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Drosophila Symplekin localizes dynamically to the histone locus body and tricellular junctions

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Abbreviations: cas, castor; CTD, RNA polymerase II C-terminal domain; HCC, histone cleavage complex; HDE, histone downstream element; HLB, histone locus body; Madm, MLF1-adaptor molecule; PAP, poly (A) polymerase; PAS, poly A signal; Rp49, ribosomal protein L32; SL, stem loop; SLBP, stem loop binding protein; sop, ribosomal protein S2; Sym, Symplekin; yps, ypsilon schachtel.

The scaffolding protein Symplekin is part of multiple complexes involved in generating and modifying the 3' end of mRNAs, including cleavage-polyadenylation, histone pre-mRNA processing and cytoplasmic polyadenylation. To study these functions in vivo, we examined the localization of Symplekin during development and generated mutations of the Drosophila Symplekin gene. Mutations in Symplekin that reduce Symplekin protein levels alter the efficiency of both poly A0 and histone mRNA 3' end formation resulting in lethality or sterility. Histone mRNA synthesis takes place at the histone locus body (HLB) and requires a complex composed of Symplekin and several polyadenylation factors that associates with the U7 snRNP. Symplekin is present in the HLB in the early embryo when Cyclin E/Cdk2 is active and histone genes are expressed and is absent from the HLB in cells that have exited the cell cycle. During oogenesis, Symplekin is preferentially localized to HLBs during S-phase in endoreduplicating follicle cells when histone mRNA is synthesized. After the completion of endoreplication, Symplekin accumulates in the cytoplasm, in addition to the nucleoplasm, and localizes to tricellular junctions of the follicle cell epithelium. This localization depends on the RNA binding protein ypsilon schachtel. CPSF-73 and a number of mRNAs are localized at this same site, suggesting that Symplekin participates in cytoplasmic polyadenylation at tricellular junctions.

Introduction

The development of complex organisms containing a diverse array of multifunctional tissues requires dynamic modulation of gene expression. While transcription regulation is a critical component of gene expression, many additional mechanisms that help control gene expression during development involve the 3' end of an mRNA, including translational regulation, mRNA localization, production of different isoforms of the same mRNA, including translational regulation, mRNA localization, production of different isoforms of the same mRNA, and regulation of 3' end formation as a component of histone mRNA synthesis.1,2

The 3' end of most eukaryotic mRNAs is generated by endonucleolytic cleavage followed by addition of a poly(A) tail. The sole known exception is the metazoan replication dependent histone mRNAs, which are not polyadenylated and instead end in a conserved stem loop structure.2 Cleavage of both types of mRNA is directed by two cis elements near the 3' end of the pre-mRNA. For polyadenylated mRNA, the signals are the AAUAAA poly(A) signal and G/U rich downstream element, with cleavage occurring between the two elements.3 These cis elements assemble the cleavage and polyadenylation machinery, which minimally consists of the scaffolding protein Symplekin and four protein complexes: CPSF, which binds the poly(A) signal, CstF, which binds the G/U rich element, and CF I and CF II, as well as poly(A) polymerase (PAP).4 CPSF-73 is the endonuclease that cleaves pre-mRNAs followed by polyadenylation by PAP.5

Cleavage of histone pre-mRNA is similarly mediated by two cis elements, the stem loop, which binds stem loop binding protein (SLBP), and the histone downstream element (HDE), which binds U7 snRNP.6,9 The Sm ring of U7 snRNP contains two proteins only found in the U7 snRNP, Lsm10 and Lsm11.10,11 The N-terminus of Lsm11 binds to FLASH, and this interaction is critical for histone pre-mRNA 3' end formation.12,13 The discovery of CPSF-73 as the endonuclease that cleaves histone pre-mRNA provided the first indication that polyadenylation factors were also involved in histone mRNA biosynthesis.14
Dominski and coworkers showed that the FLASH and Lsm11 complex binds directly to a subset of polyadenylation factors termed the histone cleavage complex (HCC). The HCC includes Symplekin, CstF64 and the entire CPSF complex. In both mammals and Drosophila, the HCC can be isolated as a component of the U7 snRNP, suggesting that the active form of the U7 snRNP complex consists of the core U7 snRNP particle plus FLASH and the HCC.15,16

Biochemical experiments additionally demonstrated that Symplekin participates in histone pre-mRNA processing. Early characterization of histone pre-mRNA processing indicated that a component of the cleavage machinery was heat sensitive.17 Almost 20 years later, Kolev and Steitz showed that complementation of a heat-inactivated extract by in vitro transcribed and translated Symplekin restored in vitro cleavage of a histone pre-mRNA substrate.18 Furthermore, Symplekin was identified as a factor required for histone pre-mRNA processing in a genomewide RNAi screen, along with poly(A) factors CPSF-73 and CPSF-100.19

How the cell assembles distinct mRNA 3' end processing complexes containing overlapping sets of components in the appropriate amounts is an open question. Symplekin is a conserved protein that contains a HEAT domain at the NH2-terminus.20 In silico predictions indicate that a short disordered region separates the HEAT domain from a series of predicted armadillo repeats that extend to the COOH-terminus. Proteins with these domains are frequently classified as scaffolds.20 As a potential scaffold, Symplekin may play a critical role in assembling distinct complexes containing the CPSF complex. Symplekin and CPSF are also required for cleavage/polyadenylation directed by STAR-PAP,22,23 as well as for cytoplasmic polyadenylation together with CPSF and cytoplasmic poly(A) polymerase.24 Curiously, Symplekin was first discovered as a protein associated with the tight junctions of mammalian polarized epithelial cells.25 Symplekin likely acts as a scaffold for multiple complexes primarily involved in mRNA metabolism, and whether it performs this role at the tight junction is unknown.

There are several known direct binding partners of Symplekin which have potential roles in various aspects of mRNA 3' end formation. The N-terminal region of Symplekin interacts with Ssu72, a RNA polymerase II C-terminal domain (CTD) phospho-Ser5 phosphatase. The role of this interaction is not definitively known, but it could help link transcription and polyadenylation.21 Symplekin likely interacts directly with CPSF during nuclear polyadenylation, and it binds tightly to CstF64 at a site that prevents subsequent binding of CstF77 and CstF50 to CstF64.26 The Symplekin/CstF64 heterodimer may specify the interaction of the HCC with the FLASH/Lsm11 complex for histone pre-mRNA processing.15,16 Disrupting the Symplekin/CstF64 interaction by mutations in Symplekin results in a reduced efficiency of histone pre-mRNA processing in vivo.27 In addition to nuclear binding partners, Symplekin interacts with CPEB as part of its role in cytoplasmic polyadenylation24 as well as with the dual localization factor ZONAB.28

The genes for the five classes of histone proteins are linked and are localized in a distinct sub-domain in the nucleus defined by the histone locus body (HLB).29 In Drosophila melanogaster, which contains a tandem repeat of 100 copies of a 5kB histone repeat unit containing one copy of each of the five histone genes, there is a single HLB that is readily visualized using antibodies against Mxc (the mammalian NPAT orthologue), U7 snRNP, FLASH, or Mute, a protein of unknown activity that may function to down-regulate histone mRNA expression.30 A likely function of the HLB is to concentrate these factors for histone mRNA biosynthesis, since they are each present at low abundance. All four of these factors are continuously present in the Drosophila HLB, including in cells that are not expressing histone mRNA. As mammalian cells approach S-phase, NPAT is phosphorylated by Cyclin E/Cdk2 to activate histone gene expression,31 and Drosophila Mxc is phosphorylated by Cyclin E/Cdk2,32 likely for the same function. Although U7 snRNP and FLASH are both present in the Drosophila HLB constitutively, it is not known whether Symplekin and other HCC components are constitutively concentrated in the HLB.

Here we have utilized a genetic system to examine how Symplekin's function in mRNA 3' end formation contributes to development, and show that Symplekin dynamically localizes to different sites during development including the HLB and tricellular junctions.

Results

Decreased levels of Symplekin affect development

To examine the contribution of Symplekin to animal development, we characterized mutations in the Drosophila Symplekin (Sym) gene. We obtained two stocks with a P-element transposon insertion at the Sym locus: SymNP2964, annotated as inserted in the second exon, and SymEY20504, annotated as inserted in the 5' UTR of the Sym gene.33 By determining the start site of Sym transcription from the experimental data of Adelman and coworkers34 and the transposon insertion site by sequencing flanking DNA, we found that SymEY20504 is located in the 97 nt 5' UTR, 63 nt upstream of the translation start site (Fig. 1A, EY). In contrast to the original annotation, our sequencing of genomic DNA flanking SymNP2964 placed the transposon in the intergenic region between Symplekin and the divergently transcribed MLF1-adaptor molecule (Madm) gene (Fig. 1A, NP). SymNP2964 is viable and fertile in trans with a genomic deletion of the Symplekin locus (Sym[SymEY20504; hereafter "SymEY"]). SymEY20504 progeny do not live past the wandering 3rd instar larval stage when SymEY is provided maternally. In contrast, when SymEY is provided paternally, 32% of the expected numbers of SymEY20504 progeny survive to adulthood (Fig. 1B).

This difference in maternal effect between SymEY and SymEY indicates that SymEY20504 (hereafter, "SymEY") is a hypomorphic allele that provides some Symplekin function.

To generate additional Sym mutants, we mobilized the SymEY P-element and recovered and analyzed 4 alleles resulting from imprecise repair events (see Experimental Procedures for details). Three of these alleles contained internal deletions of SymEY, two of which retained a substantial amount of transposon sequence in the Sym 5' UTR (6,497 nts and 9,013 nts in Sym441 and Sym619, respectively).
**Figure 1. Developmental Analysis of Symplekin Mutants (A)**. Schematic of Symplekin locus, gene structure and mutations. Symplekin is located on the 3rd chromosome in close proximity to the essential genes madm and cas. Features of the Sym gene structure are represented by intensity of grey: intergenic (light), UTRs (medium) and ORF (dark). Transcription start sites are represented with an arrow above the gene and triangles below indicate the transposon insertion sites. Outlined boxes depict the nature of each Sym mutant. The genomic deletion in Sym152 is shown above the locus diagram. The EY P element inserted in SymEY and the internal deletion of the EY P element leaving 206 nts in the Sym 5'UTR resulting in Sym58 are represented below the Sym gene structure diagram. (B). Visual representation of Sym mutant viability phenotypes. Circles indicate the proportion of Sym allele mutant (black) and control heterozygous sibling (grey) flies obtained in each experiment. The expected number of control siblings is 2/3. Thus, 1/3 black indicates full viability and the absence of black indicates lethality. The percentage of observed flies within the mutant class is labeled inside each circle. Note that the observed precise excision (Sym53) class exceeded the expected ratio as a wild type fly is healthier than control siblings carried over a balancer chromosome. For the EY genotypes, (p) indicates a paternal and (m) indicates a maternal contribution of the transposon with the other parent providing the Df chromosome. For all of the other genotypes, the Df chromosome was maternally provided. (C). Visual representation of the Sym58 delayed eclosion phenotype. The line graph represents the daily average number of mutant and control sibling flies observed within one generation. (D). Symplekin protein levels in Symplekin mutant embryos. Equal amounts of total protein from 16-20 hour embryos were resolved by SDS gel electrophoresis and Symplekin detected by Western blotting. The mutant Sym152 (lane 2) does not express zygotic symplekin and indicates the amount of maternal Symplekin remaining at this stage. The * indicates a cross-reacting band. (E). Extracts from 3rd instar larvae of each genotype were analyzed by Western blotting. 3-fold dilutions of each genotype were analyzed for Symplekin (top) and the blot re-probed for tubulin (bottom).
respectively Table S1). These two alleles weren’t considered further because they displayed the same larval lethality phenotype as SymEV (data not shown). Sym58 retained 206 nt of P-element sequence in the 5′UTR of Sym. Sym58/Sym19′ progeny developed to adulthood (Fig. 1B), but these flies were male and female sterile and also developmentally delayed, eclosing 1-6 days later than siblings that contained a single wild type copy of Sym (Fig. 1C). The fourth allele, Sym152, contains a complete deletion of the Sym coding region that extends beyond Sym to the 5′ control region of the downstream gene, castor (Fig. 1A). Sym152 causes embryonic lethality and disrupts castor function because it fails to complement castor null mutations (data not shown). We did not recover an allele that disrupts the Sym coding region without affecting the neighboring essential genes, castor and Madm. We isolated Sym53 as a precise excision event and used this allele as a wild type control in our developmental analyses to ensure that any observations were not a consequence of other lesions on the original SymEV chromosome (Fig. 1B).

To compare the levels of Symplekin expression from these alleles, we measured Symplekin protein in 16-20 h old Sym mutant embryos by western blotting (Fig. 1D). At this stage of development, Sym152 and Sym58 embryos have less Symplekin protein than wild type, while the amount in SymEV is similar to wild type (Fig. 1D). The detection of Symplekin in the Sym152 deletion, which lacks zygotic Symplekin expression, indicates there is a maternal supply of Symplekin that persists until late stages of embryogenesis and likely beyond, consistent with our results from the reciprocal matings described above. Western blot analysis indicated that both mutant alleles of Sym expressed reduced amounts of Symplekin relative to wild type. However, these alleles expressed substantial amounts of Symplekin, with Sym58 expressing more Symplekin than SymEV. In SymEV mutants, Symplekin protein was reduced to ~30% of normal as judged by the zygotic expression in embryos and to ~50% of normal in SymEV wandering 3rd instar larvae (Fig. 1D, E), consistent with allele strength as determined by the genetic results. These modest reductions in Symplekin expression indicate that relatively small changes in Symplekin protein levels have a substantial affect on development.

Pre-mRNA processing is attenuated in Symplekin mutants

When histone mRNA 3′ end processing is disrupted in Drosophila by mutation of U7 snRNP, FLASH or SLBP, longer polyadenylated histone mRNAs accumulate because of read through transcription and subsequent utilization of cryptic poly(A) signals located downstream of the HDE in all 5 histone genes.35-37 We analyzed histone mRNA 3′ end processing in SymEV and Sym58 using an S1 nuclease protection assay that distinguishes normally processed histone H2a mRNA from various mis-processed, polyadenylated H2a mRNAs as well as "read-through" transcripts that extend past the end of our probe (Fig. 2A). For both SymEV and Sym58 mutants, we analyzed total RNA from wandering 3rd instar larvae, the terminal stage of development for SymEV mutants. Because Sym58 mutants develop to adulthood, we also analyzed total RNA from Sym58 ovaries. Unlike FLASHL01692 or U7 snRNP mutants, which contain primarily misprocessed H2a mRNA,36,37 in both SymEV and Sym58 we primarily found properly processed H2a mRNA (Fig. 2A). However, each Sym mutant contained small amounts of misprocessed H2a mRNA (Fig. 2A), indicating that histone mRNA 3′ end processing was not 100% efficient. In the Sym58 ovaries we observed a higher level of accumulation of "read-through" RNA (Fig. 2A, lane 6, "R"), compared with the long polyadenylated mRNA (Fig. 2A, lane 6, "M") when compared to the U7 mutant. A similar phenotype was observed in cultured S2 cells after Sym depletion and suggests that polyadenylated transcripts don’t accumulate efficiently after histone pre-mRNA misprocessing due to disruption of the cleavage and polyadenylation machinery.38

We next asked if polyadenylation is affected in the Sym mutants. We analyzed two genes, ribosomal protein L32 (Rp49) and ribosomal protein S2 (sop), that were previously shown by RT-PCR to be sensitive to disruption of the poly(A) factor, CstF77 (Su(F)B).39 In this assay, an amplification product is detected with primers that flank the 3′ end poly(A) signals only when the pre-mRNA has not been cleaved at the normal site. Using this assay we detected misprocessed Rp49 and sop RNA in both SymEV and Sym58 mutants but not in wild type, demonstrating that 3′ end processing of these poly(A) mRNAs is sensitive to the levels of Symplekin (Fig. 2B). Because a complete failure in histone pre-mRNA processing during larval stages still permits the completion of development,40 we conclude that the lethality of the SymEV mutant and the delayed eclosion and sterility of the Sym58 mutant likely result from polyadenylation defects due to reduced Symplekin function and not to the small effects on histone mRNA 3′ end formation that we observed in Fig. 2A.

Symplekin localization to the HLB during development is dynamic

Because Symplekin is required for histone pre-mRNA processing and is a component of the HCC, we asked if Symplekin is enriched in the HLB and whether its presence in the HLB changes during development. In the early syncytial cycles of Drosophila embryogenesis, there is very little if any transcription, and there are no HLBS. Mature HLBS containing the constitutive HLB markers, Mxc, FLASH, U7 snRNP and Mute, are formed in cycle 11, corresponding to the time when there is a large increase in the expression of zygotic histone mRNA, which continues until cycle 14.32,41 During these syncytial cycles Cyclin E/Cdk2 is constitutively active, and Mxc is phosphorylated as detected by HLB staining with the MPM-2 monoclonal antibody, which recognizes a Cyclin E/Cdk2-dependent phosphorylolyte on Mxc.32 All the HLBS are MPM-2 positive once they form at cycle 11 and remain MPM-2 positive through cycle 14.41 We detect Symplekin accumulation in the HLBS of G2 phase of cycle 14 in gastrulating embryos (stage 6) (Fig. 3A). Sym positive HLBS are present in both somatic cells and the pole cells, which are germ line precursor cells that are quiescent at this time, but will re-enter the cell cycle and proliferate during larval development.42 In contrast, we do not detect Symplekin in the HLB of cells that have permanently exited the cell cycle, such as the highly polyploid salivary gland cells of wandering 3rd instar

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larvae (Fig. 3B). Thus, when cells are rapidly proliferating, have high Cyclin E/Cdk2 activity and are expressing histone genes (e.g. during early embryogenesis), Symplekin accumulates in the HLB. In contrast Symplekin is not concentrated in the HLB in differentiated cells that have exited the cell cycle.

**Symplekin localization to follicle cell HLBs is cell cycle regulated**

Two major components of the histone pre-mRNA processing system, U7 snRNP and FLASH, are constitutively present in the HLB, while a third component, SLBP, is not detected in the HLB but is present in much higher concentration throughout the nucleoplasm. It is not known whether the HCC, which contains Symplekin, CstF64 and CPSF, is present in the HLB continuously in cycling cells. Because Symplekin is localized to the HLB in rapidly proliferating cells in the early embryo and not in post-mitotic salivary gland cells, we determined whether Symplekin is localized to the HLB throughout the cell cycle or perhaps only during S phase when histone genes are expressed.

To address this question we analyzed follicle cells of stage eight ovaries. These cells are endocycling, oscillating between S and G phase resulting in polyploid cells. To address this question we analyzed follicle cells of stage eight ovaries. These cells are endocycling, oscillating between S and G phase resulting in polyploid cells.44,45 The cell cycle of these asynchronously replicating cells has been previously characterized, and they spend 30% of their time in S-phase and 70% in

![Image of Figure 2](image-url)

**Figure 2.** Depletion of Symplekin protein results in misprocessed RNA (A). S1 nuclease protection assay analysis of histone mRNA species in Symplekin mutants. The diagram indicates the location of the H2a probe. Total RNA extracted from wandering 3rd instar larvae or adult ovaries was hybridized with the 3'-labeled (star) DNA probe (P) and then incubated with S1 nuclease, which digests single stranded nucleic acids. The probe is complementary to the H2a mRNA, H2a-H4 intergenic region up to the H4 HDE and also contains vector sequence (3’ grey region). Properly processed histone H2a mRNA is cleaved between the stem loop (SL) and histone downstream element (HDE), protecting 340 bp of the DNA probe from S1 digestion (W). The H2a gene contains multiple cryptic poly(A) signals that are utilized in animals with disrupted histone pre-mRNA processing. These longer mis-processed polyadenylated mRNAs protect several discrete species (M). Any transcripts extending beyond the region of complementarity protect a single read-through product (R) that is distinct from undigested probe (P) and whose 3’ end(s) are likely heterogeneous. Protected probe products were resolved on an acrylamide/urea gel and visualized by autoradiography. (B). Processing of polyadenylated mRNAs in Sym mutants was assessed by RT-PCR with primers flanking the canonical cleavage site for three mRNAs, rp49, sop and pgk. The black bar above each gene diagram represents the amplified PCR product. Black boxes represent exons, the grey box represents the 3’UTR and the dashed line represents the downstream intergenic region, which accumulates in the Symplekin mutant. pgk transcripts were detected in each preparation. Note that the amplified product in the pgk gene spans an intron that is not present in the cDNA template but is in the genomic DNA PCR control.
Figure 3. Symplekin concentration in the HLB is dynamic during development. (A and B). Projected confocal images of Symplekin localization in a syncytial embryo (A) and salivary gland nuclei (B). The presence of Symplekin in the HLB was determined by co-localization with the constitutive component Mute. Cyclin E/Cdk2 activity was monitored by staining with MPM-2, an antibody that recognizes phosphorylated epitopes in the HLB. Note that in the embryo, Sym enrichment was detected in both rapidly cycling somatic (yellow arrow) cells and germline (white arrow) cells that are not cycling. Both types of cells have MPM-2 positive HLBs whereas in the salivary gland nucleus, neither Sym nor MPM-2 signal was present in the large Mute positive HLB (red arrowhead). Scale bars = 10 microns. The inset shows a higher magnification image of a somatic cell. (C). Quantification of Sym HLB enrichment in endocycling stage 8 ovarian follicle cells. Ovaries were stained for Mute (blue), Sym (green) and MPM-2 (red). HLBs were defined by Mute staining, and we assessed the HLBs for co-localization with Sym and/or MPM-2. A representative projected image shows each class: Mute only (blue arrow), Mute + MPM-2 (red arrow), Mute + Symplekin (green arrow) and Mute + MPM-2 + Symplekin (yellow arrow). Quantification of individual ovaries were averaged and presented as a Venn diagram (n=6, average number of cells scored per ovary was 93 +/- 21.7). The errors represent the standard deviation.
Symplekin localizes to tricellular junctions in stage 10B follicle cells

Symplekin is present in the nucleoplasm of follicle cells, which produce large amounts of mRNA and protein for eggshell production and vitellogenesis. Additionally we detected Symplekin accumulation at the follicle cell cortex during stage 10B, specifically within a region of the cortex corresponding to the tricellular junction. We further characterized this striking localization by structured illumination fluorescence microscopy (Fig. 4B, Supplemental movie 1) and confirmed that Symplekin localized to the tri-cellular junction of stage 10B follicle cells by expressing ectopic HA tagged Symplekin and visualizing it with anti-HA antibodies (Fig. 4C). We also used an antibody against gliotactin, an integral membrane protein that is found at tricellular junctions to confirm that this localization was actually at the tricellular junction. Anti-gliotactin stains the tricellular junction of stage 10B follicle cells by anti-Mute, and 72% stained only with anti-Mute, consistent with these representing cells in G-phase (Fig. 3C). The remaining 28% of cells represented three distinct populations with respect to the HLB: 13% stained only for Mute and MPM-2, 8% stained only for Mute and Symplekin, and 7% stained for Mute, MPM-2 and Symplekin (Fig. 3C). Because phosphorylated Mxc (i.e. MPM-2) is present in 20% (13% MPM-2/Mute alone + 7% all three) of the HLBS and Symplekin is enriched in 15% of the HLBS (8% Sym/Mute alone + 7% all three), these results suggest that neither MPM-2 nor Symplekin staining encompasses all of S phase. Cyclin E/Cdk2 is activated just prior to S-phase and its activity persists throughout much but not all of S-phase. MPM-2 likely stains some cells that haven’t entered S-phase, and may not stain late S-phase cells. Thus it won’t perfectly track active histone mRNA synthesis throughout S-phase. These data demonstrate that Symplekin is not a constitutive component of the HLB, but it likely cycles, and is concentrated in the HLB during at least part of S-phase.

Symplekin was first described as a protein that localized to tight junctions in mammalian cells. Symplekin was later characterized as a component of the mRNA 3′ end formation machinery. Studies of a mammalian intestinal epithelial cell line, HT-29, showed that Symplekin and the Y box protein ZONAB, co-localize at tight junctions as well as being found in the nucleoplasm. This study also showed a direct interaction between recombinant Symplekin and ZONAB, whose closest homologue in Drosophila is the Y box protein, ypsilon schachtel (Yps) (Fig. 4I).

Yps interacts with RNA trafficking proteins and is a component of the oskar mRNP, although its precise role in RNA metabolism is not clear. Like Symplekin, cytoplasmic Yps is enriched at the tricellular junctions of stage 10B follicle cells (Fig. 4G), further suggesting that mRNP(s) containing cytoplasmic polyadenylation factors accumulate at tricellular junctions. Yps is also present in the cytoplasm but is not detected in the nucleoplasm. To test if Symplekin localization depends on Yps, we stained ypsJM2/Df(3L)BK9 mutant ovaries with anti-Symplekin antibodies. Symplekin did not localize to the tricellular junction in stage 10B yps mutant follicle cells, but instead was dispersed in the cytoplasm in addition to the nucleoplasm (Fig. 4H). We conclude that Yps is required for Symplekin localization to the tricellular junction, but that the accumulation of cytoplasmic Symplekin in stage 10B follicle cells occurs independently of Yps.

Taken together these data suggest that Symplekin is present at tricellular junctions as part of a complex involved in cytoplasmic RNA metabolism. In support of this conclusion, an earlier study demonstrated that at Stage 10B of follicle cell development, a set of mRNAs encoding integral membrane proteins are concentrated and translated at the tricellular junctions, providing potential substrates for the cytoplasmic Symplekin complex.

Discussion

Our genetic data clearly demonstrate that Symplekin is essential for development and that reducing the concentration of Symplekin affects the efficiency of mRNA 3′ end formation. A small reduction in the amount of Symplekin has severe effects on development, in a concentration-dependent manner, consistent with Symplekin playing multiple roles in vivo. Symplekin participates in multiple aspects of RNA metabolism in metazoans including canonical and STAR-PAP-mediated cleavage-polyadenylation in the nucleus, cytoplasmic polyadenylation, and histone premRNA processing. In each case Symplekin acts as part of a multi-protein complex, likely performing a scaffolding role. How the cell distributes a single protein among a variety of different cellular complexes is not clear. As a potential interface between specialized trans factors and general components of 3′ end processing machinery, Symplekin may help determine the composition of each of these multi-protein complexes by binding specific trans factors to assemble the appropriate complex at the appropriate site for each 3′ end processing reaction (Fig. 5).
Figure 4. For figure legend, see page 621.
Assembling Symplekin into the histone pre-mRNA processing complex

Symplekin participates in two distinct mRNA 3’ end-processing pathways in the nucleus, cleavage of histone pre-mRNAs and cleavage/polyadenylation of all other mRNAs. Because of the very small fraction of polyadenylation factors present in the HLB, formation of the active U7 snRNP does not result in the loss of significant cleavage/polyadenylation activity. In nuclear extracts from Drosophila or mammalian cells <1% of the polyadenylation factors are present in the HCC bound to U7 snRNP. 99% of the Symplekin is likely dedicated to cleavage/polyadenylation. In nuclear extracts Symplekin can readily exchange between a polyadenylation complex and the HCC, since addition of the FLASH/Lsm11 complex to these extracts results in up to 40% of the Symplekin and CPSF being assembled into the HCC. Thus, the Lsm11/FLASH complex can drive assembly of the HCC, and the amount of the FLASH/Lsm11 complex limits how much of the HCC can form. The current evidence suggests that the Symplekin/CstF64 heterodimer is the form of Symplekin incorporated into the HCC, which then binds the CPSF complex, as well as FLASH/Lsm11. The amount of U7 snRNA (and hence the amount of Lsm11 in the U7 snRNP) or the amount of FLASH, whichever is lower, determines the maximal amount of the HCC that can be present at any particular time.

There are three potential states of the U7 snRNP: the core U7 snRNP, which can bind to histone pre-mRNA, the U7 snRNP bound to FLASH, and the active U7 snRNP containing the HCC bound to the Lsm11/FLASH complex. U7 snRNP and FLASH are constitutive components of the HLB, but Symplekin is not.

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**Figure 5.** Summary of Symplekin subcellular localization and model of HLB dynamics. Symplekin localization observed in this study is summarized in the cell drawn on the left. Grey shading represents the diffuse Symplekin accumulation in the nucleus, consistent with its role in cleavage-polyadenylation. The darker grey indicates sites of Symplekin concentration: in the tricellular junctions, where it likely participates in cytoplasmic polyadenylation leading to localized translation; and in the HLB, where it is required for histone mRNA biosynthesis. The expanded HLB depicts fluctuating concentration of Sym during the cell cycle. During S phase or when there is high activity of Cyclin E/Cdk2, Symplekin (and likely the HCC) localizes to the HLB and interacts with a unique surface generated by an interaction between FLASH and the U7 snRNP component Lsm11, creating the active form of U7 snRNP.
present in the HLB in cells that have exited the cell cycle, suggesting that the HCC normally concentrates in the HLB on the U7 snRNP/FLASH complex to activate histone pre-mRNA processing. Thus, one way to regulate processing would be to regulate the binding of the HCC to the U7 snRNP (Fig. 5), possibly as a result of activation of Cyclin E/Cdk2. We found that Symplekin is present in the HLB in early embryos when Cyclin E/Cdk2 is active, including the pole cells even when they are not replicating, indicating that ongoing histone gene transcription is not essential for Symplekin recruitment. An attractive scenario is that the HCC is present in the HLB throughout much of S phase allowing efficient expression of histone mRNA. At the end of S-phase the U7 snRNP may be inactivated by loss of the HCC (Fig. 5). This model fits our observation that Symplekin does not concentrate in the HLB of postmitotic cells, and that Symplekin localization in the HLB cycles in endoreduplicating follicle cells. Since Symplekin and the HCC complex have the potential to convert U7 snRNP, which is constitutively present in the HLB, from an inactive form to an active form, localization of Symplekin to the HLB is an essential step for activating histone mRNA biosynthesis.

What is the role of symplekin at the tricellular junction?

In stage 10B follicle cells, in contrast to younger endoreduplicating follicle cells, a substantial fraction of Symplekin is present in the cytoplasm and accumulates at the tricellular junctions together with CPSF-73 and Yps but not CstF64. The follicle cells produce proteins needed for egg development, including the yolk and eggshell, and follicle cell movement confer the characteristic shape to the oocyte. At stage 10B, when follicle cells undergo centripetal migration to encapsulate the expanding oocyte, a subset of mRNAs including dGRASP are localized to and translated at the tricellular junction (alternately termed zone of contact). At least 5 mRNAs including dGRASP are localized to and translated at the tricellular junction during this specific stage of oogenesis likely reflects the localization of a specific subset of mRNAs and the cytoplasmic polyadenylation machinery, of which Symplekin and CPSF-73 are components, resulting in regulated translation of a number of mRNAs.

Yps is part of the mRNP that traffics ask mRNA along microtubules and has been implicated in translational repression of ask mRNA. Our data show that Symplekin localization to the junction requires Yps suggesting that Yps could contribute to regulation of dGRASP both by promoting localization of the mRNA and directly interacting with the cytoplasmic polyadenylation machinery at the junction to enhance translation. ZONAB, the closest Yps homolog in mammals, interacts with Symplekin at tight junctions in mammalian cells suggesting that this strategy for synthesis of proteins at cell junctions has been conserved in evolution. The translation of at least one protein, ZO-1, which binds ZONAB, is regulated by cytoplasmic polyadenylation at the mammalian cell junctions.

Future combined genetic and cell biological approaches will help reveal the molecular details of how Symplekin assembles into different complexes in distinct subcellular compartments to facilitate its multiple functional roles in RNA metabolism.

Materials and Methods

Drosophila genetics

SymEY20504NP2964 was obtained from the Bloomington Stock Center and SymNP2964 from the Drosophila Genetic Resource Center. To generate additional Sym mutant alleles, excisions of EY20504 were recovered over Tm3 as white eyed male progeny from w1118, P[EY20504]ry506, Sh, P[y8 D2-3] fathers. These single males were crossed to w1118;Df(3R)7283/Tm3, Ser, e, p[tub-GFP] females. Males that failed to complement lethality were next crossed to y1, w7, P[lacW]casjC2/Tm3, Sh and w1118;P[EP]Madm3+/P[EP]Madm3+/TM6B, Tb1 to ensure that the excision did not disrupt vicinal, essential genes. Excision events were also characterized by PCR (see below). Of the 1,050 single males tested, we focused on four mutant excision events. Three were internal deletions of EY20504, and the other was a deletion that extended to the car promoter. Breakpoint sequences are presented in Table S1. FLASH1101602 was previously characterized, as was the Lsm11102047, [P[V5-Lsm11+]] line used for staining. UAS-Sym-HA was generated by fC31 mediated integration at the VK0033 landing site of a pUASg-HA plasmid containing the full length Sym coding sequence. The C329b-Gal4 driver was obtained from the Bloomington Stock Center. The ypsM22 and Df(3L)Bk9 mutant flies were obtained from the Wilhelm Lab. WT indicates w1118.

Sym allele analysis

The EY20504 and NP2964 insertion sites were determined by PCR. All primer sequences are documented in Table S2. DNA was prepared by single fly squash prep (10 mM Tris pH 8.2, 25 mM NaCl, 1mM EDTA, 200 µg/mL Proteinase K) or by the protocol provided by the Berkeley Drosophila Genome Project. 1 µL of either prep was used for each PCR. Primers pOUT and either CG2097 DNA 1F or CG2097 DNA 1R were used for each PCR reaction to determine the insertion site of each transposon, as well as to screen for excision of the EY20504 transposon. PCR was also used to identify the breakpoints of the four excision alleles. CG2097 DNA 1F and R were used to identify the lesion in Sym58 as well as the precise excision in Sym55. Primers pairs throughout the EY element are listed and were used to determine the Sym441 and Sym619 sequence. The primer pair 152 breakpoint primer F and 152 breakpoint primer R flank repaired DNA in Sym152. Mapping Sym55 required isolation of homozygous embryos, as the endogenous locus on the balancer interfered with the PCR. Primer sequences are summarized in Table S2.

Western blot

16-20h embryos homozygous for WT, Sym152, SymEY, or Sym58 were isolated by lack of GFP expression with a COPAS-Select Embryo Sorter (Union Biometrica). 100 embryos per genotype were boiled in SDS loading buffer followed by shearing 25 times with a 27.5 gauge needle. 7.5 embryos per genotype were resolved on a 10% gel by SDS-
PAGE. Alternatively ten mutant larvae of each genotype were identified by lack of GFP and dissected brains, larval imaginal discs and salivary glands were boiled in 100 μL SDS loading buffer and sheared as described above with a 27.5 gauge needle and 30 μL of each sample was resolved on a 10% gel by SDS-PAGE. After transfer to a PVDF membrane, the blot was probed for Symplekin with an anti-rabbit Symplekin antibody (1:1000), and donkey anti-rabbit HRP (1:10,000; GE Healthcare). After exposure of the blot with ECL Prime (Amersham), or SuperSignal West Dura Chemiluminescent Substrate (Pierce) signal to film, the larval blot was probed for H2a (Amersham), or SuperSignal West Pico Chemiluminescent Substrate (Pierce) to capture signal from the loading control on film. The relative amounts of Symplekin in SymEY and Sym58 mutant animals were determined by gel analysis of film exposures with Image J software.

**RT-PCR**

Total RNA was extracted from whole control and mutant wandering 3rd instar larvae that were identified by lack of GFP with Trizol Reagent (Invitrogen). 2 μg of total RNA was treated with DNase (Fermentas). Samples were divided in half, and a master mix of all of the reagents for reverse transcriptase (except the RT enzyme) was added to each sample (cDNA Synthesis Kit, Fermentas). Reverse transcriptase was added to one sample per genotype. After cDNA synthesis, 1:5 dilutions of sample were used for each 25 μL PCR reaction. Genomic DNA prepared from WT larvae (as described above) was used as a positive control for the PCR reaction. Indicated primers were used with TAQ DNA Polymerase (NEB), resolved on a 2% agarose gel and visualized by ethidium bromide staining. The sequences are included in Table S2.

**S1 protection assay**

5 μg of RNA for each genotype was used for the S1 nuclease protection assay. The probe and method used have been previously described. Briefly, the probe was generated by end labeling a BspEI cut H2a DNA with α-32P-dCTP and Klenow Polymerase (NEB). After release from the TOPO TA vector (Invitrogen) by digestion with HindIII (NEB), the probe was gel purified and hybridized to total RNA from the indicated genotype. Following digestion by S1 nuclease (Promega), protected DNA fragments were resolved on a 6% acrylamide-7M urea gel and visualized by autoradiography.

**Immunofluorescence**

WT embryos were dechorionated, fixed in a 1:1 mixture of 7% formaldehyde/heptane for 25 min, and incubated with primary and secondary antibodies overnight at 4°C and for 1 h at 25°C, respectively. Salivary glands were dissected in PBS + 0.1% TritonX-100 and ovaries were dissected in Grace’s medium (GIBCO). Both tissues were fixed in 7% formaldehyde for 25 min and permeabilized in a mixture of PBS and 0.2% Tween for 15 min. Samples were blocked for 30 min with Enhancer (Invitrogen) and incubated with primary and secondary antibodies overnight at 4°C and for 1 h at 25°C, respectively. Primary antibodies used were polyclonal rabbit anti-Symplekin (1:1000), polyclonal rabbit anti-YPD (1:1000), polyclonal rabbit anti-CPSF73 (1:1000), polyclonal guinea pig anti-Mute (1:5000), monoclonal mouse anti-MMP1 (Millipore), monoclonal mouse anti-Disc Large (1:1000, Developmental Studies Hybridoma Bank), monoclonal mouse anti-Gliotakin (1:300), monoclonal mouse anti-HA (1:1000; UNC Hybridoma) and monoclonal mouse anti-V5 (1:1000; Invitrogen). Secondary antibodies used were goat anti-mouse IgG-Cy3 (Jackson Immuno Research Laboratories), goat anti-guinea pig IgG-Cy5 (Jackson Immuno Research Laboratories) and goat anti-rabbit Alexafluor 488 (Invitrogen). DNA was detected by staining samples with 4,6-diamidino-2-phenylindole (DAP) (1:1000 of 1 mg/mL stock, Dako North America) for 1 min. Images, with the exception of panel 4B, were obtained on a Zeiss 510 or 710 Confocal Microscope using a 40x objective lens. Images were prepared with Lsm Software (Zeiss) and Photoshop (Adobe). The SIM structured illumination fluorescence microscopy images presented in panel 4B were obtained with a DeltaVision OMX (GE Healthcare) imaging system. Images were collected with a 60X oil objective [Olympus Plan/Apo 60X, 1.42NA]. The OMX images were processed with the Imaris software and Photoshop. Images shown are max intensity projections of several z-slices. The reconstructed 3D image (sup. movie 1) was generated by the OMX software (softWoRx).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Acknowledgments**

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**Supplemental Material**

Supplemental data for this article can be accessed on the publisher’s website: http://www.tandfonline.com/kncl

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4. Mandel CR, Bai Y, Tong L. Protein factors in pre-

3. Martin F, Schaller A, Eglite S, Schumperli D, Muller B.

2. Martin F, Schaller A, Eglite S, Schumperli D, Muller B.

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4. Mandel CR, Bai Y, Tong L. Protein factors in pre-

3. Martin F, Schaller A, Eglite S, Schumperli D, Muller B.

2. Martin F, Schaller A, Eglite S, Schumperli D, Muller B.

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Table S1. *Sym* Allele DNA Sequences

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The flanking 5’ sequence (lowercase) and 3’ sequence (UPERCASE) of each insertion (*) or breakpoint (/).

Table S2. Primer Sequences

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