Dynamic expression pattern of kinesin accessory protein in *Drosophila*

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We have identified the *Drosophila* homologue of the non-motor accessory subunit of kinesin-II motor complex. It is homologous to the SpKAP115 of the sea urchin, KAP3A and KAP3B of the mouse, and SMAP protein in humans. *In situ* hybridization using a DmKAP specific cRNA probe has revealed a dynamic pattern of expression in the developing nervous system. The staining first appears in a subset of cells in the embryonic central nervous system at stage 13 and continues till the first instar larva stage. At the third instar larva stage the staining gets restricted to a few cells in the optic lobe and in the ventral ganglion region. It has also stained a subset of sensory neurons from late stage 13 and till the first instar larva stage. The DmKAP expression pattern in the nervous system corresponds well with that of Klp64D and Klp68D as reported earlier. In addition, we have found that the DmKAP gene is constitutively expressed in the germline cells and in follicle cells during oogenesis. These cells are also stained using an antibody to KLP68D protein, but mRNA *in situ* hybridization using KLP64D specific probe has not stained these cells. Together these results proved a basis for further analysis of tissue specific function of DmKAP in future.

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1. Introduction

The 115 kDa kinesin accessory protein (SpKAP115) was first identified in the sea urchin as an integral component of the heterotrimeric kinesin-II motor complex (Wedaman *et al* 1996). Subsequently homologous proteins were identified in *Caenorhabditis elegans* (CeKAP), the mouse (KAP3A, KAP3B; Yamazaki *et al* 1996), and humans (SMAP; Shimizu *et al* 1996). The predicted amino acid sequences of SpKAP115 and SMAP contain several 40 amino acid long armadillo like repeat elements, which are likely to be involved in protein–protein interactions *in vivo*. A recent report has also suggested that the SMAP protein can be bound to the APC tumour suppressor protein through the armadillo like repeat elements. This interaction appears to be important for the formation of APC clusters in the plasma membrane protrusions of MDCK and L cells (Jimbo *et al* 2002). In addition, immunoprecipitation experiments and electron microscopic studies of the kinesin-II protein complex suggest that KAP is likely to associate with the C-terminal domains of kinesin-II motor subunits (Wedaman *et al* 1996; Yamazaki *et al* 1996). Studies in *C. elegans* have further suggested that KAP is involved in intra-cellular transport in the cilia. It is found to move into the cilia at a rate similar to kinesin-II motor subunits (Signor *et al* 1999). This is also supported by co-purification of KAP homologous protein with the FLA10 motor subunit from *Chlamydomonas* flagella (Cole *et al* 1998). All these results clearly suggest that KAP is an adaptor for cargo association with the kinesin-II motor subunits. However, immunoprecipitation experiments from mouse tissue extracts revealed that only 70% of the KAP3A was associated with KIF3A/KIF3B complex in the testis (Yamazaki *et al* 1995, 1996). Furthermore, a yeast two-hybrid interaction study suggested that the human homologue of KAP (SMAP) could associate with the human small G-protein GDP dissociation stimulator (SmgGDS), and was also found to associate with the human chromatin-associated-proteins of the HCAP/XCAP-E/SMC-1 family in the nui-

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clear fraction of COS-7 cells (Shimizu *et al* 1996, 1998). This evidence suggests that KAP may have many functions *in vivo*. Therefore it is important to establish a system where one could analyse the KAP function *in vivo*.

The availability of the *Drosophila* genome sequence and a complete cDNA library provided a good opportunity to identify and clone homologous genes *in silico* (Adams *et al* 2000). The fly community (www.flybase.org) has also developed a collection of P-transposon insertion stocks in identified genomic locations, thus allowing easy genetic manipulation of cloned genes. Homologues of each one of the kinesin-II motor subunits, as well as the non-motor accessory subunit are found in the *Drosophila* genome. The motor subunit homologues were previously identified as KLP64D and KLP68D respectively (Stewart *et al* 1991; Pesavento *et al* 1994; Ray *et al* 1999). We have further identified an ORF in the *Drosophila* genome (BDGP gene annotation No. CG11759, predicted ORF CT4241, Flybase acc. No. Fban 0011759) that could code for a 935 amino acid long polypeptide (figure 1A), and is homologous to CeKAP of *C. elegans*, SpKAP115 of the sea urchin, KAP3A and KAP3B of the mouse, and the human SMAP protein, respectively (figure 1C, and table 1). Further, analysis of the predicted amino acid sequences of the ORF CT4241 using the motif scan programs revealed three armadillo/***b***-catenin like repeat elements at the C-terminal domain and a conserved tyrosine phosphorylation site at position 767, respectively (figure 1A). In addition, the N-terminal domain contained a predicted coiled coil structure (figure 1B), and similar coiled coil structures are also predicted for the mouse and human homologues, respectively. Together, all this suggested that the predicted ORF CT4241 of *Drosophila* was the likely homologue of the KAP protein and therefore we labelled it DmKAP. For further characterization of the function of the DmKAP gene, we studied its expression pattern at different stages of development using the mRNA *in situ* hybridization technique and compared it with the expression patterns of Klp64D and Klp68D, respectively. The results of our experiments are presented below.

2. Materials and methods

2.1 *mRNA* in situ *hybridization*

The DmKAP cDNA in pBluescript vector was obtained from Genomes System Inc (clone No. LD13052). We had it further sequenced to confirm that it matched with the previously reported sequence (Bangalore Genei (P) Ltd., India). The C-terminal 1·2 kb fragment of DmKAP cDNA (obtained by digesting the clone with Pst1) was used to make antisense RNA probe using T3 RNA polymerase as described before (Ray *et al* 1999). We could not use the same strategy to make KLP64D probe due to some inconsistencies in our cloned DNA. Therefore we made an anti-sense cDNA probe by amplifying the 1·5 kb C-terminal fragment of intronless Klp64D coding sequence from genomic DNA using specific primers. The amplified DNA fragment is then used as a template to make an anti-sense cDNA probe by 30 cycles of PCR reaction with a combination of three anti-sense primers in 200 μM dNTP mixtures and ~ 30 μM dig-dUTP (Boehringer GmBH). The relative probe concentrations were determined visually in the following manner. We first spotted a small alliquote of all the probes on a nylon membrane (PVDF) in a denaturing condition, then washed it with water and naturally dried it to fix the DNA samples on the membrane. It was then washed with PBS and incubated with 1 : 200 Anti-dig: AP (Boehringer GmBH) for 40 min, followed by several washes in PBS with 0·3% Triton X100, and then developed using a mixture of 0·3 mg/ml NBT and 0·17 mg/ml BCIP in 0·1 M Tris (pH 9·0) and NaCl buffer. The staining in embryos and larva were done according to established protocols (Ray *et al* 1999). The corresponding sense strand specific probes were used as control, which did not produce any staining. The ovaries were dissected in PBS and first fixed using 4% paraformaldehyde (PF) in PBS with 10% dimethylsulfoxide (DMSO), and three volumes of heptane, for 30 min at room temperature. Next, they were transferred to 4% PF in PBS for 20 min, then washed in PTX and incubated with 4–30 μg of proteinase K in 1 ml PBS for 2–5 min. The proteinase K digestion was stopped by several washes in PTX and again fixed in 4% PF solution as before. The hybridization was done for 16 h at 45°C in Hyb-B containing 50% formamide, 5X SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml heparin and 0·1% Tween 20. The samples were washed with several changes of Hyb-B followed by PTX, then incubated with 1 : 200 Anti-dig: AP (Boehringer GmBH) and developed with NBT and BCIP as described above. The tissue samples were then dehydrated in ascending grades of alcohol and mounted with a drop of DPX.

2.2 *Immunostaining ovaries*

Ovaries from 3–4 day old females were dissected and fixed as described above. They were washed three times for 15 min each in PBT (0·3% Triton X100), incubated 1 h in PBS with 0·3% Triton X100 and 0·5% BSA (blocking buffer), and then incubated overnight in a 1 : 50 dilution of purified rabbit anti-KLP68D in the

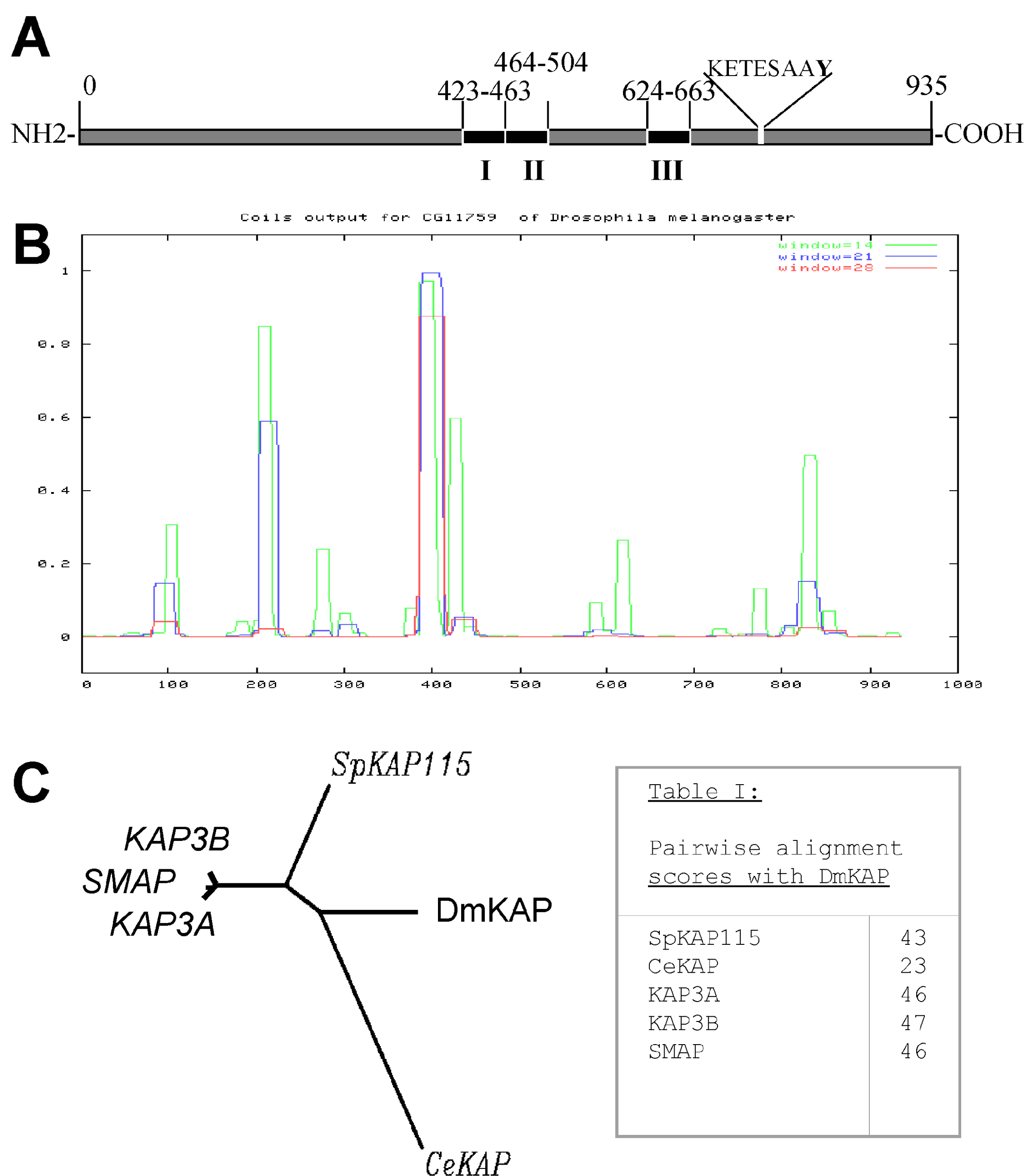

Figure 1. (**A**) The thick gray line represents the length of DmKAP polypeptide with amino acid positions marked for different structural features. Flybase prediction analysis suggests that DmKAP gene codes for a 935 amino acid polypeptide. Sequence analysis using "ScanProsite" (http://www.expasy.ch/tools/scanprosite/) suggests a potential tyrosine phosphorylation site at residue 767 (Ket.Esaa**Y**), which is also conserved in SMAP and KAP3A respectively. In addition, analysis using "MotifScan" program (http://hits.isb-sib.ch/cgi-bin/PFSCAN_parser) suggests three armadillo/***b***-catenin like repeat elements at positions 423–463 (I), 464–504 (II), and 624–663 (III) respectively (marked by thick black lines in the figure). DmKAP polypeptide has a predicted isoelectric point 5·61 and molecular weight 106·29 kDa. (**B**) The probability of ***a***-helical coiled coil structure along the length of DmKAP polypeptide. The probability scores are plotted along Y-axis while the X-axis indicates the amino acid positions form the N-terminus (http://www.ch.embnet.org/software/COILS_form.html). Secondary structure predictions using GOR IV (Garnier *et al* 1996; http://pbil.ibcp.fr/) also suggest that DmKAP is a globular protein rich in alpha helical conformation (48·66% overall). In addition, analysis according to the algorithm of Lupas *et al* (1991) suggests that the region between the residues 385 and 412 has a high probability to form a coiled coil structure. (**C**) The predicted DmKAP polypeptide is homologous to the mouse KIF3A and KIF3B, human SMAP, SpKAP115 of sea urchin and CeKAP of *C. elegans* as shown in this figure and in table 1, respectively. The figure shows an unrooted dendrogram of all the KAP homologous sequences as produced by ClustalW 1·8 and the corresponding similarity scores are presented in table 1 (Higgins *et al* 1996).

blocking buffer. Samples were washed three times for 15 min in the blocking buffer and then incubated for 1 h in Alexa 488 coupled secondary antibody (1 : 500 in PBT). They were further washed with several changes of blocking buffer and then mounted with a drop of Vectashield® (Vector Labs, Inc. USA).

3. Results

3.1 *DmKAP is expressed in a pattern similar to KLP64D and KLP68D in the nervous system*

In situ hybridization using a specific cRNA probe and a standard non-radioactive detection technique revealed that the DmKAP gene is expressed in different types of cells including the neurons of the central nervous system (CNS) as well as a subset of sensory neurons. The zygotic expression begins at stage 6 when a high level of staining is observed in the cells around the morphogenetic furrows and in the neuro-ectoderm cells at this stage (data not shown). The staining gradually condenses in a subset of cells in the CNS (arrows in figure 2A) and they appear in every segment in the presumptive ventral ganglion (VNG) region of the developing brain. This staining persists until the end of embryogenesis and here a strong staining is observed in the brain lobes and the ventral ganglion (figure 2B, arrow head and arrow respectively). The DmKAP staining also appears in a subset of sensory neurons in the peripheral nervous system (PNS) at stage 13, and continues till late stage 17 when the embryo is completely developed. At this stage we found expression in: (i) the lateral chordotonal organs (figure 2C), (ii) the bolwig organ (figure 2D), (iii) and other anterior sense organs (data not shown). In addition, there is staining in certain sensory neurons of the ventral and dorsal clusters of the chordotonal organ (data not shown). The pattern of sensory neurons stained by DmKAP probe is identical to that of KLP68D and KLP64D as reported earlier (Pesavento *et al* 1994; Ray *et al* 1999) and we have further verified this, using a KLP64D specific cDNA (single stranded) probe (figure 2E–H).

The DmKAP expression in the CNS is found to continue to the first instar larva stage (figure 3A) when a strong staining is observed in most of the ventral ganglion (arrow, figure 3A) and brain lobe cells (arrowhead, figure 3A). In the third instar larva stage the staining is restricted to a few cells in the presumptive optic lobe region (arrowhead, figure 3B) while a weak staining is visible in the ventral cortical region (arrow, figure 3B). Our examination has revealed that DmKAP positive cells in the optic lobe are present at the insertion site of the optic stalk (arrow, figure 3C). This region is known to have cell bodies of cholinergic inter-neurons (Yasuyama *et al* 1995). A similar pattern of staining was seen earlier with the KLP64D specific probe (Ray *et al* 1999). Our data suggests that DmKAP could act in association with KLP64D and KLP68D in the neurons of CNS and PNS. This hypothesis is based on co-localization of the respective gene expression in different neurons. Some direct experimental evidence would be necessary to establish this hypothesis. In addition to the nervous system cells, the epithelial cells of the imaginal disc peripodium are also stained at the third instar larva stage (arrow, figure 3D). This indicates that DmKAP may play a role in imaginal disc development as well. DmKAP in the disc epithelial cells may function in association with other kinesin-II components or it may function independent of KLP64D and KLP68D. This can be resolved by studying the corresponding expression pattern of these two genes in the imaginal disc and other non-neural tissue.

3.2 *DmKAP expression pattern in the ovary only overlaps with that of KLP68D*

Since KLP64D and KLP68D were earlier reported to have dissimilar levels of expression in the ovary (Stewart *et al* 1991), we analysed the *in situ* hybridization pattern in this tissue using both the DmKAP cRNA (figure 4A–D) and KLP64D cDNA (figure 4E) probes, respectively. In addition, we studied the KLP68D localization in this tissue using a purified antibody (figure 4F). Our RNA *in situ* hybridization data has revealed that DmKAP is expressed in follicle cells and in nurse cells at all stages of oogenesis (figure 4). There is, however, no staining in other somatic tissue like the stalk cells that links individual vitellarium (big arrows, figure 4A), and cells of the terminal filament of an ovariole (big arrow, figure 4A). DmKAP mRNA is also present in stem cells and in all developing cyst cells inside germarium (figure 4A). The staining in nurse cells (marked by a white asterisk in figure 4A) and in follicle cells (arrowhead, figure 4A) has persisted through out development. However, the level of expression in nurse cells is elevated from stage 10b (figure 4C, a single nurse cell is marked by dotted lines). This is likely to contribute to the maternal component to oocyte. DmKAP gene expression is also observed in all follicle cells that cover the egg chambers at this stage. This is visible at a higher magnification as shown in a stage 10b egg chamber in figure 4D. This data suggests that DmKAP may play an important role during oogenesis. In contrast, the KLP64D cDNA probe (single stranded, antisense) revealed no staining during the early developing stages of oogenesis (arrowheads in figure 4E). Only the nurse cells of stage 10b egg chamber stained for KLP64D mRNA and this is likely to contribute as maternal component to oocyte. This

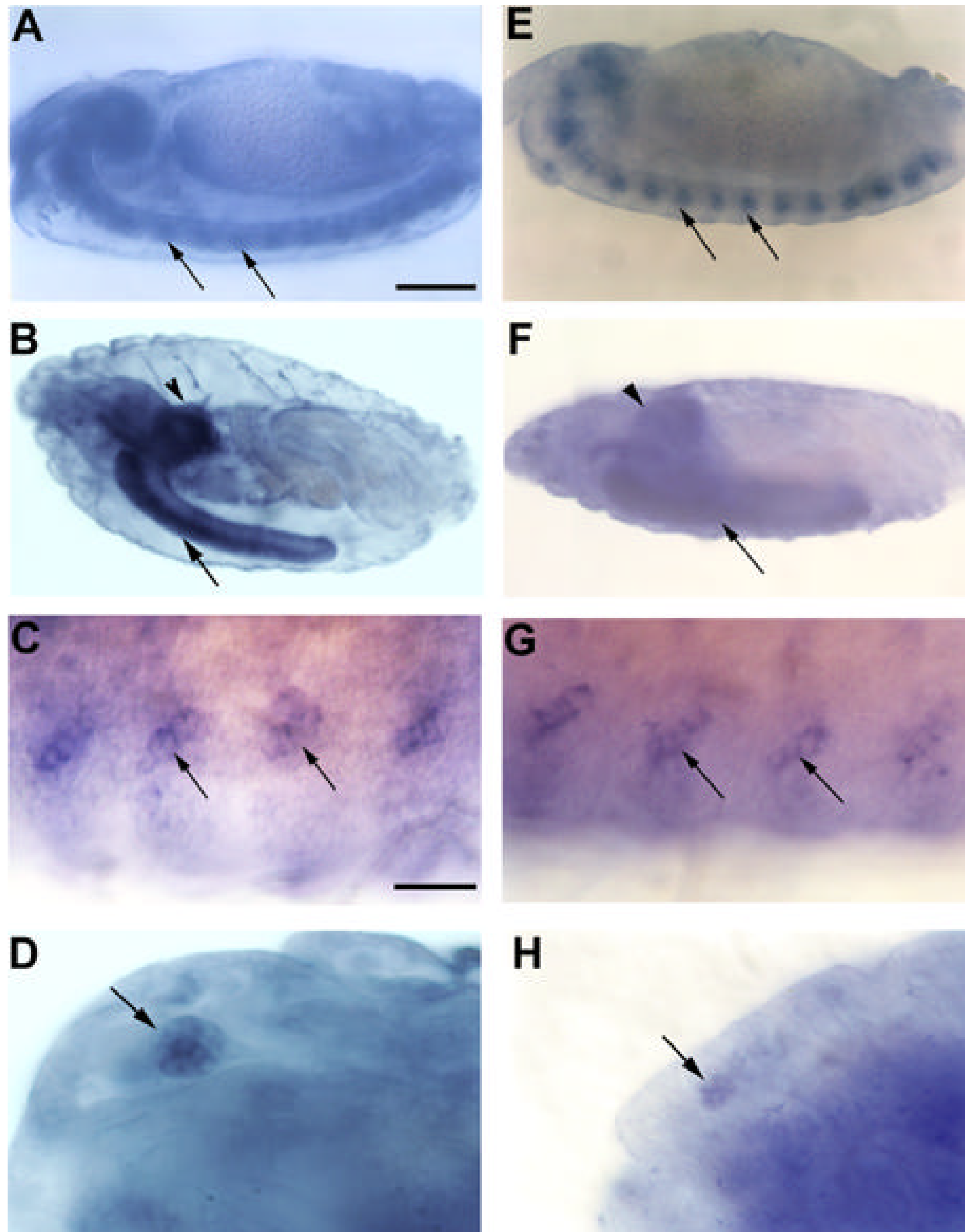

Figure 2. DmKAP mRNA expression pattern during embryogenesis as revealed by mRNA *in situ* hybridization with a specific DIG labelled cRNA probe made from the 1·2 kb 3′ Pst1 fragment. The probe for KLP64D is a specific cDNA probe. The anterior side of the embryo is towards the left and the ventral side faces downward. (**A**) The staining in a stage 13 embryo. A relatively higher level of staining is observed in the central nervous system (CNS, arrows) at this stage and it appears condensed in some cells in each segment of developing ventral ganglion (arrows) in a pattern similar to what has been seen with the KLP64D specific probe (**E**). The expression continues throughout embryogenesis and till the first instar larva stage. (**B**) The staining in the CNS (arrow) of a late stage 17 embryo. (**C**) The staining in the peripheral nervous system (PNS) at this stage is limited to a subset of sensilla, which includes the abdominal lateral chordotonal organ (lch5) as shown in the figure (arrows). (**D**) We have also found staining in the sensilla at the anterior side of the embryo. The staining in bolwig organ neurons (arrow). **E**, **F**, **G** and **H** show the mRNA expression pattern of the KLP64D gene at similar stages of embryogenesis as described in **A**, **B**, **C** and **D** respectively. The embryonic stages and sense organs are identified according to Campos-Ortega and Hartenstein (1997). The scale bar is 100 µm for **A** to **F** and 25 µm for **C** to **H** respectively.

pattern is again found to be very consistent even when KLP64D probe concentration is about two- to three-fold higher than that of the DmKAP cRNA probes as estimated by colorimetric methods. We have also shown that the KLP64D cDNA probe stains in an exactly identical pattern in the embryos as reported previously using cRNA probes (Ray *et al* 1999). Furthermore, both the DmKAP cRNA and KLP64D cDNA probes stained in an identical pattern in the embryonic and larval nervous system. This suggests the DmKAP and KLP64D staining patterns in the ovary is a true reflection of tissue specific expressions of the respective genes. We therefore

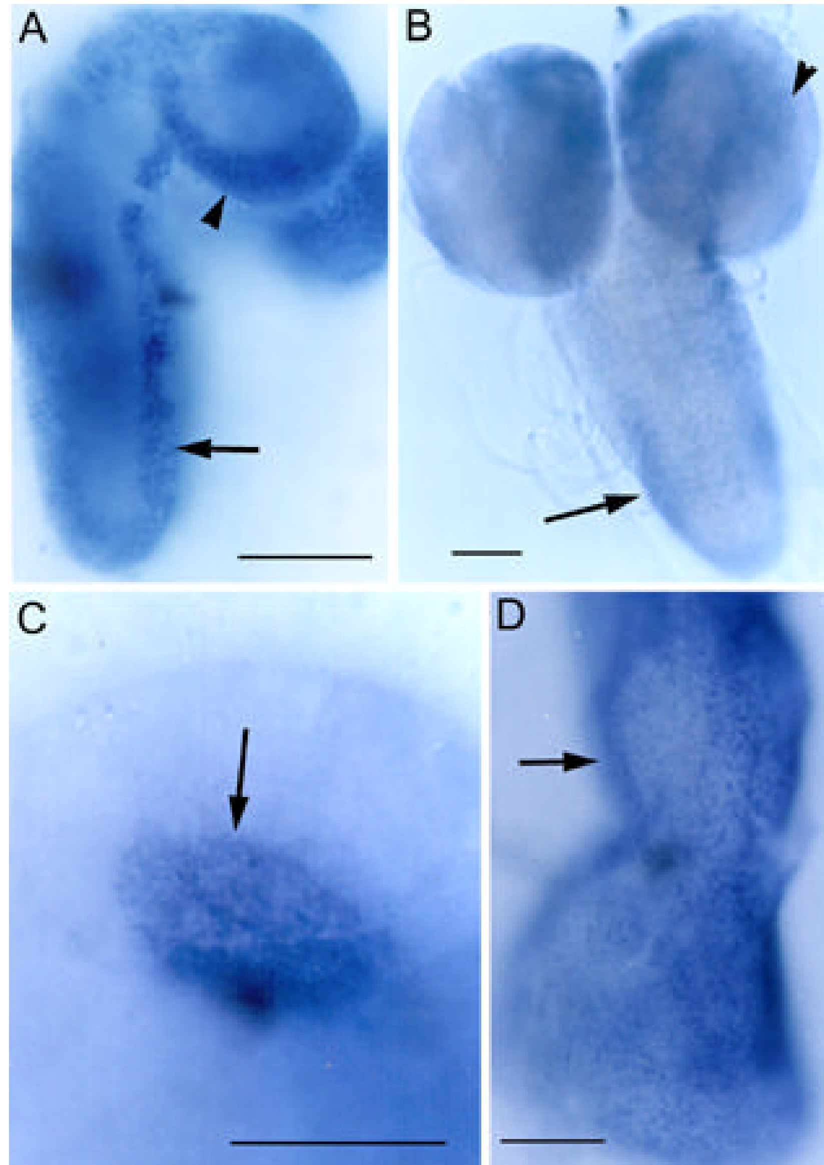

Figure 3. DmKAP mRNA *in situ* hybridization in the larval brain (**A**, **B**, **C**) and imaginal disc (**D**) also indicate a dynamic pattern of expression. (**A**) A majority of the cells in a first instar larva brain are stained with the DmKAP specific probe. (**B**) The pattern, however, gets restricted to a few cells in the ventral cortical region (arrows) and in the optic lobe region at the third instar larva stage (arrowhead). (**C**) A high magnification image of the optic lobe shows specific staining in a set of cells around the optic stalk region (arrow) in the brain. (**D**) In addition, a high level of staining is found in the peripodial cells (arrow) of the eye-antennal disc as well. The pattern is similar in all other imaginal discs. Scale bar represents 50 μm in all figures.

conclude that the KLP64D does not express itself during early stages of oogenesis.

This observation has raised the important question of whether DmKAP may function independent of kinesin-II in the ovary. We have searched the *Drosophila* genome sequence for predicted open reading frames that could code for kinesin-II homologous motor proteins. We have only found KLP64D and KLP68D as the closest match to any known kinesin-II motor. Since transcription precedes translation, the presence of a protein in a specific tissue could be used as an indication of corresponding gene expression in those cells. We therefore stained the ovary with an antibody to KLP68D. This was previously reported to stain in a pattern similar to the KLP68D mRNA

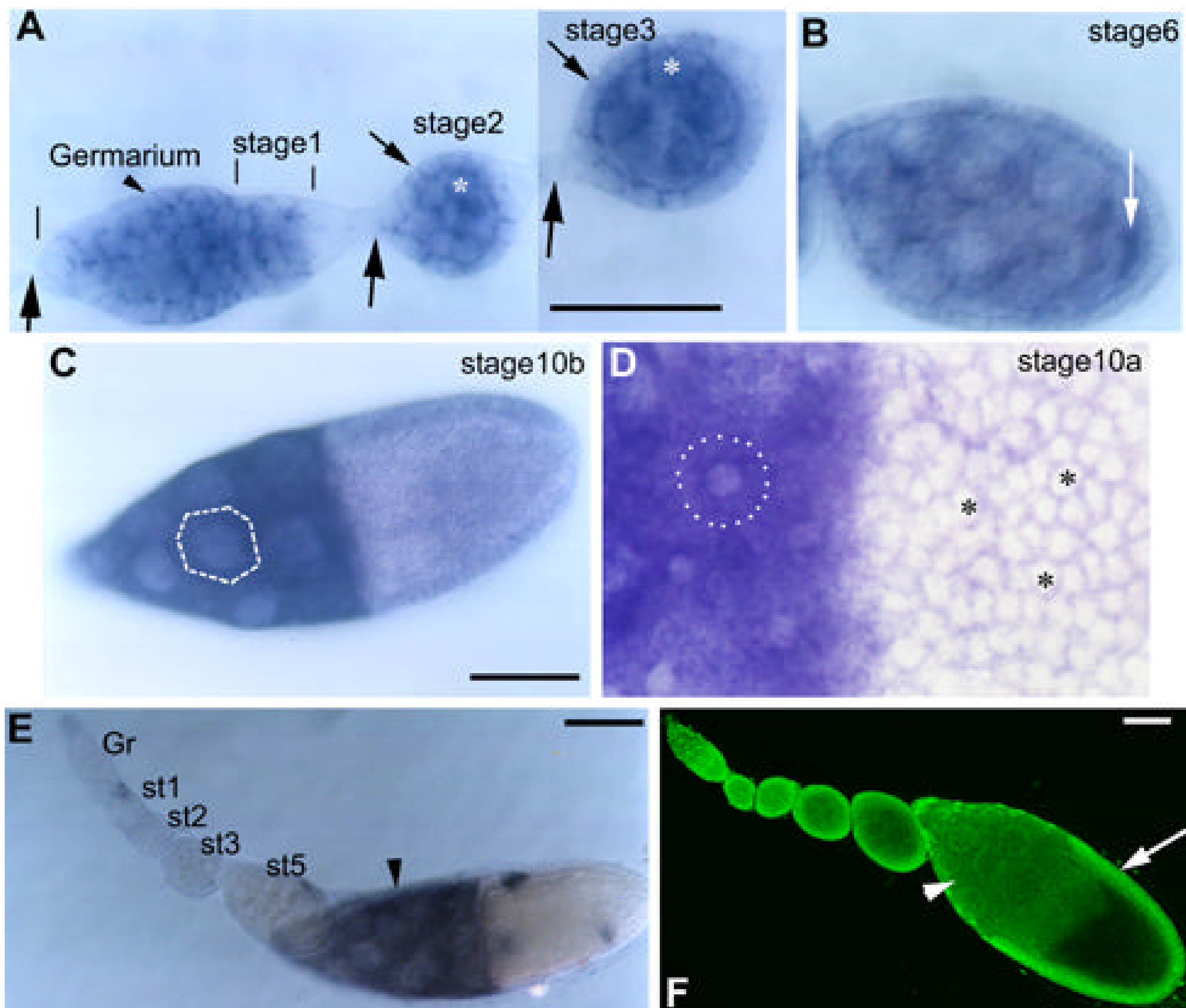

Figure 4. mRNA *in situ* hybridization pattern in the ovary using DmKAP (**A**, **B**, **C**, **D**) and KLP64D (**E**) specific probes. It also shows the distribution of KLP68D antigen in the ovary (**F**). (**A**) DmKAP staining is observed in germarium cells (arrowhead), and in all the cells of stage 1, stage 2, and stage 3 egg chambers. Each such egg chamber contains 15 nurse cells (marked by a white asterisk) one oocyte, and several follicle cells at the periphery (fine arrows). No staining has been observed in the terminal filament cells connecting different egg chambers (arrows). (**B**) The staining continues at later stages and the figure shows staining in a stage 6 egg chamber (the oocyte is marked by an arrow). (**C**) A relatively high level of DmKAP staining is observed in the nurse cells (marked by dotted lines) of a stage 10b egg chamber. (**D**) In addition, we have also seen staining in the follicle cells surrounding the oocyte (asterisks) and in the nurse cells (dotted circle). (**E**) No KLP64D mRNA expression is seen during early stages of oogenesis. The staining is only observed in the nurse cells of late stage 10b egg chambers (arrowhead). The figure shows a picture of a single ovariole containing germarium (Gr), stage 1 (st1), stage 2 (st2), stage 3 (st3), stage 4 (st4), stage 5 (st5) and a stage 10b (st10b) egg chambers respectively. (**F**) A purified KLP68D antiserum has been used to stain all the nurse cells and follicle cells at all stages of oogenesis as shown in the picture. The scale bar indicates 50 μm in **A**, 100 μm in **C** and **E** and 70 μm in **F**.

probe in the embryos (Ray *et al* 1999). Our immunostaining experiments revealed that the KLP68D antigen was present in all the germ line cells in the ovary (figure 4F) in a pattern very similar to that of DmKAP mRNA (figure 4). This suggests that KLP68D and DmKAP genes are simultaneously expressed in a subset of neurons and in all germline cells. Further, genetic analysis using mutants in the KLP68D and DmKAP gene would reveal their functions in the respective tissues.

4. Discussion

We have reported the identification and characterization of *Drosophila* homologue of SpKAP115 in this paper. Homology search and secondary structure analysis with the predicted DmKAP amino acid sequence revealed both sequence and structural similarities between DmKAP and it homologues. We found a coiled coil motif at the N-terminus region of DmKAP (figure 1B), which was similar to the ones predicted for other KAP homologous proteins. Since KAP homologues in other systems are known to associate with kinesin-II motor subunits through the N-terminal domains (Shimizu *et al* 1996), it is likely that DmKAP and KLP68D/KLP64D could form the functional kinesin-II in *Drosophila*. We have further shown that DmKAP, KLP64D and KLP68D express in an identical set of cells in the nervous system at late stages of development while only DmKAP and KLP68D show a similar pattern of expression during oogenesis. This indicates that the composition of kinesin-II could be different in the nervous system and ovary.

Analysis of gene expression patterns during development and in adults often provides important clues about the probable function of corresponding proteins. For instance, the KIF3A and KIF3B genes are expressed in a diverse range of tissues including neurons in the mouse (Yamazaki *et al* 1995; Marszalek and Goldstein 2000), and they are essential for cilia morphogenesis during early development (Nonaka *et al* 1998). The KIF3A is expressed in the photoreceptor cells of the retina (Murasen *et al* 1998) and is found to transport specific proteins to the outer compartment of photoreceptor cells (Marszalek *et al* 2000). Although KLP64D and KLP68D mRNAs are found in many different tissues by Northern analysis (Stewart *et al* 1991), the mRNA *in situ* hybridization technique revealed a higher level of expression in a subset of mature neurons in the embryos (Pesavento *et al* 1994; Ray *et al* 1999). Further, genetic studies suggested that both these proteins are involved in the axonal transport process (Ray *et al* 1999). The Northern blot experiment also revealed that in certain tissue types, like in ovary and testis, the KLP68D gene expression is much more than that of KLP64D (Stewart *et al* 1991). We have further shown that DmKAP is expressed in a pattern identical to that of both the kinesin-II motor subunits in the nervous system, whereas its expression in the ovary coincides only with that of KLP68D. The actual cellular and developmental implication of this result is still unclear. It is highly likely that the composition of kinesin-II would be important in selecting the cargo in the respective cell types. Previous genetic analysis has already suggested that expression of KLP64D and KLP68D in the cholinergic neurons is linked to their role in transporting specific cargoes to the synapse (Ray *et al* 1999). The identity of such cargoes in the ovary is not clear at this point, but our results certainly indicate that the transport will have an important bearing on the function of these cells during oogenesis.

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