

Drosophila Larp associates with poly(A)-binding protein and is required for male fertility and syncytial embryo development

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ABSTRACT

As the influence of mRNA translation upon cell cycle regulation becomes clearer, we searched for genes that might specify such control in *Drosophila*. A maternal-effect lethal screen identified mutants in the *Drosophila* gene for Larp (*La*-related protein) which displayed maternal-effect lethality and male sterility. A role for La protein has already been implicated in mRNA translation whereas Larp has been proposed to regulate mRNA stability. Here we demonstrate that Larp exists in a physical complex with, and also interacts genetically with, the translation regulator poly(A)-binding protein (PABP). Most mutant alleles of *pAbp* are embryonic lethal. However hypomorphic *pAbp* alleles show similar meiotic defects to *larp* mutants. We find that *larp* mutant-derived syncytial embryos show a range of mitotic phenotypes, including failure of centrosomes to migrate around the nuclear envelope, detachment of centrosomes from spindle poles, the formation of multipolar spindle arrays and cytokinetic defects. We discuss why the syncytial mitotic cycles and male meiosis should have a particularly sensitive requirement for Larp proteins in regulating not only transcript stability but also potentially the translation of mRNAs.

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Introduction

Rapid changes in cellular physiology during cell cycle progression are regulated primarily by reversible protein phosphorylation, a process that can dramatically change the properties of target proteins. Additionally, periodic proteasome-mediated destruction of key regulatory proteins provides another means of controlling their activity. Cells prepare for passage through particular phases of the cell cycle by initiating the translation of specific regulatory proteins from mRNA. Together with transcriptional control, these mechanisms all contribute to the regulation of cell cycle progression during development. While many key mechanisms that regulate the tissue specific and periodic transcription of genes within the cell cycle have been characterised, much less is known about the mechanisms regulating the stability and translation of mRNAs during cell cycle progression. Such processes might also have particular importance at developmental stages that place high demands upon the translational machinery to provide proteins essential for progression through the cell cycle.

The majority of proteins are synthesised by cap-dependent translation in which the 5'-(m7GpppN) mRNA cap is bound by initiation factors eIF4E, eIF4A and eIF4G, to form the trimolecular eukaryote initiation factor complex 4F (eIF4F). This facilitates the recruitment of the 43S pre-initiation complex (the 40S sub-ribosomal particle plus initiation factors) which migrates along the mRNA until reaching the initiation codon whereupon 80S ribosome assembly occurs and translation is initiated (Hershey and Merrick, 2000).

Poly(A)-binding protein (PABP) attaches to both the 3' poly(A) tail (Deo et al., 1999) and eIF4G (Imataka et al., 1998) which in turn binds eIF4E bringing the 3' and 5' ends of the mRNA together to form a circular mRNA loop (Wells et al., 1998). By forming a "closed-loop" structure, PABP acts synergistically with the 5' cap complex to stimulate translation, whilst maintaining mRNA stability (reviewed by Gallie, 1998). Once translation has been completed, the mRNA is degraded by shortening of the poly(A) tail (deadenylation) and loss of PABP, followed by removal of the 5' cap by the Dcp1/2 complex and bidirectional exonucleolytic degradation (Shyu et al., 2008). PABP protects mRNA from both 5' de-capping and 3'-5' deadenylation, the latter through inhibition of deadenylase enzymes such as poly(A) ribonuclease (PARN) (reviewed by Gorgoni and Gray, 2004). Translation can be thus be indirectly controlled by proteins that regulate PABP both in its interaction with eIF4G (such as PAIP1 and PAIP2 (Martineau et al., 2008; Craig et al., 1998; Khaleghpour et al., 2001))

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or that regulate the interaction between PABP and the poly(A) tail (such as AUF1 (Sagliocco et al., 2006)). In addition, PABP may negatively regulate its own expression at mRNA level (Wu and Bag, 1998).

During mitosis, cellular stress, apoptosis or viral infection, cap-mediated translation is repressed in favour of cap-independent forms (Spriggs et al., 2005, 2008), such as internal ribosome entry site (IRES)-mediated translation. Here the ribosome binds directly to the IRES upstream of the start codon within target mRNA, where a translation initiation complex is then assembled. This process requires IRES *trans-acting* factors (ITAFs), such as La protein (Yoo and Wolin, 1994). The switching from cap-dependent to cap-independent forms of translation is essential for mitotic progression, and a number of crucial G₂/M regulatory proteins such as p58^{PITSLRE} kinase and *c-myc* carry an IRES within their 5' UTR. How this switch is controlled remains unknown, but may be activated through sequestration of the cap-dependent translation regulators 4E-BP1, via mTOR activation, and eIF4B, through binding 14-3-3 σ (Sivan and Elroy-Stein, 2008).

La-related proteins (Larps) are so named because they carry a La domain in common with La protein (Sjogren's syndrome type B antigen, SS-B or La autoantigen). La protein is believed to be a transcription termination factor that acts by binding to 3' termini of nascent polymerase III transcripts (Teplova et al., 2006). La protein carries an N-terminal La domain and an RNA recognition motif (RRM). In contrast, Larp proteins carry a more centrally positioned La domain and lack a classical RRM. The Larp family of proteins are conserved in metazoans, with five human Larp subfamilies identified so far (Larp1, Larp2, Larp4, Larp5 and Larp7) (HUGO Gene Nomenclature Committee at the European Bioinformatics Institute, <http://www.genenames.org>). In addition to their La domain, Larp1 members carry a conserved C-terminal region containing DM15 repeats (Ponting et al., 2001) (Fig. 1).

Two Larp proteins have been identified so far in *Drosophila melanogaster*, Larp (CG42551) from the Larp1 subfamily and CG11505 from the Larp5 subfamily. Recent studies in *Caenorhabditis elegans* suggest that certain mRNAs, specifically those encoding Ras and MAP kinase, are stabilised in the germ-line of *LARP-1* mutants. Together with the localization of *LARP-1* in P-bodies, this has led to the suggestion that Larp participates in mRNA translation via regulation of mRNA decay (Nykamp et al., 2008).

Though *Drosophila larp* has been identified as a target of the HOX gene, Ubx (Chauvet et al., 2000), suggesting it functions during development, the precise cellular role of Larp protein has remained unknown. Ichihara et al. (2007) reported meiotic defects in *Drosophila larp* mutants and have suggested, from its association with mitochondria, that Larp is involved in multiple meiotic processes including mitochondrial transmission. Here we show that *Drosophila larp* exists in a complex with cytoplasmic poly(A)-binding protein (PABP), that male sterile alleles of the *pAbp* locus show similar meiotic defects to *larp* mutants, and that combined *larp* and *pAbp* mutants show enhanced phenotypes. We also characterise mitotic defects in syncytial embryos derived from *larp* mothers. We discuss the possibility that Larp has multiple roles that can include regulation of translation in addition to mRNA stability. The high demands of the syncytial nuclear division cycles and the requirements for dramatic cell growth preceding male meiosis may make these developmental stages particularly sensitive to changes in the regulation of gene expression.

Materials and methods

Fly husbandry

The mutant *larp*^{mt^rw} corresponds to *l(3)S003108*^{l(3)S003108}, *larp*^{mt^r1} to *l(3)S022529*^{l(3)S022529}, and *larp*^{mt^r2} to *P{PZ}l(3)0648*⁰⁶⁴⁸⁷ as described by Ichihara et al. (2007). We generated a null allele (referred

to in this paper as *larp*^{mt^r null}) following FLP-FRT recombination of the Exelixis transposon insertions, *P{XP}larp[d00350]* (minus) and *PBac{WH}f03059* (plus). Fig. S1 shows the position of these insertions. The genetics of the crossing scheme is as described (Parks et al., 2004). From this, 19 lines were established. Homozygous adult flies survived in 17 lines: bristles were thinner and shorter than wild-type and were occasionally missing. A slight rough eye phenotype was also present. All 17 lines were tested by western blotting to confirm the loss of the Larp protein (data not shown).

The remaining two lines (#15 and #19) did not survive beyond the embryonic stage. However, if these lines were made heterozygous with *larp*^{mt^r null} the resulting transheterozygotes were female and male sterile and their external phenotype resembled *larp*^{mt^r null} homozygous flies. From this we conclude that lines #15 and #19 carry a second site mutation in addition to the *larp* deficiency.

Five *pAbp* alleles were identified: *P{lacW}l(2)SH1908*^{SH1908} (referred to in this paper as *pAbp*^{SH1908}); *yw;P{lacW}pAbp[k10109]* (referred to in this paper as *pAbp*^{k10109}); *yw;pAbp[EY11561]* (referred to in this paper as *pAbp*^{EY11561}); *P{EP}pAbp[EP310]* (referred to in this paper as *pAbp*^{EP310}); and *P{RS5}5-SZ-4029* (referred to in this paper as *pAbp*⁴⁰²⁹) (Ryder et al., 2004). These were uncovered by the deficiency *Df(2R)Pcl7B*.

Production of the larp antibody and western blotting

An antibody was raised against the central portion of the Larp protein using primers (24_Centr_Mtr_ab_F ATGGATCCGGCTACTA-CACCACCACAACAAC and 25_Centr_Mtr_ab_R ATAAGCTT-GAGTTTCCTCTTCTGGCG) to generate a PCR fragment of 885 base pairs corresponding to the 295 amino acid sequence located between amino acids 710–1004 in Larp (shown in Fig. S1).

PCR fragments were cloned in frame with the pET23b (Novagen) vector to incorporate a six-Histidine tag at the C-terminal end of the Larp protein. Recombinant protein was expressed in BL21 pLysS cells (Novagen) and purified from the insoluble pellet using BugBuster Protein Extraction Reagent (Novagen). The solubilized protein was refolded using the Novagen Protein Refolding Kit and dialysed into Novagen Dialysis Buffer (1 M Tris-HCl pH 8.5), before injection into rabbits. Polyclonal antibodies were raised in two rabbits, 9346 and 9347 (Harlan UK). Larp-Central 9347 recognized a single band at around 212 kDa in protein extracted from both larval neuroblasts (see Fig. 1) and testes (data not shown). To check the specificity of anti Larp-Central 9347 testes from the *larp*^{mt^r null} background were immunostained. This revealed the loss of the Larp signal present in spermatocytes in the extended G₂ phase and the mitotic divisions in the tip of the testes. However, centriole-like structures were recognized in the meiotic divisions indicating this antibody recognized proteins other than Larp. In order to remove this contaminant the antibody was affinity purified against the recombinant protein using standard procedures, which successfully removed the contaminant.

Western blotting of Larp-Central 9347 used the non-affinity purified antibody at a concentration of 1:1500. Western Blotting was done following standard procedures. Anti-actin antibody (Sigma A-2066, 1:200) was used to compare loading. We will refer to this antibody as anti-Larp central.

Immunostaining of embryos, larval neuroblasts, D-Mel cells and testes

Embryos were collected from Oregon R females allowed to lay for 2 h, aged for 1 h, washed and dechorionated in 50% bleach for 2 min. They were fixed with 14.8% formaldehyde, 55.6% Heptane, 1 \times PBS for 7 min. The fixative was replaced by methanol to completely remove the viteline membrane and embryos were rehydrated with 1 \times PBS, blocked using PBSTB (1 \times PBS, 0.1% Triton X-100, 1% BSA) and immunostained using standard procedures. Larval neuroblasts were

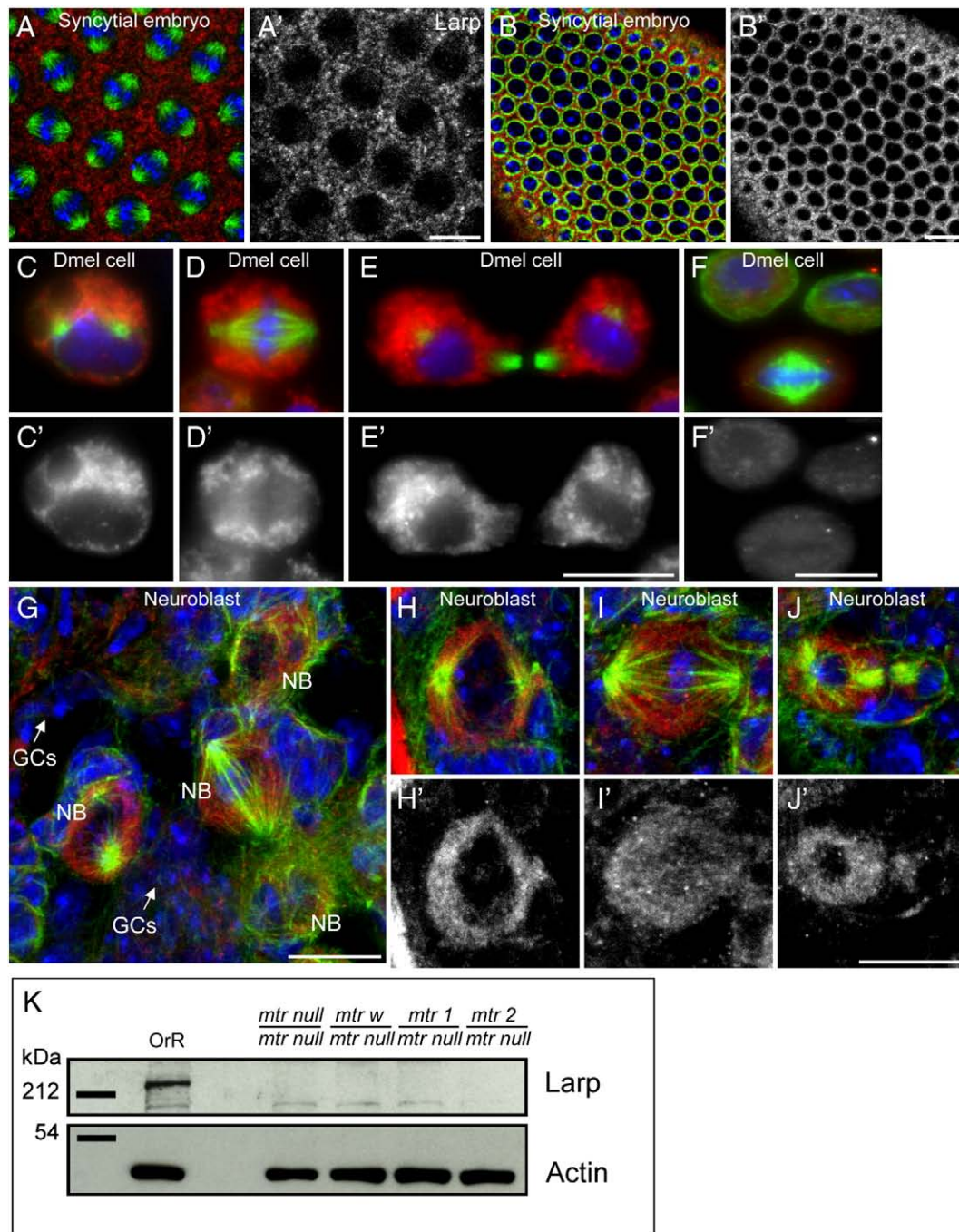


Fig. 1. Localization of Larp in *Drosophila*. The Larp antibody was used to immunostain a variety of cells and tissues in order to investigate the subcellular localization of Larp. This revealed that Larp is cytoplasmic in syncytial embryos, which are undergoing mitosis (A) and is also cytoplasmic in syncytial embryos during interphase (B). In D-Mel cells, Larp is excluded from the nucleus and enriched in the cytoplasm throughout cell division (C–E). Larp is absent in cells treated with dsRNA directed against Larp (F). In larval neuroblasts Larp is found at high levels in the cytoplasm of mitotically active neuroblasts (NB), but its levels are low in the surrounding ganglion cells (GCs; G–J). Microtubules are shown in green, Larp in red (also shown in monochrome) and DNA in blue (scale bars = 10 μ m). (K) Western Blotting of larval brains from both wild-type OrR and *larp* mutant alleles using the same antibody as for the immunolocalization. A band at the expected size for Larp (~212 kDa) can be seen in the wild-type control but this band disappears in homozygous null and transheterozygous *larp* mutant alleles. Actin (~50 kDa) was used as a loading control.

dissected in PBS then fixed for 20 min at room temperature in 10% formaldehyde, 1 mM EGTA, 1 \times PBS. After washing (1 \times PBS) they were permeabilised with 0.3% Triton X-100 for 10 min then blocked in PBSTB for 40 min at room temperature. Neuroblasts were incubated with primary and secondary antibodies following standard procedures. DNA was stained using Toto-3-iodide. D-Mel cells were allowed to settle onto glass coverslips for 1 h then fixed with 4% formaldehyde in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 4 mM MgCl₂). Cells were permeabilised and blocked using PBSTB (PBS

containing 0.1% Triton X-100 and 1% BSA before immunostaining. Testes, dissected from pharate adult males, were prepared for immunostaining using standard methods (methanol–acetone fixation) (Cenci et al., 1994) and Toto-3-iodide was used to stain the DNA.

The following antibodies were used for immunostaining: anti-tyrosinated α -tubulin (1:10, YL1/2, Harlan Sera-Labs); anti Larp-Central diluted 1:100; γ -Tubulin was localized using the monoclonal antibody GTU88 (Sigma) diluted 1:50. Toto-3-iodide (T-3604, Molecular Probes) was used to counterstain DNA in embryos, larval

neuroblasts and testes. Vectorshield mounting medium containing DAPI (Vector Laboratories) was used to counterstain DNA in D-Mel cells. Secondary antibodies were obtained from Jackson Immunochemicals and used according to the supplier's instructions.

Images of embryos, larval neuroblasts and testes were acquired on a Nikon Microphot microscope fitted with a MRC1024 scanning confocal head (Biorad) using a 63× (N.A. 1.4) objective lens. Images of D-Mel and HeLa cells were acquired on a Zeiss Axiovert 200 (Carl Zeiss Microimaging) running Metamorph (Universal Imaging) using a 100× (N.A. 1.4) objective lens. Figures shown are the maximum-intensity projection of optical sections acquired at 0.5 μm steps.

Imaging of onion-stage spermatids using phase-contrast microscopy

For phase-contrast imaging of onion-stage cysts, testes were dissected in testis buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl pH 6.8, 1 mM EDTA) (Gonzalez and Glover, 1993) and gently squashed under an 18 × 18 mm coverslip until the appropriate degree of flattening was attained. Specimens were screened for intact cysts of primary spermatocytes using phase-contrast on a Nikon Microphot-FX microscope at low magnification (25×), and the morphology and number of cells in those cysts were analysed. Images were acquired with a Spot RT camera (Diagnostic Instruments) running the included software package on a PC.

Time lapse imaging of larv embryos expressing EGFP-tagged β-tubulin or Polo

Embryos were visually dechorionated using 50% bleach and immediately washed in water following removal of the chorion. A chamber was constructed to allow time lapse imaging of embryos. Briefly, a drop of Voltalef 10s oil (Elf Atochem) was placed onto a clean No. 1.5 coverslip (22 × 40 mm). Embryos were transferred to the Voltalef oil and covered with a gas-permeable membrane (bioFOLIE VivaScience). A 1:1:1 mixture of Vaseline, lanolin and paraffin (VALAP) was used to 'glue' a metal slide, which had a central circular portion removed to create an opening, onto the coverslip, effectively holding the membrane in position. This ensured that there was no slippage between the membrane and the coverslip and generated an open cavity through which gas could permeate. Time lapse images were acquired at 15 s intervals and the resulting image is the maximum-intensity projection of 10 sections (1 μm step size). Images were acquired on a Perkin Elmer Ultraview RSIII attached to a Zeiss Axiovert 200 using a 63× (N.A. 1.4) objective lens. Software used was the proprietior software, Ultraview. Alternatively, embryos were viewed with a 63× (N.A. 1.4) oil immersion objective, on a BioRad MRC1024 confocal microscope. Images were collected using LaserSharp V3 software (BioRad) as single sections or stacks of 3-sections at 10-second intervals and were animated using VideoMach 2.4.0 (Gromada.com).

Cell culture, RNAi and flow cytometry

D-Mel cells were cultured in Serum Free Media supplemented with glutamine, penicillin and ampicillin (all from Gibco). RNAi was performed essentially as described (Bettencourt-Dias et al., 2004a) using 30 μg of gene-specific dsRNA and 20 μl of Transfast reagent (Promega) for 1 million cells. After 4 days, cells were harvested and processed for flow cytometry as described (Bettencourt-Dias et al., 2004b).

Expression vectors and stable cell lines

Larp cDNA was cloned by PCR using primers designed to amplify the cDNA (described in Chauvet et al., 2000) where the non-conventional Isoleucine start codon was replaced with a Methionine

codon. The primers were designed with 5' overhangs to generate PCR products without a stop codon and allowing cloning into pDONR221 using a BP recombination reaction (Gateway technology, Invitrogen). The pDONR221-LARP entry clone was used in LR Gateway reactions to generate the pAC5C-LARP-PRA and pAC5C-LARP-EGFP expression vectors, allowing constitutive expression of the fusion proteins from the Actin 5C promoter. D-Mel cells were co-transfected with the expression vector and with pCoBlast (Invitrogen). Three days later, medium was replaced with medium containing blasticidin at 30 μg/ml, and five days later, medium containing blasticidin at 50 μg/ml was added for selection. A week later, cell lines were maintained in presence of blasticidin at 20 μg/ml.

Protein A-affinity purifications

Approximately 5 × 10⁸ D-Mel cells stably expressing Larp-PrA or Polo-PrA were harvested by centrifugation, washed with PBS containing EDTA-Free Complete protease inhibitors (Roche), and frozen in liquid nitrogen. Cells were thawed in 5 ml of ice-cold RIPA-type extraction buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 0.25% sodium deoxycholate, 1 mM DTT, Complete inhibitors [Roche]). Cells were lysed by 4 cycles of freezing and thawing in extraction buffer. The lysates were clarified by centrifugation at 12,000 rpm in a SS34 rotor. Two hundred microliters of rabbit Dynabeads (Invitrogen) conjugated to rabbit IgG (MP Biochemicals) were added to the supernatants for a 4 h incubation with agitation at 4 °C. Beads were washed 4 times for 10 min in 10 ml of extraction buffer and once for 10 min in three-fold diluted extraction buffer. Proteins were eluted from the beads using 0.5 M NH₄OH, 0.5 mM EDTA. Eluates were lyophilized and resuspended in Laemmli SDS-PAGE sample buffer (Sigma).

RNase treatments

For the RNase resistance experiment presented in Fig. 3B, The following enzymes were used in 100 μl respective buffers: RNase A (Qiagen, 1 μg) in PBS; RNase One (Promega, 30 U) in manufacturer's buffer; RNase H (Ambion; 30 U) in 20 mM K-HEPES pH 8.0, 50 mM KCl, 4 mM MgCl₂, 1 mM DTT, 50 μg/ml BSA; RNase V1 (Ambion, 0.3 U) in RNA Structure Buffer (Ambion). All samples were incubated for 20 min at 37 °C. In addition, one sample was incubated in PBS without RNase. Another sample was kept on ice. Ribosomal RNA was extracted from D-Mel cells using RNeasy (Qiagen).

Mass spectrometry

In the experiment shown in Fig. 3A, protein bands were excised from Coomassie-stained gels, digested with trypsin and resulting peptides analysed by LC-MS/MS using a nanoLC (Waters) coupled to a Q-TOF2 mass spectrometer (Waters). Uninterpreted fragmentation data was used to search the NCBI database using the MASCOT search engine (Matrix Science) allowing identification of the protein(s) within a gel band. In the experiment shown in Fig. 3B, samples were analysed without prior separation by SDS-PAGE.

Results

Isolation of mutants in the gene encoding Larp

The early *Drosophila* embryo undergoes rapid cycles of nuclear divisions that are dependent upon maternal supplies of mRNA and proteins. For this reason, mutations in the mother that cause embryonic lethality often affect genes involved in the cell cycle. We identified the gene encoding Larp in a collection of P-element mutants causing maternal-effect lethality as described by Ichihara et al. (Figure S1 in Supplemental Data). As well as these previously

Table 1
Fertility tests of *larp* alleles.

Allele	No. of females tested	Female – % sterile	No. of males tested	Male – % sterile
<i>larp^{mtr null} / TM6C</i>	15	7%	27*	0%
<i>larp^{mtr null} / larp^{mtr null}</i>	23	100%**	10	100%
<i>larp^{mtr w} / larp^{mtr null}</i>	51	71%	49	100%
<i>larp^{mtr 1} / larp^{mtr null}</i>	35	45%	45	100%
<i>larp^{mtr 2} / larp^{mtr null}</i>	34	6%	48	100%

* 27 males were tested in six vials. All vials were fertile.

** Females do not lay.

identified P-element insertions in the first intron of *larp* upstream of its initiation codon (*larp^{mtr w}*, *larp^{mtr 1}*, and *larp^{mtr 2}*), we also generated a null allele *larp^{mtr null}* (see Materials and methods). *larp^{mtr null}* homozygotes survived to adulthood but had bristle and eye defects and both male and female flies were sterile (Table 1). This small deficiency also allowed us to determine that, when hemizygous with *larp^{mtr null}*, *larp^{mtr w}*, *larp^{mtr 1}*, and *larp^{mtr 2}* all survived to adulthood, were male sterile, but demonstrated varying degrees of female infertility (Tables 1 and 2). By examining the number of progeny produced we rated the severity of the mutant alleles as: *larp^{mtr null}* > *larp^{mtr w}* > *larp^{mtr 1}* > *larp^{mtr 2}*. These results confirm that *larp* is essential for male and female fertility (Ichihara et al., 2007).

Larp is enriched in cells with potential to divide

The maternal effect of *larp* mutants led us to examine the distribution of the Larp protein in the syncytial embryo. We demonstrated that the protein was present in the cytoplasm during both mitosis and interphase (Figs. 1A and B). Its presence throughout syncytial development suggests a continued requirement for its function to permit progression of the nuclear division cycles during this developmental stage. We also examined the distribution of Larp in the D-Mel cells, cultured embryonic cells that resembles those of the later cellularised embryo. This revealed a similar localization of the protein in the cytoplasm of both interphase and mitotic cells (Figs. 1C–E) that was absent from cells treated with dsRNA against *larp* (Fig. 1F). The specificity of our antibody was also shown by western blotting of extracts of larval brains where it recognized a band at around 212 kDa that was not present in homozygous *larp^{mtr null}* and hemizygous mutant larval brains (Fig. 1K). We then determined how Larp was distributed in the developing central nervous system of third instar larvae. The larval brain comprises a complex set of differentiated neural and glial cells together with mitotically active neuroblasts that divide asymmetrically to produce ganglion mother cells. The latter undertake one further division and their progeny then differentiate. High levels of Larp could be found within the cytoplasm of neuroblasts and ganglion mother cells but not within differentiated cell types such as surrounding non-mitotic ganglion cells (Fig. 1G). The protein

remained cytoplasmic at all mitotic stages, appearing to diffuse slightly through the fenestrated spindle envelope during anaphase (Figs. 1H–J). Overall, the levels of Larp appeared to be higher in dividing cells or in cells with the potential to divide.

Maternal requirement for *Larp* for mitosis in the syncytial embryo

The partial fertility of hemizygous *larp* females allowed us to study the effects of the loss of Larp upon development of the syncytial embryo in some detail. The nuclear division cycle of the wild-type syncytial embryo is highly asynchronous (Figs. 2A–D). Centrosomes, revealed by gamma-tubulin staining, are tightly attached to the poles of the mitotic spindle and begin to separate during telophase in anticipation of the next division cycle (Fig. 2D). The embryos of hemizygous *larp^{mtr}* mothers frequently showed spindles that were multipolar or apparently conjoined, i.e. sharing a common pole (Figs. 2E and F). Some embryos had areas devoid of nuclei but containing numerous free centrosomes (Fig. 2F), some of which could nucleate microtubules (Fig. 2G). There was frequently a polyploid complement of chromosomes associated with mitotic spindles that were abnormally condensed with evidence of chromosome bridging during telophase and the synchrony of the mitotic cycles throughout the mutant syncytial embryo was disrupted (Fig. 2H).

Time lapse imaging of embryos expressing GFP-tagged β -tubulin (Inoue et al., 2004) revealed the origins of these defects. In wild-type embryos, nuclei took approximately 6–7 min to traverse the mitotic cycle (Fig. 2I, Movie S1). Although successive division cycles were observed in some mutant embryos laid by hemizygous *larp^{mtr 1}* females (Fig. 2J, Movie S2), progression through mitosis in other embryos appeared to stall at metaphase (Movie S3). Centrosomes could be seen to successfully duplicate following the previous division cycle, however the movement of the centrosomes around the nuclear membrane was hindered (Fig. 2J, arrowheads). Nonetheless, a spindle formed between two groups of centrosomes (note that in some cases there are two centrosomes at each pole) (Fig. 2J, arrowheads). Nuclear spacing was also disrupted and two spindles, positioned next to each other, fused to form a common spindle, which was able to divide (Fig. 2J, arrow). Although the cell cycle was longer in this embryo (11 min) the nuclei were able to successfully pass through mitosis (Fig. 2J). Interestingly, unlike wild-type embryos where after each successive division the number of spindles doubled, the number of spindles in this mutant embryo did not increase geometrically making it impossible to judge which division cycle the embryo was in (Movie S2).

To observe these events in more detail, we imaged mitotic progression in *larp*-derived embryos expressing Polo-GFP (Logarinho and Sunkel, 1998). The Polo kinase has a dynamic pattern of localization that reflects its multiple mitotic functions. It is enriched at centrosomes as they migrate around the nuclear envelope in prophase (Fig. 2K 0–110 s); it then enters the nuclear space and

Table 2
Number of progeny produced from females of different *larp* genotypes.

Allele	No. of females tested*	Total no. of female progeny	Total no. of male progeny	Total no. of progeny	Average no. of progeny per female
<i>larp^{mtr null} / TM6C</i>	15	1373	1282	2655	177
<i>larp^{mtr null} / larp^{mtr null}</i>	23	0	0	0	0
<i>larp^{mtr w} / larp^{mtr null}</i>	15	6	1	7	0.5
<i>larp^{mtr w} / larp^{mtr null} – repeat[^]</i>	18	27	18	45	3
<i>larp^{mtr 1} / larp^{mtr null}</i>	15	51	29	80	5
<i>larp^{mtr 2} / larp^{mtr null}</i>	15	382	269	651	43

Individual females were mated with three OrR males (Day 0, vial 1) and left for five days before transfer to a new vial (Day 5, vial 2). After a further five days, the flies were discarded (Day 10). The number of progeny that eclosed was counted at day 12, 14 and 17 for vial 1 and day 17, 19 and 22 for vial 2.

* Females were tested individually (one female with three OrR males).

[^] 22% of progeny had abdominal defects (10/45).

associates with kinetochores and transiently with the nuclear envelope (Fig. 2K, 400–490 s); it remains on kinetochores as they separate in mitosis (Fig. 2K, 640–660 s); and briefly redistributes into telophase nuclei before concentrating again on centrosomes (Fig. 2K, 740–1000 s). In a typical nucleus from *larp*-derived embryos, Polo was enriched on centrosomes but these failed to separate around the nuclear envelope (Fig. 2L). Polo kinase failed to flood into the nucleus and showed no association with the nuclear envelope. The self-organizing capacity of microtubules and their associated proteins permits formation of an acentrosomal spindle pole and anaphase was eventually initiated. Thus the pattern of Polo kinase localization points towards defects in mitotic progression resulting from the loss of Larp function. Taken together these data indicates a requirement for Larp in the development of the syncytial embryo that includes but does not limit it to mitotic functions.

Mutant *larp* males show defective meiosis

Ichihara et al. (2007) have previously described defects in male meiosis in *larp* mutants. We have also examined male meiosis in various allelic combinations of *larp* and concur with their findings (Figure S2). The “onion-stage” spermatids, the products of the meiotic divisions, displayed defects indicating that the loss of Larp affected both karyokinesis and cytokinesis during male meiosis. Moreover, the immunostaining of fixed preparations of *larp^{mtr null}* spermatocytes revealed aberrancies in male meiosis similar to those reported (Ichihara et al., 2007). In contrast, our findings on the pattern of distribution of Larp throughout the testes showed some differences with their report. Both antibody used by Ichihara et al. (2007) and the one that we generated identified high levels of Larp in the cytoplasm of all cysts near the tip of the testes. These cells undergo mitosis to form cysts of 16 inter-connected spermatocytes (Figure S2) suggesting a requirement for the protein throughout this stage. Levels of Larp then appeared to intensify in premature spermatocytes but abruptly decreased during the later stages of this extended G2 phase (Figure S2 M, N). However, whereas Ichihara et al. (2007) were able to detect Larp in association with mitochondria in meiotic cells and spermatids, we were unable to do so. This could be because of some differences in the recognition of Larp epitopes in the different antibodies used by the two groups. Alternatively it could reflect the possibility that the antibody used by Ichihara et al. might recognize proteins other than Larp, a possibility supported by our observations of the ability of preimmune sera to stain the mitochondrial aggregates in this tissue. Nevertheless, the abundant expression of Larp protein during spermatogenesis recognized by both antibodies and the mutant defects indicate a requirement for the protein in meiotic progression.

Larp exists in a complex with poly(A)-binding protein (PABP)

As La and its related proteins have been implicated in interactions with a variety of RNAs to regulate their processing or translation, we wished to determine whether Larp might associate with proteins required for either of these processes. To this end we generated a stable D-Mel cell line expressing a fusion of Protein-A with the C-terminus of Larp (Larp-PrA). We then carried out affinity-purification of Larp-PrA on IgG beads under conditions that allowed co-purification with its interacting proteins. The purified complexes were separated by SDS-polyacrylamide gel electrophoresis (Fig. 3A) and bands were excised for identification by mass spectrometry (Table S1). The most prominent band at around 70 kDa, absent from a Polo-PrA purification performed in parallel under the same conditions as a control, was identified by mass spectrometry as PABP (Fig. 3A). Two less characterised RNA-binding proteins, Yu and Growl, were also found to be specifically associated with Larp-PrA (data not shown), albeit with lower scores than PABP. These results are consistent with

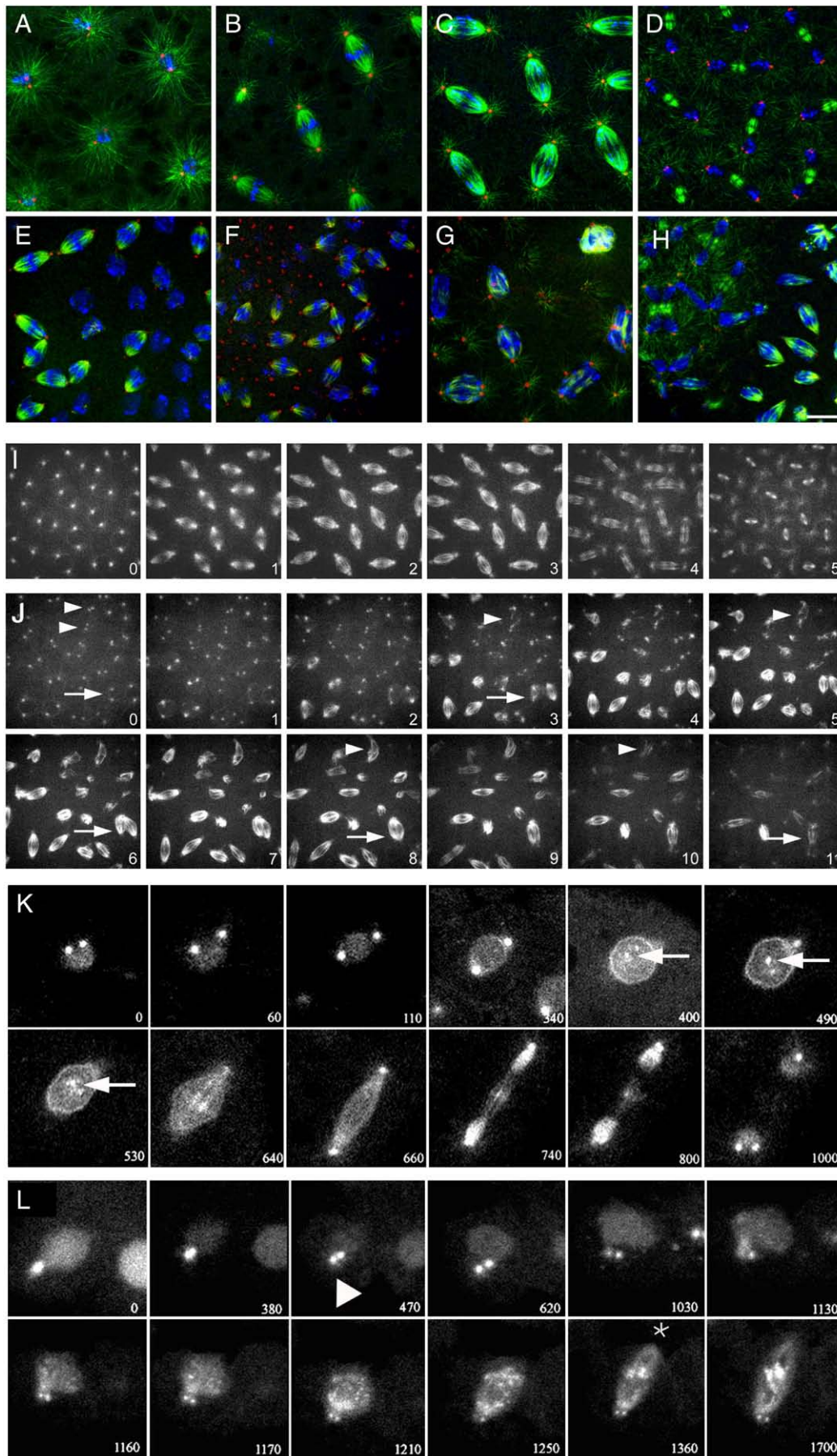
the idea that Larp and PABP are associated for a function at the level of RNA that may involve additional proteins.

We next considered the possibility that the association of PABP with *Drosophila* Larp occurred indirectly through an RNA intermediate. To test whether this was the case we prepared Larp-PrA complexes and incubated these with four ribonucleases of differing specificity (see legend to Fig. 3) and analysed part of the digestion product by gel electrophoresis and part by gel-free mass spectrometry. The 70 kDa band persisted in all treatments in which we could clearly resolve it (Fig. 3B; note it is obscured by BSA from the buffer required for RNaseH digestion) and PABP was identified by mass spectrometry with the next highest Mascot score among specific Larp interactors in each case. Thus the interaction between Larp and PABP appears independent of RNA.

PABP plays an important role in both the initiation of translation and in conferring mRNA stability. We therefore expected that its depletion would have lethal consequences for the cell. Indeed, depletion of PABP by RNAi caused cell death as assessed by flow cytometry analysis (see sub2C population, Fig. 3C, left panel; arrow). Following PABP RNAi, there appeared to be a higher proportion of cells with 4C DNA content compared to cells with 2C DNA along with a greater proportion of polyploid cells. In order to determine if these 4C DNA cells were in G₂ or mitosis we stained the cells using an antibody directed against the mitotic protein, phospho-Histone 3. This demonstrated that very few cells were mitotic, suggesting the cells were in G₂ phase and had not progressed into mitosis (data not shown). In contrast, downregulation of Larp did not affect the mitotic index and flow cytometry analysis showed only a slight alteration of the DNA content profile (Fig. 3C, right panel). This suggests that Larp is only required for a subset of the functions of PABP. The depletion of Larp was verified by western blot (Fig. 3C).

We then questioned the consequence of reduced function of *pAbp* in the fly. We found that two independent P-element insertions into the *pAbp* locus, *pAbp^{SH1908}* and *pAbp^{k10109}*, resulted in embryonic lethality when hemizygous over the deficiency *Df(2R)Pcl7B* which uncovers *pAbp* (Fig. 4A). As these P-insertions are inserted into exons of the *pAbp* gene, they are likely to result in reduced PABP function from early stages of development. However, two other P-element insertion alleles, *pAbp⁴⁰²⁹* and *pAbp^{EY11561}* (Fig. 4A), when placed over the deficiency *Df(2R)Pcl7B* allowed survival to adulthood and female fertility, but caused male sterility. In order to confirm that the P-insertions *pAbp⁴⁰²⁹* and *pAbp^{EY11561}* were affecting *pAbp* they were made heterozygous with *pAbp^{SH1908}* whereupon they were again found to result in male, but not female, sterility. An EP-insertion mutant, *pAbp^{EP310}*, an insertion into the first 3' non-coding exon also resulted in male sterility when homozygous or heterozygous with *pAbp^{k10109}*. Together this suggests that the *pAbp⁴⁰²⁹*, *pAbp^{EY11561}*, and *pAbp^{EP310}* insertion elements that do not interrupt the *pAbp* open reading frame are weak hypomorphic mutants.

Phase-contrast microscopy was used to examine the “onion-stage” spermatids in the *pAbp* mutant allele *pAbp⁴⁰²⁹*. Following two meiotic divisions in wild-type spermatocytes each cyst contains 64 onion-stage spermatids (Fig. 4B). However, when *pAbp⁴⁰²⁹* was made hemizygous with the deficiency all complete cysts contained only 32 onion-stage spermatids (32 nuclei associated with 32 Nebenkern), indicating that only one meiotic division had occurred. When heterozygous with *pAbp^{SH1908}* all complete cysts also only contained 32 nuclei (Fig. 4D). Moreover, a ratio of two nuclei to one Nebenkern revealed a failure of cytokinesis in 7% of these onion-stage spermatids. When heterozygous with *pAbp^{k10109}*, cysts of 64 onion-stage spermatids were observed. However, some cysts contained spermatids with nuclei and Nebenkern which were twice the size of the surrounding spermatids, suggesting that these cells had gone through only one meiotic division (Fig. 4C). Cytokinesis also appeared to be affected in this combination leading to the presence of spermatids with a 2:1 and



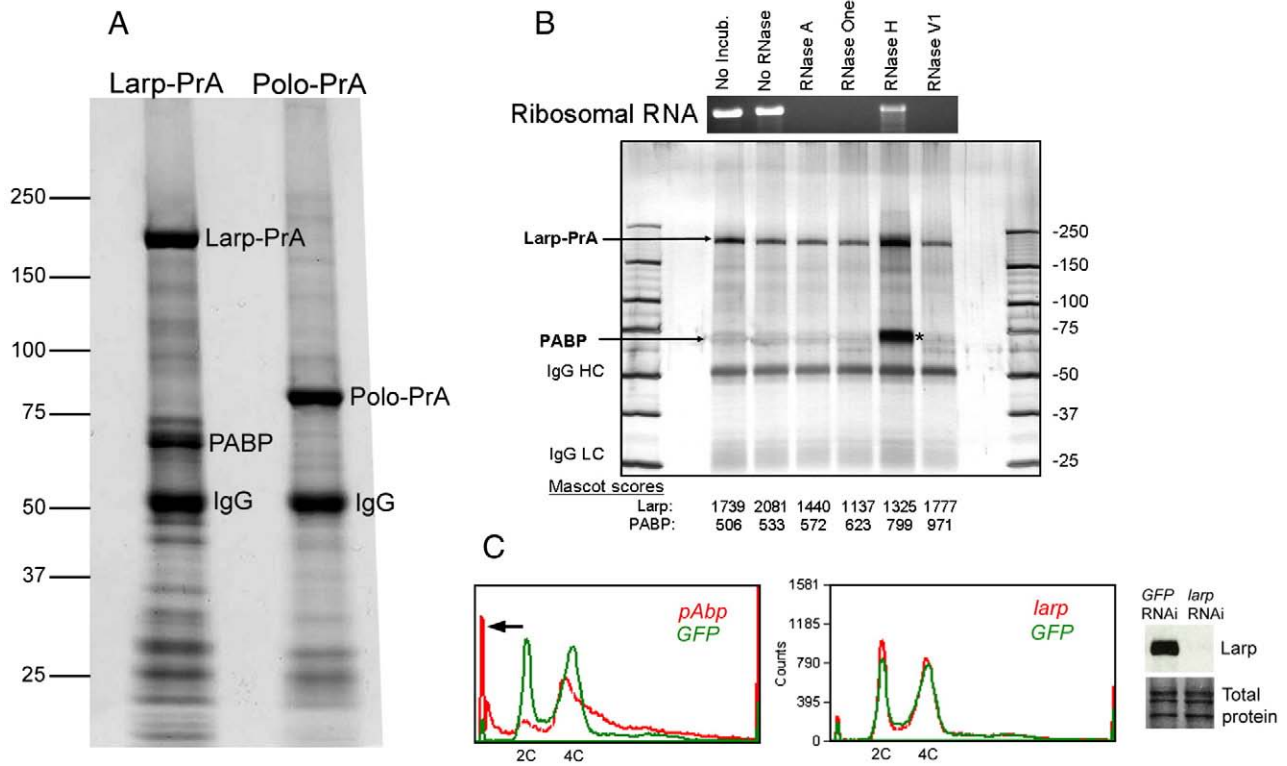


Fig. 3. Larp is in a complex with PABP. (A) Cell lysates of D-Mel cells stably expressing Larp-PrA and Polo-PrA were submitted to Protein A-affinity purification as described in Materials and methods. Proteins were identified by mass spectrometry. Bands corresponding to Larp-PrA, Polo-PrA and PABP are indicated, as is the IgG heavy chain. The gel shown was stained with colloidal Coomassie blue stain. (B) The Larp-PABP complex is resistant to RNase treatments. Larp-PrA was purified in complex with PABP from stably transfected D-Mel cells (as in panel A). Beads were incubated in the presence of RNase A (specific for ssRNA), RNase One (broad specificity), RNase H (RNA:DNA duplexes), RNase V1 (dsRNA) or no RNase, or kept on ice (no incubation). Beads were then washed in RIPA buffer and eluted in 1 M NH₄OH. A third of the product was visualized on a silver-stained gel (Middle). Bands corresponding to Larp-PrA and PABP are indicated. The molecular weight markers (first and last lanes) are labeled in kiloDaltons. The asterisk indicates residual BSA from the RNase H digestion buffer. IgG heavy and light chains (HC and LC) are indicated. Bottom: Two thirds of the products were analyzed gel-free by mass spectrometry. Protein identification Mascot scores for Larp and PABP in all samples are indicated. Note that PABP was identified robustly in all samples. Top: As a control for RNase activity, ribosomal RNA extracted from D-Mel cells was treated under the same conditions and visualized on an agarose gel. Ribosomal RNA is resistant to treatment by RNase H (specific for RNA:DNA duplexes). (C) Left panel: Flow cytometry profile after 4 days of PABP RNAi in D-Mel cells. There is a marked increase in cell death (arrow). GFP dsRNA was used as the control. Right panel: Flow cytometry profile after 4 days of RNAi against Larp in D-Mel cells shows little change in DNA content even though western blotting indicated a complete knockdown of Larp. GFP dsRNA was used as the control.

3:1 ratio of nuclei:Nebenkern (note that the wild-type situation is a 1:1 ratio) (Fig. 4C). Overall, although these males were sterile it appears that this combination has a weaker phenotype than that seen in the *pAbp*⁴⁰²⁹ / *pAbp*^{SH1908} combination.

Immunostaining of testes from hemizygous *pAbp*⁴⁰²⁹ or *pAbp*-flies revealed a number of meiotic defects. In wild-type spermatocytes,

the paired centrioles detach from the plasma membrane to associate as centrosomes with the nuclear envelope around which they migrate in the first meiotic prophase (Figure S2). In contrast centrosomes of the hypomorphic *pAbp* mutant spermatocytes failed to become positioned at the nuclear envelope in prophase (Fig. 4E). Moreover, whereas the spindles in wild-type primary spermatocytes

Fig. 2. Larp is required for syncytial divisions in the embryo. (A) – (D) Immunostaining of syncytial divisions in wild-type embryos. In wild-type embryos, centrosomes are positioned opposite each other on the nuclear envelope at prophase (A). As the nuclear envelope breaks down, centrosomes nucleate spindle microtubules resulting in the formation of a bipolar spindle (B). The spindle elongates at anaphase (C) and a central spindle forms during telophase/cytokinesis (D). Centrosomes duplicate, migrate around the nuclear envelope at the end of cytokinesis and position opposite each other ready for the next division cycle (D). (E) – (H) Immunostaining of embryos laid by *larp*^{mtr w} / *larp*^{mtr null} (E, G, H) and *larp*^{mtr 1} / *larp*^{mtr null} (F) mutant mothers. In embryos laid by *larp* transheterozygous mothers a variety of defects can be observed. These include chains of linked spindles (E, F), free centrosomes (F, G), multipolar spindles (G), chromosome segregation defects (H) and a loss of synchrony throughout the syncytial divisions (H). Microtubules are shown in green, γ -tubulin in red and DNA is in blue (scale bar = 10 μ m). (I) Selected images from Movie S1 showing one division cycle in a wild-type syncytial embryo expressing EGFP-tagged β -tubulin. Centrosomes are positioned opposite each other on the nuclear envelope at prophase (0 min). Following nuclear envelope breakdown, marked by an influx of tubulin to the centrosomes (0 min), centrosomes nucleate spindle microtubules and form a bipolar spindle (1–2 min). The spindle elongates at anaphase (3 min) and a central spindle is formed during telophase/cytokinesis (4–5 min). Time between frames is 1 min. (J) Selected images from Movie S2 showing mitotic cell division in a syncytial embryo laid by a *larp*^{mtr 1} / *larp*^{mtr null} mother expressing EGFP-tagged β -tubulin. In this embryo we see normal bipolar spindle formation as well as the formation of a spindle containing two centrosome at each poles which resulted from a failure centrosome movement around the nuclear envelope (arrowheads). A multipolar spindle is also seen to result from the merging of two adjacent spindles (arrows). Time between frames is 1 min. (K) In wild-type GFP-polo embryos the centrosomes are at the nuclear periphery during interphase. These separate and migrate around the nuclear periphery until they occupy positions at opposing nuclear poles. At this stage (prophase, 110–340 s) Polo is localized to the nucleus, and later to the nuclear periphery (400–530 s). Polo decorates the kinetochores during prometaphase, and also to the spindle midbody as the nucleus splits during late telophase/cytokinesis (740–800 s). In the daughter nuclei, Polo can again be seen within the nucleus and centrosomes (1000 s). (L) The failure of the movement of the centrosomes around the nuclear envelope was observed in time lapse imaging of GFP-*polo*; *mtr*¹ derived embryos. Centrosomes (arrowhead), although distinct from one another, appear unable to migrate around the nuclear rim and remain together and loosely attached to the edge of the nucleus. The nuclear periphery itself appears distorted in shape compared to its regular and clearly circumscribed appearance in the wild-type embryo. The kinetochores marked by GFP-Polo appeared to “congress” to a metaphase plate-like structure (1250 s) whereupon they stabilised microtubules to form a distinct bipolar spindle as has been described in other *Drosophila* cells with centrosome separation defects.

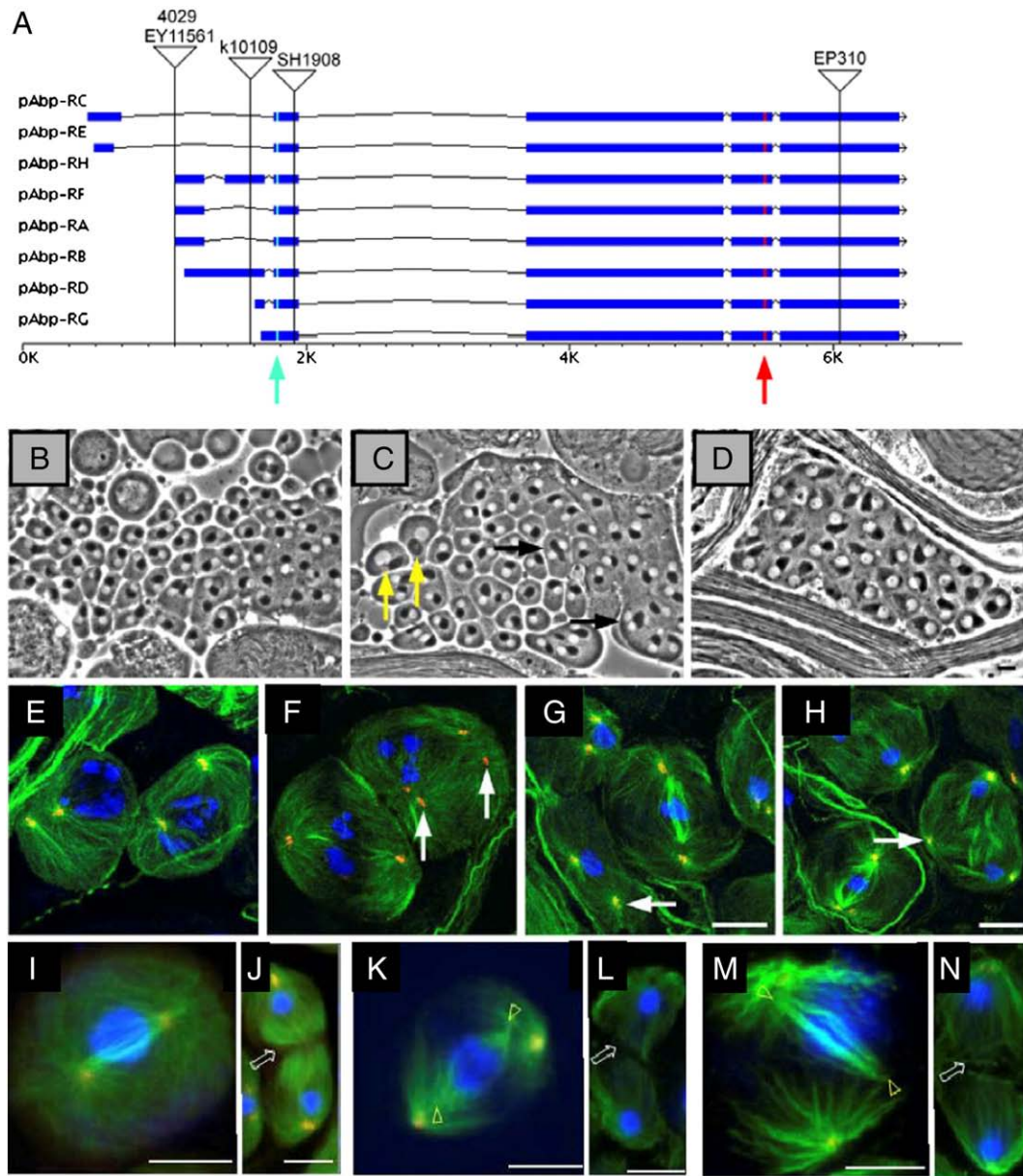


Fig. 4. Hypomorphic alleles of *pAbp* display similar phenotypes to *larp* in male meiosis. (A) Schematic representation of the *pAbp* locus indicating the location of P-element insertions used in this study (modified from FlyBase). Although there is variation in the 5' UTR of the mRNAs the coding region is identical in all transcripts (as indicated by the start and stop codon, green and red arrow, respectively). (B) Phase-contrast image of wild-type (OrR) onion-stage spermatids showing the expected 1:1 ratio of nuclei to Nebenkern. (C) Phase-contrast image of *pAbp*⁴⁰²⁹/*pAbp*^{k10109} onion-stage spermatids. A single enlarged Nebenkern associated with multiple nuclei (black arrows) is indicative of a failure of cytokinesis. Onion-stage spermatids in which both nuclei and Nebekern are larger than expected are also present (yellow arrows). (D) Cysts which contain only 32 onion-stage spermatids are evident in *pAbp*⁴⁰²⁹/*pAbp*^{SH1908} testes (there are 64 onion-stage spermatids in a wild-type cyst). Note that the size of nuclei and Nebekern are larger than those seen in the OrR onion-stage spermatids (scale bar = 10 μ m). (E–H) Mutant spermatocytes from hemizygous *pAbp* alleles, *pAbp*⁴⁰²⁹/*Df(2R)Pcl7B* (H) and *pAbp*^{EY11561}/*Df(2R)Pcl7B* (F–H), show a number of defects during male meiosis. Centrosomes fail to become positioned at the nuclear envelope in prophase (E). Meiotic spindles with a wild-type appearance are observed (F) although the short spindle phenotype, similar to those seen in *larp* mutant spermatocytes, can also be observed in some cells (G). Precocious separation of centrioles occurs in some cells (arrow in F and G). Some cells appear to enter meiosis II but attachment of centrosomes to the nuclear envelope is compromised (arrow in G). This was also observed in *larp* mutant spermatocytes. Microtubules are shown in green, γ -tubulin in red and DNA in blue. Scale bar = 10 μ m. (I–N) Comparison between *larp* and *pAbp* phenotypes. (I) Wild-type metaphase; (J) Wild-type telophase; (K) *larp*^{mtc null} metaphase; (L) *larp*^{mtc null} telophase; (M) *pAbp*^{EY11561}/*pAbp*^{k10109} metaphase, (N) *pAbp*^{EY11561}/*pAbp*^{k10109} telophase. Open arrowheads indicate positions of focused poles of spindles that are displaced from centrosomal asters in mutants; open arrows in L and N indicate central regions of telophase spindles that are incorrectly formed in mutants.

extended to the centrosomes, some spindles in the *pAbp* mutant were shorter than in wild-type and did not extend to the centrosomes although this phenotype was not as penetrant as that seen in *larp* mutant spermatocytes (Fig. 4K). Finally, the maintenance of centriole cohesion appeared to be compromised in *pAbp* mutant spermatocytes and premature separation of centrioles was observed in some cells (Figs. 4F and H). Some of these phenotypes were similar to those we observed for loss of function of *larp* (see above). We cannot exclude

the possibility that these mutations may also affect other biological processes at different developmental stages. However, the results suggest that these *pAbp* mutations have an effect upon male meiosis, presumably as a result of compromised gene expression in the developing spermatocyte.

In order to investigate whether a genetic interaction between *larp* and *pAbp* occurred, we examined meiotic cells in the *pAbp*^{EP310} mutation in combination with two different *larp* alleles (Table 3).

Table 3
Quantification of meiotic defects in spermatocytes with combinations of *larp* and *pAbp* mutant alleles.

Genotype	Meiotic cells scored	Abnormal meiotic cells				Total abnormal meiotic cells (%)	
		Abnormal meiotic cells associated with detached spindles		Other meiotic abnormalities			
			(%)		(%)		
Wild-type	98	0	0.0	4	4.1	4	4.1
<i>pAbp</i> ^{EP310} /CyO	93	6	6.5	0	0.0	6	6.5
<i>pAbp</i> ^{k10109} /CyO	155	6	3.9	2	1.3	8	5.2
<i>larp</i> ^{mtr null} /TM6C	108	2	1.9	2	1.9	4	3.7
<i>larp</i> ^{mtrw} /TM6C	135	6	4.4	3	2.2	9	6.7
<i>pAbp</i> ^{EP310}	152	21	13.8	5	3.3	26	17.1
<i>pAbp</i> ^{EP310} / <i>pAbp</i> ^{k10109}	128	20	15.6	3	2.3	23	18.0
<i>larp</i> ^{mtr null}	62	20	32.2	10	16.1	30	48.4
<i>larp</i> ^{mtrw}	93	16	17.2	17	18.3	33	35.4
<i>pAbp</i> ^{EP310} /CyO ; <i>larp</i> ^{mtr null} /TM6B	126	26	20.6	3	2.4	29	23.0
<i>pAbp</i> ^{EP310} /CyO ; <i>larp</i> ^{mtrw} /TM6B	123	15	12.2	5	4.1	20	16.3
<i>pAbp</i> ^{EP310} ; <i>larp</i> ^{mtr null} /TM6B	70	15	21.4	7	10.0	22	31.4
<i>pAbp</i> ^{EP310} ; <i>larp</i> ^{mtrw} /TM6B	58	12	20.7	5	8.6	17	29.3
<i>pAbp</i> ^{EP310} /CyO ; <i>larp</i> ^{mtrw}	50	13	26.0	11	22.0	24	48.0
<i>pAbp</i> ^{EP310} ; <i>larp</i> ^{mtrw}	98	35	35.7	31	31.6	66	67.3

The enhancement revealed when each mutant is heterozygous with its appropriate balancer strongly suggests involvement of the two genes in a common process. Note that this combination of balancers does not show any significant mutant phenotype when against wild-type chromosomes. Moreover, the translocation balancer, *T(2;3)TSTL, CyO: TM6B, Tb¹*, derived from the two balancer chromosomes we use in this study, has been extensively used to balance second chromosome mutants in other studies of second chromosome mutations affecting mitosis or male meiosis. We cannot rule out that the phenotypes of homozygous mutants might be affected by second site mutations. However, the phenotype of the *pAbp*^{EP310} homozygote is almost indistinguishable from that of *pAbp*^{EP310}/*pAbp*^{k10109}. Moreover the enhancements of phenotypes scored when homozygotes were placed against heterozygotes of the other gene were those male meiotic phenotypes associated with these mutations. Thus taken together with the enhancement of phenotype seen with when each mutant is heterozygous with its wild-type gene, these observations are consistent with the biochemical interactions identified between Larp and pAbp.

When *pAbp*^{EP310} was either homozygous or heterozygous with *pAbp*^{k10109} it resulted in 17% or 18% meiotic defects respectively (Table 3). The most common abnormal phenotype was detachment of the meiotic spindles from astral microtubules. As this phenotype was common to both *larp* and *pAbp* mutants, it was separately scored. Other abnormalities included small or wavy spindles, disorganized asters and monopolar spindles that, although observed in both mutants, were present at a lower frequency. A single mutant copy of *pAbp* (against the CyO balancer) or *larp* (against TM6C) showed less than 6% meiotic defects, a level comparable to wild-type. When the genome contained a single mutant copy of both genes, the frequency of meiotic defects rose to between 16 and 23% depending upon the allelic combination (Table 3). Similarly a single mutant copy of *larp* increased the frequency of meiotic defects in *pAbp*^{EP310} mutants from 17.1% to 29.3% (for *larp*^{mtrw}) or 31.4% (for *larp*^{mtr null}). A single copy of *pAbp*^{EP310} increased the frequency of meiotic defects of *larp*^{mtrw} mutants from 35.4% to 48%. Thus, partial loss of function of PABP and Larp enhance each other, suggesting that these proteins function in the same process.

Discussion

Larp is a conserved family of proteins present in all metazoans. The expression of Larp either in actively proliferating cells or in those with an extended G₂ phase suggests that, in *Drosophila*, it is required for correct progression through M phase. This is borne out by our finding of mitotic defects in *Larp* mutants alongside the meiotic defects previously described. We present both biochemical and genetic evidence for an interaction between Larp and PABP in *Drosophila*. This, together with the previously described roles of the wider family of proteins carrying La motifs in RNA metabolism and translation, suggests that Larp exerts its effects through a similar mechanism.

The ability of null mutants of *larp* to develop to adulthood indicates that Larp is dispensable for cell cycle progression at many developmental stages even though it is absolutely essential for syncytial embryo development and for male meiosis and the late stages of spermatogenesis. Although the predominating phenotype we see in *larp* mutants is upon the mitotic cycle of the syncytial embryo and male meiosis, this does not rule out the involvement of

Larp in other developmental and cellular processes as might be expected for a protein of this family. Indeed we cannot rule out the possibility that some of the mitotic defects observed in syncytial embryos are secondary to other events either in cell cycle progression or in cellular metabolism. Indeed the demands placed upon the syncytium by the nuclear division cycles are evident from the findings that depletion of metabolic enzymes such as glutamine synthase (Frenz and Glover, 1996) and mitochondrial ATP synthase (Kidd et al., 2005) result in mitotic defects in the syncytial cycles. Nevertheless, specific defects, such as the mislocalization of the Polo mitotic kinase, the failure of centrosomes to separate, and the mis-orientation of the meiotic spindle do seem to point towards specific M phase functions being affected. Moreover, when we down-regulate the counterpart of Larp in cultured human cells, this leads to striking defects in chromosome segregation (data not shown). Thus a requirement for Larp in the regulation of RNA stability or translation of genes important for cell cycle progression cannot be excluded from a more general requirement at these developmental stages.

Although of clear importance in development, neither translational control nor processes controlling mRNA stability have been intensively studied in *Drosophila*. Translational control is known, however, to be crucial during spermatogenesis where primary spermatocytes undergo a prolonged G₂ phase in preparation for meiosis and sperm differentiation. Meiotic entry requires the expression of a germ-line form of a Cdc25 protein phosphatase, Twine, to activate Cdk1 (Alphay et al., 1992; Courtot et al., 1992). Translation of *twine* mRNA is under the direct control of a cytoplasmic protein Boule. Boule is a conserved protein known as DAZ (Deleted in Azoospermia) in man. It has recently been shown that DAZ-like proteins regulate translation directly through an interaction with PABP (Collier et al., 2005). As meiotic entry takes place in *larp* mutants, it suggests that Larp must be regulating mRNAs other than *twine*. Just as a more general role for Larp in regulating gene expression cannot be ruled out, the same is true for spermatogenesis. The extended G₂ phase in *Drosophila* spermatocytes is a stage of dramatic cell growth that places high demands on the primary spermatocytes. 2D-Difference Gel Electrophoresis (2D-DIGE) revealed that the expression of proteins involved in a variety of cellular processes, particularly general metabolism and protein folding, was

perturbed in *Larp* mutant testes (Figure S3). Thus *Larp* appears to be required in the testes for a broader range of cellular functions than simply cell cycle progression.

Of all previous studies of *Larp* protein function, the recently reported work of Nykamp et al. (2008) in *C. elegans* has perhaps been the most informative. These authors noticed that the phenotypes of *larp-1* mutant worms in oogenesis resembled those of mutants with a hyperactive Ras-MAPK signaling pathway. Consistently, *larp-1* mutants partially suppressed germ-line effects of reduced MPK1. Moreover, levels of mRNAs for the Ras-MAPK signaling pathway were increased in *larp-1* mutants. This together with the localization of LARP-1 in cytoplasmic bodies associated with P-body markers CAR-1 and DCAP-1, but not with the storage granule marker PGL-1, would suggest that LARP-1 was required to mediate the decay or stability of mRNA transcripts (Squirrell et al., 2006; Rajyaguru and Parker, 2009). Ichihara et al. did describe a spindle association of *Drosophila* *Larp* with their anti-*Larp* antibody which overlapped with mitochondria. However, as this region of the meiotic spindle is membrane rich it is also possible that this could represent other bodies such as P-bodies. We did not observe this localization perhaps because it reflects an epitope in *Larp* that is not well recognized by our own antibody.

In mammalian cells, when mRNA is translationally inactive, it is packaged (in a protein complex) into mRNP and sequestered to cytoplasmic processing granules (P-granules), comprising P-bodies and stress granules. Within P-bodies, mRNA undergoes deadenylation, decapping, and exonuclease digestion or is stored for subsequent translation (Parker and Song, 2004). Stress granules contain mRNAs that have stalled during translation initiation and also contain initiation factors such as eIF4E, eIF4G, eIF4A, eIF3, eIF2 along with PABP. They are closely associated with P-bodies within the cytoplasm and there may be exchange of mRNA between the two (Kedersha et al., 2005). Whilst P-bodies contain proteins required for translational repression (Aizer et al., 2008), stress granules are believed to temporarily harbour mRNA while the cell is exposed to stress so that translation can be resumed once it is removed (Mollet et al., 2008). Our finding that *Drosophila* *Larp* is a partner of PABP could suggest that *Larp* may control translation indirectly, via regulation of mRNA within cytoplasmic granules. Alternatively, by interacting with PABP at the 3' end of mRNA, *Larp* may affect mRNA stability by modulating the interaction between PABP and the poly-A tail or with other proteins that regulate this interaction. Such a mechanism would be supported by experiments demonstrating that the tethering of PABP1 near a premature termination codon can stabilise mRNA and propagate translation (Amrani et al., 2006).

Cytoplasmic PABPs have been shown to be essential for viability in *Saccharomyces cerevisiae* (Sachs et al., 1987) and, in *Drosophila*, strong alleles of PABP are embryonic lethal (Sigrist et al., 2000). We show here that weaker alleles also have a male meiotic phenotype with defects in spindle formation, chromosome segregation and cytokinesis. However, whereas the strong alleles of PABP are embryonic lethal, our null allele of *larp* is viable, albeit with male and female sterility. This suggests that unlike PABP, which has been described as a canonical translation initiation factor (Kahvejian et al., 2005), any role that *Larp* might have in translation could be directed towards translation of a specific subset of proteins. The possibility that *Larp* might indeed have a direct and conserved role in translation is supported by our recent finding that downregulation of its human counterpart (*Larp1*) results in an increase in the hypophosphorylated form of 4E-BP1 which has greater affinity for eIF4E and represses cap-mediated translation (Blagden, in preparation). However, this may be secondary to the regulation of MAP kinase (Nykamp et al., 2008) and the ability of MAPK signaling to affect the phosphorylation status of 4E-BP1 (Kelleher et al., 2004). In either event, the consequences would be to reduce eIF4F complex formation and thereby directly attenuate cap-dependent mRNA translation.

A future challenge will be to unravel the networks of interactions between the poly(A) tail, PABP and its binding proteins, and the many factors that regulate the translation and stability of mRNAs required for embryogenesis and spermatogenesis. Moreover, further studies will be required to elucidate the conserved role played by *Larp* and evaluate its contribution to this process. From a broader perspective, the finding of an interaction between PABP and *Larp* suggests that it is timely to re-examine the broader role of translational control in relation to cell cycle progression and cellular physiology, not in the least because of its potential importance in understanding proliferative disease.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2009.07.016.

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