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Up-regulation of the Ku heterodimer in *Drosophila* testicular cyst cells

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Abstract **In *Drosophila*, developing germline cysts in testis are enveloped by two somatic cyst cells essential for germline development and male reproduction. The cyst cells continue development along with the germline. However, the mechanisms of somatic gene expression in testes are poorly understood. We report transcriptional up-regulation of the Ku heterodimer in cyst cells. The initial up-regulation is independent of germline, and transcription is further augmented during spermatogenesis. Abundance of Ku in the cyst cell cytoplasm suggests the role for Ku subunits in the regulation of sperm individualization.**

Keywords: Testes; Transcription; Somatic cells; Ku heterodimer

1. Introduction

Testicular somatic cells represent an essential component of male reproductive system. In mammals, germline cells maintain interactions with the somatic Sertoli cells throughout development. In insects, groups of developing germline cells are encased in the envelope composed of the two somatic cyst cells that are functionally similar to the Sertoli cells. Interactions between cyst cells and germline are required for differentiation of germline cells throughout spermatogenesis and spermiogenesis. Genetic studies have demonstrated that a specific cyst cell/germline interaction that involves dpp (TGFβ/BMP) signaling plays critical role in differentiation of spermatogonia into spermatocytes [1]. Further, mutations that affect cyst cells impair spermatocyte development, indicating requirement for the cyst cells at this stage [2]. Finally, transcriptional dysregulation in the cyst cells leads to failure in sperm maturation [3].

Therefore, cyst cells appear to play different, essential roles at every major stage of germline development. This evolution of the functional role is accompanied by significant changes in cell morphology. In the beginning of spermatogenesis, two cyst cells provide the envelope around the cyst of dividing spermatogonia and establish contacts with the cells within cyst by extending the processes between the germ cells. This general organization remains in place during spermatogenesis, but spermatid differentiation leads to complete displacement of the cyst cells to the surface of the spermatid bundles where the tail cell forms a thin sleeve around the bundle [4].

Enhancer trap studies have demonstrated that gene expression pattern in cyst cells may be changing during germline development [5]. These observations imply existence of the somatic gene expression program in testes that parallels the germline gene expression in spermatogenesis. The underlying mechanisms that may control somatic gene expression in testes are poorly understood. Perhaps the furthermost advance in this direction is provided by the studies of Fabrizio et al. [2] who demonstrated that the transcriptional regulators *eya* and *so* are required in cyst cells for proper spermatocyte development. Identification of the role of endopeptidase NEP2 in cyst cells [6] provides an example of a possible downstream target of the transcriptional regulatory mechanism. However, the very limited numbers of the known putative transcriptional regulators and target genes impedes further understanding of the mechanism of somatic gene expression in testes. Here, we report transcriptional up-regulation of the Ku heterodimer subunits in cyst cells. Our data indicate that up-regulation occurs primarily in the testicular somatic cells independent of germline, and then transcription is further augmented during spermatogenesis. Thus, the Ku subunit genes represent the targets for transcriptional regulatory mechanism(s) that may establish identity of testicular somatic cells and control further developmental changes in these cells that accompany development of the germline.

2. Materials and methods

2.1. Fly stocks

Drosophila melanogaster stocks were maintained on the yeast-molasses media at room temperature. The wild-type flies used were Oregon-R for transcription assays, and the mixture of Oregon-R and *y w* for biochemical purification of proteins. The stocks carrying the mutations aly^{1} [7], sa^{1} [7], $TAF12L^{KG00946}$, and tud^{1} [8] were obtained from the Bloomington Drosophila Stock Center at Indiana University.

2.2. Reverse transcription and real-time PCR

RNA was extracted from manually dissected tissues and from adult flies with the Trizol reagent (Invitrogen). Each sample represented the combined tissues from 50 to 100 animals. Reverse transcription reactions were performed using 1 μg of total RNA as a template, with the PowerScript reverse transcriptase (Clontech). 0.5% of the reverse transcription reaction was used as a template for a 20 μl real-time

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Abbreviations: SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; NFDM, non-fat dry milk; PBST, phosphate buffered saline with 0.05% Tween-20; PBS, phosphate buffered saline; BSA, bovine serum albumin; EMSA, electrophoretic mobility shift assay; NIB, nuclei isolation buffer; PCR, polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; NHEJ, non-homologous end-joining; DSB, double strand break; HR, homologous repair

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polymerase chain reaction (PCR) reaction. Reactions were run in triplicates in the ABI 5700 Sequence Detector, using SYBR Green chemistry (Applied Biosystems).

2.3. Western analysis

Proteins were separated by SDS–PAGE in the 4–15% gradient precast minigels (Bio-Rad) and transferred onto the Nybond-C membrane (Amersham) by electroblotting in the Tris–glycine buffer containing 20% methanol. Membranes were blocked overnight at 4 °C in 5% non-fat dry milk (NFDM) solution in phosphate buffered saline containing 0.05% Tween-20 (PBST). The primary polyclonal antibodies were raised in chicken against the peptide ERKVIYDNDKEDKMLKDK derived from the Ku80 sequence and were affinity-purified at the Aves Labs, Inc. On the Western blot of whole *Drosophila* adults, the antibody recognized a single band of ca. 80 kDa, consistent with the size of Ku80 protein. After blocking, membranes were incubated with 1 μg/ml of the primary antibodies in PBST containing 2.5% bovine serum albumin (BSA) for 1 h at 4 °C, and washed extensively in PBST. Secondary antibodies (goat anti-chicken horseradish peroxidase conjugated, Aves Labs) were used at concentration of 20 ng/ml, in PBST containing 2% NFDM. After 1 h incubation with the secondary antibodies at 4 °C, membranes were washed in PBST and developed using the SuperSignal West Femto substrate (Pierce).

2.4. Indirect immunofluorescence

Testes were dissected in phosphate buffered saline (PBS) and fixed in 4% formaldehyde, 8% sucrose in PBS for 25 min on ice, then washed three times with cold PBS. Fixed testes were placed on the Superfrost/Plus microscope slide (Fisher) in drop of PBS and let to attach to the glass. Most of the PBS was removed by aspiration, testes were covered by another Superfrost/Plus slide and the resulting sandwich was frozen in liquid nitrogen. Slides were pried apart with razor, and incubated in ice-cold PBS containing 4% formaldehyde and 0.1% Triton X-100 for 30 min, then washed three times with PBS and blocked overnight in PBS containing 3% BSA, 0.03% Triton X-100 and 0.05% Tween-20 at 4 °C. Primary antibodies were diluted in the blocking solution and incubated with the slides overnight at 4 °C. The chicken polyclonal antibody against Ku80 (described above) was diluted to 0.7 μg/ml; the rat monoclonal antibody DCAD2 against D-cadherin (obtained from the Developmental Studies Hybridoma Bank at University of Iova) was diluted 1:200. The slides were washed five times for 20 min in PBST supplemented with 1% BSA at room temperature and incubated for 1.5 h at 4 °C with the secondary antibodies (goat anti-chicken AlexaFluor 594 conjugated, and rabbit anti-rat AlexaFluor 488 conjugated, Molecular Probes) diluted to 4 μg/ml in the blocking solution. Slides were washed four times as described above followed with overnight incubation in the washing solution at 4 °C, briefly air-dried and mounted in the DAPI-containing VectaShield medium (Vector). Fluorescence was observed in the Leica TCS SP2 confocal microscope coupled with the Coherent Mira 900 femtosecond laser at GRASP center, Tufts/NEMC. Images were imported into Adobe Photoshop and processed using automated level correction.

2.5. Protein extracts from dissected tissues and EMSA

Testes, heads, and ovaries were manually dissected from adult flies in phosphate buffered saline (PBS), and homogenized in microcentrifuge tubes in ice-cold extraction buffer (20 mM Tris–HCl, 1.5 M KCl, 2 mM EDTA, 0.4% Triton X-100, 0.04% β-mercaptoethanol, 10% glycerol, 1 mM PMSF, and 1 μg/ml of each of pepstatin, leupeptin, and trypsin inhibitor, pH 7.5) using disposable pestles. Debris was sedimented by centrifugation at $16000 \times g$ for 10 min at 4 °C, and supernatants were dialyzed against the binding buffer (20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 0.05% Triton X-100, 0.04% β-mercaptoethanol, 10% glycerol, pH 7.9) using the 10 kDa MWCO Slide-a-lyser cassettes (Pierce). Protein concentrations were determined with the Bradford assay. Double-stranded oligonucleotide AGCTTTGATCGTAGTGTGCCTTTGGGGGAAATTCTG labeled to specific activity of 3×10^6 dpm/pmol with polynucleotide kinase (Invitrogen) and [γ-^{32}P] ATP (Perkin Elmer) was used as the probe for electrophoretic mobility shift assay (EMSA). EMSA (20 μl) reactions contained 5 μg of protein extracted from the tissues, 2 μg of acetylated BSA (New England Biolabs), 1.0×10^5 dpm of labeled probe, and 0.5 μg poly-dIdC (Sigma) in the binding buffer. After 25 min of incubation at room temperature, reaction products were separated by electrophoresis in 5% acrylamide gels containing 0.5× TBE and 10% glycerol. For analysis of fractions during purification of the Ku heterodimer, a fixed volume of fraction (1 μl) rather than a fixed amount of protein was added to reactions.

2.6. DNA affinity matrix

DNA beads were prepared using the streptavidin-coated magnetic beads (Dynabeads M-280, Dynal). The sequence AGCTTTGATCGTAGTGTGCCTTTGGGGGAAATTCTG (the "TSE" sequence) was flanked with the TC overhang at the 5′-end and with the AG complementary overhang at the 3′-end, to drive the "head-to-tail" concatenation. Single-stranded oligonucleotides TSE-U and TSE-L (obtained from Integrated DNA Technologies) were phosphorylated at the 5′-end. One of the oligonucleotides (TSE-Lbio) was also synthesized with the 3′-biotin-TEG modification. A mixture of 10 parts of TSE-U, 9 parts of TSE-L, and 1 part of TSE-Lbio was annealed to generate the double-stranded TSE oligonucleotides, and these were concatenated by DNA ligase (New England Biolabs). Ligation products were analyzed by gel electrophoresis, to confirm polymerization. The average product length was 300–400 bp, which corresponds to ten tandem repeats of the TSE sequence. Biotinylated DNA (200 pmol) fragments were loaded onto 2 mg of streptavidin-coated magnetic beads, according to the manufacturer's recommendations.

2.7. Purification of the non-specific dsDNA-binding activity

Adult *D. melanogaster* (300 g) frozen at −80 °C were homogenized in 1 l of the ice-cold nuclei isolation buffer (NIB) (20 mM HEPES, 50 mM KCl, 5 mM $MgCl_2$, 0.04% β-mercaptoethanol, 10% glycerol, 1 mM PMSF, and 1 μg/ml of each of pepstatin, leupeptin, and trypsin inhibitor, pH 7.9) by blending in the Kitchenaid KSB5MC4 blender for 4 min at 4 °C at the highest speed. The nuclei were pelleted by centrifugation at $3000 \times g$ for 20 min at 4 °C, resuspended in 1 l of ice-cold NIB, and pelleted again. Nuclei were extracted in 600 ml of ice-cold extraction buffer (30 min at 4 °C), and the extracts were cleared by centrifugation at $15000 \times g$ for 20 min at 4 °C. Solid ammonium sulfate was gradually added to the supernatant to concentration of 1.2 M, and precipitated proteins were removed by centrifugation at $15000 \times g$ for 20 min at 4 °C. The supernatant was diluted with the buffer Q (20 mM Tris–HCl, 50 mM KCl, 2 mM EDTA, 0.05% Triton X-100, 0.04% β-mercaptoethanol, 10% glycerol, pH 7.5) to final 1 M concentration of ammonium sulfate, cleared by centrifugation again as described above, and loaded on to the 250 ml Octyl-Sepharose column (XK50/20, Amersham) at 15 ml/min using the BioLogic LP system (Bio-Rad). The column was washed by 700 ml of the buffer Q containing 1 M ammonium sulfate, and the bound proteins were eluted with the buffer Q in the 300 ml volume. The solution was desalted on the Sephadex G-50 column (1 l bed volume, XK50/60, Amersham) at 15 ml/min, with the buffer being exchanged to the buffer S (20 mM MES, 50 mM KCl, 0.05% Triton X-100, 0.04% β-mercaptoethanol, 10% glycerol, pH 7.0), and loaded on the 50 ml SP-sepharose column (XK26/20, Amersham) at 2 ml/min. The bound proteins were eluted with the buffer S containing 0.5 M KCl in the 30 ml volume. The solution was desalted on the 70 ml Sephadex G-50 column (XK26/20, Amersham) with the buffer being exchanged to the buffer D (10 mM K-Phosphate, 50 mM KCl, 5 mM $MgCl_2$, 0.05% Triton X-100, 0.04% β-mercaptoethanol, 10% glycerol, pH 7.0), and loaded onto the 20 ml Heparin-Sepharose column (XK16/20, Amersham) at 2 ml/min. For this and the further steps we used the BioLogic DuoFlow system (Bio-Rad). The column was developed with 300 ml of the 0–1 M linear gradient of KCl in the buffer D. The 2 ml fractions were analyzed by EMSA, and the fractions containing the dsDNA-binding activity were pooled, desalted on the 70 ml Sephadex G-50 column (XK26/20, Amersham), and loaded on the Uno Q-1 anion exchange column (Bio-Rad) at 0.5 ml/min. The proteins were eluted by 15 ml of the 0–1 M linear gradient of KCl in the buffer D. The 1 ml fractions positive for the dsDNA-binding activity by EMSA were pooled and dialyzed against the buffer D in the 10 kDa MWCO Slide-a-lyser cassettes (Pierce). The solution was supplemented with $CaCl_2$ to 1 mM and with the poly-dIdC to 20 μg/ml (as described for EMSA), and incubated with 0.2 mg of the DNA affinity beads per 1 ml of reaction volume for 30 min at room temperature. The beads were captured using the magnetic stand (Promega), washed four times with the buffer D containing 1 mM $CaCl_2$ and 20 μg/ml of the poly-dIdC, then three

times with the buffer D containing 1 mM $CaCl_2$, and eluted with the 50 μl volumes of buffer D containing increasing concentrations of KCl ranging from 0.1 M to 3 M.

2.8. Size exclusion chromatography

The solution (0.5 ml) containing the dsDNA-binding activity after the UnoQ purification step (see above) was loaded onto the Superdex-200 gel filtration column (Amersham), and eluted with the buffer D at 0.2 ml/min. The 1 ml fractions were analyzed for the dsDNA-binding activity by EMSA. The column was calibrated using the set of molecular weight standards (Sigma) that includes the Blue dextran (MW ca. 2000 kDa), β-amylase from sweet potato (200 kDa), alcohol dehydrogenase from yeast (150 kDa), bovine serum albumine (66 kDa), bovine carbonic anhydrase (29 kDa), and horse cytochrome *c* (12.4 kDa).

2.9. LC/MS/MS

Proteins were fractionated by SDS–PAGE in the 4–15% gradient gel and stained using colloidal Coomassie Blue (Invitrogen). The protein bands were excised with razor blade, soaked in 50% acetonitrile/water and sent for identification to Harvard Microchemistry Facility (Cambridge, MA) using liquid chromatography/tandem mass spectrometry (LC/MS/MS).

3. Results

3.1. Transcriptional up-regulation of the Ku heterodimer subunits in testes is independent of the meiotic arrest genes

Our studies that identified a number of Drosophila testes-biased genes using expressed sequence tag database analysis [9] suggested testes-biased transcription pattern for the *Ku80* subunit of the Ku heterodimer. To confirm the observations and to extend them on the other Ku subunit, the *Ku70* and the *Ku80* transcripts were quantitated in testes and in other tissues using reverse transcription followed with real-time PCR. Constitutive transcripts of the genes *Rpl9* and *His3.3A* were used as the cDNA template loading controls: the observed differences in the amounts of the Ku subunit transcripts were normalized by the average of the differences observed for *Rpl9* and *His3.3A*. The analysis showed that the *Ku80* and the *Ku70* transcripts in testes are up-regulated 10-fold or more as compared to the other adult tissues including heads, ovaries, and whole gonadectomized males (Table 1).

The vast majority of testes-biased genes are up-regulated in the male germline where their transcription is under the control of the testes-specific components of transcriptional machinery, including the putative chromatin remodeling Aly-containing complex and the testes-specific TFIID subunits (tTAFs) [7]. In addition, we recently found that a number of testes-specific genes are positively regulated by the multifunctional protein Modulo [10]. However, real-time RT-PCR experiments showed that the levels of *Ku80* and *Ku70* transcripts in testes were not drastically affected by the mutation in *aly*: the *Ku80* transcript was down-regulated two to three-fold (0.41; S.D. 0.12), while the apparent level of the *Ku70* transcript in the *aly* mutant testes was even slightly higher than in the wild type (1.8; S.D. 0.6). For comparison, germline-specific transcripts that depend on the Aly complex are down-regulated in the *aly* group mutants hundreds- to thousands-fold [10]. The Ku subunit transcripts also appear to be independent of tTAFs: the *Ku70* transcript level in testes of the mutant for the tTAF *sa* is higher than in the wild-type (2.1; S.D. 0.9). We also have shown that the *Ku80* transcription in testes does not depend on tTAFs Sa and Rye, or specific transcription factor Modulo [10]. Thus, the drastic up-regulation of the Ku subunit transcripts in testes is not under the control of the major transcriptional mechanism that operates in male germ line, raising the possibility that up-regulation occurs in other cell types within testes.

Table 1
Relative levels of transcripts for the Ku subunits in *Drosophila* tissues

RNA source	*Ku80*		*Ku70*	
	Average	S.D.	Average	S.D.
Adult gonadless male	1.0	0.2	1.0	0.1
Adult testes	17.5	1.4	18.2	2.1
Adult heads	1.0	0.1	1.0	0.1
Ovaries	1.0	0.1	2.4	0.6

Amounts of transcripts were measured by real-time RT-PCR relative to the levels in gonadectomized males, and normalized to the levels of constitutively expressed transcripts *Rpl9* and *His3.3A* used as the template loading references. S.D. values were calculated for the repeated real-time PCR assays.

3.2. Abundant Ku80 expression in testicular somatic cells

In order to localize the Ku80 protein within testes, we developed an anti-peptide polyclonal antibody against Ku80. Western analysis confirmed up-regulation of Ku80 in testes. A protein of expected size of ca. 80 kDa was detected. Even though more material from the gonadectomized males had to be loaded to obtain appreciable signal (as demonstrated by the control silver staining), the signal in testes was still stronger than in gonadectomized males (Fig. 1A). Semi-quantitative studies (Fig. 1B) showed that the levels of Ku80 in testes are approximately 10 times higher than in gonadectomized males, consistent with 17-fold up-regulation of the transcript (Table 1). Immunostaining of the male accessory glands revealed nuclear localization typical of the Ku heterodimer (Fig. 2a–c); control treatments in the absence of the primary antibody did not produce appreciable staining (data not shown).

In contrast, in testes the observed distribution of Ku80 was rather unusual. The protein was up-regulated in the zone

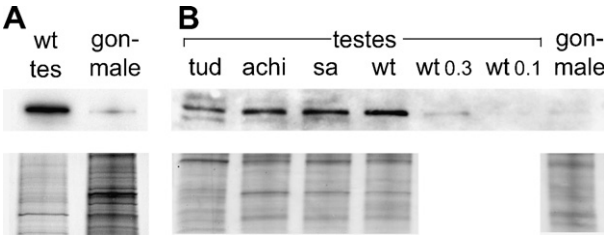

Fig. 1. Up-regulation of Ku80 in testicular somatic cells. Hand-dissected testes of the tud[1], achi/vis[1], and *sa*[1] mutants and of the wild type and the whole gonadectomized males were homogenized in the Laemmli SDS–PAGE loading buffer. Proteins were separated in 4–15% gradient SDS–PAGE gels. Upper panel: after electrophoresis, proteins were analyzed by Western blotting using anti-Ku80 antibody. Lower panel: identical gels were stained using (A) SilverSnap reagents (Pierce) or (B) colloidal Coomasie Blue (Invitrogen) to show protein loading on the lanes. (A) Ku80 is overexpressed in wild type testes (wt tes) as compared to the gonadectomized males. (B) Expression of Ku80 in the mutant and wild type testes, and gonadectomized males. For semi-quantitative analysis, the samples containing 30% (wt 0.3) and 10% (wt 0.1) of the amount of wild type testes extract loaded on the lane "wt" were also analyzed.

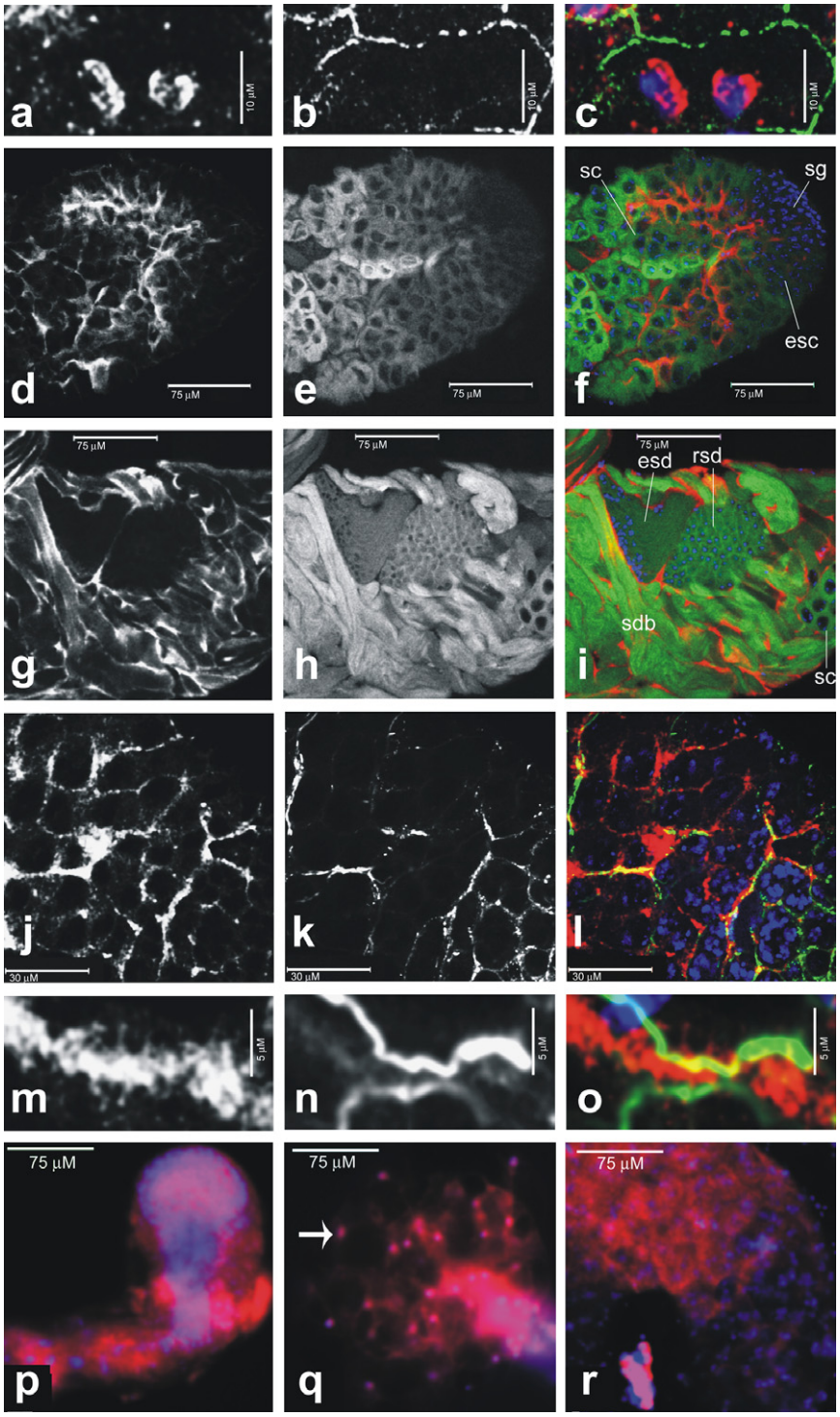

Fig. 2. Localization of the Ku80 protein in testes by immunofluorescence. Testes of the *y w* males (a–c, j–o), of the transgenic *y w* males carrying male germline-specific *Sdic::GFP* transgene [11] (d–i), of the sons of *tud*^{1} mutant mothers (p,q) and of the *TAF12L*^{KG00946} mutant (r) were stained using the anti-Ku80 antibody. This staining was detected in the red channel using the AlexaFluor 594-conjugated secondary antibody (left column). In addition, preparations in (a–c, j–o) were stained using the anti-D-cadherin antibody that was visualized in the green channel using the AlexaFluor 488-conjugated secondary antibody. GFP expression in germline (d–i) was also detected in the green channel (middle column). Right column: the red and the green channels merged with the DAPI nuclear stain (blue). (a–c) control staining of the accessory gland epithelium reveals nuclear localization of Ku80. (d–f) Ku80 is expressed at relatively low level at the tip of testis occupied by spermatogonia (sg), but is up-regulated in somatic cells associated with early (esc) and maturing (sc) spermatocytes marked by the Sdic::GFP expression. (g–i) Prominent Ku80 signal outlines cytoplasm of the cyst cells that encase the cysts of round (rsd) and elongating (esd) spermatids and form the sleeves around spermatid bundles (sdb). (j–l) Ku80 signal co-localizes with the D-cadherin staining that outlines the cyst cell/germline junctions at the periphery of germline cysts. (m–o) zoom-in view of the area between two germline cysts shows that Ku80 is localized distal to the adherens junctions revealed by the anti-D-cadherin staining, i.e. outside the germline cells. (p,q) Ku80 is abundantly expressed in somatic cells that fill the *tudor* mutant testes; cells released from the disrupted testis (q) show nuclear localization of Ku80 (arrow), (r) in the meiotic arrest mutant *TAF12L*^{KG00946} Ku80 is observed around the spermatocyte cysts, similar to the wild type.

occupied by early spermatocytes, and expression level further increased with progression of spermatogenesis and spermiogenesis: the highest signal was detected in the area of testes filled with the bundles of elongating spermatids (Fig. 2d–i). Analysis of testes in which male germline was marked by expression of the *Sdic::GFP* transgene [11] (Fig. 2d–i) showed that up-regulation of Ku80 does not occur within the cells of male germline, and the protein is not accumulated in the nuclei of spermatogonia, spermatocytes, or round spermatids. Instead, the signal was confined to the periphery of the cells, in particular periphery of the cysts containing spermatocytes and round spermatids. During spermatid elongation, accumulation of Ku80 at the periphery of cysts was further rectified so that the spermatid bundles were encompassed by the thin Ku80-rich envelope.

To further localize Ku80 at the periphery of the germline cells, testes were co-stained with the antibodies against Ku80, and against D-cadherin that is localized at the cell surface (within the cells) at the adherens junctions. Distribution of adherens junctions in testes outlines the contacts between germline and cyst cells that encase developing spermatocyte cysts [2]. General pattern of the staining revealed co-localization of Ku80 with the zones enriched with adherens junctions (Fig. 2j–l). At high magnification, it was evident that Ku80 is localized not at the adherens junctions formed by the germ line cells, but distal to the junctions and thus outside the germ cells (Fig. 2m–o).

Thus, Ku80 is enriched at the periphery (and, in fact, outside) of germ line cells in the areas that contain extensive adherens junctions. In particular, the Ku80 signal outlines the outer envelope of spermatocyte and spermatid cysts and bundles. This localization is matching the pattern expected from the testicular cyst cells that are associated with the germline cysts and form the thin sleeve around spermatid bundles. Therefore, the Ku heterodimer is probably up-regulated in the somatic cyst cells of testes, rather than in germline. To test this possibility, we analyzed testes of the *tudor* mutant progeny that lack germline [12]. Western analysis demonstrated that testes of sons of the *tudor* mutant mothers still contained elevated amounts of the Ku80 protein. The amount of Ku80 was ca. 2 times less than in wild type testes, but at least 3 times higher than in the tissues of gonadectomized males (Fig. 1B). Immunostaining of testes of the *tudor* mutant progeny showed abundant expression of Ku80 in the somatic cells present within the testes, however the protein was predominantly localized to the nuclei (Fig. 2p and q). In contrast, in testes of the meiotic arrest mutant $TAF12L^{KG00946}$, Ku80 was observed at the periphery of arrested spermatocyte cysts, similar to its localization around spermatocytes in the wild type (Fig. 2r).

3.3. Ku80 is further up-regulated in cyst cells during germline development

Analysis of testes of the meiotic arrest mutants *achi/vis*[1] and *sa*[1] using Western blots revealed the levels of Ku80 expression in testes that were similar to the wild type and exceeded the levels observed in the *tudor* mutants (Fig. 1B). However, the testes of the meiotic arrest mutants are overfilled with premeiotic spermatocytes that significantly contribute to the total protein content of the testis. The proportion of the material derived from the somatic cyst cells in these testes is quite modest, as opposed to the *tudor* mutant testes that are filled exclusively with somatic cells. Thus, the difference in Ku80 expression in somatic testicular cells between the *tudor* and the meiotic arrest mutants is even higher than observed on Western blots. These observations suggest that Ku80 is up-regulated in cyst cells during spermatogenesis, and this additional up-regulation requires interaction with germline. Immunostaining of testes with the antibody against Ku80 supports this hypothesis, since intensity of the cyst cell staining increases with progression of germline from spermatogonia to maturing spermatocytes (Fig. 2d–f).

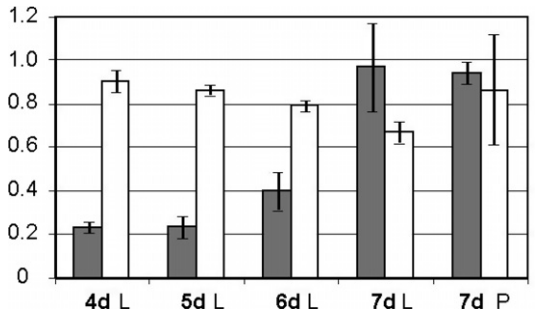

Fig. 3. Real-time RT-PCR analysis of testes of developing larvae shows up-regulation of *Ku80* (but not *Ku70*) during spermatogenesis. Testes were dissected from larvae (L) or pupae (P) of different age ranging from four (4 d) to seven (7 d) days in culture, total RNA isolated, converted into cDNA and analyzed by real-time PCR. Amounts of the *Ku70* (white bars) and *Ku80* (grey bars) transcripts in the samples, as compared to the adult testes, are shown; vertical bars represent S.D.

Up-regulation of Ku80 could result from transcriptional activation or from the post-transcriptional effects. To identify the mechanism, we analyzed the *Ku80* transcript levels in testes of developing larvae. As soon as the testes are readily discernible in the second instar larvae (the 4th day of culture in our conditions), they already contain young spermatocytes. Maturation of the first wave of spermatocytes continues through the third instar of larvae, and the cells commit to meiosis at the onset of pupation or soon thereafter [13]. Testes of the larvae of different age were dissected and analyzed by real-time RT-PCR, as described above. We found that the *Ku80* transcript is fourfold up-regulated prior to pupation (meiosis). At the same time, the levels of the *Ku70* transcript in testes did not substantially change throughout larval development (Fig. 3). Thus, Ku80 (but not Ku70) is up-regulated during spermatogenesis at the level of transcription.

3.4. Up-regulation of the Ku subunit transcripts in testicular cyst cells manifests increased activity of the functional Ku heterodimer

Transcripts of the both subunits of the Ku heterodimer are up-regulated in testes to the similar extent. Using antibody against Ku80, we confirmed its accumulation at the protein level and localized it to the testicular cyst cells. To demonstrate that the observed effects reflect up-regulation of the functional Ku heterodimer, we assayed testes extracts for the presence of non-sequence-specific double stranded DNA (dsDNA)-binding activity that is characteristic for Ku [14]. We found that testes are enriched with dsDNA-binding activity that is not suppressed in the presence of poly-dIdC, but is inhibited by addition of heterologous double stranded oligonucleotides or PCR fragments (Fig. 4A). The representative example of an

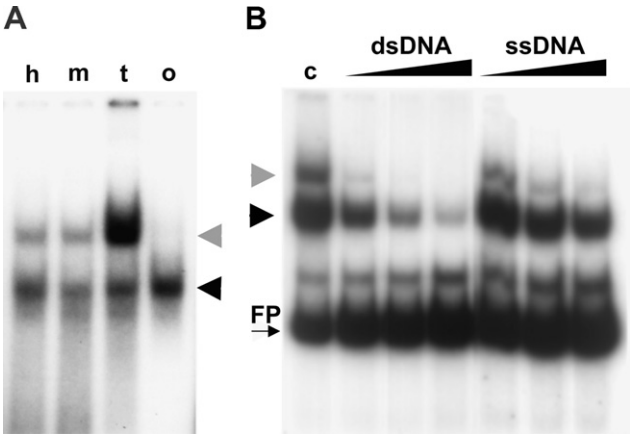

Fig. 4. Non-sequence specific dsDNA binding activity is abundant in *Drosophila* testes. (A) Protein extracts from heads (h), whole gonadescomized males (m), testes (t) and ovaries (o) were incubated with radiolabeled oligonucleotide TSE probe and analyzed by EMSA in the presence of poly-dIdC competitor. Arrowheads indicate two protein:DNA complexes enriched in testes. (B) Extracts from testes were incubated with radiolabeled 200 bp PCR fragment in the presence of poly-dIdC alone (c), or in the presence of poly-dIdC and increasing concentrations of double-stranded DNA (dsDNA, the 100 bp DNA ladder, New England Biolabs) or single-stranded sheared salmon sperm DNA (ssDNA). Each of the two DNA competitors was used in amounts of 0.5, 1.5, and 5.0 ng (corresponding to ca. 3×, 10×, and 30× excess to the labeled probe), increasing as shown above.

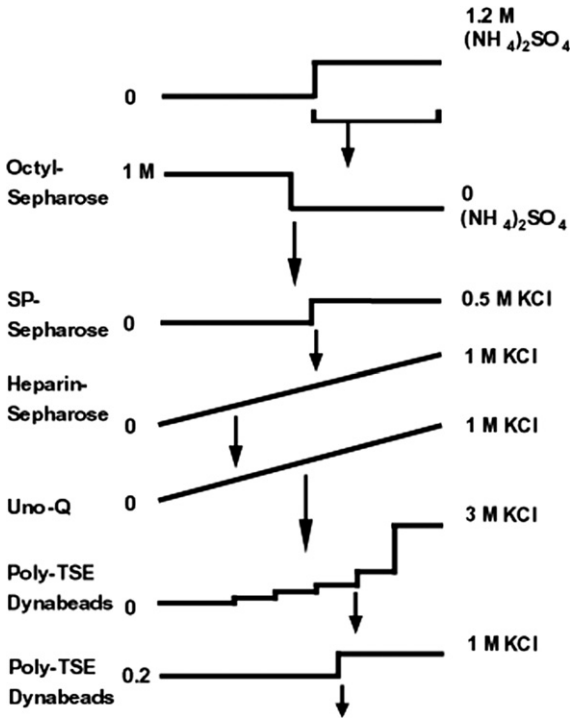

Fig. 5. Outline of purification of the dsDNA-binding activity. The media used for purification are indicated on the left.

inhibition experiment is shown on Fig. 4B. Denatured, sheared single stranded salmon sperm DNA (ssDNA) was about 10 times less efficient in inhibition than the double-stranded fragments, indicating strong preference of the binding protein to dsDNA. The extent of inhibition was the same for the unlabeled probe (the specific competitor) and for any other double-stranded DNA fragments (including five different double-stranded oligonucleotides and eleven PCR fragments, data not shown), indicating that the observed dsDNA-binding activity is not sequence-specific.

The observed traits of the DNA-binding activity up-regulated in testes, including lack of sequence specificity, strong preference to dsDNA, and lack of specificity towards poly-dIdC, are characteristic for Ku. In the vast majority of somatic tissues of flies and mammals alike, Ku heterodimer has been localized in the nucleus. Thus, we analyzed subcellular distribution of the observed dsDNA-binding activity in somatic tissues (whole adult gonadectomized flies) and, as expected, found that it is enriched in the nuclei rather than in the cytoplasm (data not shown). To further confirm the identity of testes-enriched dsDNA binding activity, we developed a biochemical procedure for its purification (Fig. 5). Whole flies were used for the purification; therefore nuclei were isolated as the first step. Proteins extracted from the nuclei with 1.5 M salt were fractionated by ammonium sulfate precipitation and purified by series of hydrophobic interaction, ion exchange, and heparin affinity chromatography. Purification of the dsDNA-binding activity was monitored by EMSA. The partially purified protein was applied on the magnetic beads covered with dsDNA, and eluted with 0.5–1.0 M salt. This final step yielded two protein species, one with the mobility in SDS–PAGE corresponding to about 75 kDa and another of about 80 kDa, present in approximately equal amounts (Fig. 6). Size exclusion chromatography showed that the dsDNA binding activity elutes with the fractions corresponding to the molecular weight of 150–200 kDa (Fig. 7), indicating that both the ca. 75 kDa and the ca. 80 kDa proteins contribute to the activity by forming the heterodimer, as is expected for the Ku subunits. The observed mobility of the purified proteins was consistent with the predicted size of the proteins encoded by *Ku70*/*Irbp* (72.5 kDa) and by *Ku80* (79.8 kDa). Analysis of the purified protein species by nano-LC/MS/MS confirmed their identity as the *Drosophila* Ku80 and Ku70, providing more than 50% sequence coverage for each protein.

4. Discussion

4.1. Up-regulation of the Ku subunits in cyst cells reveals somatic gene expression program in testes

We demonstrated that both Ku subunits are strongly up-regulated in testes. Independence of this up-regulation of the major germline-specific transcriptional regulators indicates expression outside of the germline. Localization of the Ku80 protein in testes is consistent with this suggestion and shows high level of expression in cyst cells. Interestingly, analysis of the *tudor* mutant testes reveals elevated expression of Ku80. In these testes, germline cells are absent. Therefore, up-regulation of Ku80 in testicular somatic cells occurs independently of the germline. Such up-regulation may manifest the regulatory mechanism that defines the identity of cyst cells in testes, and suggests that such mechanism may not require interaction with germline. However, such interaction may be necessary for cytoplasmic localization of Ku80 in somatic cells, because in the absence of germline (in *tudor* mutant testes) Ku80 is localized to the nuclei of somatic cells.

Analysis of the *Ku70* and *Ku80* transcription during spermatogenesis shows that these two genes are regulated

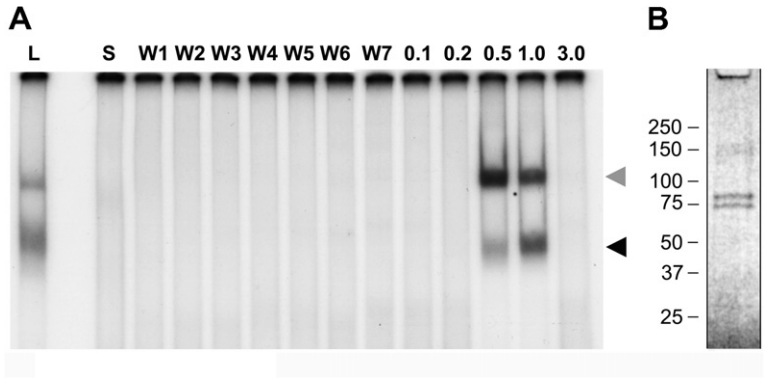

Fig. 6. Final stage of purification of the dsDNA-binding activity on the DNA affinity magnetic beads. (A) Collected fractions analyzed by EMSA. Arrowheads indicate two protein:DNA complexes enriched in testes. L, protein sample loaded onto the beads; S, supernatant containing proteins that did not bind to the beads; W1–W7, washes; 0.1–3.0, elution with the KCl concentration indicated in Moles. (B) SDS–PAGE analysis of the proteins eluted from DNA beads in 1 M KCl. Proteins were separated in 4–15% gradient gel and stained with colloidal Coomassie Blue (Invitrogen). Positions of the protein size standards (Bio-Rad) are indicated on left.

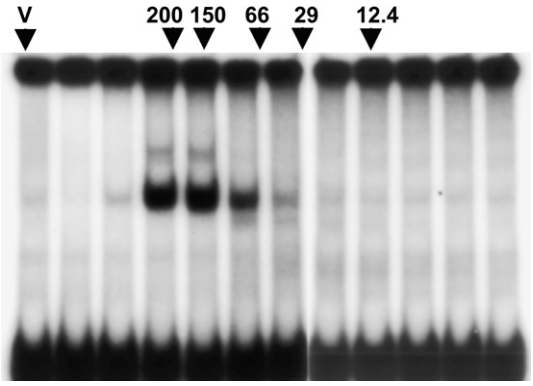

Fig. 7. Size of the protein that corresponds to the dsDNA-binding activity in testes, determined by size exclusion chromatography. Proteins were separated on the Superdex-200 gel filtration column, and fractions assayed by EMSA. The void volume (v) and the elution volumes for the protein size standards (Sigma) are indicated on top.

differently. Transcription of *Ku80* is up-regulated during spermatogenesis, and is followed with up-regulation of the Ku80 protein. In contrast, the level of *Ku70* transcript does not substantially change during germline development. Considering that both transcripts are up-regulated in adult testes to similar extent, these data indicate that up-regulation of *Ku70* is completed in early cyst cells, perhaps using the same mechanism that mediates the initial germline-independent up-regulation of *Ku80*. In addition, *Ku80* is subject to the additional germline-dependent regulation that further augments its expression during spermatogenesis.

4.2. Ku is expressed in testes as functional heterodimer

We demonstrated that transcriptional regulation of the Ku subunits leads to up-regulation of the functional heterodimer that, in fact, represents the major non-specific dsDNA end-binding activity in *Drosophila* testes. Different expression profiles of the Ku subunit genes during spermatogenesis indicate that at early stages of development, Ku70 is present in excess and the Ku80 levels draw alongside later. Therefore Ku80 may be the limiting subunit of the Ku heterodimer, hence the pattern of the Ku activity may follow the pattern of the Ku80 expression. In agreement with this suggestion, overexpression studies have demonstrated that Ku70 is not the limiting factor for the non-homologous end-joining (NHEJ) DNA repair mediated by the Ku heterodimer [15]. In mammals, Ku70 is present in excess and is needed to stabilize the newly synthesized Ku80 by forming the heterodimer; otherwise Ku80 is rapidly degraded [16]. In addition, at early stages of spermatogenesis abundant Ku70 can be involved in interactions with proteins other than Ku80 in cytoplasm.

4.3. Paucity of Ku80 in male germline nuclei

The major documented activity of Ku in the NHEJ DNA repair requires nuclear localization. However, we have not detected appreciable Ku80 signal in germline nuclei even though control staining of the accessory gland epithelium demonstrated that the used antibody is able to recognize nuclear Ku80 protein. A similar observation has been made in mammals where a marked paucity of Ku70 [17,18] and Ku80 [18] in late spermatogonia and primary spermatocytes has been observed. It has been proposed that down-regulation of Ku in meiotic prophase funnels double strand break (DSB) repair into the HR pathway, thus facilitating meiotic recombination [17]. Ku reappears later in spermatogenesis, where it acts in collaboration with members of the HR pathway to complete DSB repair before the meiotic checkpoint [18,19]. Interestingly, in *Drosophila* male germline meiotic recombination is absent [20], nevertheless Ku80 is still underrepresented in the nuclei. Thus, paucity of Ku in male germline nuclei may be conserved between distant species and not necessarily linked to the meiotic recombination.

4.4. Abundant Ku80 expression in cytoplasm of the cyst cells

Coordinated up-regulation of the Ku subunit genes in testes suggests the functional role for the protein complex. It is possible that Ku is involved in the NHEJ DNA repair pathway. In addition, Ku is capable of regulation of template-associated protein kinase that phosphorylates C-terminal domain of DNA polymerase II [21] and may be involved in transcriptional activation in spermatogenesis, similar to its role in gene regulation in mammalian development [22,23]. However, observed abundant presence of Ku in the cyst cell cytoplasm suggests other possible roles. Extranuclear activities of Ku have

been reported, in particular extracellular membrane-bound Ku has been implicated in intercellular signaling, cell adhesion and motility, and in tissue remodeling through recruitment of matrix metalloproteinase [24–30]. In addition, studies in mammals showed that cytoplasmic Ku70 is involved in regulation of apoptosis [31]. Ku70 binds pro-apoptotic factor Bax of Bcl protein family, sequestering it in cytoplasm and preventing its localization to mitochondria [31]. If not sequestered, Bax relocates into mitochondria and starts a cascade of events leading to release of cytochrome *C*, caspase activation, and apoptosis in mammalian cells (reviewed in [32]).

The similar mechanism of apoptosis was discovered in *Drosophila*. Bax homolog Debcl contains the membrane anchor (MA) domain and localizes to mitochondria when overexpressed [33,34]. Overexpression of Debcl in mammalian cells induces release of cytochrome *C* and activation of caspases in the same way as mammalian Bax [34]. Although cytochrome *C* release has not been documented in *Drosophila*, the other homologs of mammalian apoptosome complex, including caspases Drice and Dronc, appear to localize in proximity of mitochondria upon their activation [35].

It has been shown that the elements of apoptotic machinery are necessary at certain stages in spermatogenesis [36]. In *Drosophila*, the same caspases as involved in apoptosis – Drice and Dronc are expressed in spermatid bundles during individualization, and the caspase activity is necessary for normal individualization of spermatids [36]. The mechanisms of the timely caspase activation have not been discovered yet. However, drawing parallels with mammalian cells, we can propose the regulatory role for Ku70 and Ku80 in this process. We suggest that early in spermatogenesis Ku70 binds Bax homolog Debcl, preventing it from entering mitochondria. With progression of spermatogenesis, however, the expression of Ku80 increases and Ku70 is increasingly recruited to the Ku heterodimer, therefore releasing Debcl and allowing activation of the apoptotic pathway. The observed expression profiles of Ku70 and Ku80 as well as the peak expression of the Ku80 protein at the elongated spermatid stage – right before individualization – support the proposed scenario.

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