Dm-JNK* for the *Drosophila* genes. We choose this nomenclature to avoid confusion with the multiple names used in *Drosophila* (e.g., *jra* for *jun, kay* or *fra* for *fos,* and *bsk* for *JNK*).

Similar to Dm-Jun, the Tc-Jun ORF (227 amino acids) is encoded by a single exon, and the basic-region leucine zipper (bZIP) domain is located at the C-terminus of the protein. Three conserved serine/threonine-proline (S/TP) phosphorylation sites (T47, T55, T70) and a conserved tyrosine residue (Y116) were found in Tc-Jun. Although the entire protein homology is lower than 50%, the bZIP domain is well conserved (Fig. 6).

The structure of the *Tc-fos* gene is highly similar to *Dm-fos.* The bZIP domain is located in the middle of the protein and conserved S/TP phosphorylation sites were found at its both ends (Fig. 6). Tc-Fos is much smaller than Dm-Fos (366 aa vs. 595 aa), and is more similar in size to four mammalian Fos proteins (ranging from 271 to 380 aa). Unlike Tc-Fos, Dm-Fos carries an N-terminal region similar to a delta domain in Dm-Jun. Two JNK-specific S/TP phosphorylation sites at the amino-end (T37, T41) are conserved as well as four of seven JNK/ERK phosphorylation sites at the carboxy-end (S226, T308, T328, S354). We therefore assume that Tc-Fos, as well as Tc-Jun, is a potencial target of Tc-JNK and/or Tc-ERK. Surprisingly, as in *Drosophila*, one leucine within the leucine zipper is substituted (by tyrosine in Tc-Fos and by methionine in Dm-Fos).

The *Tribolium* ortholog of Jun N-terminal kinase is the best conserved of the three studied genes both in sequence and length (Fig. 6). The only low-homology parts are found at the very beginning and at the end of Tc-JNK. Based on genome data, two alternative first exons may occur, predicting almost identical-size proteins with an ATP-binding motif. The central part of Tc-JNK has a typical MAP kinase signature with a serine/threonine protein-kinase active site including a conserved aspartic acid residue. A dual-specificity threonine-proline-tyrosine phosphorylation motif resides in the middle of the protein.

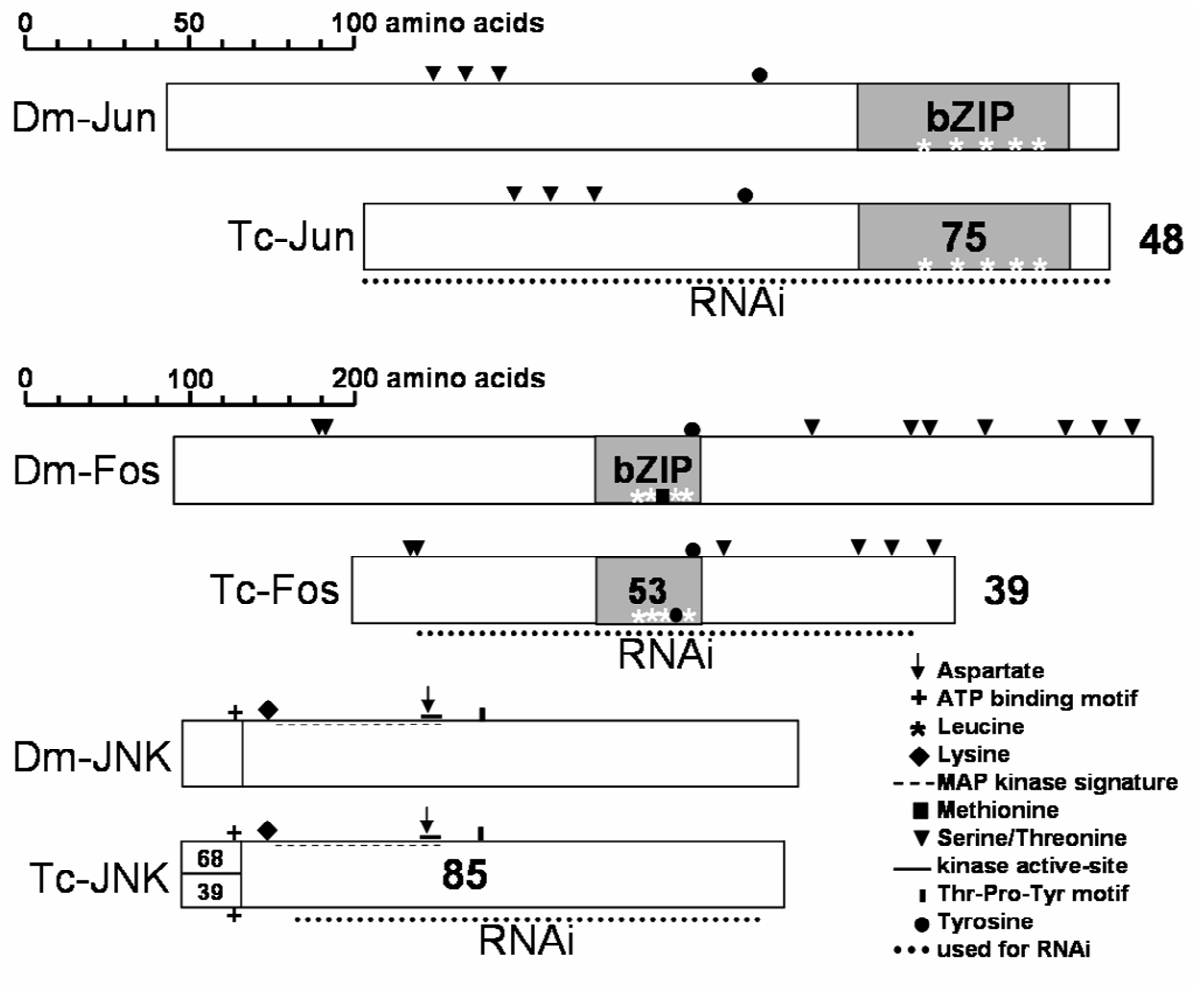

Fig. 6. **Similarity of *Tribolium* Jun, Fos and JNK to *Drosophila* orthologs**. Schematic representations of Jun, Fos and JNK proteins compared to *Drosophila* orthologs. Numbers indicate amino acid identities of whole proteins and within bZIP domains, respectively. In Tc-JNK, small numbers indicate amino acid identities between each of the alternative *Tc-JNK* exons and the corresponding region of Dm-JNK, in which the "+" symbols represent ATP binding motifs. The lines with arrows (aspartic acid residues) denote the serine/threonine protein kinases active-site signature. Other conserved features are marked with specific symbols.

3.2 Developmental expression of *Tc-jun* and *Tc-fos*

Tc-jun and *Tc-fos* were expressed throughout *Tribolium* development (Fig. 7A). The levels of both mRNAs became higher during the pupal stage and in freshly hatched adults; then the expression seemed to decline with age. Continuous expression of both genes was observed in three age groups of reproductively active females (Fig. 7B). RT-PCR with dissected ovaries confirmed that both *Tc-jun* and *Tc-fos* mRNAs were expressed in ovaries (Fig. 7C). *Tc-JNK* was not detected by RT-PCR until the number of cycles was increased to 30 (data not shown) and therefore it is possible that a basal *Tc-JNK* expression level in the ovary is relatively low.

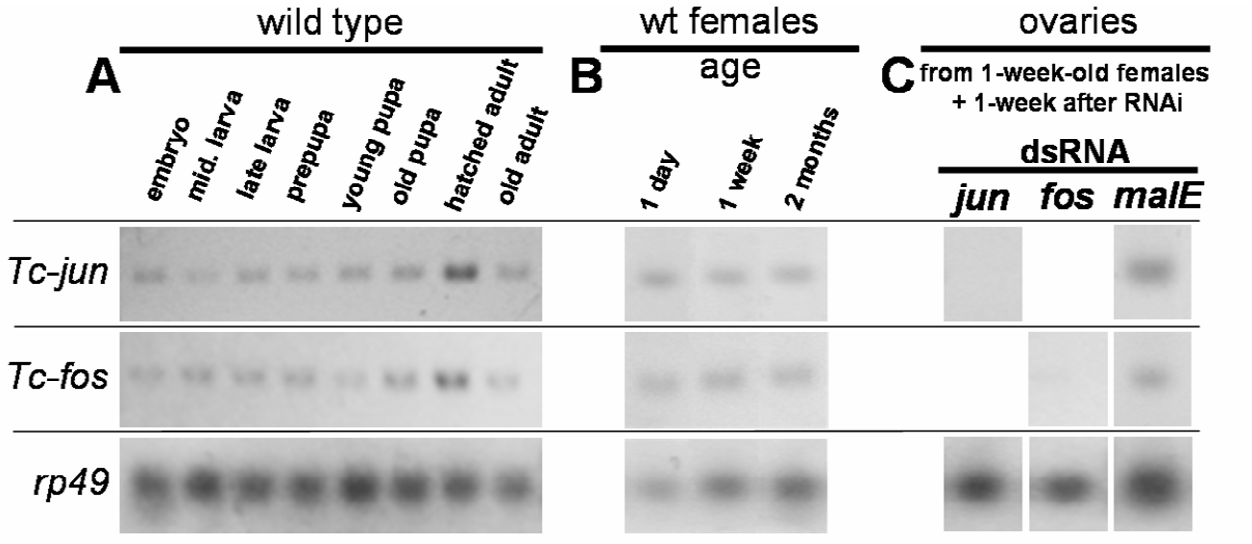

Fig. 7. **Developmental expression and knockdown of *Tc-jun* and *Tc-fos*.** Total RNA was isolated from indicated stages, treated with DNase, and used for RT-PCR with 24 temperature cycles; amplification of *rp49* mRNA served as a loading control. (**A, B**) *Tc-jun* and *Tc-fos* were expressed in all developmental stages and in reproductive females. (**C**) RNAi silenced *Tc-jun* and *Tc-fos* gene expression in ovaries as well as in whole beetles (not shown). Expression of *Tc-jun* and *Tc-fos* was unaffected in control *malE* dsRNA ovaries.

3.3 RNAi silencing of *Tc-jun* and *Tc-fos* disrupts *Tribolium* oogenesis

Tribolium females mate and start laying eggs shortly after eclosion and continue for at least several months of their adult life. One-week-old females injected with a control dsRNA (*malE*, a heterologous gene from *E. coli*) quickly recovered from the injection and resumed laying eggs (Fig. 8, top). In contrast, females injected with *Tc-jun* or *Tc-fos* dsRNA stopped laying eggs within 2-3 days post injection, and did not recover during the observed period of 10 days (Fig. 8). The few eggs laid by females after *Tc-jun* or *Tc-fos* RNAi treatment likely escaped the RNAi, possibly because they might have matured before RNAi took effect and were retained by the females. Also, the knockdown may not have been equally effective on all ovarioles, allowing some eggs to develop. RT-PCR showed depletion of *Tc-jun* and *Tc-fos* mRNAs in *Tc-jun*(*RNAi*) and *Tc-fos*(*RNAi*) ovaries, respectively, and in whole females (not shown) compared to controls (Fig. 7C).

Surprisingly, *Tc-JNK* RNAi had no effect on egg laying when compared with the *malE* dsRNA control. However, almost no larvae hatched from the *Tc-JNK*(*RNAi*) eggs, indicating a requirement of Tc-JNK for embryogenesis (Fig. 8). Larvae hatched normally from embryos produced by females injected with control *malE* dsRNA.

Since perturbed function of many genes is known to block oviposition, rather than oogenesis, in *Tribolium* (M. Schoppmeier, personal communication), we dissected ovaries from *Tc-jun*(*RNAi*) and *Tc-fos*(*RNAi*) females to determine the cause of the egg-laying defect.

Staining of the ovaries with the DNA-specific dye DAPI revealed that both *Tc-jun*(*RNAi*) and *Tc-fos*(*RNAi*) females had ovaries with massively degenerating follicles, where none or very few mature oocytes proceeded to the oviduct (Fig. 9). Ovaries from neither control *malE* nor from *Tc-JNK*(*RNAi*) females showed such degeneration (Fig. 9).

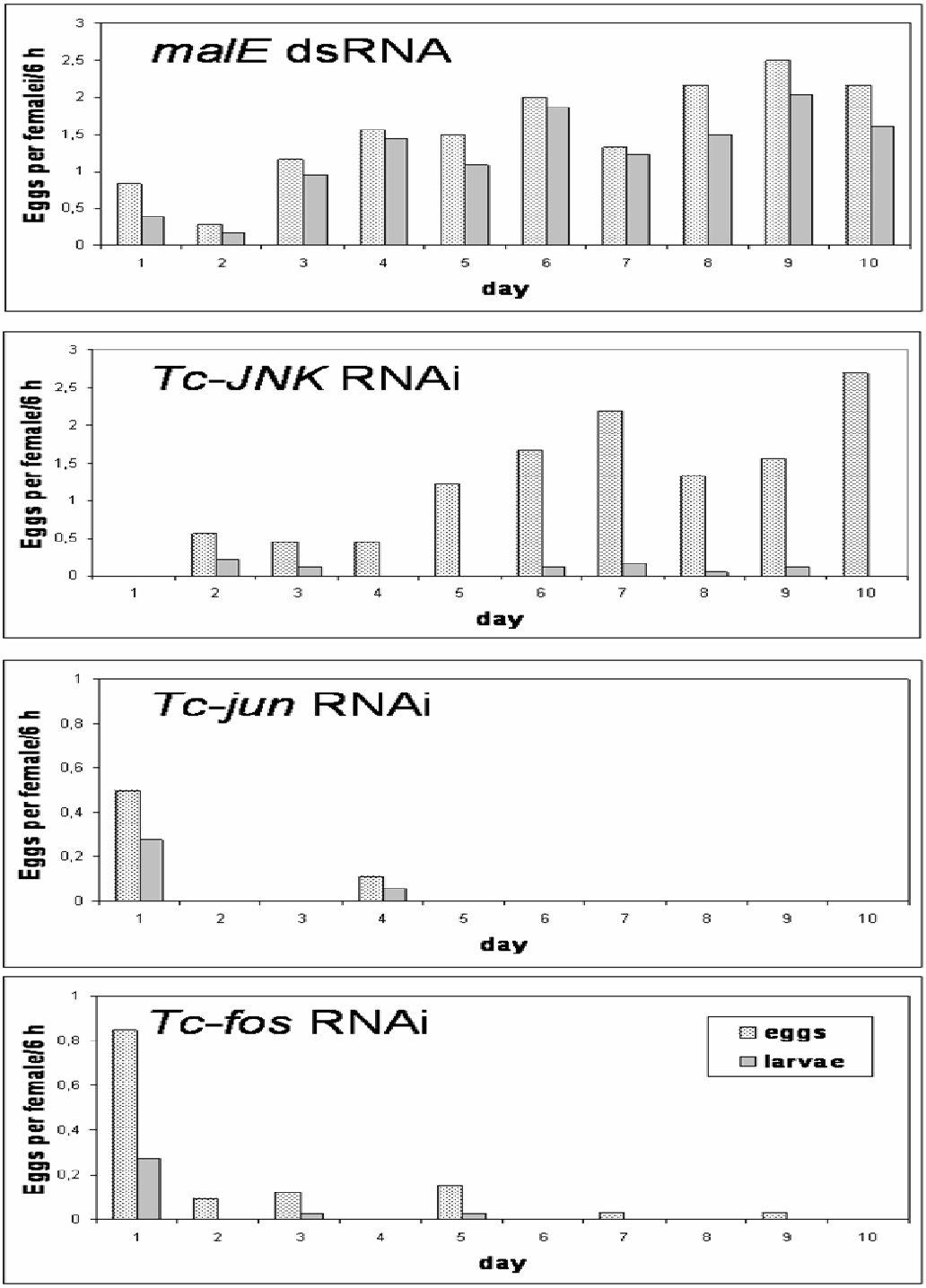

Fig. 8. **Loss of *Tc-jun* and *Tc-fos* blocks egg laying**. One-week-old mated females were injected with dsRNA, recovered on wholemeal flour, mixed with males and allowed to lay eggs for 6 h each day on white flower, then they were fed on wholemeal flour for the remaining 18 h. Laid eggs and hatched larvae were counted and values presented as average per female and 6 h. Numbers of females were: 18 for each *malE, Tc-jun* and *Tc-JNK;* 33 for *Tc-fos*. Note the different Y scales.

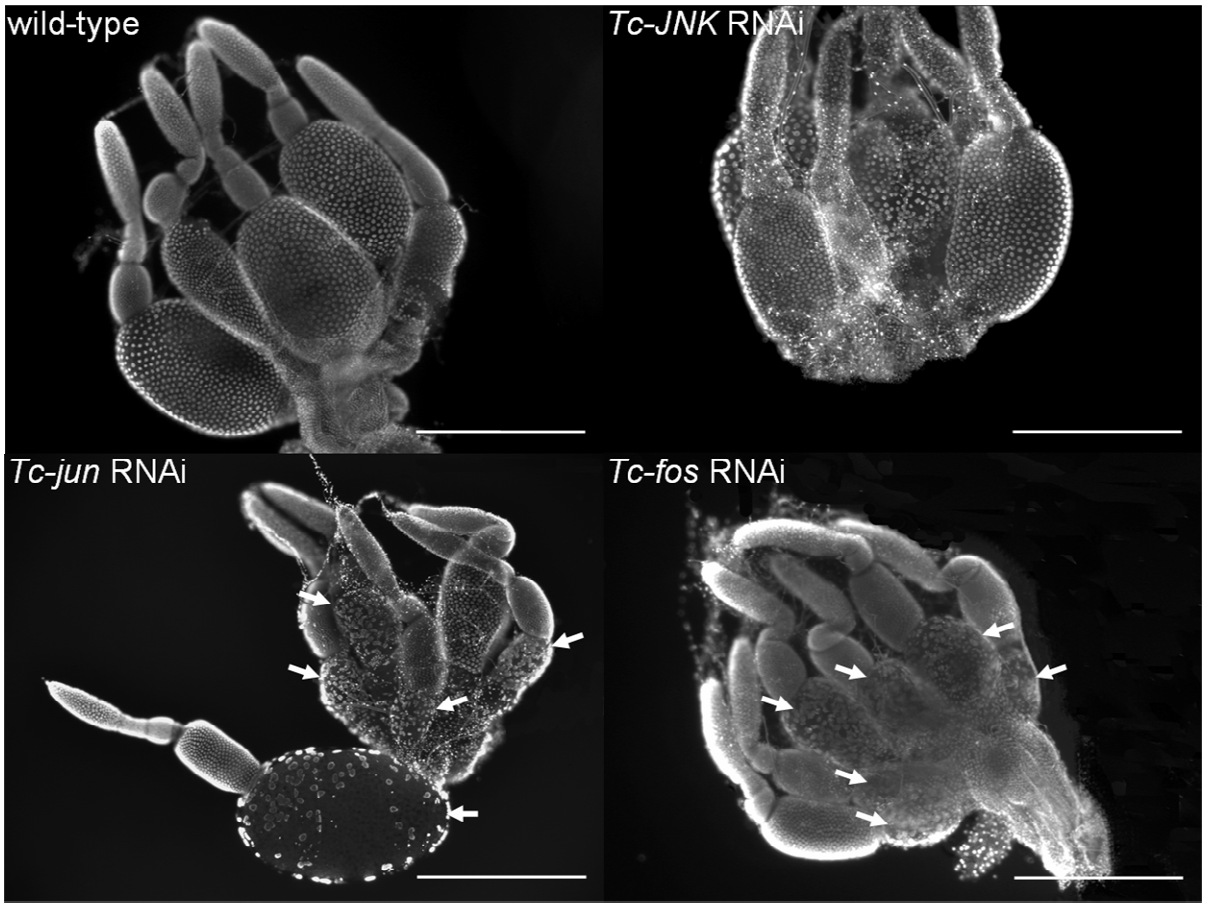

Fig. 9: *Tc-jun* and *Tc-fos* RNAi disrupts *Tribolium* oogenesis. Ovaries were dissected from females 7 days after dsRNA injection and stained with DAPI. Wild-type ovaries from untreated females (top left), from *malE* dsRNA-injected females (not shown) and *Tc-JNK(RNAi)* ovaries (top right) produced normal mature oocytes. *Tc-jun(RNAi)* and *Tc-fos(RNAi*) ovaries showed degeneration of follicle cells (arrows; compare with wild-type follicle cells) and almost no large terminal oocytes (one shown in *Tc-jun(RNAi)*, bottom). Scale bars = 500 µm.

3.4 Follicle cells in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females proliferate normally but degenerate by apoptosis at mid-oogenesis

Figure 9 suggests that follicles lacking *Tc-jun* or *Tc-fos* function develop normally during early steps of oocyte development. To understand which phase of oogenesis failed upon *Tc-jun* or *Tc-fos* knockdown, we first examined whether the FC proliferated normally. FC proliferate in the zone of prefollicular cells and during the previtellogenic stage of a growing oocyte (Trauner & Büning, 2007). Staining of anti-phospho-Histone H3 that marks mitotic cells during prophase and disappears at telophase showed that FC divided normally, within the natural variability observed among ovarioles (Trauner & Büning, 2007), in both *Tc-jun(RNAi)* and *Tcfos(RNAi)* females (Fig. 10). Incorporation of bromo-deoxyuridine (BrdU) confirmed that also DNA synthesis in both dividing and endoreplicating FC showed no difference from control ovaries (Fig. 10).

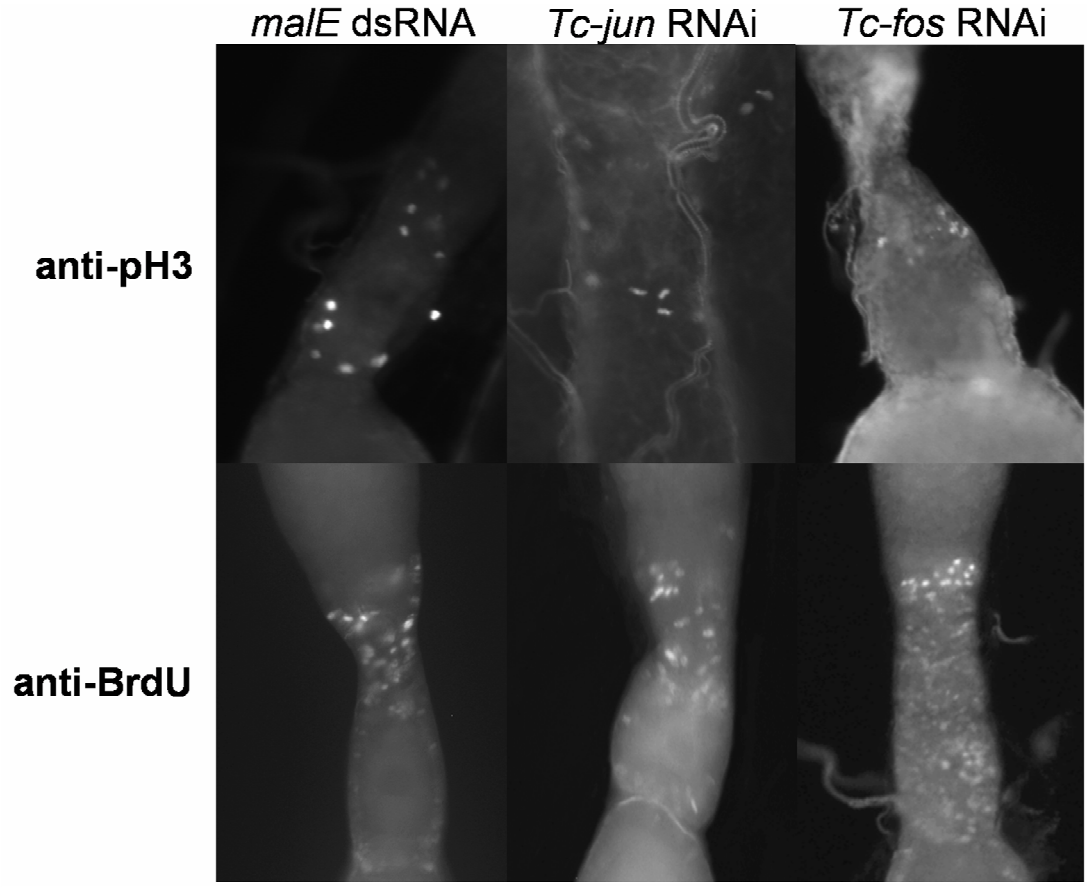

Fig. 10. **Follicle cells proliferate normally in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* ovarioles.** Posterior parts of tropharia and anterior parts of vitellaria stained for phospho-Histone-H3 and for incorporated BrdU showed normal proliferation and replication, respectively, of FC in control *malE* and *Tc-jun(RNAi)* or *Tc-fos(RNAi)* females. The region of BrdU-stained FC extends more posteriorly because BrdU incorporation also marks endoreplicating FC. The observed differences in the number of pH3 or BrdU positive cells are within natural variability observed in wild-type ovarioles and are also caused by focusing on only some of the signals. Anterior is up in all panels.

Nuclear morphology of FC in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* ovaries (Figs. 9 and 11) indicated that these FC might be undergoing apoptosis in growing follicles. We tested that by using the TUNEL technique, in which DNA breaks typical for apoptosis become labeled by incorporation of fluorescent dUTP. TUNEL showed positive staining in mid-stage follicles of ovaries from *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females but not from *wild-type* or *malE* control females (Fig. 12 and not shown). The TUNEL signals often corresponded with apparent chromatin fragmentation that was visible by DAPI staining. Moreover, *Tc-jun* and *Tc-fos* RNAi caused disruption of actin cytoskeleton in the FC epithelium (Fig. 12, compare phalloidin staining).

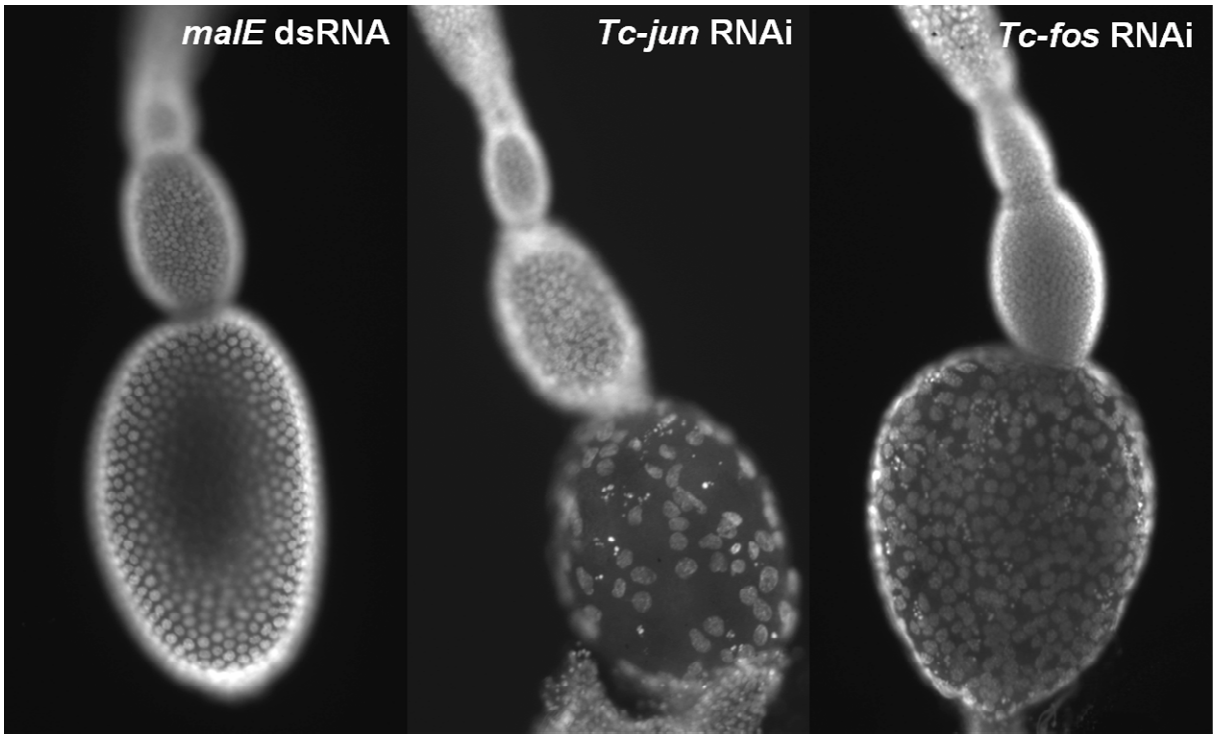

Fig. 11. **Degenerated follicle cells in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females.** DAPI staining of *Tc-jun(RNAi)* and *Tcfos(RNAi)* ovarioles showed massive degeneration of FC of mid-vitellogenic oocytes. Note the irregular spacing and shape of FC nuclei and the apparent DNA fragmentation. Anterior is up.

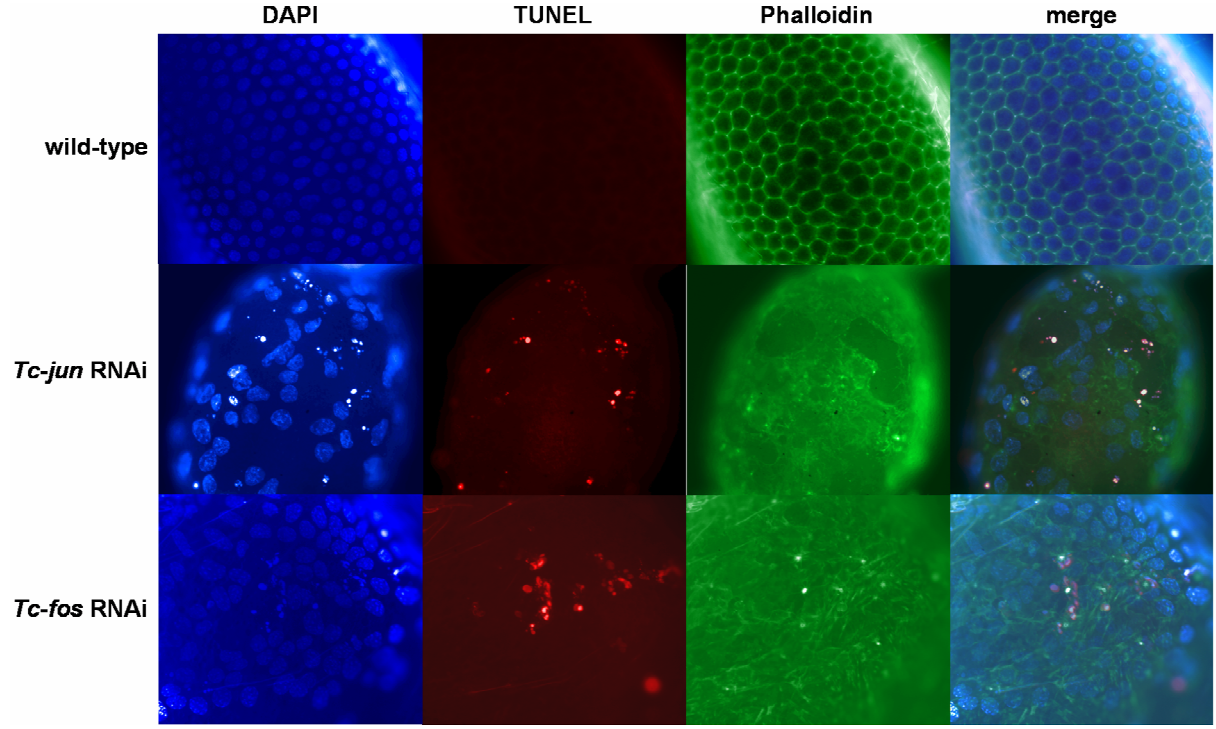

Fig. 12. ***Tc-jun* and *Tc-fos* RNAi causes apoptosis of follicle cells during mid-oogenesis**. Apoptotic TUNEL-positive bodies are present in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* follicles (red). The actin cytoskeleton (green) of the follicle epithelium is completely disorganized. The follicles are of similar stage, although the *Tc-jun(RNAi)* follicle is slightly younger (with a smaller oocyte) than the other two examples.

3.5 Apoptosis due to loss of *Tc-jun* and *Tc-fos* requires caspase activity

To test whether degeneration of FC in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females occurs through caspase-dependent cell death, we blocked the canonical apoptotic pathway by silencing of *Tc-ICE*. We have identified and cloned *Tc-ICE* as a *Tribolium* ortholog of the effector Caspase-3 (drICE in *Drosophila*). For RNAi, we used a sequence corresponding to 239 amino acids with a 61% identity to drICE.

When co-injected into adult females together with *Tc-jun* or *Tc-fos* dsRNAs, the *Tc-ICE* dsRNA suppressed FC apoptosis in mid-vitellogenic follicles (Figs. 13 and 14). In contrast to silencing of *Tc-jun* or *Tc-fos* alone (Fig. 12), no TUNEL staining was observed in the *Tc-jun; Tc-ICE* or *Tc-fos; Tc-ICE* double-RNAi FC at the same or later stages (Figs. 13 and 14). Although *Tc-ICE* RNAi restored follicle development in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females through mid-oogenesis, it was insufficient to rescue egg production completely, and the FC cell morphology eventually became defective in *Tc-jun; Tc-ICE* (Fig. 14) as well as in *Tc-fos; Tc-ICE* double-RNAi ovarioles. These results suggest that depletion of Jun and Fos causes caspase-dependent apoptosis of FC at mid-oogenesis, but that both proteins (or Tc-ICE) might have additional roles in later follicle development.

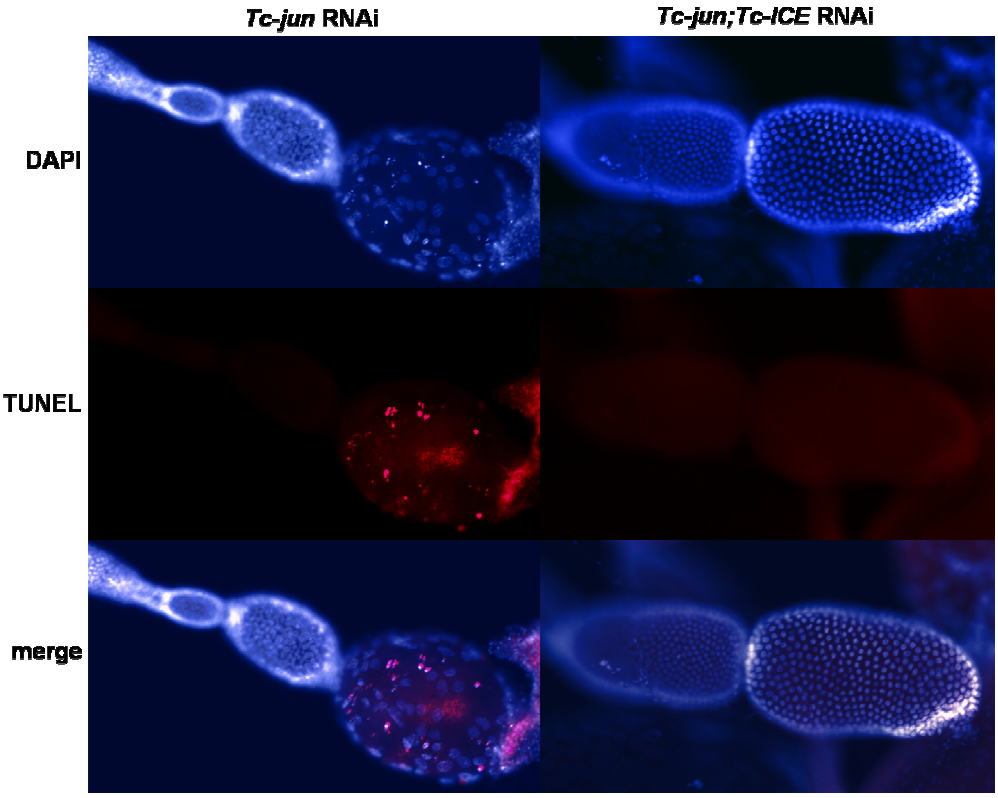

Fig. 13. **Apoptosis of *Tc-jun(RNAi)* follicle cells requires caspase activity**. Co-injection of *Tc-ICE* dsRNA prevented *Tc-jun* RNAi from causing degeneration and TUNEL staining (red) in FC of mid-stage follicles. Anterior is to the left.

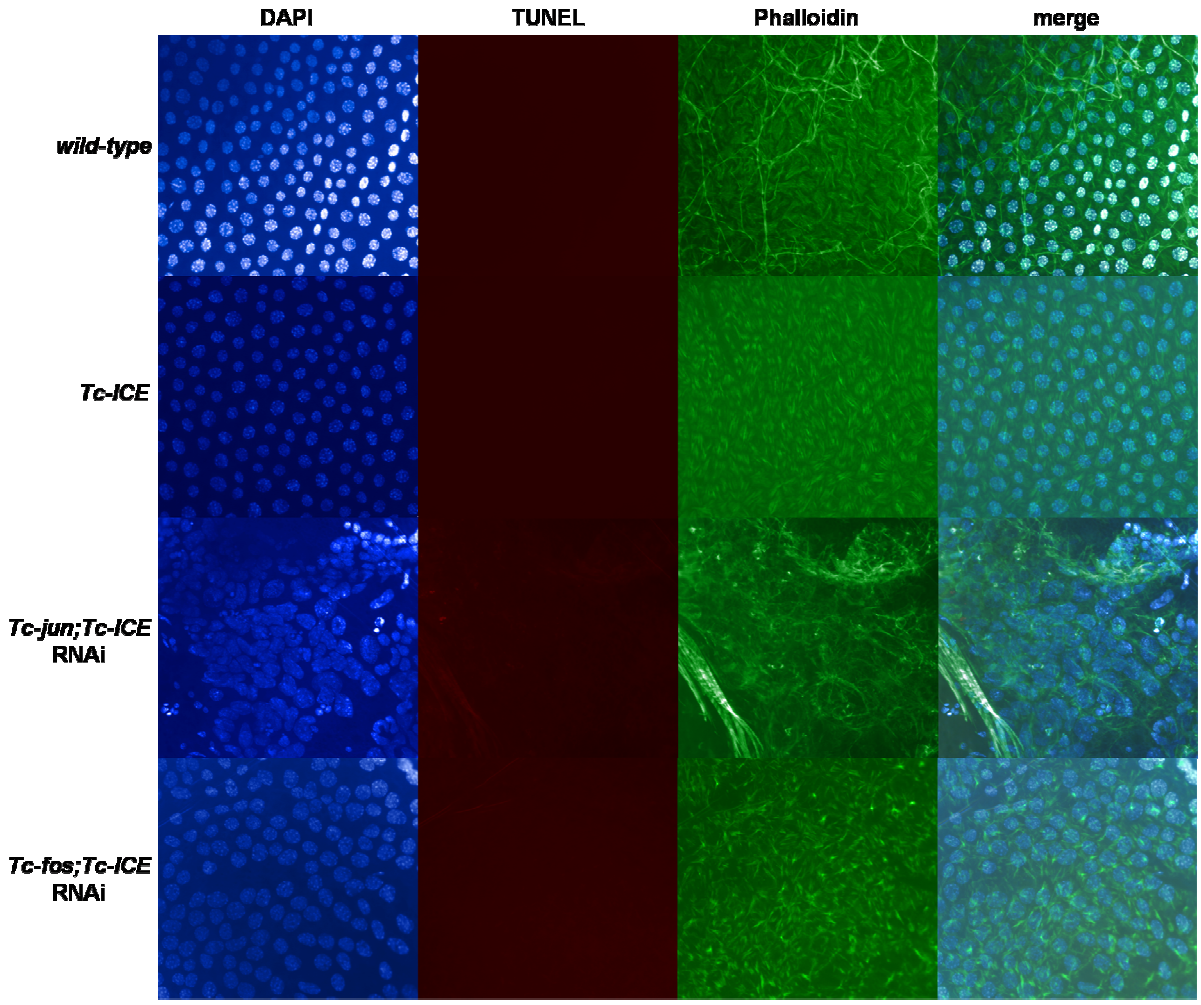

Fig. 14. ***Tc-ICE*** **RNAi blocks apoptosis but does not rescue follicle cells lacking Jun and Fos function during late oogenesis**. Late-oocyte FC in wild-type or in *Tc-ICE(RNAi)* females showed no TUNEL staining. Silencing of *Tc-ICE* prevented apoptosis in *Tc-jun; Tc-ICE* and *Tc-fos; Tc-ICE* RNAi FC, although the follicle epithelium still could not develop normally to its terminal stage. *Tc-jun; Tc-ICE* follicle is of slightly later stage.

4. Discussion

This study has revealed new and unexpected function of the AP-1 transcription factors Jun and Fos during insect oogenesis. Unlike in the polytrophic ovary of *Drosophila*, Jun and Fos are necessary for survival of follicle epithelial cells during oocyte development in the telotrophic ovary of *Tribolium*. Without proper function of Tc-Jun and Tc-Fos, the FC degenerate by caspase-dependent cell death during mid-oogenesis in the beetle.

4.1 Structure and function of *Tribolium* Jun, Fos and JNK

High degree of homology between Tc-Jun, Tc-Fos and their respective *Drosophila* and mammalian counterparts within the conserved bZIP domains suggest that we have cloned orthologs of the Jun and Fos transcription factors. We have found no genes with a closer homology to Jun and Fos in the *Tribolium* genome sequence. Importantly, positions of the MAP kinase target phosphorylation sites known from *Drosophila* proteins (Peverali et al., 1996; Ciapponi et al., 2001) are conserved in both Tc-Jun and Tc-Fos, suggesting that these may be true targets of Tc-JNK. The putative *Tribolium* JNK is highly similar to its *Drosophila* ortholog, and presence of the essential protein-kinase active site indicates that Tc-JNK is a member of the MAP kinase family. Similar to the situation in *Drosophila melanogaster* (Perkins et al., 1990; Riesgo-Escovar et al., 1996), Jun, Fos and JNK are encoded by single genes also in *Tribolium*.

Whether *Tribolium* Jun and Fos proteins form a heterodimer and act together as the canonical Activator protein-1 (AP-1) known from mammals or *Drosophila* remains to be tested. However, the nearly identical phenotypes observed in the ovaries of *Tc-jun(RNAi)* and *Tc-fos(RNAi)* females suggest that this may be the case, at least during oogenesis. In contrast to the clear overlap between *Tc-jun* and *Tc-fos* RNAi phenotypes observed in *Tribolium*, the common developmental defects caused by loss of *jun* and *fos* function in *Drosophila* are limited to failed embryonic dorsal closure (Kockel et al., 1997; Riesgo-Escovar & Hafen, 1997) and incomplete adult thorax closure (Zeitlinger and Bohmann, 1999; Jindra et al., 2004). However, effects of *jun* mutations on *Drosophila* oogenesis are quite subtle and different from those of *fos* (Suzanne et al., 2001). Surprisingly, silencing of *Tc-JNK* did not affect oogenesis, suggesting that Jun and Fos might be part of another pathway in the *Tribolium* ovary.

While in this study we focused on *Tribolium* oogenesis, we also noticed additional defects upon *Tc-jun*, *Tc-fos* and *Tc-JNK* dsRNA injection. First, parental *Tc-JNK* RNAi

disrupted embryogenesis. It will be interesting to examine these embryos in detail, since in *Drosophila*, JNK, Jun and Fos are all required for dorsal closure, a process that occurs somewhat differently in the short germ band beetle embryo. Since parental RNAi of *Tc-jun* and *Tc-fos* blocked oogenesis and thus precluded observation of embryos, examination of the potential role of these genes in *Tribolium* dorsal closure will require embryonic RNAi. We were also interested in JNK, Jun and Fos function during metamorphosis, which we tested by dsRNAi injections into last or penultimate instar larvae. *Tc-jun* (but not *Tc-fos*) RNAi often compromised larval-pupal ecdysis, and some animals were unable to emerge properly from the pupal cuticle. Problems with adult eclosion were also observed in *Tc-JNK(RNAi)* and *Tc-fos(RNAi)* animals, and based on experience from our laboratory and from others, we suspect that this might be a nonspecific defect common to many genes. Since *Tribolium* thorax is not built from imaginal discs, we have not seen phenotypes similar to the *Drosophila* thorax closure defect. There were also no signs of perturbed planar polarity upon *Tc-jun, Tc-fos* or *Tc-JNK* silencing. Surprisingly however, injection of adults with high doses (1-2 μg) of *Tc-jun* dsRNA invariantly killed the beetles within several days.

4.2 Jun and Fos are critical for *Tribolium* oogenesis

Follicle cells (FC) are critical for oogenesis from the time a follicle is created until the vitelline membrane and chorion are deposited. Loss of Jun and Fos caused disruption of the follicle epithelium during mid-vitellogenic stages as judged by nuclear morphology and disorganized actin cytoskeleton. TUNEL staining and the ability of Tc-ICE/caspase 3 knockdown to suppress the FC degeneration strongly suggested an untimely trigger of apoptotic death mechanism in *Tc-jun(RNAi)* or *Tc-fos(RNAi)* ovaries. Unfortunately we could not document this effect more directly because the available anti-active Caspase 3 did not seem to work in the *Tribolium* ovary.

Drosophila FC undergo apoptosis naturally at late stages of follicle development, when apoptotic bodies are phagocytosed at the entry to the oviduct (Nezis et al., 2002). A similar situation can be observed in *Tribolium* where FC become TUNEL positive after a complete egg leaves the follicle (V. Smykal, unpublished). *Tc-ICE* RNAi alone seemed to suppress also this natural apoptosis in the terminal-stage follicles. In combination with *Tc-jun* or *Tc-fos* RNAi, silencing of *Tc-ICE* averted the mid-vitellogenic ectopic FC apoptosis, then in the terminal-stage follicles a disruption of cytoskeleton but no positive TUNEL staining was observed, indicating that the apoptotic program might have been triggered but could not be executed (Nezis et al., 2006).

Based on our results, the role of JNK signaling in oogenesis is quite different between *Tribolium* and *Drosophila*. This is likely because of major differences in the organization of the beetle telotrophic versus the fly polytrophic ovaries (Trauner & Büning, 2007). While the *Drosophila* follicle epithelium consists of several functional sub-populations of FC that undergo complex morphogenetic movements, only two types of FC have been distinguished in *Tribolium,* of which the main body FC remain stationary and only change shape during vitellogenesis. In *Drosophila,* JNK signaling is needed to stimulate FC proliferation in early-stage follicles (Jordan et al., 2006), while in *Tribolium Tc-JNK* (or Jun and Fos) RNAi had no influence on FC divisions. In *Drosophila,* Dm-JNK and Dm-Fos are required for nurse cell dumping of maternal components into the oocyte (Dequier et al., 2001) and for morphogenesis of specialized FC that will ensure proper differentiation of the dorsal appendages; Dm-Jun seems to play only a minor role in the latter process (Dobens et al., 2001; Dequier et al., 2001; Suzanne et al., 2001).

In a striking contrast to *Tribolium* Jun and Fos RNAi phenotypes, neither Dm-JNK nor Dm-Jun and Dm-Fos are required for FC survival or maintenance during vitellogenic stages, since FC clones homozygous for mutations in these genes survive normally. However, we cannot distinguish whether Tc-Jun and Tc-Fos function is strictly autonomous to the FC, because the systemic nature of RNAi in the beetle affects the germline and other tissues at the same time.

4.3 Role of Jun and Fos in maintenance of follicle cells

Why do follicle cells die prematurely without Tc-Jun and Tc-Fos? As judged from normal mitotic pH3 marker staining and BrdU incorporation, it is unlikely due to insufficient proliferation or growth by endoreplication of the *Tc-jun(RNAi)* or *Tc-fos(RNAi)* FC. Encapsulation of the oocyte as well as FC morphology appear unaffected until the mid-vitellogenic stage, when Tc-Jun and Tc-Fos become necessary for FC survival.

During vitellogenesis, the FC must withstand an enormous mechanical stress caused by the rapidly growing oocyte. The formerly columnar FC epithelium becomes stretched, without increasing the number of cells, to cover the expanding oocyte surface. How is this physical stress resistance ensured is not clear. We hypothesize that Tc-Jun and Tc-Fos act in the vitellogenic FC to increase their mechanical stress resistance and allow the major cell shape change. To support this idea, we sought some way of limiting the oocyte growth. Knockdown of yolk protein or vitellogenin receptor genes proved ineffective (K. Takaki and M. Jindra, unpublished observations). However, silencing of the ecdysone receptor gene *Tc-*

EcR in adult females completely blocked the oocyte growth (Fig. 15A; K. Takaki and M. Jindra, in preparation). In accord with our prediction, knockdown of *Tc-jun* or *Tc-fos* in females that had been treated with *Tc-EcR* dsRNA did not lead to FC apoptosis at the mid-vitellogenic stages (Fig. 15A). Although the small mutliple follicles were relatively old, no changes of FC morphology or TUNEL staining were observed in them. Moreover, ovarioles from females injected with diluted *Tc-EcR* dsRNA and concentrated *Tc-fos* dsRNA showed TUNE-positive FC specifically in larger, i.e. growing follicles but not in the small ones (Fig. 15B). This further supports our idea that FC apoptosis resulting from Tc-Jun or Tc-Fos deficiency directly correlates with the physical stretching of the follicle epithelium.

In our preliminary model (Fig. 16), mechanical stress resulting from oocyte growth activates a pathway where the AP-1 transcription factors Jun and Fos ensure cytoskeletal flexibility and thus promote epithelial cell-shape changes, necessary for FC survival. Such a function would correspond to the role of JNK and AP-1 during epithelial reshaping processes such as the embryonic dorsal closure and adult thorax closure in *Drosophila,* or wound healing in both *Drosophila* and vertebrates (Rämet et al., 2002; Harden, 2002; Galko and Krasnow, 2004; Homsy et al., 2006). Because *Tc-JNK* knockdown did not produce the same defect as *Tc-Jun* and *Tc-Fos* RNAi, and since a single well-conserved JNK is predicted in the beetle genome, it is likely that some other kinase pathway regulates AP-1 in the ovary. One candidate is Tc-Erk, which would be expected to phosphorylate the *Tribolium* AP-1 proteins. We are currently testing whether Tc-Erk or other kinases known to mediate resistance to mechanical stress in mammals might be required for follicle cell survival.

In conclusion, we have shown that the function of Tc-Jun and Tc-Fos in the telotrophic ovaries of *Tribolium* differs strikingly from the function in the polytrophic ovaries of *Drosophila*. While the *Drosophila* ovary is one of the best studied insect organs, almost nothing is known about genetic regulation of different types of insect oogenesis. Studies in *Tribolium* can substantially improve our understanding of molecular principles governing insect reproduction.

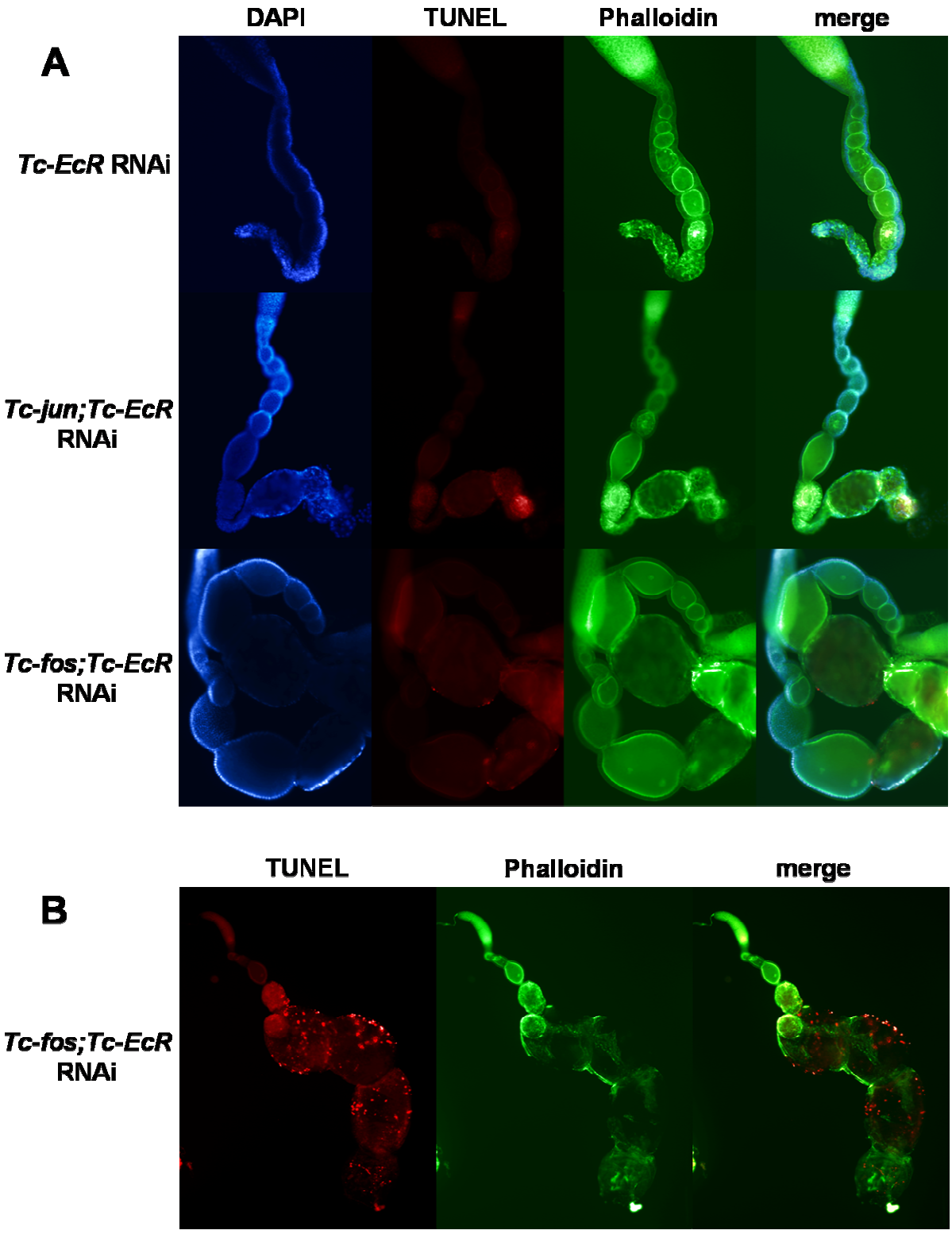

Fig. 15. **Block of oocyte growth prevents apoptosis in *Tc-jun(RNAi)* and *Tc-fos(RNAi)* follicles**. (**A**) *Tc-EcR* RNAi inhibits oocyte growth and FC in *Tc-jun; Tc-EcR* and *Tc-fos; Tc-EcR* double-RNAi ovarioles do not undergo apoptosis. Note the absence of TUNEL label in the small follicles. The red spot in TUNEL-stained *Tc-jun; Tc-EcR* RNAi ovariole is an artefact due to damage of the ovariole during dissection. (**B**) In *Tc-fos(RNAi)* follicles that grew larger than those shown in (A), TUNEL signal appeared. Anterior is up.

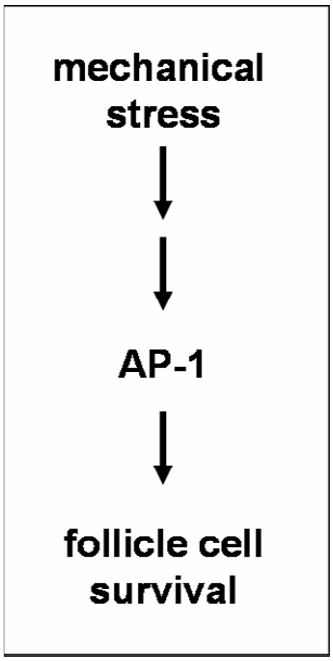

Fig. 16. **Model for AP-1 function in follicle cell survival**. Mechanical stress resulting from oocyte growth requires AP-1 to ensure epithelial tissue integrity. Molecules acting upstream of Jun and Fos remain to be identified.

5. Conclusions

1. *Tribolium* genes encoding Jun N-terminal kinase and its target proteins Jun and Fos were cloned. Sequence analysis confirmed that all three genes are true orthologs of *Drosophila JNK, jun* and *fos*.

2. Systemic *jun* and *fos* RNAi in *Tribolium* disrupted oogenesis and led to massive follicle cell (FC) degeneration in mid-stage follicles. The FC death caused by both *jun* and *fos* RNAi could be suppressed by a simultaneous knockdown of an effector caspase. Thus we showed that, unlike in *Drosophila*, Jun and Fos are necessary for survival of the FC during *Tribolium* oogenesis.

3. To our surprise, *JNK* RNAi did not affect either egg-laying or FC survival, but instead it completely disrupted embryogenesis. So we assume that another kinase might act upstream of Jun and Fos to ensure FC survival.

4. FC surrounding early oocytes proliferated and survived normally, and FC also survived in Jun or Fos RNAi females when the oocyte growth had been prevented. Based on our results, we suggest that both Jun and Fos are required to withstand the physical stretching imposed on FC during the rapid oocyte expansion.

6. Materials and methods

6.1 Keeping beetles

Wild-type *Tribolium castaneum*, strain San Bernardino (obtained from G. Bucher, Georg August University, Goettingen) was reared in plastic boxes at 32°C in constant darkness. The food was wholemeal wheat flour with 5% yeast powder and 0.03% of the anti-sporozoal Fumagilin-B (Medivet Pharmaceuticals). For egg collections, presieved (with a 250-µm sieve) white wheat flour (type 405) was used. Adult beetles – females and males – were placed in a plastic box with white flour for 6 hours in darkness, and eggs were sifted from flour with a 300-µm sieve. Eggs were then placed into Petri dishes and supplied with wholemeal flour. For single-female egg-lays, glass "fly" vials with a 2-cm layer of presieved white flour were used. Eggs were collected after 6 hours, counted and placed into wells of a cell culture plate, then checked daily to mark the number of hatched larvae. Between egg collections, beetles were fed on wholemeal flour to provide complete nutrition.

6.2 Identification of Tribolium gene orthologs

Orthologs of *JNK, jun, fos and caspase-3* genes were searched in the *Tribolium* genome database (www.bioinformatics.ksu.edu/BeetleBase) by using translated nucleotide search (TBLASTN) with *Drosophila* JNK, Jun, Fos and Caspase-3 (termed drICE) protein sequences. One copy of each gene was found in the *Tribolium* genome. Homologous parts of the genes were identified with the Clustal W program and specific primers were designed against the best conserved parts of the open reading frames (Table 2).

Table 2. **Primers used for cloning of *Tribolium JNK, jun, fos and ICE***. Restriction sites are underlined.

Target sequence	Forward primer (5` - 3`)	Reverse primer (5` - 3`)
Tc-JNK	ATGTAGCGATTAAGAAATTGAG	ATGGATCCTTCCATTGTTCGACAG
Tc-jun	ACGGATCCTACTTTAACTCCGACAA	CACTCGAGCACGAAGGCACCAAG
Tc-fos	TACTCGAGCGGACCCAACTTTG	TTGGATCCCGCAATGATGGGACA
Tc-ICE	TACAATATGAATCATAAGAACC	ATACTCATCACGCAAGGAAT

6.2.1 RNA isolation

Three adult beetles in a 1.5-ml eppendorf test tube were frozen in liquid nitrogen and homogenized in 1 ml of TRIzol solution (Invitrogen) with a plastic pestle. The homogenate was incubated for 5 min at room temperature (RT), then 0.2 ml of chloroform was added and

the emulsion was shaken by hand for 20 sec, incubated 3 min at RT and centrifuged 15 min at 12,000*g* at 4°C. Aqueous phase was precipitated with 0.5 ml of isopropanol for 10 min at RT and then centrifuged 10 min, 12,000*g* at 4°C. RNA pellet was washed by vortexing with 1 ml of chilled 75% ethanol (-20°C), centrifuged for 5 min at 7,500*g* at 4°C and dissolved in 40 µl of diethylpyrocarbonate-treated water (DEPC-water).

RNA was treated with DNase I (10 units/µl; Roche) to avoid genomic DNA contamination. 2 µg of total RNA were mixed with 1 µl of 10x incubation buffer [100 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 8.4], 1 µl of DNase I and DEPC-water to 10 µl final volume. After 15 min of incubation at RT, DNase I was inactivated by addition of 1 µl of 25 mM EDTA and by 15-min incubation at 65°C. Reaction was then cooled on ice, collected by brief centrifugation and used for cDNA synthesis.

6.2.2 cDNA synthesis and amplification

Complementary DNA was synthesized with the reverse-transcription system SuperScript II RT (Invitrogen). 1 µg of purified total RNA was mixed with 1 µl of oligo d(T) primers, 0.8 µl of deoxyribonucleotides (dNTP, a mix of 12.5 mM each nucleotide, TaKaRa) and sterile distilled water to 12 µl end volume. The mixture was incubated for 5 min at 65°C, cooled on ice and centrifuged briefly. Then 8 µl of mixture containing 4 µl 5x First-Strand Buffer, 2 µl 0.1 M DTT, 1 µl RNaseOUT (RNase inhibitor) and 1 µl of SuperScript II reverse transcriptase were added and cDNA was synthetized at 42°C for 50 min. The reaction was stopped at 70°C for 15 min. cDNA was stored at 4°C.

Gene-specific primers were used for amplification of the homologous parts of the *Tribolium JNK, jun, fos and drICE* genes. Four 50-µl reactions were prepared for each gene. Each reaction contained 37 µl of water, 5 µl of 10x Taq Complete Buffer (Top-Bio), 4 µl deoxyribonucleotides mix (2.5 mM of each nucleotide, TaKaRa), 2 µl of each primer (10 µM; Table 2), 0.4 µl of Taq DNA polymerase (Top-Bio) and 1 µl of template cDNA (50 µg/µl). Temperature profile of the PCR was: 95°C, 5 min; [94°C, 1 min; 60°C (*ICE*), 58°C (*JNK*, *fos*) or 56°C (*jun*), 1 min; 72°C, 1 min] x 34 cycles; 72°C, 5 min. PCR products were purified with MicroSpin Sephacryl S-200 HR columns (Amersham) and stored at 4°C overnight.

6.2.3 Cloning

The primers used for cDNA isolation (Table 2) contained restriction sites for *Bam* HI or *Xho* I (in case of *Tc-JNK*, an innate *Eco* RI site downstream of the forward primer was utilized). *Tc-ICE* cDNA was amplified with primers directly containing the T7 promoter

sequence TAATACGACTCACTATAGGG on 5' ends and therefore it did not need to be cloned into a plasmid vector.

Purified PCR products of *Tc-JNK*, *Tc-jun* and *Tc-fos* cDNAs (22.5 µl per reaction) and approximately 1 µg of the pBluescriptII-SK plasmid (Stratagene) were digested with appropriate restriction endonucleases (Table 3; all from TaKaRa) in 25-µl reactions for 2 h at 37°C. Reactions were stopped by heating for 15 min at 65°C, and DNA was separated on a 1% SeaPlaque (Cambrex) low-melting-temperature agarose gel in 1x TAE buffer (Sambrook et al., 1989) for 2 hrs at 50V. DNA fragments were cut out from the gel under a long-wavelength UV light.

Table 3. **Sizes and cloning sites of cDNA products.**

Gene	**Restriction enzymes**	**Buffer**	**Size of restriction fragment**
Tc-JNK	*Eco* RI + *Bam* HI	K	918
Tc-jun	*Xho* I + *Bam* HI	K	680
Tc-fos	*Xho* I + *Bam* HI	K	916

Gel pieces with cleaved DNA fragments were melted at 65°C and combined in 0.5-ml test tubes as follows: 3 µl of pBluescript II-SK, 7 µl of a PCR product and 2 µl of water were mixed and placed for another 10 min in 65°C. 2 µl of T4 DNA Ligase Reaction Buffer (TaKaRa), 1 µl of T4 DNA Ligase (350 units/µl; TaKaRa) and 7 µl of distilled water were mixed in a separate tube on ice. The liquefied DNA mixture was then allowed to cool briefly (a few seconds) in a pipette tip and added to the T4 DNA Ligase mixture by pipetting up and down. The reaction was incubated at 18°C overnight.

20 µl from the ligation reaction was diluted with 20 µl of distilled water and 20 µl of mixture was added to 200 µl of competent cells (*E. coli* DH5α) and incubated for 30 min on ice. Transformation was achieved by a heat shock (42°C for 80 s) in a water bath. 0.8 ml of LB medium (Luria-Bertani) was added after 2 min of cooling the mixture on ice and cells were incubated for 45 min at 37°C with shaking. Approximatelly 100 µl of the bacterial suspension was spread on a LB-agar plate containing ampicillin (0.1 mg/ml), previously treated with a solution of 40 µl of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside; 200 mg/ml in dimethyl-formamide) and 4 µl of IPTG (isopropyl-β-D-thiogalactoside; 20 mg/ml in water) for the blue-white selection of recombinant colonies. Plates with bacteria were incubated for 16 h at 37°C.

Colonies carrying recombinant plasmid of desired size were found with „cracking gel“. The method is based on lysis of small amount of bacterial cell suspension in the well of a denaturating gel containing SDS (sodium dodecylsulfate). Circular plasmid molecules are released and their size can be compared with a supercoiled circular DNA standard. Cracking gel is prepared by adding SDS to a final concentration of 0.05% into 0.8% agarose gel solution (at about 50°C) containing 1x TBE [10x stock solution of TBE: 108 g Tris base, 55 g boric acid, 40 ml 0.5 M EDTA (pH 8.0) in 1000 ml of water]. Single white colonies were resuspended in 5 µl of freshly prepared protoplasting buffer [20% sucrose, 30 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), 50 mM NaCl, 2.5 µl RNase A (10 mg/ml) and 1 µl lysozyme (50 mg/ml)]. 3 µl of lysis buffer (20% sucrose, 1x TBE, 2% SDS and bromphenol blue) were pipetted into the wells of the cracking gel (not submersed) and the bacterial suspension in the protoplasting buffer was added to the wells immediately. The gel was then immersed into 1x TBE, 0.05% SDS, and DNA was separated for 2 h. After separation, DNA was detected with ethidium bromide.

6.2.4 Plasmid DNA isolation

Bacterial clones carrying recombinant plasmids were inoculated into 5 ml of LB media with ampicillin and incubated for 16 hours at 37°C with shaking. Plasmid DNA was isolated with QIAprep Spin Miniprep kit (Qiagen). The method is based on permeabilisation and alkaline lysis of bacteria with precipitation of proteins and chromosomal DNA. 5 ml of bacterial suspension were pelleted by centrifuging 10 min at 5,400*g* at 4°C and bacterial cells were resuspended in 250 µl P1 Buffer (containing RNase A, 0.1 mg/ml) and transferred to a 1.5-ml eppendorf tube. 250 µl of P2 Buffer was added and mixed thoroughly by inverting the tube. 350 µl N3 Buffer was added and mixed immediately. White pellet was removed by centrifugation for 10 min at maximum speed. The supernatant was applied onto a QIAprep spin column and centrifuged for 60 s at maximum speed (this step was repeated until all of the supernatant was applied). The flow-through was discarded. The column was washed by adding 0.75 ml of PE Buffer and centrifuged for 60 s. The flow-through was discarded and column was centrifuged for another 1 min to remove residual buffer. 50 µl of DEPC-water (37°C) was added to the center of the column, and after 1 min centrifuged to collect the eluate in a fresh 1.5-ml tube. Plasmid DNA concentration was measured at λ = 260 nm, and DNA was stored at -20°C.

6.2.5 DNA Sequencing

Sequencing of DNA cloned in pBluescript was performed with the dideoxynucleotide ABI Prism BigDye Terminator Kit (Applied Biosystems) and with universal primers M13F (5‘-GTAAAACGACGGCCAGT-3’) and M13R (5‘-GGAAACAGCTATGACCATG-3’). *Tc-ICE* was sequenced with its specific primers (Table 2). Sequencing reactions were prepared by mixing 4 μl of Terminator Ready Reaction Mix, 4 μl of 2.5x a Sequencing buffer (200 mM Tris-HCl, 5 mM $MgCl_2$, pH 9.0), 1 μl of 10 μM primer (M13F or M13R), 1 μg of plasmid DNA and water to final volume 20 μl. Reactions were run in a thermal cycler with a program: 96°C, 1 min; [96°C, 10 s; 60°C, 4 min] x 26 cycles. The products were transferred into 1.5-ml eppendorf tubes and precipitated with 80 μl of 80% isopropanol for 15 min at RT. Supernatant was removed after 30 min of centrifugation at full speed (16,000*g*, 4°C), and the pellet was washed with 200 μl of 80% isopropanol at RT. All liquid was then withdrawn and the pellet was dried completely for several minutes at RT. The sequencing reactions were analyzed with automatic sequencer ABI PRISM 310 (Applied Biosystems). Data were processed with Chromas, BioEdit and Gene Runner programs.

6.3 mRNA expression analysis by RT-PCR

Total RNA was isolated from embryos, larvae, prepupae, pupae, adult beetles and ovaries with TRIZol as described above. RNA was extracted once more with phenol-chloroform: DEPC-water was added to 200 μl end volume, mixed with 200 μl of phenol-chloroform-isoamylalcohol (25:24:1), vortexed and centrifuged for 10 min at 16,100*g* (maximum speed) at 4°C. The same volume of chloroform-isoamylalcohol (24:1) was added to the aqueous phase, vortexed and centrifuged again. The aqueous phase was collected, 1/10 volume of 3 M NaOAc (pH 5.2) was added, and RNA was precipitated overnight at -20°C with 2 volumes of 100% ethanol, then pelleted and washed with 75% ethanol as above. The pellet was dried briefly and resuspended in 10 or 20 μl of nuclease-free water. Optical density was measured at 260 nm, RNA concentration was set to 500 ng/μl and RNA was stored at -70°C.

RNA was treated with DNase I and used for cDNA synthesis and RT-PCR with gene-specific primers (Table 4). The temperature profile of the reaction was: 95°C, 5 min; [94°C, 1 min; 62°C (*rp49*), 58°C (*fos*) or 56°C (*jun*), 1 min; 72°C, 30 s (*rp49*), 80 s (*fos*) or 1 min (*jun*)] x 24 cycles; 72°C, 5 min. PCR products were separated on 1% agarose gel and stained with ethidium bromide.

Table 4. **Primers used for RT-PCR.**

Target mRNA	Forward primer (5` - 3`)	Reverse primer (5` - 3`)
Tc-jun	ATGTAGCGATTAAGAAATTGAG	ATGGATCCTTCCATTGTTCGACAG
Tc-fos	TACTCGAGCGGACCCAACTTTG	TTGGATCCCGCAATGATGGGACA
rp49	TTATGGCAAACTCAAACGCAAC	GGTAGCATGTGCTTCGTTTTG

6.4 Preparation of dsRNA

Double-stranded RNA for RNAi was prepared with the MEGAscript T7 and T3 kits (Ambion) (Bucher & Klingler, 2004; Tomoyasu & Denell, 2004). Control bacterial *malE* PCR-product (Tan & Palli, 2008) or sequences cloned in pBluescript II-SK and flanked with T7 and T3 RNA polymerase promoters were amplified in a large-scale PCR (200 µl total in four 0.2-ml PCR tubes) with M13F and M13R primers (annealing at 60°C for 1 min). The 720-bp *Tc-ICE* PCR product with T7 RNA polymerase promoters on both ends was obtained with specific primers (Table 2) under the same conditions. The PCR product was purified with MicroSpin Sephacryl S-400 HR columns (Amersham) according to the manufacturer's instructions. Reactions were then pooled and extracted with phenol-chloroform-isoamylalcohol (as described above for RT-PCR), precipitated and resuspended in DEPC-water. Concentration of the purified PCR product was measured at 260 nm, and an aliquot was tested on a 1% agarose gel. The DNA was stored at -20°C and used as template for transcription.

Sense and anti-sense ssRNA strands were synthesized with T7 and T3 RNA polymerases in separate test tubes. Both ssRNAs of *Tc-ICE* were synthesized with T7 RNA polymerase in one tube. Reactions were assembled at RT to avoid precipitation of the reaction buffer and consisted of 8 µl of NTP mixture (75 mM each nucleotide), 2 µl of 10x Reaction buffer (T7 or T3), 2 µl of Enzyme Mix (T7 or T3), 1 µl of template DNA (500-700 ng/µl) and nuclease-free water to 20 µl. Reactions were mixed by pipetting and incubated for 4-6 hrs at 37°C. 1 µl of TURBO DNase (2 U/µl, Ambion) was added into each reaction and incubated for 15 min at 37°C to remove the template DNA. Reactions were collected briefly, RNA was transferred to 1.5-ml eppendorf tubes, and precipitated by adding 30 µl of nuclease-free water and 30 µl of 7.5 M LiCl, 50 mM EDTA solution and mixing thoroughly. RNA was chilled for 1 h at -20°C and centrifuged for 30 min at maximum speed at 4°C. Supernatant was removed, pellet was washed with 1 ml of 70% ethanol, re-centrifuged and ethanol was removed. RNA

pellet was dried briefly and resuspended in 15 μl of nuclease-free water, and RNA concentration was determined. ssRNA was stored at -70°C.

To produce dsRNA, complementary RNA strands were annealed (according to protocol kindly provided by Bucher & Klingler) as follows: 50 μg of each, sense and anti-sense ssRNA were mixed with 4 μl of 5x RINGER injection buffer and nuclease-free water to final volume 20 μl. (5x concentrated RINGER stock: 7.5 g NaCl, 0.1 g KCl, 0.2 g $CaCl_2$ in 180 ml of H_2O, treated with DEPC and autoclaved was mixed with 20 ml of DEPC-treated and separately autoclaved 1% $NaHCO_3$). Eppendorf tube with RNA was placed for 2 min in a 95°C heating block, then placed in a boiling water bath that was allowed to cool down to about 70°C (ca. 15 min), and finally again for 5 min in a 95°C heating block. The whole heating block with the tubes was then removed from the heater and was allowed to cool down for 40 min to RT. dsRNA was store at -20°C (or -70°C for longer storage).

6.5 RNAi and ovary dissection

One-week old adult beetles were anesthetized with flowing CO_2 on a CO_2 pad and sexed. Females or males were attached laterally to the CO_2 pad with a double-stick tape under continual CO_2 flow. Glass capillaries (type G-100, Narishige; pulled on a PC-10 needle puller, Narishige) were filled with dsRNA solution and attached on an MN-151 micromanipulator (Narishige). About 0.3 μl of liquid was injected dorso-laterally between second and third abdominal segments until beetles visibly bloated. Beetles were then placed into Petri dishes on filter paper and wholemeal flour was added. Beetles were kept at 32°C.

For dissection of ovaries, adult females were anesthetized with CO_2, two last abdominal segments were cut off with a razor blade, and females with open abdomen were immersed in 1x Phosphate Buffer Saline [10x PBS: 40 g NaCl, 1 g KCl, 1 g KH_2PO_4, 7.21 g $Na_2HPO_4.2H_2O$, ddH_2O to 500 ml, pH 7.4] in a Petri dish. Ovaries were pulled out with forceps and fixed for 30 min in 4% formaldehyde in PBS and then washed 2 times for 15 min with PBS. Ovaries were then rid of sheaths with forceps (in PBST), separated into single ovarioles, and further processed.

6.6 Antibodies and immunocytochemistry

Primary rabbit IgG anti-phospho-Histone H3 (anti-pH3, Upstate; Trauner & Büning, 2007) and mouse monoclonal anti-BrdU (anti-5-bromo-2-deoxy-uridine, Roche) antibodies were diluted 1:200. Secondary antibodies used for visualization were: Cy3-conjugated goat anti-rabbit IgG (Amersham; diluted 1:500); Cy2-conjugated goat anti-rabbit IgG (Jackson

Immunoresearch; diluted 1:500) and sheep anti-mouse IgG-fluorescein (Roche; diluted 1:200).

Dissected and fixed ovarioles were blocked with 3% BSA [Bovine Serum Albumine, Fraction V (Serva)] in PBST [1x PBS, 0.1% Triton X-100 (Gerbu)] for 1 h at RT on a rotator. Primary antibody was added into the blocking solution and incubated with ovaries overnight at 4°C on a rotator. Ovarioles were washed 6 times for 10 min in PBST, blocked in 3% BSA in PBST for 30 min at RT. Secondary antibody was added and incubated for 2 h at RT (Cy2- and Cy3-conjugated antibodies) or overnight at 4°C (anti-mouse Ig). Tissues were washed 6 times for 10 min in PBST. For visualization of the actin cytoskeleton, ovarioles were stained with Oregon Green 488-labeled Phalloidin (2 µg/ml in PBST; Invitrogen) for 30 min and then washed 15 min in PBST. Ovarioles were transfered to slides, mounted in 80% glycerol containing 2% propyl-gallate and 4',6'-diamino-2-phenylindol dihydrochloride (DAPI, 1 µg/ml, Molecular probes), and stored at 4°C in darkness. Images were captured on a Zeiss Axioplan 2 with an F-View II camera (AnalySIS).

6.7 BrdU incorporation

DNA synthesis in dividing or endoreplicating cells was detected with 5-bromo-2-deoxyuridine (BrdU) Labeling and Detection Kit I (Roche). Bromide-labeled deoxy-uridine is incorporated into DNA in place of thymidine and detected with a specific anti-BrdU antibody. Adult females were injected with undiluted 10 mM BrdU in PBS. Ovaries were dissected two days after injection, fixed and separated into single ovarioles. Cellular DNA was denatured by incubating ovarioles in 2 N HCl with 0.5% Triton X-100 for 30 min at 37°C. Ovarioles were then rinsed once with PBST and neutralized by two 10-min washes in 0.1 M Na_2BO_4.10 H_2O, 0.5% Triton X-100 on a rotator. Neutralization solution was washed out with PBST (3 times 10 min). Ovarioles were stained with the anti-BrdU antibody as described above. BrdU incorporation was performed according to protocol kindly provided by Daniel Bäumer (Bäumer, unpublished)

6.8 Apoptosis detection with TUNEL

Apoptotic cells were stained with In Situ Cell Death Detection Kit, TMR Red (Roche). The method is known as TdT-mediated dUTP nick end labeling (TUNEL) and it is based on labeling of DNA strand breaks by a terminal deoxynucleotidyl transferase (TdT), which adds labeled nucleotides (tetramethylrhodamine-dUTP) to free 3'-OH DNA ends. Incorporated labeled nucleotides are then detected by direct fluorescence microscopy. Ovaries were

dissected and fixed as described above. Permeabilization of cells was achieved by incubation in 0.1 M sodium citrate, 0.1% Triton X-100 for 10 min at RT on a rotator and for another 30 min at 37°C on a water bath. Ovarioles were washed 2 times for 15 min with PBST. PBST was replaced with 50 µl of TUNEL reaction mixture (5 µl TdT enzyme mix plus 45 µl Label solution), and reaction was incubated for 2 h at 37°C in darkness. The solution was removed and ovarioles were washed 2 times for 15 min in PBST in darkness (test tubes wrapped in aluminum foil). Ovarioles were then mounted and microscoped.

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