

The role of *Drosophila hyperplastic discs* gene in spermatogenesis

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Abstract

In *Drosophila*, the ubiquitin ligase Hyd (hyperplastic disc) is required for regulation of cell proliferation during development [Martin et al. (1977) *Dev Biol* **55**, 213–232; Mansfield et al. (1994) *Dev Biol* **165**, 507–526]. Earlier, we demonstrated that the *Drosophila* tumour suppressor Merlin participates not only in imaginal discs proliferation control, but also performs a separate Nebenkern structural function in *Drosophila* spermatogenesis [Dorogova et al. (2008) *BMC Cell Biol* **9**, 1. Here, we show that the *hyd* mutants also have spermatogenesis defects: chromosome condensation and attachment to the spindle, centrosome behaviour and cytokinesis in meiosis. The process of spermatid elongation was also greatly affected: nuclei were scattered along the cyst and had an abnormal shape, Nebenkern–axoneme angular relation and attachment was distorted, axonemes themselves lost correct structure. Since Hyd and pAbp protein families share a common PABC [poly(A)-binding protein C-terminal] protein domain, we also studied spermatogenesis in *pAbp* homozygotes and found defects in cytokinesis and spermatid elongation. However, our study of *hyd* and *pAbp* genetic interaction revealed only the phenotype of defective nuclei shape at the final stage of spermatid differentiation. So, the PABC domain is unlikely to be responsible for meiotic defects. Thus, our data document that, in addition to the tumour suppressor Merlin, another tumour suppressor, Hyd, also has a function in spermatogenesis.

Keywords: Hyd; pAbp; spermatogenesis; meiosis; spermatid; tumour suppressor

1. Introduction

In *Drosophila*, Hyd (hyperplastic disc) is required for the regulation of cell proliferation during development (Martin et al., 1977; Mansfield et al., 1994), with mutations in the *hyd* gene resulting in developmental abnormalities that include adult sterility caused by germ cell defects (Callaghan et al., 1998). A later study showed that in eye imaginal disc, Hyd function differs from what is expected for conventional tumour suppressor behaviour (Lee et al., 2002). It happens that in eye disc, homozygous *hyd* mutant clones induce non-autonomous overproliferation of nearby tissue directed by ectopic expression of Hh morphogene within the clones mediated by Hh regulator Ci. However, in the posterior compartment of the wing disc, this is not the case, and homozygous *hyd* mutation induces cell autonomous clone overgrowth by a separate mechanism (Lee et al., 2002). The tumour suppressor nature of Hyd protein was supported by the studies in mammals (Callaghan et al., 1998; Clancy et al., 2003; Fuja et al., 2004).

Structurally, Hyd protein family ligases contain an ubiquitin-associated domain at their N-termini, two nuclear localization signals, a zinc-finger-like UBR domain involved in recognition of type 1 N-terminal regions (Tasaki et al., 2005), a domain highly homologous with the PABC [poly(A)-binding protein C-terminal] domain and a HECT domain at their extreme C-termini. While the HECT domain is a common feature of E3 ubiquitin ligases, the presence of PABC in Hyd is unique. PABC domains are

commonly found in PABP proteins, whose function is related to mRNA translation.

A number of studies document that the function of PABC is clearly different from the function of ubiquitin ligases (Khaleghpour et al., 2001; Kozlov et al., 2001; Roy et al., 2002; Uchida et al., 2002). Our evolutionary study showed that the PABC domain was transferred to the Hyd protein at the time when primitive metazoa emerged (Omelyanchuk et al., 2009a).

The study of tumour suppressor genes in *Drosophila melanogaster* showed that such genes are active in a variety of tissues, but that only one, or at most two, cell types become malignantly transformed by any particular mutation (Gateff, 1994). This means that tumour suppressor proteins may have different functions in different tissues. Earlier, we demonstrated that *Drosophila* tumour suppressor Merlin participates not only in imaginal discs proliferation control, but also performs a separate Nebenkern structural function in *Drosophila* spermatogenesis (Dorogova et al., 2008). Here, we asked similar question for the *Drosophila* tumour suppressor Hyd.

2. Materials and methods

2.1. Fly stocks

Flies were maintained at 25°C in standard cornmeal yeast–agar medium. This and our other experimental procedures used, followed

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Abbreviations: GFP, green fluorescent protein; Hyd, hyperplastic disc; PABC, poly(A)-binding protein C-terminal.

internationally recognized guidelines. We used Bloomington stocks: *kn1^{fl-1} hyd¹⁵ e¹/TM3, Sb¹ (3718)* and *y¹ w¹¹¹⁸; PBac{3HPy+}hyd^{CO17}/TM3, Sb¹ Ser¹ (16256)* as the source of *hyd* alleles. In addition, we used an allele *hyd^{hs1}* obtained from P. Bryant (University of California). Following Bloomington, stocks were used as a source of *pAbp* alleles: *w¹¹¹⁸; P{EP}pAbp^{EP310} (17261), y¹ w^{67c23}; P{EPgy2}pAbp^{EY11561} (20684), y¹ w^{67c23}; P{lacW}pAbp^{k10109}/CyO (10970)*.

GFP (green fluorescent protein)-tag PTT insertion (37-2) for the *pAbp* (CG5119) gene was kindly provided by A. Debec (Université Pierre et Marie Curie, Observatoire Océanologique, Villefranche-sur-mer, France) and was balanced by the following way: *w¹¹¹⁸; P{w+mC=PTT-GA}pAbp/CyO, y⁺*.

2.2. Antibody staining

The procedures of testis preparation and mounting was described earlier (Dorogova et al., 2008). We used a mouse anti- γ -tubulin antibody (1:500 dilution; Sigma Chemicals) and a rabbit anti- α -tubulin antibody (1:100 dilution; Sigma Chemicals). After slide washing with 0.1 M PBS three times, a secondary antibody conjugate [Alexa 488-conjugated anti-mouse IgG (1:400 dilution) and a FITC-conjugated anti-rabbit antibody IgG (1:100 dilution)] was added for 2 h at room temperature. In some experiments, nuclei were stained with DAPI (4',6-diamidino-2-phenylindole) (1.5 μ g/ml).

2.3. Visualization of mitochondria using MitoTracker Red

Dissected testes were fixed in a drop of 0.25% glutaraldehyde in 100 mM sodium cacodylate buffer, pH 7.4, for 1 min. Then, testes were additionally fixed in 1.85% formaldehyde in 0.1 M PBS, pH 7.4, for 2 min. Preparations were washed once in 0.1 M PBS containing 0.5% Triton X-100 and then also washed twice in 0.1 M PBS. The stock 10 mM MitoTracker Red solution was diluted 1000 times in

0.1 M PBS and then was used to stain the testes for 5 min at room temperature. Preparations were then washed in 0.1 M PBS with 4 mM Pipes (pH 7.2) for 20–30 min, stained by DAPI and mounted as described in the work of Dorogova et al. (2008).

2.4. Electron microscopy

The procedure coincides with that already described in the work of Dorogova et al. (2008).

2.5. Insertion site

GFP-tag for the *pAbp* gene gives an opportunity to visualize the localization of the protein in the cell. To determine the location of artificial GFP exon within the *pAbp* gene, we extracted genomic DNA from *w¹¹¹⁸; P{w+mC=PTT-GA}pAbp/CyO, y⁺* flies, cut it with Kzo 9I restrictase, ligated it into circular form and amplified DNA fragments with internal PTT primers CCTTTCACCTCG-CACTTATT and GTGAGACAGCGATATGATTGT. The resulting DNA fragment was amplified with the same primers and sequenced with the TATCGCTGTCTCACTCAG primer. Sequence analysis showed that the PTT element was inserted into 2R chromosome arm at 14029525 bp of chromosome contig AE013599.4. In other words, protein-tag P-element is located in the second intron of *pAbp* gene at the position 1942 when counting from the gene transcription start.

3. Results

3.1. *pAbp* phenotype and localization

Flies carrying hetero-allelic combinations of three available *pAbp* alleles are viable, and their spermatogenesis can be analysed by fluorescence microscopy (see Materials and methods section). Among homozygotes, viable flies can be found only in the case of

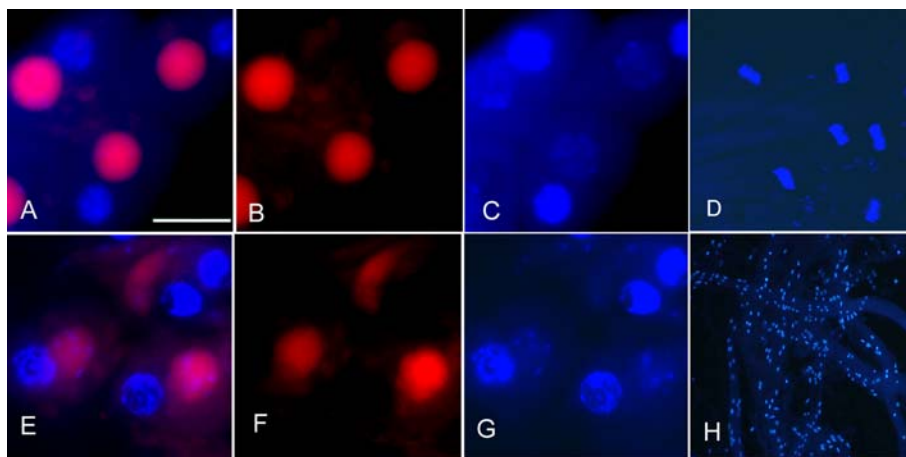


Figure 1 'Onion' and post-elongation stages in wild-type and in *pAbp* mutant spermatids

In comparison with wild-type (A–C) mutant 'onion' stage spermatids do not have distinct spherical shape of Nebenkern and uniform DNA spreading (E–H). (D) In wild-type, nuclei cluster and form a common band on the basal end of the cyst during elongation stage. (H) In mutant, nuclei are scattered through the elongated cysts and have a round instead of needle shape. For (A, B, C, E, F and G), the scale bar represents 10 μ m; for (D and H) the scale bar represents 30 μ m.

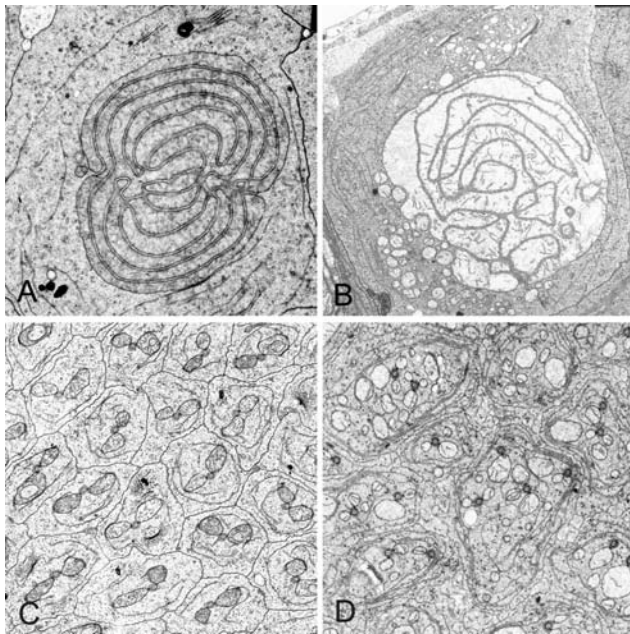


Figure 2 Abnormalities of Nebenkern formation in *pAbp* mutant (A, B) (A) Normal onion-like structure of Nebenkern in wild type. (B) In *pAbp* cells, Nebenkern does not form the correct onion-like structure. Some mitochondrial bodies are excluded in the Nebenkern sphere. Mitochondrial cysts remain inside. Morphology of the mitochondrial derivatives during the elongation stage in wild-type (C) and in the *pAbp* mutant (D). (C) Elongating mitochondrial derivatives keep their integrity. (D) Mitochondrial derivatives undergo disintegration and fragmentation.

pAbp^{EP310} allele. Most spermatogenesis abnormalities were found in the case of *pAbp*^{k10109}/*pAbp*^{EY11561} heterozygotes. Those include absence of meiotic cytokinesis (5% cases, 25 among 500 meiotic cells), resulting in formation of polyploid spermatids (Figures 1E–1G) and defects in spermatid elongation (in about 100% of the cases, 1000 spermatids) (Figure 1H). Nuclei fail to elongate, keep a round shape and are dispersed along the cyst instead of grouping at the head of the cyst. Normally, mitochondrial DNA can be visualized by DAPI staining (Figures 1A–1C). In the case of *pAbp*^{k10109}/*pAbp*^{EY11561} Nebenkern DAPI staining appears to be non-uniform, probably reflecting the effect of mutation on mitochondrial DNA organization at least in 60% of the cases (about 1000 cells inspected) (compare Figures 1A and 1G). The MitoTracker staining reveals no essential differences between the mutant and control showing that mitochondrial membranes keep their integrity,

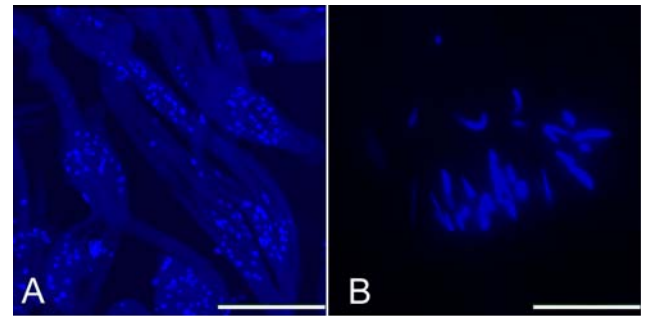


Figure 4 *Hyd* mutant cysts of late elongating spermatids (A) Nuclei are scattered through the elongated cysts and have abnormal sizes and shapes; (B) Rarely, nuclei- and mini-nuclei-rich basal end of cyst. For (A), the scale bar represents 20 μ m; for (B) the scale bar represents 5 μ m.

but in a mutant, the onion-stage Nebenkern looked larger than in a control, and its morphology differs from a perfect round (Figures 1B and 1F). Electron microscopic observations of Nebenkern structure in elongating onion and elongating spermatids of *pAbp*^{k10109}/*pAbp*^{EY11561} males reveal abnormal mitochondrial membranes at onion-stage spermatids in all the cases studied (Figure 2B). Figure 2(D) also documents the abnormal shape of mitochondrial derivatives and its defective attachment to axonemes in the elongating spermatids.

We detected the presence of a chimaeric protein in the cytoplasm of spermatocytes (Figure 3). In meiosis and onion stage spermatids, the level of the protein greatly decreased. Despite low viability, homozygotes for *P{w+mC =PTT-GA}pAbp* insertion can be found. We have not found any spermatogenesis abnormality in those homozygotes. The intracellular localization of the chimaeric protein in homo- and heterozygotes is the same. Thus, the chimaeric pAbp protein is fully functional in spermatogenesis, and its localization reflects the true pAbp distribution within the cell.

3.2. *hyd* phenotype

In the case of three *hyd* alleles, viable flies were seen in the *hyd*^{hs1}/*hyd*^{C017}, *hyd*¹⁵/*hyd*^{C017}, *hyd*^{C017} cases. Spermatogenic cells in *hyd*^{C017} homozygotes could not develop up to spermatocytes.

Qualitatively, the abnormalities in the case of *hyd*^{hs1}/*hyd*^{C017} are the same with *hyd*¹⁵/*hyd*^{C017}, but are expressed stronger than in the former case [100% of cysts (among 100 inspected) contain

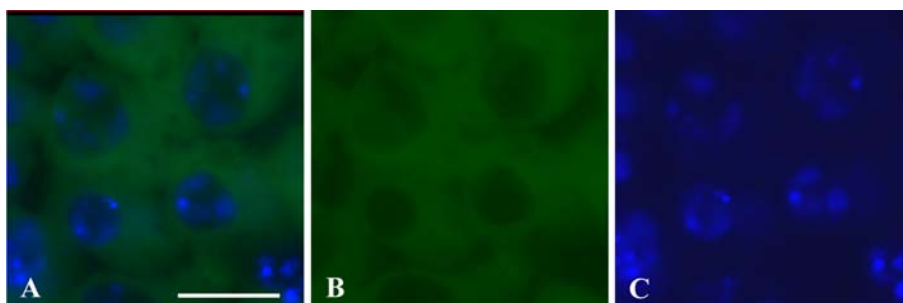


Figure 3 *pAbp*–GFP chimaeric protein localization during premeiotic interphase in spermatocytes *pAbp* is marked GFP (green), and chromosomes are stained DAPI (blue). The scale bar represents 10 μ m.

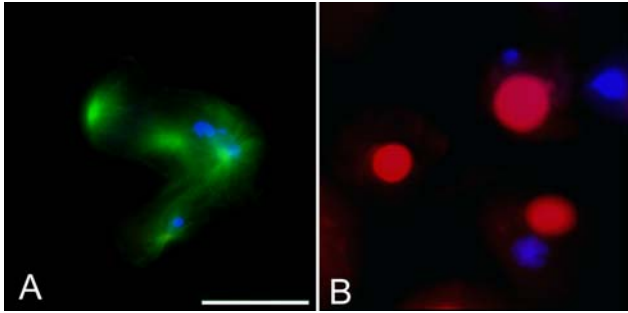


Figure 5 Consequences of defects in chromosome segregation and of cytokinesis failure in *hyd* mutant meiosis

(A) Abnormal spindle structure in anaphase II. Two spindles in common cytoplasm and one of them lose chromosomes. (B) Neberkern formation at onion stage. Spermatids differ in Neberkern size. Arrow show cell without nucleus. The scale bar represents 5 μ m.

at least one type of defect]. Figure 4(A) shows nuclei scattered through the elongated cysts and have a round shape instead of a needle one. In rare cases, the group of needle-shaped nuclei can be found in the correct place in the cyst; however, some of the nuclei are evidently incorrectly condensed (Figure 4B). About 90% of nuclei and micronuclei do not reach the correct cyst pole (totally about 100 cysts inspected).

The formation of two spindles in one cell in meiosis we found in the case of *hyd*¹⁵/*hyd*^{CO17} shows the defect of meiotic cytokinesis (Figure 5A). We also note that the meiotic products in the mutants may have unequal amounts of chromatin or mitochondrial material. The example given in Figure 5(B) shows that onion-stage spermatid nuclei and Neberkerns have unequal size. Centrosome behaviour is also affected. Figure 6 demonstrates the meiotic cell bearing four centrosomes, four asters per one nucleus. The formation of such a cell must be the consequence of incorrect centrosome disjunction. Really, in wild-type, the formation of meiotic asters takes place just before meiosis (Figure 7A). Some cells in the mutant at this stage completely lack an opportunity to organize meiotic cytoskeleton and particularly spindle asters (Figure 7B). Figure 7(C) depicts two cells, the upper one lacking a nuclei representing a product of meiosis-1 connected with the chromatin bearing a cell with two centrosomes and two asters separated just before the second meiotic division. Those examples show that meiotic chromosomes were not correctly attached to the spindle (this takes place in 50% of the meiotic figures: 100 among 200 meiotic cells in analysis).

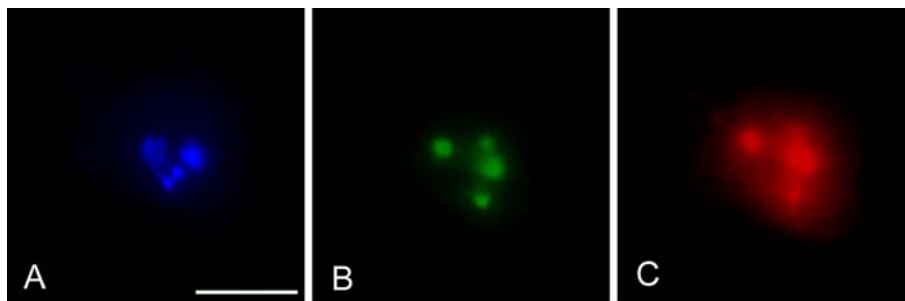


Figure 6 Meiosis II in *hyd* mutant

Cell contains four centrosomes, as a result of abnormal centrosome dynamics and failure of cytokinesis. The scale bar represents 5 μ m.

Spermatid nuclei vary in size; this is evident from the observation of different spermatogenesis stages, including the late ones. Elongated spermatid cyst in Figure 7(D) gives an example. Some of the nuclei contain only a small amount of chromatin and definitively constitute a single chromosome.

We studied the process of spermatid differentiation in *hyd*¹⁵/*hyd*^{CO17} males by electron microscopy. Cross-sections depicted in Figures 8(A) and 8(B) document a cyst, containing less than 64 spermatids. Since the axoneme grows from a single centrosome attached to spermatid nuclei (Lindsley and Tokuyasu, 1980), the cysts shown in Figure 8 may be the consequence of abnormal centrosome distribution in meiosis. It can also be seen that one axoneme can be attached to a few mitochondrial bodies (Figure 8B), and one mitochondrial body can be attached to two axonemes (Figure 8A). All this shows that, in addition to the focus of action in meiosis, *hyd* is also functioning in the process of spermatid differentiation.

3.3. *hyd* and *pAbp* Genetic interaction

Since pAbp and Hyd proteins have a common PABC domain, we checked the genetic interaction of their mutation in the diheterozygous condition. All possible diheterozygous combinations of *pAbp* and *hyd* alleles were fertile. Among those combinations, only *pAbp*^{k10109}/*hyd*^{hs1} and *pAbp*^{k10109}/*hyd*¹⁵ variants demonstrate some degree of spermatogenesis defects. In the case of *pAbp*^{k10109}/*hyd*^{hs1}, 30% of head bundles in the sperm contain both round- and needle-shaped nuclei and look like those shown in Figure 4(B) (100 among 300 cysts). Nuclei scattering was mentioned but at a low frequency. In the case of *pAbp*^{k10109}/*hyd*¹⁵, the morphology of nuclei in the sperm head bundle was indistinguishable from the control, but the nuclei were frequently scattered instead. Also, in those two diheterozygous combinations, some preponderance of cytokinesis abnormalities (in comparison with other diheterozygous combinations) were found.

4. Discussion

Human pAbp protein was identified on the basis of homology with *Drosophila* Hyd protein (Callaghan et al., 1998). *D. melanogaster* has a homologous protein pAbp (P21187), constituting the CG5119

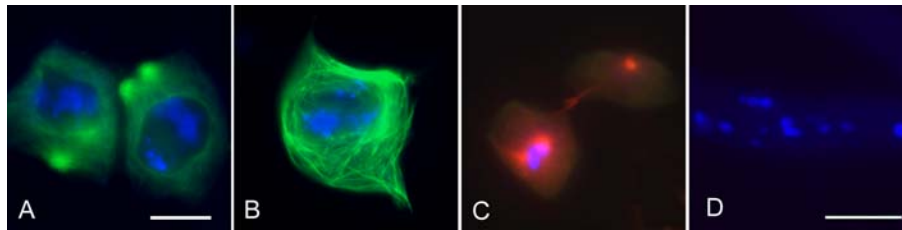


Figure 7 Defects of spindle pole organization in *hyd* mutant meiosis at late interphase stage (A, B)

(A) In normal meiosis, a bipolar spindle structure with asters is formed, microtubules are anchored on spindle poles and distribute around the nucleus. (B) Mutant cell without poles and asters, microtubules are disintegrated and are misoriented in the cytoplasm. (C) Cytokinesis in *hyd* mutant cell with normal bipolar structure: chromosomes are contained in only one daughter cell, other daughter cells are separated with the centrosome. (D) Mutant cyst with nuclei and mini-nuclei. For (A–C), the scale bar represents 7 μm ; for (D), the scale bar represents 10 μm .

ORF (open reading frame), whose molecular function was defined upon protein domains (four RRM and one PABC domain) binding to the 3' poly(A) tail of mRNA and have very important roles in the pathways of gene expression. The protein provides a scaffold on which other proteins can bind and mediate processes such as export, translation and turnover of the transcripts. Moreover, pAbp acts as antagonists to the binding of factors that allow mRNA degradation, regulating mRNA longevity.

Our analysis of pAbp function in *Drosophila* spermatogenesis reveals defects in cytokinesis and spermatid elongation. Male meiotic spindle defect in the *pAbp* (*duo*) mutant was described earlier (Fasulo et al., 1999). However, we have not found disconnection of asters and central mini-spindle, described in this thesis, despite trying to find this effect. The authors also described cytokinesis defects in this case, and this agrees with our finding. Insignificant discrepancy between those data may be a consequence of the different allelic combinations used. Moreover, in the RNAi (RNA interference) screening for cytokinesis regulators in *Drosophila* tissue culture, the *pAbp* was identified as one of the essential cytokinesis genes (Eggert et al., 2004). Thus, all the data agree well and show that protein has cytokinesis function both in meiosis and mitosis.

The localization of pAbp in spermatogenesis almost perfectly coincides with that found in the case of the mRNA localization protein Imp (Nerusheva et al., 2009). The only difference found was that the Imp concentrates at the caudal end of fully elongated cysts, while pAbp did not. This structure, we named 'pottle' is

currently thought of as the major protein synthetic activity site during spermatid elongation (Lindsley and Tokuyasu, 1980; Fuller, 1993). Thus, in general, pAbp intracellular localization follows the expected from its protein domain structure. If so, we must attribute the effect of *pAbp* mutations in meiotic cytokinesis and in the spermatid elongation to the disruption of its function in mRNA translation.

The range of spermatogenesis abnormalities in the case of *hyd* mutants was significantly wider: chromosome condensation and attachment to the spindle, centrosome behaviour and cytokinesis in meiosis are greatly affected. Despite of the efforts done, we were not able to draw a conclusion as to what process is the primary target of *hyd* mutation in meiosis; it looks like all the processes were disrupted simultaneously, and the general picture of the abnormalities qualitatively differ from those observed in the cases where the structural meiotic protein was destroyed. The process of spermatid elongation was also greatly affected, nuclei are scattered along the cyst and have abnormal shapes; Nebenkern–axoneme angular relation and attachment is distorted; axonemes, themselves, may lose their correct structure. All this shows the presence of an additional *hyd* target in spermatid differentiation.

Our study of *hyd* and *pAbp* genetic interaction revealed only the phenotype of defective nuclei shape at the final stage of spermatid differentiation. This suggests that, despite of the presence of common PABC domain, the proteins may have the only common target in spermatid differentiation, while all other spermatogenesis targets may differ. Looking at the spectrum of phenotypes of *pAbp* and *hyd* mutants, it is tempting to suppose that their effects may both be due to mRNA translation and the PABC domain. It would be more adequate to relate Hyd effects with its ubiquitin–ligase activity. This point agrees well with the already established role of the ubiquitin-dependent proteolytic system in spermatogenesis of mammals (Baarends et al., 2000). In *Drosophila*, the phenotype of scattered nuclei produced by the mutation in a gene *cbx*, coding for ubiquitin–ligase was also described (Fabrizio et al., 1998).

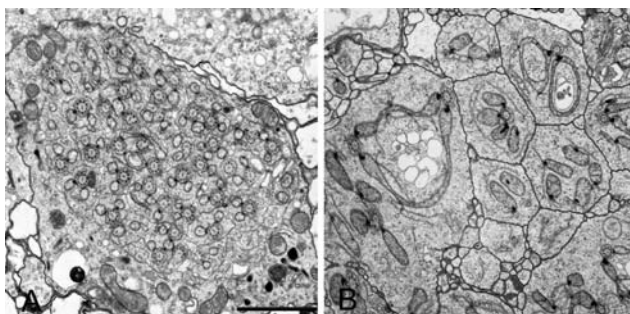


Figure 8 Elongating cysts of *hyd* mutant

(A) Cyst contains reduced number of spermatids. Spermatids show a number of mitochondrial derivatives and axoneme higher than normal. (B) Several spermatids do not contain axonemes (arrow). The scale bar represents 2 μm .

Author contribution

Julia Pertceva and Natalia Dorogova performed the cytological study of *hyd* and *pAbp* mutants and studied the phenotype of *pAbp*–*hyd* genetic interaction. Elena Bolobolova carried out the electron microscopy. Olga Nerusheva performed a pilot study of

pAbp-GFP protein distribution in spermatogenesis and *pAbp-hyd* genetic interaction. Svetlana Fedorova performed the Feulgen-Giemsa study of *hyd* and *pAbp-hyd* mutants, supporting the data presented in the manuscript. Leonid Omelyanchuk helped in the design, co-ordination, writing, submission and correction of the manuscript.

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