Developmental Cell, Vol. 2, 343–353, March, 2002, Copyright ©2002 by Cell Press

half pint Regulates Alternative Splice Site Selection in *Drosophila*

Cheryl Van Buskirk and Trudi Schüpbach[1]
Howard Hughes Medical Institute
Department of Molecular Biology
Princeton University
Princeton, New Jersey 08544

Summary

Alternative splicing is used by metazoans to increase protein diversity and to alter gene expression during development. However, few factors that control splice site choice in vivo have been identified. Here we describe a factor, Half pint (Hfp), that regulates RNA splicing in *Drosophila*. Females harboring hypomorphic mutations in *hfp* lay short eggs and show defects in germline mitosis, nuclear morphology, and RNA localization during oogenesis. We find that *hfp* encodes the *Drosophila* ortholog of human PUF60 and functions in both constitutive and alternative splicing in vivo. In particular, *hfp* mutants display striking defects in the developmentally regulated splicing of *ovarian tumor* (*otu*). Furthermore, transgenic expression of the missing *otu* splice form can rescue the ovarian phenotypes of *hfp*.

Introduction

Alternative exon selection is a means of producing multiple transcripts, often encoding distinct polypeptides, from a single gene. At least 35% of human genes are alternatively spliced (Croft et al., 2000), leading in some cases to a remarkable diversity of transcripts (Gravely, 2001). Changes in splicing have been shown to alter the activities of transcription factors and cell death regulators, the ligand binding specificities of growth factor receptors and cell adhesion molecules, and the subcellular distribution of proteins (Lopez, 1998; Jiang and Wu, 1999). Despite the prevalence of alternative splicing, however, little is known about its regulation in vivo. One notable exception comes from the sex determination pathway of *Drosophila*, in which the Sex-lethal (Sxl) protein catalyzes a series of sex-specific alternative splicing events that lead to a mode of sexual differentiation and dosage compensation in keeping with the X:A ratio of the animal (Schütt and Nöthiger, 2000). Interestingly, the *male-specific lethal-2* (*msl-2*) gene is regulated by Sxl at two levels: Sxl binds within and suppresses the removal of an intron in the 5′UTR of *msl-2* and also represses translation of the unspliced, exported message (Bashaw and Baker, 1997; Kelley et al., 1997). From this and other examples, it is becoming clear that several aspects of RNA metabolism, from RNA export to translation, are connected with splicing regulation (Gebauer et al., 1997; Reed and Magni, 2001).

The *gurken* (*grk*) gene of *Drosophila*, a mammalian TGFα homolog, has been used to study many aspects of RNA regulation, including nuclear export, RNA localization, and translational control (for review, see Nilson and Schüpbach, 1999). During midoogenesis, *grk* RNA becomes restricted to one side of the oocyte, where it produces a localized source of protein that induces EGF receptor activation in the overlying follicle cells, establishing the dorsal side of the egg shell and embryo (Schüpbach, 1987; Neuman-Silberberg and Schüpbach, 1993). The importance of *grk* RNA localization is evidenced by the defects in dorsoventral (DV) polarity that result when the *grk* transcript is not correctly localized. In egg chambers mutant for *squid* (*sqd*), *grk* RNA is not restricted to the dorsal-anterior corner of the stage 9 oocyte and instead forms an anterior ring (Neuman-Silberberg and Schüpbach, 1993), resulting in activation of the EGF receptor around the circumference of the oocyte and leading to the production of dorsalized eggs (Kelley, 1993). The *sqd* gene encodes three isoforms of hrp40, a *Drosophila* hnRNP protein found within the nuclei and cytoplasm of all cells in the egg chamber (Kelley, 1993; Matunis et al., 1994), and has been proposed to function in the regulated nuclear export and translational repression of *grk* mRNA (Norvell et al., 1999).

The *encore* (*enc*) gene is also required for proper *grk* mRNA localization (Hawkins et al., 1997). However, in *enc* mutant egg chambers, Grk protein levels are reduced, and these females lay ventralized, not dorsalized, eggs. Thus it appears that *enc* is not only required for proper *grk* mRNA localization but for its translation as well. *enc* encodes a large, cytoplasmic protein of unknown function that becomes concentrated at the dorsal side of the oocyte in stage 9 egg chambers, where it colocalizes with *grk* mRNA (Van Buskirk et al., 2000). While the DV defects of *enc* are cold sensitive, *enc* females raised at higher temperatures show a highly penetrant extra mitosis in the germline, producing oocytes that are associated with 31 nurse cells instead of the normal 15 (Hawkins et al., 1996). However, the effectors that mediate this extra mitosis are not known.

Here we describe a factor, isolated in a yeast two-hybrid screen with Enc, that affects *grk* RNA localization and germline mitosis. We have named this factor Half pint (Hfp) based on the mutant phenotype, which includes the production of short eggs and the formation of egg chambers that contain eight instead of sixteen germline cells. Surprisingly, *half pint* encodes the *Drosophila* homolog of human PUF60, an RNA binding protein characterized as a general splicing factor. We find that *Drosophila* Hfp does indeed affect splicing, but that it specifically regulates the alternative splicing of a subset of genes within the ovary.

Results

half pint (*hfp*) Encodes a Predicted Splicing Factor

The *encore* (*enc*) gene is required for the regulation of germline mitosis, karyosome formation, and establishment of dorsoventral (DV) polarity of the *Drosophila* egg

[1]Correspondence: gschupbach@molbio.princeton.edu

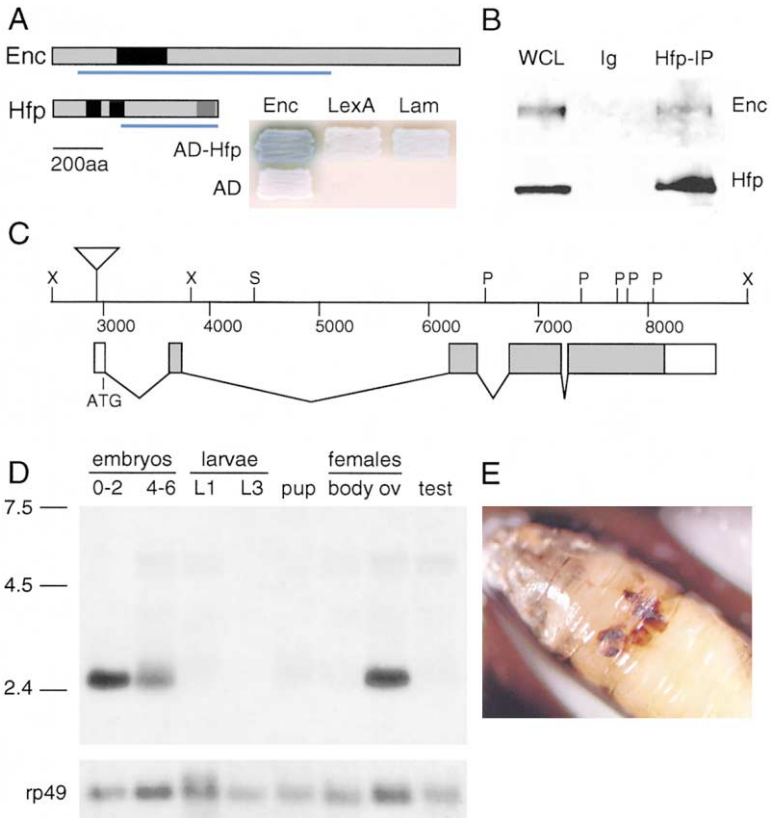

Figure 1. Encore Interacts with Half pint, a Predicted RNA Binding Protein

(A) The 1548-amino acid (aa) Enc open reading frame, with the conserved R3H domain-containing region in black and the portion used as bait in the two-hybrid screen in blue. The 637 aa Hfp open reading frame is shown with its two consensus RRMs in black, the C-terminal RRM-like domain in dark gray, and the portion isolated in the two-hybrid screen with Enc in blue. The Hfp-AD (activation domain) fusion interacts with the Enc-LexA fusion but not with LexA alone or with a LexA-Lamin fusion, and the Enc-LexA bait does not interact with the AD alone.

(B) Western blot of an Hfp immunoprecipitation (IP) from ovarian lysate showing copurification of Enc and Hfp proteins. WCL, whole-cell lysate (one-tenth volume); Ig, mouse immunoglobulin control.

(C) The ovarian *hfp* cDNA aligned with the genomic sequence of the BDGP P1 DS02734 (accession number AC004343). The transposon insertion site for *EP(3)3058* is shown. X, XhoI; S, SalI; P, PstI.

(D) *hfp* developmental Northern: 0–2 and 4–6 hr embryos, first and third instar larvae (L1 and L3), pupae, female body, ovary, and testis. The major 2.5 kb transcript is highly enriched in ovaries and early embryos.

(E) Pupal lethal phenotype of *EP(3)3058/DfAr14-8* animals showing melanized patches of tissue on the larval body.

and embryo (Hawkins et al., 1996, 1997; Van Buskirk et al., 2000). *enc* encodes a large cytoplasmic protein with a single conserved region that contains an R3H motif (Van Buskirk et al., 2000), which is implicated in RNA binding (Grishin, 1998). A portion of the Enc protein containing this conserved domain was used as bait in a two-hybrid screen for interacting proteins. A single factor that showed a specific interaction with Enc was recovered (Figure 1A), and we have confirmed this interaction through coimmunoprecipitation (Figure 1B). We have named the gene encoding this interacting protein *half pint* (*hfp*).

The *hfp* genomic region and cDNA are shown in Figure 1C. The 2.5 kb *hfp* cDNA corresponds to the observed size of the transcript, which is highly enriched within the ovary and early embryo (Figure 1D) but which must also be present at other stages of development, given the lethal *hfp* phenotype described below. The predicted Hfp open reading frame encodes a 637-amino acid, 68 kDa protein. The central portion of the protein contains two consensus RNA recognition motifs (RRMs), found in a number of RNA binding proteins, including poly(A) binding proteins and splicing factors (Burd and Dreyfuss, 1994). The C terminus contains an RRM-like domain that possesses lower similarity to the consensus RRM motif. Database searches reveal that *hfp* is identical to *Drosophila* poly(U) binding splicing factor *(pUbsf)*, a gene identified by homology to human PUF60, which was isolated in a screen for poly(U) binding factors that could stimulate splicing in vitro (Page-McCaw et al., 1999). We were surprised to have isolated a predicted splicing factor in our two-hybrid screen, given the cytoplasmic localization of Encore, and we sought to investigate the function of *hfp* during oogenesis through genetic analysis.

The P element insertion line *EP(3)3058* (Rorth, 1996) harbors a recessive lethal insertion in the 5′UTR of *hfp*, and the deficiency *Df(3L)Ar14-8* (61C7;62A8) fails to complement this lethality. The *EP(3)3058/Df* animals are slower to develop than their siblings and die shortly after pupariation, displaying melanized patches of tissue (Figure 1E). To determine the requirement for *hfp* during oogenesis, we needed to circumvent this pupal lethality. To this end, we isolated several P element excision lines, with the aim of generating small excisions within the 5′UTR of the gene that might function as hypomorphic alleles. The majority of imprecise excisions were lethal but several were semiviable and sterile. Of these, the hypomorphic alleles hfp^{13} and hfp^{9} were the most amenable to study. While hfp^{13} homozygotes are fertile, hfp^{13}/*Df* males and females are sterile. The hfp^{9} allele is homozygous male and female sterile and hemizygous lethal. PCR analysis shows that the excisions do not delete neighboring genes and, in fact, are internal to the P element itself, leaving behind an insertion of 74 nt in the case of hfp^{13} and 481 nt in the case of hfp^{9}.

hfp Mutations Affect Germline Mitosis and Nuclear Morphology

The *Drosophila* egg chamber is composed of a cluster of 16 interconnected germline cells (15 nurse cells and an oocyte) surrounded by a layer of somatic follicle cells

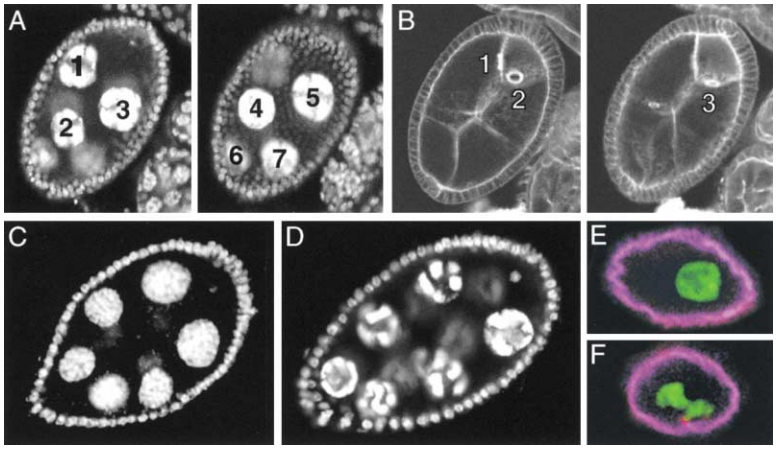

Figure 2. *hfp* Mutants Display Defects in Germline Mitosis and Nuclear Morphology

(A) Two focal planes of an *hfp*^{13} mutant egg chamber, stained with OliGreen to visualize DNA, showing seven nurse cells and an oocyte.

(B) The same egg chamber, stained with rhodamine-phalloidin, showing three oocyte-associated ring canals.

(C and D) Wild-type (C) and *hfp*^{13} (D) egg chambers stained with Oligreen. *hfp* nurse cell chromosomes fail to undergo normal dispersal and remain polytene throughout oogenesis.

(E and F) Wild-type (E) and *hfp*^{9} (F) oocyte nuclei stained with Oligreen and wheat germ agglutinin (purple) to visualize nuclear membranes. *hfp* mutant karyosomes can appear diffuse or fragmented instead of compact and spherical as in wild-type.

(Spradling, 1993). The ovary contains several strings of developing egg chambers, or ovarioles, that can be divided into two regions: the germarium, where egg chambers are formed, and the vitellarium, in which egg chambers mature. Within the germarium, a germline cystoblast undergoes four rounds of mitosis with incomplete cytokinesis to produce a cluster of 16 cystocytes that are linked by actin-rich connections called ring canals. One of the two cells with four ring canals becomes the oocyte, while the other 15 differentiate into polyploid nurse cells. *hfp* egg chambers commonly contain eight germline cells: seven nurse cells and an oocyte (Figure 2A) or, in some cases, eight nurse cells. At room temperature, 24% of *hfp*^{9} egg chambers and 45% of *hfp*^{13}/*Df* egg chambers contain eight germline cells. Viable females of stronger *hfp* alleles have rudimentary ovaries that contain few, undifferentiated egg chambers. To determine whether the eight-cell *hfp* egg chambers arise from a defect in germline mitosis, we examined the number of oocyte-associated ring canals. An oocyte possesses one ring canal for each round of cell division within the cyst and hence is normally associated with four ring canals. However, in *hfp* mutant egg chambers that contain seven nurse cells and an oocyte, the oocyte invariably has three ring canals (Figure 2B), revealing that these cysts have undergone only three rounds of mitosis.

In addition to having fewer cells per egg chamber, *hfp* mutants also display defects in germline nuclear morphology. During the early endocycles of wild-type nurse cells, the replicated chromosomes are polytene but disperse by midoogenesis (Dej and Spradling, 1999; Figure 2C). In *hfp* mutant egg chambers, nurse cell chromosomes remain polytene throughout oogenesis, giving them a striking banded appearance (Figure 2D; Table 1). In addition, we observe less penetrant defects in the morphology of the oocyte nucleus. The DNA of a wild-type oocyte nucleus forms a compact sphere, or karyosome, which persists from shortly after egg chamber formation until the late stages of oogenesis (Figure 2E). In a fraction of *hfp* mutant oocytes, however, the karyosome appears abnormally shaped, diffuse, or fragmented (Figure 2F; Table 1).

hfp Mutant Females Produce Short Eggs with Defects in DV Polarity

hfp mutant females lay few eggs, most of which are shorter than wild-type eggs and display dorsal appendage defects ranging from an expansion to a reduction of dorsal appendages (Figures 3A–3D). A wild-type egg is approximately 500 μm in length, while the eggs produced by *hfp* mutant females (hereafter referred to as *hfp* mutant eggs) range from 300 to 500 μm. An examination of late stage *hfp* egg chambers reveals that, in many cases, the nurse cells fail to transfer all of their cytoplasm into the oocyte prior to egg shell deposition (Figure 3E). This defect in nurse cell to oocyte transport may explain the shortness of *hfp* mutant eggs.

The dorsal appendage phenotypes of *hfp* mutant eggs are suggestive of perturbations in DV patterning of the egg chamber. As Hfp interacts with Enc, which is required for *grk* mRNA localization and Grk protein accumulation (Hawkins et al., 1997), we asked whether *grk* expression is also affected in *hfp* mutants. In wild-type

Table 1. Rescue of *hfp*^{9} Phenotypes by Otu104 and Hfp-GFP Transgenes

	Percentage with Polytene Nuclei	Percentage with Abnormal Karyosomes	Percentage with *grk* RNA Mislocalization
hfp^{9}	96.0	15.3	52.5
hfp^{9}; 2 × pOtu104	4.0	5.5	7.3
hfp^{9}; 4 × pOtu104	0.0	1.3	0.0
hfp^{9}; 1 × pHfp-Gfp	0.0	0.0	0.0

Females were raised at room temperature, and ovaries were stained with Hoechst to determine the percentage of egg chambers with polytene nuclei ($n > 100$ for each genotype; stages 6–10 scored) and abnormal karyosome morphology ($n > 40$; stages 6–9 scored). For *grk* in situ hybridizations, stage 10 and late stage 9 egg chambers were scored ($n = 200$ for *hfp*^{9}; $n > 30$ for other genotypes).

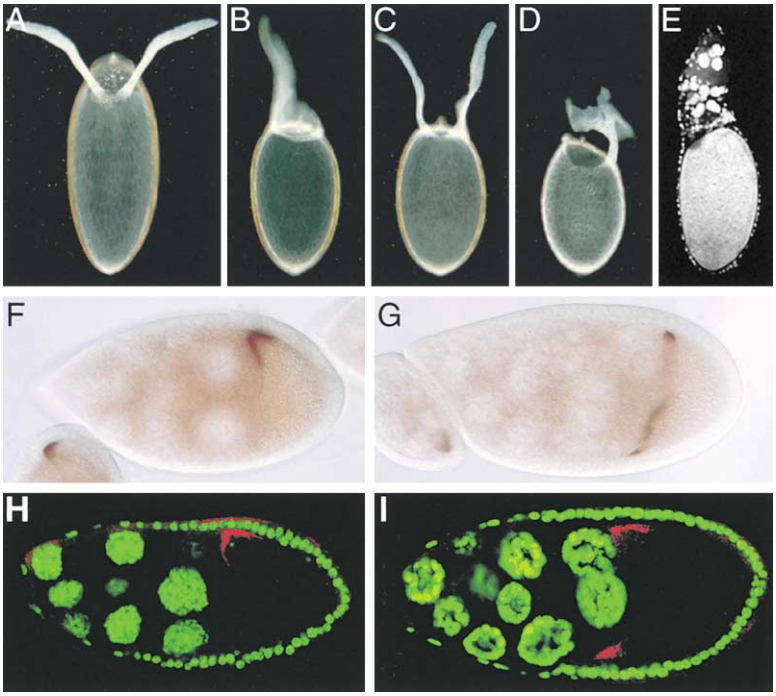

Figure 3. *hfp* Mutants Lay Short Eggs with Defects in Dorsoventral (DV) Patterning

(A) A wild-type egg with two respiratory appendages marking the dorsal anterior surface of the eggshell.

(B–D) hfp^{9} females lay eggs that are shorter than wild-type eggs and display defects in DV polarity ranging from dorsalized (B) with a ring of appendage material to two appendages (C) to partially ventralized with a single dorsal appendage (D).

(E) A Hoechst-stained late stage hfp^{13}/Df egg chamber with nurse cells that have failed to expel their contents into the oocyte prior to eggshell deposition.

(F–I) *grk* in situ hybridizations (F and G) and Grk antibody/Oligreen stainings (H and I) of stage 9 egg chambers. In wild-type (F and H), *grk* RNA and protein are restricted to the dorsal-anterior corner of the oocyte. In hfp^{9} mutants (G and I), *grk* RNA and protein can be detected in an anterior ring.

stage 9 egg chambers, *grk* mRNA is localized to a region overlying the oocyte nucleus, and Grk protein is thus restricted to the adjacent plasma membrane, where it induces dorsal follicle cell fates (Neuman-Silberberg and Schüpbach, 1993, 1996; Figures 3F and 3H). In a small fraction of *hfp* mutant egg chambers, *grk* RNA appears to be poorly expressed or undetectable, which would account for the production of eggs with reduced dorsal egg shell structures. More striking, however, is that, in 52% of the stage 9/10 hfp^{9} mutant egg chambers in which *grk* mRNA can be detected, the transcript is present in an anterior ring (Figure 3G). Thus, *hfp* appears to be required for both the production of wild-type levels of *grk* transcript and, like *enc,* for proper *grk* mRNA localization. However, unlike *enc*, this mislocalized *grk* mRNA in *hfp* mutants gives rise to detectable Grk protein (Figure 3I). This ring of Grk protein would be predicted to induce dorsal fates in all anterior follicle cells, accounting for the observed dorsalized eggs of *hfp*.

As *hfp* encodes a predicted splicing factor, we sought to determine whether the *grk* RNA mislocalization in *hfp* is due to a splicing defect that leads to the production of a *grk* transcript lacking proper localization signals. The *grk* gene encodes a single identified transcript (Neuman-Silberberg and Schüpbach, 1993; Figure 4A), and, in RT-PCR analysis of poly(A) RNA from wild-type ovaries, a single *grk* product is detected (Figure 4B). In *hfp* mutants, a larger product is also detected, suggestive of inefficient removal of one of the *grk* introns (Figure 4B). Using a series of primers, we detect maintenance specifically of the third intron in a fraction of *grk* transcripts in *hfp* mutants (Figure 4C). This type of splicing defect is unlikely to account for the mislocalized *grk* RNA observed in *hfp* oocytes, as the unspliced transcript does not lack any sequences found in the wild-type transcript. Furthermore, previous studies have shown that insertion of a fragment of the *lacZ* gene into the *grk* cDNA at a position analogous to the third intron does not disrupt normal RNA localization (Thio et al., 2000). Thus, it appears that, while Hfp is required for efficient *grk* splicing, this is not the cause of the RNA localization defect.

sqd Splicing Is Developmentally Regulated, but Unaffected by Hfp

The dorsalized eggs laid by *hfp* mutants are similar to those produced by females mutant for *squid* (*sqd*), which encodes an RRM-containing protein that has been shown to directly bind *grk* RNA (Kelley, 1993; Norvell et al., 1999). The *sqd* gene encodes three splice forms that contain the same N-terminal RNA binding domain but have divergent C termini (Kelley, 1993; Figure 4D) and display distinct activities with respect to *grk* RNA localization and translational control (Norvell et al., 1999). We wished to determine whether the dorsalized eggs of *hfp* could be caused by defects in the splicing of *sqd*. Using RT-PCR analysis of stage-dissected ovaries, we find that, in wild-type oogenesis, *sqd* splicing is developmentally regulated. While the *sqdA* and *sqdB* transcripts are produced constitutively, the *sqdS* transcript is detected only in later stages (Figure 4E). We detect no alterations in *sqd* splicing in hfp^{9} (Figure 4E) or in the stronger allelic combination hfp^{13}/Df (data not shown). Furthermore, we detect no differences in Sqd protein localization or levels in *hfp* mutants (data not shown), and, thus, the defects in *grk* RNA localization seen in *hfp* are not likely due to alterations in *sqd* expression.

Hfp Regulates the Alternative Splicing of *ovarian tumor*

The polytene nurse cell chromosome morphology of *hfp* mutants is strikingly similar to that observed in females

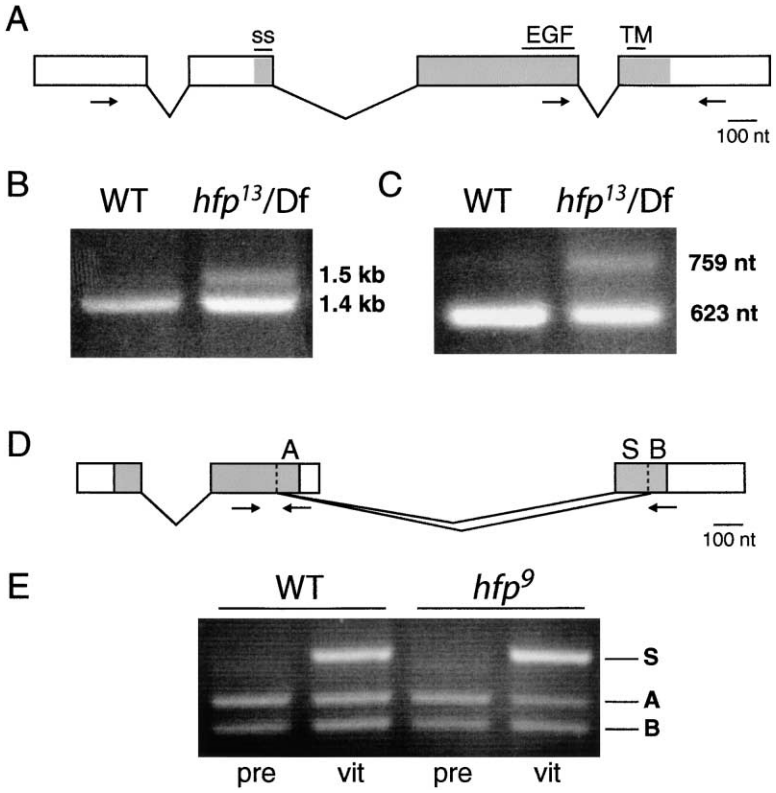

Figure 4. *grk* and *sqd* Splicing in Wild-Type and *hfp* Ovaries

(A) The *grk* gene encodes a single known transcript encoding a protein with a signal sequence (ss), an EGF domain, and a transmembrane (TM) region.

(B) RT-PCR analysis, using primers in the first and last *grk* exons, showing a single product in wild-type and an additional larger product in *hfp* mutant ovaries.

(C) RT-PCR analysis, using primers within the third and last *grk* exons, showing that the larger transcript retains the last *grk* intron.

(D) Three isoforms of Sqd are produced from alternative splicing.

(E) RT-PCR analysis of the *sqd* gene, showing that, in wild-type, SqdA and SqdB are expressed in both early (previtellarial) and later stage (vitellarial) egg chambers, but the transcript encoding SqdS is detectable only in later stages. This pattern of expression is unaffected in *hfp* mutants.

mutant for certain alleles of *ovarian tumor* (*otu*; King and Riley, 1982). *otu* also plays a role in germline cell division, as *otu* egg chambers can have too few or too many germline cells. The *otu* transcript is subject to alternative splicing, resulting in the production of a 98 kDa protein and a less abundant 104 kDa protein, depending on the incorporation of a 126 bp alternatively spliced exon (Steinhauer and Kalfayan, 1992; Figure 5A). The biochemical functions of the Otu isoforms are unknown, but they share an N terminus that contains a cysteine protease-like domain and a C terminus that possesses weak similarity to microtubule-associated proteins (Tirronen et al., 1995). Intriguingly, the alternatively spliced exon encodes a tudor domain, a sequence element found multiply repeated in the *Drosophila* Tudor protein and also present in proteins with putative RNA binding functions in several species (Ponting, 1997). Genetic and molecular analysis reveal distinct requirements and expression patterns for the two Otu isoforms (Storto and King, 1988; Steinhauer and Kalfayan, 1992; Sass et al., 1995), suggesting that the splicing of the *otu* transcript may be developmentally regulated. Indeed, RT-PCR shows that expression of the transcript encoding the 104 kDa isoform is restricted to the early stages of oogenesis (Figure 5B).

In order to determine whether the alternative splicing of *otu* is dependent on *hfp* function, we analyzed the expression of *otu* in *hfp* mutants. RT-PCR analysis of RNA isolated from early stage egg chambers shows that the relative abundance of the larger *otu* transcript is decreased in *hfp*, and Western analysis shows that the levels of the corresponding 104 kDa Otu isoform are significantly decreased (Figures 5C and 5D). Severe loss of Otu104 activity results in the production of tumorous egg chambers (Steinhauer and Kalfayan, 1992), which we do not observe in *hfp*. Therefore, some residual Otu104 protein must be present, possibly because our mutants represent partial loss-of-function alleles. To determine which, if any, of the *hfp* phenotypes are due to an effect on *otu* splicing regulation, we introduced a hybrid genomic-cDNA transgene encoding exclusively the 104 kDa Otu isoform (Sass et al., 1995) into an *hfp* mutant background. We found that *hfp* mutants expressing two copies of the Otu104 transgene produce egg chambers with predominantly wild-type nurse cell nuclear morphology (Figure 5E; Table 1) and that expression of four copies of the transgene completely rescues the nurse cell and oocyte nuclear morphology (Table 1) as well as the germline division defect (Figure 5F). Expression of Otu104 also restores normal *grk* mRNA localization (Table 1). This was unexpected, as defects in *grk* RNA localization have not been previously described in *otu* mutants, and we do not observe *grk* RNA localization defects in otu^{7} homozygotes or otu^{7}/otu^{11} mutants, which give rise to differentiated egg chambers. However, otu^{11} females, though often producing tumorous germaria, will under certain conditions produce rare eggs, and these show defects, including the expansion of dorsal appendages associated with mislocalization of *grk* RNA, very similar to those of hfp^{9} mutant eggs (data not shown). Interestingly, otu^{11} is a point mutation in the alternatively spliced exon and hence specifically affects the activity of the 104 kDa Otu isoform (Steinhauer and Kalfayan, 1992).

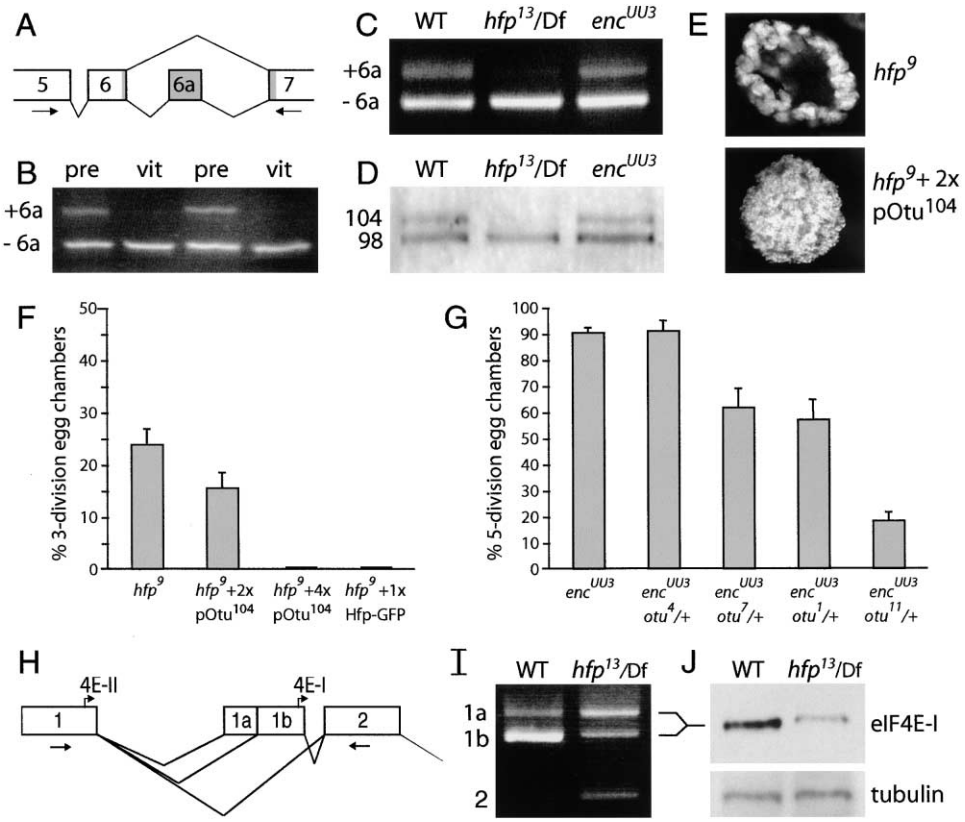

Figure 5. *hfp* Regulates the Splicing of *otu* and *eIF4E*

(A) Portion of the *otu* gene surrounding the alternatively spliced exon 6a. The tudor domain (shaded) and the positions of primers used in PCR are shown.

(B) RT-PCR analysis of previtellarial versus vitellarial stage wild-type egg chambers. The larger *otu* transcript containing exon 6a is detected exclusively in earlier stages.

(C and D) RT-PCR (C) and Western analysis (D) of previtellarial egg chambers. The ratio of the larger to the smaller *otu* splice form is dramatically decreased in *hfp*, while *otu* expression appears normal in *enc* females raised at the nonpermissive temperature for the division defect.

(E) Oligreen-stained nurse cell nuclei from hfp^{9} and from an hfp^{9} mutant strain carrying two copies of an Otu104-expressing transgene ($pOtu^{104}$). The rescue of the polytene nuclear phenotype of *hfp* is shown.

(F) The percentage of egg chambers with eight germline cells in hfp^{9} and in hfp^{9} females carrying two or four copies of $pOtu^{104}$ or an Hfp-GFP transgene.

(G) The percentage of egg chambers with 32 germline cells in enc^{UU3} and in enc^{UU3} females that are also heterozygous for *otu* alleles. The otu^{11} mutation, which specifically affects the activity of the Otu104 isoform, significantly suppresses the *enc* mitotic defect. Error bars indicate standard error of the mean.

(H) The *Drosophila* eIF4E gene encodes three transcripts that give rise to two different protein isoforms. Splicing to the more proximal 1a or 1b splice sites produces eIF4E-I, while splicing to exon 2 gives rise to eIF4E-II.

(I) In wild-type ovaries, the 1b splice acceptor site is used preferentially. In *hfp*, this preference is lost.

(J) A Western blot using eIF4E-I-specific antibodies shows that the levels of this isoform are significantly decreased in *hfp* ovaries.

As Half pint was isolated based on its ability to interact with Encore and these two genes have opposite effects on germline mitosis, we hypothesized that Enc may act to antagonize Hfp's role in splicing and that the extra cell division seen in *enc* mutants may be a result of an overproduction of Otu104. However, we find that *otu* expression in *enc* appears to be normal by RT-PCR and Western analysis (Figures 5C and 5D), and it is therefore unlikely that Enc acts to regulate the splicing activity of Hfp. However, we do observe suppression of the *enc* extra division phenotype by reducing the dose of *otu* (Figure 5G). Of the *otu* alleles tested, the suppression was most significant with the 104 kDa isoform-specific mutation otu^{11}. Thus, while Otu expression is not affected in *enc*, Otu104 activity is critical in mediating the *enc* extra division.

Hfp Affects Splicing of *eIF4E*

We analyzed the expression of several other alternatively spliced ovarian transcripts in *hfp* mutants. One potential target that was examined was eukaryotic initiation factor 4E (*eIF4E*), which binds to the seven-methylguanosine cap at the 5′ end of messenger RNAs and is a limiting factor in translation initiation (Gingras et al., 1999). The *Drosophila eIF4E* gene produces three ovarian transcripts through alternative splicing (Lavoie et al., 1996; Figure 5H). Two of these give rise to the eIF4E-I isoform, while the other gives rise to eIF4E-II. Both isoforms have been shown to have cap binding activity in vitro, and transgenic expression of eIF4E-I is sufficient to rescue the phenotype of several loss-of-function *eIF4E* alleles (Lavoie et al., 1996; Lachance et al., 2002); hence, the requirement for the different splice forms is unclear.

The major splice form in wild-type utilizes the 1b splice acceptor site, producing eIF4E-I. In *hfp* mutants, the bias for the 1b acceptor is lost and all three splice variants are produced roughly equally, with a slight preference for the more proximal 1a splice acceptor site (Figure 5I). Using antibodies specific for eIF4E-I, we find that the levels of this isoform are decreased in *hfp* (Figure 5J). We were surprised to find the levels of eIF4E-I so drastically affected, since the 1a transcript also gives rise to this isoform. This reduction in eIF4E-I protein levels may reflect either that the combined levels of eIF4E-I-encoding transcripts are lower in *hfp* than in wild-type or that the 1a transcript is not efficiently translated. The latter possibility would point to alternative splicing as a means of regulating *eIF4E* protein levels. As the misregulation of *otu* splicing appears to account for all of the known ovarian defects of *hfp*, the significance of the regulation of *eIF4E* splicing during oogenesis is unclear. However, it is possible that Hfp's role in *eIF4E* splicing is important at other stages of development and that decreased eIF4E-I levels may contribute to the reduced viability of *hfp* mutants.

Thus, Hfp acts to regulate alternative splice site choice within at least two genes in the ovary. Hfp does not appear to participate in all cases of splice site selection, however, as several alternatively spliced genes are not affected by *hfp* mutations (data not shown). Among these are *par-1* (Shulman et al. 2000), *pipsqueak* (Horowitz and Berg, 1996), and, as mentioned above, *sqd*. We did observe defects in the splicing of the sex-determining gene *Sex-lethal* (*Sxl*), detecting some of the male-specific transcript in hfp^{13} hemizygous females (data not shown), but, as *otu* is known to affect *Sxl* splicing (Pauli et al., 1993), *hfp*'s role in *Sxl* expression may be indirect.

Hfp Protein Localization

In order to determine the subcellular localization of Hfp during oogenesis, monoclonal antibodies to Hfp were generated. Using these, we detect Hfp protein within the nuclei of all germline cells and also within the follicle cell nuclei (Figure 6A). In *hfp* ovaries, the expression of Hfp is decreased within the germline of early stage egg chambers, while expression in the surrounding somatic follicle cells appears normal (Figure 6B). The reduction in Hfp expression specifically within the germline of *hfp* mutants suggests that different *cis*-acting sequences control germline and somatic expression of *hfp* and also that the observed *hfp* phenotypes are due to loss of *hfp* function in the germline. Consistent with this, we find that germline clones of the strong allele hfp^{38} result in developmental arrest early in oogenesis. Interestingly, the polyclonal serum obtained from the immunized animal prior to hybridoma production also detects cytoplasmic aggregates in the germline, which are particularly abundant within the oocyte (Figure 6C). This polyclonal serum, like the monoclonal antibodies, recognizes one major band that is less abundant in *hfp* mutant ovaries on Western blots (Figure 6D). However, since none of the monoclonal antibodies show this nucleo-cytoplasmic distribution and the rescuing Hfp-GFP fusion protein is detectable only in nuclei (Table 1; Figure 6E), we have been unable to determine the significance of the cytoplasmic staining.

Discussion

Hfp Regulates Alternative Splicing in the Ovary

Multiple transcripts produced from alternative splicing can encode proteins with different or even opposing functions (Lopez, 1998; Jiang and Wu, 1999), and, hence, the regulation of alternative splicing can have profound biological consequences. However, few factors that regulate splice site selection in vivo have been identified, and much of our knowledge of splicing regulation comes from in vitro studies (Manley and Tacke, 1996). The difficulty in isolating factors with effects on alternative splicing in vivo has, in part, led to the hypothesis that splice site choice is influenced by many factors that act combinatorially, each exerting a small influence on any one splicing event (Smith and Valcárcel, 2000). Hfp, however, represents a factor with a significant role in alternative splicing regulation. Reductions in Hfp activity disrupt the developmentally regulated splicing of *ovarian tumor*, splice site usage within *eIF4E* and the efficiency of *grk* splicing. However, several other splicing events appear to be unaffected. The specificity of Hfp function is highlighted by our observation that, though both the *otu* and *sqd* splicing patterns shift between early and late oogenesis, *otu*, but not *sqd*, splicing is regulated by Hfp.

Our in vivo analysis of Hfp function along with in vitro studies on the human homolog, PUF60, demonstrate a role for this factor in splicing. However, the human protein has been implicated in a number of other processes. It has been identified as Ro-BP1, a component of RNP complexes of unknown function found in both nuclear and cytoplasmic compartments (Peek et al., 1993). It has also been isolated as FBP-interacting repressor (FIR), shown to block activated transcription by interacting with the TFIIH complex (Liu et al., 2000). PUF60/Ro-BP1/FIR therefore appears to represent an unusually multifunctional protein, participating directly in transcriptional repression and pre-mRNA splicing and also associating with RNA-protein complexes in the cytoplasm.

The Role of *otu* in Germline Mitosis and RNA Localization

The observation that all of the ovarian phenotypes of *hfp* can be rescued by expression of the missing 104 kDa Otu splice form underscores the importance of *otu* splicing regulation and reveals previously undetected aspects of Otu function. While some *otu* egg chambers that have too few or too many germline cells have been observed, and the tumorous phenotype of strong *otu* mutations indicates a role in cell proliferation, none of the *otu* mutations specifically prevent the fourth mitosis of the germline cluster. The observations that Otu104 can rescue this defect in *hfp* and that the otu^{11} mutation can suppress the *enc* mitotic defect point to a role for Otu in the precise regulation of the four rounds of germline mitosis. Similarly, while *otu* egg chambers have been shown to display defects in the localization of *oskar* RNA to the posterior pole of the oocyte (Tirronen et al., 1995), a defect that we also observe in *hfp* (C.V.B., unpublished data), a role for *otu* in the localization of *grk* mRNA has not been detected. Our results suggest

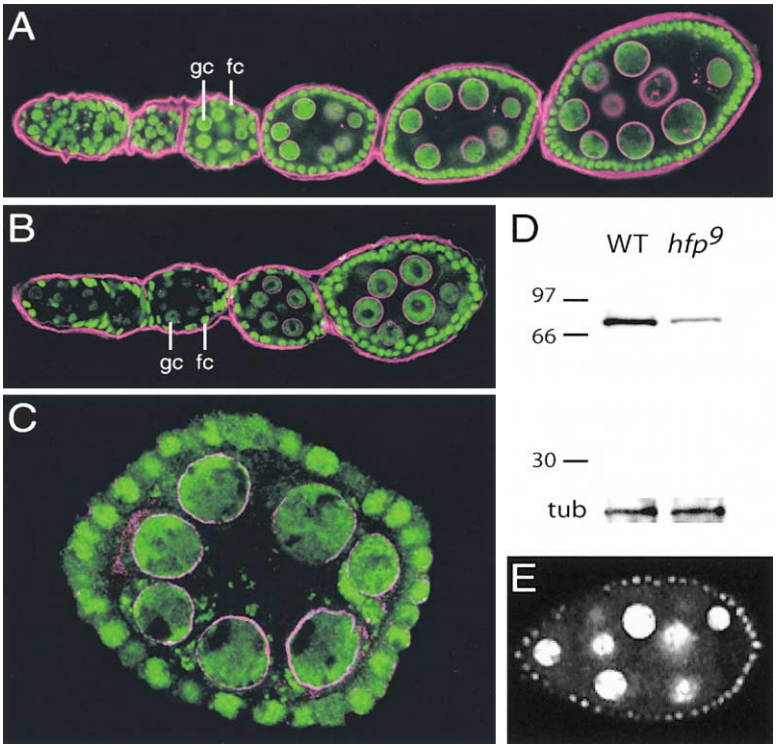

Figure 6. Germline Expression of Hfp Is Reduced in *hfp* Mutants

(A and B) Ovarioles stained with anti-Hfp monoclonal antibody (green) and wheat germ agglutinin (purple).

(A) In wild-type, Hfp is expressed in all germline-cell (gc) and follicle-cell (fc) nuclei.

(B) An hfp^{7} mutant ovariole showing a reduction in germline expression of Hfp during the early stages of oogenesis.

(C) Mouse polyclonal anti-Hfp antibody staining of an early stage wild-type egg chamber. Nuclear and cytoplasmic immunoreactivity are shown.

(D) Mouse polyclonal anti-Hfp Western blot of early stage-enriched ovaries. A single band of predicted size that is decreased in *hfp* is shown.

(E) Unfixed egg chamber from a wild-type female expressing an Hfp-GFP fusion protein under the control of the germline expression vector pCOG.

that *otu* does indeed play a role in *grk* RNA localization and that it is the 104 kDa isoform that performs this function. How does Otu participate in these diverse processes, from the regulation of germline mitosis to the dispersal of polytene chromosomes to the localization of RNAs? Mutations in *otu* are associated with aberrations in actin distribution (Rodesch et al., 1997), including ectopic accumulation of actin filaments within the fusome, a germline organelle implicated in the control of cystocyte division (Deng and Lin, 1997; Lilly et al., 2000). Thus, *otu* may affect germline mitosis by regulating the interaction between fusome components and actin microfilaments. Likewise, *otu* may participate in mRNA localization by regulating the interaction of localized RNAs with the cortical actin cytoskeleton, which may act to anchor transcripts at their subcellular destinations. A direct role for *otu* in RNA localization is supported by its cofractionation with RNP complexes (Glenn and Searles, 2001).

The Relationship between Hfp and Enc

Both *hfp* and *enc* affect the regulation of germline mitosis. While *hfp* mutant egg chambers often contain eight germline cells, *enc* mutations result in an extra round of division, resulting in egg chambers with 32 germline cells. We have shown that overexpression of Otu104 can rescue the division defect of *hfp* and that reduction of Otu104 activity can suppress the *enc* extra division. However, though Otu104 levels are strongly decreased in *hfp*, we do not detect a converse overproduction of this isoform in *enc* mutants, and, thus, *enc* does not appear to antagonize Hfp's splicing activity. It is possible that *enc* regulates germline mitosis through a pathway independent of *hfp*. However, the observed interaction between Hfp and Enc proteins raises the possibility that these genes function in a common pathway. If Enc does not antagonize Hfp's splicing activity, though, under what circumstances do these proteins interact? It may be useful to consider the Hfp-Enc interaction in terms of their roles in DV patterning. *hfp* and *enc* mutants both show defects in *grk* RNA localization, and *enc* is required for the accumulation of Grk protein (Hawkins et al., 1997). We have also shown that *hfp* plays a role in *grk* splicing. As has been observed for other splicing factors (Reed and Magni, 2001), it is possible that Hfp remains associated with its targets during nuclear export and perhaps delivers the spliced *grk* transcript to Enc in the cytoplasm. Enc may in turn be required for both anchoring and translation of the *grk* message. We thus propose that Hfp may have two roles: one in splicing regulation and one in the subcellular targeting of RNAs.

Since Otu104 overexpression can rescue the RNA localization defect of the hypomorphic allele hfp^{9}, the delivery of *grk* RNA to the anchoring machinery must also be dependent on *otu*. Otu's tudor domain and observed cofractionation in RNP complexes suggest that it might associate with mRNAs during transport or anchoring. We therefore propose that Hfp may also be present within the RNP complexes with which Otu fractionates. In support of this, the vertebrate homolog of Hfp has been isolated as a component of nucleo-cytoplasmic RNP complexes (Peek et al., 1993), and Hfp has also been found to copurify with proteins found in cytoplasmic RNP particles (J. Wilhelm, personal communication). This putative cytoplasmic function of Hfp may involve an interaction with Enc. For instance, Enc may be required to release *grk* RNA from an intermediate Hfp-/Otu-dependent stage of transport to an anchored, translationally competent state.

The Mechanism of Hfp's Splicing Activity

Much of our knowledge of splicing regulation in vivo comes from the study of the Sex-lethal (Sxl) protein of *Drosophila*. This factor has been shown to control splice site usage by blocking U2snRNP auxiliary factor (U2AF) from binding to the polypyrimidine tract upstream of the 3′ splice acceptor site (reviewed in Gebauer et al., 1997). Thus, Sxl binding represses usage of the proximal 3′ splice site, and a competing splice acceptor is used. How does Hfp function to regulate splice site choice? In the case of the *otu* transcript, does *hfp* promote splicing to exon 6a or repress selection of exon 7? In vitro studies with the human homolog of *hfp*, PUF60, point to a stimulatory role (Page-McCaw et al., 1999). In these studies, PUF60 [60 kDa *p*oly(*U*) binding *f*actor] was isolated as part of a complex that could stimulate efficient splicing of several introns in Hela nuclear extracts. This complex, along with U2AF, was found to facilitate the association of U2snRNP with the pre-mRNA, thus promoting spliceosome assembly.

The rat homolog of Hfp, Siah-BP, was isolated in a three-hybrid screen for factors that could bind to a splicing enhancer, a short RNA sequence required for neuron-specific alternative splicing of the amyloid precursor protein (APP) gene (Poleev et al., 2000). Siah-BP was also found to directly bind the large subunit of U2AF, consistent with studies on PUF60 suggesting that it acts in conjunction with U2AF in the early steps of splicing (Page-McCaw et al., 1999). The ability of the human PUF complex to stimulate splicing of all introns assayed suggests that it acts constitutively, facilitating spliceosome assembly at all polypyrimidine tracts. Half pint, however, appears to possess sequence specificity, as it is capable of influencing splice site choice and affects only a subset of genes analyzed. This apparent discrepancy between the human and *Drosophila* homologs may come from the artificial nature of the in vitro splicing assay, in which addition of perhaps nonphysiological levels of splicing factors may stimulate the splicing of targets that would not normally be affected in vivo. Alternatively, the orthologs may indeed differ in their target range, a point that could be addressed through in vitro analysis of Hfp activity.

Little is known about the *cis*-acting regulatory elements that control alternative splicing, posing a challenge for genomic protein prediction models (Black, 2000). The recognition element for Hfp is likely to be somewhat degenerate, as the known Hfp targets do not share an obvious binding site. RNA-protein crosslinking studies and/or the identification of several more Hfp target genes may define this binding site, providing insight into the elusive *cis*-acting sequences that control alternate splice site selection during development.

Experimental Procedures

Isolation of *hfp* cDNAs

The two-hybrid screen was performed using the Matchmaker LexA System (Clonetech). A LexA-Enc bait was constructed by cloning the 2.8 kb EcoRI-BamHI fragment of the *enc* cDNA into the pLexA plasmid, fusing the LexA protein with amino acids 133–1080 of Enc. The ovarian cDNA prey library, ovo1b, was provided by J. Großhans (Großhans et al., 1999). A single prey, which we later named Half pint, passed all tests of specificity, and DNA sequence from the interacting clone was found to correspond to EST clot 24 from the Berkeley *Drosophila* Genome Project (BDGP) database. The *hfp* cDNA LD27486 (accession number AF479079) is 2.5 kb and appears to be of full length as judged by Northern analysis. The genomic region surrounding *hfp* has been cloned into a P1 (DS02734), sequenced (accession number AC004343), and mapped by in situ hybridization to polytene bands 62A1–A2 on the third chromosome. The sequence of this 33 kb P1 is oriented proximal to distal, as determined by STS mapping of the P1 termini with respect to other clones in the region.

Drosophila Stocks and Mapping of *hfp*

The lethal P element insertion line *EP(3)3058* (Rorth, 1996) contained additional background lethals, which were removed through outcrossing to a white strain, generating 3058rec1. The deficiency *Df(3L)Ar14-8*, which fails to complement the insertion, has breakpoints of 61C4 and 62A8. The deficiencies tested and found to complement the lethality of the insertion are *Df(3L)Aprt-133* [62A2–B1; 62C2–D1] and *Df(3L)ru22* [61F8; 62A3–A5], though the latter should in theory uncover *hfp*, given the polytene analysis above. The discrepancy is likely due to error in mapping DS02734 to 62A1–A2: this P1 overlaps with other clones that have been mapped more proximally by in situ hybridization, suggesting that *hfp* is most likely in 62A3–A5. This is consistent with the estimated map position of the genomic scaffold AE003471, which contains *hfp*, to 62A4. The hfp^{9} chromosome used in these studies was marked with $rosy^{-}$, and hfp^{9} mutant lines carrying multiple copies of a $rosy^{+}$-marked *otu104* transgene (a gift of L. Searles) were generated by recombination and P element hopping.

Developmental Northerns and In Situ Hybridizations

Tissues were collected and frozen prior to RNA isolation using the Trizol Reagent (Gibco BRL). Approximately 20 μg of total RNA were loaded per lane on a 1.2% agarose gel containing formaldehyde and transferred in 10XSSC to Zeta-Probe nylon membrane (Bio-Rad). cDNA LD27486 was used as probe for *hfp*, and the Northern blot was reprobed for rp49 as a loading control. For in situ hybridizations, ovaries were dissected and teased apart in cold PBS and fixed in 200 μl 4% paraformaldehyde in PBS, 20 μl DMSO, and 600 μl heptane for 20 min at room temperature. Subsequent steps were performed as described in Tautz and Pfeifle (1989).

Germarial Western Analysis and RT-PCR

For Western analysis, germaria from ten females were dissected in cold PBS and transferred directly into 10 μl protein loading buffer (5 M Urea, 0.125 M Tris [pH 6.8], 4% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.1% Bromophenol Blue). The samples were boiled and pelleted, and 5 μl of the lysate was loaded per lane onto a 10% SDS polyacrylamide gel (Bio-Rad). After standard transfer to nitrocellulose, the blot was blocked in 5% milk/TBST and incubated with guinea pig anti-Otu antibody (a gift of Lillie Searles) or rabbit anti-eIF4E-I (kindly provided by Paul Lasko) at 1:1000 in TBST. HRP-conjugated secondary antibodies (Amersham) were used at 1:2000, and bands were visualized using the ECL-Plus chemiluminescent detection system (Amersham). For RT-PCR analysis, dissected germaria of ten females were transferred directly into 10 μl of RNA binding/lysis buffer of the Dynabead RNA isolation kit (Dynal). RNA isolation and first strand synthesis was performed as described in Tomancak et al. (1998). For PCR, the first-strand-containing Dynabeads were used as template in conjunction with Ready-To-Go PCR beads (Amersham). PCR was performed for 32 cycles, with an annealing temperature of 62°C. Forward primers include: 5′-AAAC CAGGAGGAGCGAAAGGCATC-3′ (*otu*), 5′-GCCCAGTAACCTACG CAGCTTGAG-3′ (*eIF4E*), 5′-GCGCAACAAGACCTAAAATCAAAGTG GAGC-3′ (*grk*, exon 1), 5′-CCCACACCGGAGAACCGC CACC-3′ (*grk*, exon 3), and 5′-GGTGGCTACAACAACCAGTG-3′ (*sqd*). Reverse primers include: 5′-ATAGCGGAATGGCAACGACC-3′ (*otu*), 5′-CTCGTTTTGCATGTCCTCCCAGG-3′(*eIF4E*), 5′-CTTGTGCGTCTT TTGCAAC-3′ (*grk*), 5′-GGCTCTACTTAGAACTGCTG-3′ (*sqd A*), and 5′-TTTTAGTAGGGCTGATGCCG-3′ (*sqd B* and *S*).

Hfp Antibody Production, Antibody Stainings, and IP Westerns

The C-terminal half of Hfp, corresponding to amino acids 277–637, was fused to GST, and purified fusion protein was used for mouse

monoclonal antibody production. For antibody staining, ovaries were dissected in cold PBS and fixed for 20 min in 200 μl 4% paraformaldehye in PBS and 600 μl heptane plus 1 μl 20% NP-40. After several rinses in PBST (PBS plus 0.2% Tween), ovaries were incubated for 1 hr in PBS plus 1% Tween 20 then blocked for 1 hr in PBST plus 1% BSA. Polyclonal Hfp serum was used at 1:1000, and monoclonal supernatant was used at 1:20 in PBST. Alexa^{568}-conjugated anti-mouse secondary antibody was used at 1:1000. To counterstain nuclear membranes, Alexa^{488}-conjugated wheat germ agglutinin was used at 1:1000. To visualize DNA, ovaries were incubated in 1:5000 Oligreen (Molecular Probes) plus 20 μg/ml RnaseA in PBS for 1 hr. Counterstains were performed during secondary antibody incubation. Immunoprecipitation was performed as described in Van Buskirk et al. (2000), using polyclonal mouse anti-Hfp as the precipitating antibody. Samples were loaded onto 10% and 7.5% Ready Gels (Bio-Rad), transferred, and probed, respectively, with monoclonal anti-Hfp at 1:20 and rat anti-Enc at 1:1000.

Acknowledgments

We would like to thank Lillie Searles for providing anti-Otu antibodies and pOtu104 transgenic lines. Many thanks to Pascal Lachance and Paul Lasko for sharing unpublished observations and anti-eIF4E antibodies. We are grateful to the BDGP for cDNAs, the P1 clone containing *hfp*, and the *EP(3058)* insertion line and to Tapio Heino and Brian Oliver for *otu* alleles. We also thank Jim Wilhelm for performing Hfp co-IPs with Yps and Exu, Nate Hafer for construction of the Hfp-Gfp fusion, and Roshan Jain for assistance with the *hfp* phenotypic analysis. Many thanks go to Laura Nilson, Andrew Swan, and Jennifer Zallen for their critical comments on the manuscript, Joe Goodhouse for assistance with confocal microscopy, and Marty Marlow for monoclonal antibody production. This work was supported by the Howard Hughes Medical Institute and by NIH grant PO1 CA41086.

Received: November 8, 2001
Revised: January 29, 2002

References

Bashaw, G., and Baker, B. (1997). The regulation of the *Drosophila msl-2* gene reveals a function for *Sex-lethal* in translational control. Cell *89*, 789–987.

Black, D.L. (2000). Protein diversity from alternative splicing: a challenge for bioinformatics and post-genome biology. Cell *103*, 367–370.

Burd, C.G., and Dreyfuss, G. (1994). Conserved structures and diversity of functions of RNA-binding proteins. Science *265*, 615–621.

Croft, L., Schandorff, S., Clark, F., Burrage, K., Arctander, P., and Mattick, J.S. (2000). ISIS, the intron information system, reveals the high frequency of alternative splicing in the human genome. Nat. Genet. *24*, 340–341.

Deng, W., and Lin, H. (1997). Spectrosomes and fusomes anchor mitotic spindles during asymmetric germ cell divisions and facilitate the formation of a polarized microtubule array for oocyte specification in *Drosophila.* Dev. Biol. *189*, 79–94.

Dej, K.J., and Spradling, A.C. (1999). The endocycle controls nurse cell polytene chromosome structure during *Drosophila* oogenesis. Development *126*, 293–303.

Gebauer, F., Merendino, L., Hentze, M.W., and Valcárcel, J. (1997). Novel functions for 'nuclear factors' in the cytoplasm: the Sex-lethal paradigm. Semin. Cell Dev. Biol. *8*, 561–566.

Gingras, A.-C., Raught, B., and Sonenberg, N. (1999). eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu. Rev. Biochem. *68*, 913–963.

Glenn, L., and Searles, L. (2001). Distinct domains mediate the early and late functions of the *Drosophila ovarian tumor* proteins. Mech. Dev. *102*, 181–191.

Gravely, B.R. (2001). Alternative splicing: increasing diversity in the proteomic world. Trends Genet. *17*, 100–107.

Grishin, N.V. (1998). The R3H motif: a domain that binds single-stranded nucleic acids. Trends Biochem. Sci. *23*, 329–330.

Großhans, J., Schnorrer, F., and Nusslein-Volhard, C. (1999). Oligomerisation of Tube and Pelle leads to nuclear localization of Dorsal. Mech. Dev. *81*, 127–138.

Hawkins, N.C., Thorpe, J., and Schüpbach, T. (1996). *encore*, a gene required for the regulation of germ line mitosis and oocyte differentiation during *Drosophila* oogenesis. Development *122*, 281–290.

Hawkins, N.C., Van Buskirk, C., Grossniklaus, U., and Schüpbach, T. (1997). Post-transcriptional regulation of *gurken* by *encore* is required for axis determination in *Drosophila*. Development *124*, 4801–4810.

Horowitz, H., and Berg, C.A. (1996). The *Drosophila pipsqueak* gene encodes a nuclear BTB-domain-containing protein required early in oogenesis. Development *122*, 1859–1871.

Jiang, Z.-H., and Wu, J.Y. (1999). Alternative splicing and programmed cell death. Proc. Soc. Exp. Biol. Med. *220*, 64–72.

Kelley, R.L. (1993). Initial organization of the *Drosophila* dorsoventral axis depends on an RNA-binding protein encoded by the *squid* gene. Genes Dev. *7*, 948–960.

Kelley, R.L., Wang, J., Bell, L., and Kuroda, M.I. (1997). Sex-lethal controls dosage compensation in *Drosophila* by a non-splicing mechanism. Nature *387*, 195–199.

King, R.C., and Riley, S.F. (1982). Ovarian pathologies generated by various alleles of the *otu* locus of *Drosophila melanogaster.* Dev. Genet. *3*, 69–89.

Lachance, P.E.D., Miron, M., Raught, B., Sonenberg, N., and Lasko, P. (2002). Phosphorylation of eukaryotic initiation factor 4E (eIF4E) is critical for growth. Mol. Cell. Biol. *22*, in press.

Lavoie, C.A., Lachance, P., Sonenberg, N., and Lasko, P. (1996). Alternatively spliced transcripts from the *Drosophila eIF4E* gene produce two different cap-binding proteins. J. Biol. Chem. *271*, 16393–16398.

Lilly, M.A., de Cuevas, M., and Spradling, A.C. (2000). Cyclin A associates with the fusome during germline cyst formation in the *Drosophila* ovary. Dev. Biol. *218*, 53–63.

Liu, J., He, L., Collins, I., Ge, H., Libuttti, D., Li, J., Egly, J.-M., and Levens, D. (2000). The FBP interacting repressor targets TFIIH to inhibit activated transcription. Mol. Cell *5*, 331–341.

Lopez, A.J. (1998). Alternative splicing of pre-mRNA: developmental consequences and mechanisms and of regulation. Annu. Rev. Genet. *32*, 279–305.

Manley, J.L., and Tacke, R. (1996). SR proteins and splicing control. Genes Dev. *10*, 1569–1579.

Matunis, E.L., Kelley, R.L., and Dreyfuss, G. (1994). Essential role for a heterogenous nuclear ribonucleoprotein (hnRNP) in oogenesis: hrp40 is absent from the germ line in the dorso-ventral mutant *squid.* Proc. Natl. Acad. Sci. USA *91*, 2781–2784.

Neuman-Silberberg, F.S., and Schüpbach, T. (1993). The *Drosophila* dorsoventral patterning gene *gurken* produces a dorsally localized RNA and encodes a TGFα-like protein. Cell *75*, 165–174.

Neuman-Silberberg, F.S., and Schüpbach, T. (1996). The *Drosophila* TGFα-like protein Gurken: expression and cellular localization during *Drosophila* oogenesis. Mech. Dev. *59*, 105–113.

Nilson, L., and Schüpbach, T. (1999). EGF receptor signaling in *Drosophila* oogenesis. Curr. Top. Dev. Biol. *44*, 203–243.

Norvell, A., Kelley, R.L., Wehr, K., and Schüpbach, T. (1999). Specific isoforms of Squid, a *Drosophila* hnRNP, perform distinct roles in Gurken localization during oogenesis. Genes Dev. *13*, 864–876.

Page-McCaw, P.S., Amonlirdviman, K., and Sharp, P.A. (1999). PUF60: a novel U2AF65-related splicing activity. RNA *5*, 1548–1560.

Pauli, D., Oliver, B., and Mahowald, A.P. (1993). The role of the *ovarian tumor* locus in *Drosophila melanogaster* germ line sex determination. Development *119*, 123–134.

Peek, R., Prujin, J.M., van der Kemp, A.J.W., and van Venrooij, W.J. (1993). Subcellular distribution of Ro ribonucleoprotein complexes and their constituents. J. Cell Sci. *106*, 929–935.

Poleev, A., Hartmann, A., and Stamm, S. (2000). A trans-acting factor, isolated by the three-hybrid system, that influences alternative splicing of the amyloid precursor protein minigene. Eur. J. Biochem. *267*, 4002–4010.

Ponting, C.P. (1997). Tudor domains in proteins that interact with RNA. Trends Biochem. Sci. *22*, 51–52.

Reed, R., and Magni, K. (2001). A new view of mRNA export: separating the wheat from the chaff. Nat. Cell Biol. *3*, E201–E204.

Rodesch, C., Pettus, J., and Nagoshi, R.N. (1997). The *Drosophila ovarian tumor* gene is required for the organization of actin filaments during multiple stages in oogenesis. Dev. Biol. *190*, 153–164.

Rorth, P. (1996). A modular misexpression screen in *Drosophila* detecting tissue-specific phenotypes. Proc. Natl. Acad. Sci. USA *93*, 12418–12422.

Sass, G.L., Comer, A.R., and Searles, L.L. (1995). The Ovarian tumor protein isoforms of *Drosophila melanogaster* exhibit differences in function, expression, and localization. Dev. Biol. *167*, 201–212.

Schüpbach, T. (1987). Germ line and soma cooperate during oogenesis to establish the dorsoventral pattern of egg shell and embryo in *Drosophila melanogaster*. Cell *49*, 699–707.

Schütt, C., and Nöthiger, R. (2000). Structure, function and evolution of sex-determining systems in Dipteran insects. Development *127*, 667–677.

Shulman, J.M., Benton, R., and St. Johnston, D. (2000). The *Drosophila* homolog of *C. elegans* Par-1 organizes the oocyte cytoskeleton and directs *oskar* mRNA localization to the posterior pole. Cell *101*, 377–388.

Smith, C.J.W., and Valcárcel, J. (2000). Alternative pre-mRNA splicing: the logic of combinatorial control. Trends Biochem. Sci. *25*, 381–388.

Spradling, A.C. (1993). Developmental genetics of oogenesis. In The Development of *Drosophila melanogaster*, M. Bate and A. Martinez-Arias, eds. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press), pp. 1–70.

Steinhauer, W.R., and Kalfayan, L.J. (1992). A specific *ovarian tumor* protein isoform is required for efficient differentiation of germ cells in *Drosophila* oogenesis. Genes Dev. *6*, 233–243.

Storto, P.D., and King, R.C. (1988). Multiplicity of functions for the *otu* gene products during *Drosophila* oogenesis. Dev. Genet. *9*, 91–120.

Tautz, D., and Pfeifle, C. (1989). A non-radioactive *in situ* hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals a translational control of the segmentation gene *hunchback*. Chromosoma *98*, 81–85.

Thio, G.L., Ray, R.P., Barcelo, G., and Schüpbach, T. (2000). Localization of *gurken* RNA in *Drosophila* oogenesis requires elements in the 5′ and 3′ regions of the transcript. Dev. Biol. *221*, 435–446.

Tirronen, M., Lahti, V.-P., Heino, T.I., and Roos, C. (1995). Two *otu* transcripts are selectively localised in *Drosophila* oogenesis by a mechanism that requires a function of the Otu protein. Mech. Dev. *52*, 65–75.

Tomancak, P., Guichet, A., Zavorsky, P., and Ephrussi, A. (1998). Oocyte polarity depends on regulation of *gurken* by Vasa. Development *125*, 1723–1732.

Van Buskirk, C., Hawkins, N., and Schüpbach, T. (2000). *encore* encodes a member of a novel class of proteins and affects multiple processes during *Drosophila* oogenesis. Development *127*, 4753–4762.

Accession Numbers

The GenBank accession number for the hfp cDNA LD27486 sequence reported in this paper is AF479079.