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TROPOMYOSIN IS AN INTERACTION PARTNER OF THE DROSOPHILA COILED COIL PROTEIN YURI GAGARIN

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Abstract

The *Drosophila* gene *yuri gagarin* is a complex locus encoding three protein isoform classes that are ubiquitously expressed in the organism. Mutations to the gene affect processes as diverse as gravitactic behavior and spermatogenesis. The larger Yuri isoforms contain extensive coiled-coil regions. Our previous studies indicate that one of the large isoform classes (Yuri-65) is required for formation of specialized F-actin-containing structures generated during spermatogenesis, including the so-called actin “cones” that mediate spermatid individualization. We used tandem affinity purification of a tagged version of Yuri-65 (the TAP-tagging technique) to identify proteins associated with Yuri-65 in the intact organism. Tropomyosin, primarily as the 284-residue isoform derived from the ubiquitously expressed *Tropomyosin 1* gene was thus identified as a major Yuri interaction partner. Co-immunoprecipitation experiments confirmed this interaction. We have established that the stable F-actin cones of spermatogenesis contain Tropomyosin 1 (Tm1) and that in mutant *yuri*^{F64}, failure of F-actin cone formation is associated with failure of Tm1 to accumulate at the cone initiation sites. In investigating possible interactions of Tm1 and Yuri in other tissues, we discovered that Tm1 and Yuri frequently colocalize with the endoplasmic reticulum. Tropomyosin has been implicated in actin-mediated membrane trafficking activity in other systems. Our findings suggest that Yuri-Tm1 complexes participate in related functions.

Keywords

tropomyosin; coiled coil proteins; endoplasmic reticulum; *Drosophila*; muscle; spermatogenesis; golgins; actin

INTRODUCTION

Dynamic remodeling of the actin cytoskeleton underlies a wide range of cellular activities including movement [1], cell-cell interactions, and morphogenesis [2]. Although all actin filaments have the same fundamental monomer organization, other features such as filament length, bundling, branching, and turnover all contribute to the generation of discrete cytoskeletal elements that perform specialized functions. An ever-increasing catalog of

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actin-binding proteins is involved in generating these different actin structures. The actin-binding protein tropomyosin (Tm), originally identified in muscle, is now recognized as a significant element in the generation of this functional diversity [3,4]. In muscle, where rod-like coiled-coil Tm dimers lie along the actin filaments, tropomyosin regulates access of the myosin heads to their actin binding sites. In non-muscle cells, the precise mechanisms for tropomyosin control of actin function have not yet been defined. However, tropomyosin is associated with the more stable, linear, actin filaments in cells and is thought to prevent filament branching [5] and severing [6]. There is also evidence that tropomyosin acts to bring myosin II to this type of F-actin structure [7]. Multiple gene copies and alternative splicing events combine to produce a large number of Tm isoforms (>40) in mammals [8]. Further, alternative promoter usage results in production of low molecular weight Tm species of ~248 amino acids in length, in addition to the high molecular weight, 284-residue “conventional” Tms [8]. This plethora of Tm variants and the highly specific roles of some of these isoforms in F-actin function [9-11] suggest a complexity and richness to Tm regulation of actin filament dynamics that has yet to be fully explored.

In studying gravitactic behavior in *Drosophila*, we identified the locus *yuri gagarin* through a mutation to the gene (*yuri^{c263}*) that alters responses to gravity [12]. *yuri* shows widespread expression in the organism [13], but *yuri^{c263}* may specifically affect gravity-related behavior because it changes *yuri* activity in certain mechanosensory neurons. The *yuri* locus generates three major classes of isoforms (of 30, 65 and 100 kDa) with the two larger classes representing progressive addition of C-terminal sequences to the 30-kDa isoform [13]. The amino acid sequences of the Yuri isoforms provide little information as to molecular function, although the longer isoforms are largely composed of putative coiled-coil regions, suggesting homo- and/or heterodimerization capabilities.

To gain more insight into the function of *yuri*, we generated further mutant alleles of the gene, and one of these, the *yuri^{F64}* allele, has been particularly informative. *yuri^{F64}* results in loss of expression of the 65-kDa isoforms of the protein in all tissues examined, as well as the loss of the 30-kDa isoform specifically in the testis. The only developmental phenotype associated with the *yuri^{F64}* mutation is complete male sterility [13], which could indicate functional redundancy between the 65- and 100-kDa isoforms in other tissues.

The defects in spermatogenesis caused by the *yuri^{F64}* mutation indicate that Yuri is intimately associated with F-actin function. After meiosis, the single centriole of each developing spermatid attaches to the nuclear membrane and differentiates into the basal body, from which the sperm tail axoneme grows. This nuclear association of the centriole involves the formation of a distinctive structure on the nuclear surface – the so-called “dense complex”. The dense complex lies between the nuclear envelope and a layer of endoplasmic reticulum (ER) that forms as a cap on one hemisphere of the maturing nucleus. Our analysis revealed that both Yuri and F-actin are components of the dense complex, and that Yuri is required for F-actin accumulation in this structure. Thus, in the *yuri^{F64}* mutant, both Yuri and F-actin fail to accrete on the nuclear surface resulting in aberrant centriole attachment and displacement of the final basal body and axoneme relative to the nucleus.

Loss of Yuri function in the *yuri^{F64}* mutant also results in failure to form a second F-actin structure during spermatogenesis. After spermatid elongation, an individualization process is initiated in which the 64 syncytial spermatids in each cyst are converted into individual sperm. Individualization involves the formation of a “cone” of F-actin around each of the 64 spermatid basal bodies embedded in the apical tips of the condensed spermatid nuclei [14-16]. The actin cones are the only F-actin elements detectable in the spermatogenic cysts at this stage. The cones move up the spermatid axonemes, pushing excess cytoplasm ahead of them and organizing the formation of individual plasma membranes around each sperm.

We established that Yuri is an integral component of the mature, moving F-actin cones [13]. Further, we determined that in *yuri*^{F64} animals, F-actin cone formation fails, and, as a result, no spermatid individualization takes place.

Given the widespread expression of Yuri in the organism, these findings led us to speculate that Yuri has a role in the assembly of F-actin structures in other tissues in addition to the testis. Further, the association of Yuri and F-actin with ER membranes in dense complex formation suggested a particular role for Yuri in F-actin formation at membranes. As a critical step in understanding the molecular action of Yuri in the organism, we undertook to determine what interacting proteins might mediate its roles in F-actin function. To isolate Yuri interaction partners, we chose the TAP-tag approach [17,18]. The specific advantage of this method is that it identifies binding partners for a given protein in tissues native for its expression. A version of the protein fused to two tags that permit serial affinity purification is expressed *in vivo* and then purified from suitable extracts, along with any bound interaction partners. Mass spectroscopy (Mass Spec.) is used to identify these interacting proteins after separation by SDS-PAGE. Using this approach, we have identified the ubiquitously expressed F-actin binding protein Tropomyosin 1 (Tm1) as a major interaction partner for the Yuri-65 isoform. We show for the first time that, like F-actin, Tm1 accumulates at the site of formation of the F-actin cones and is lost from this site in the *yuri*^{F64} mutation. We have also established that Yuri and Tm1 colocalize with F-actin at known sites of the ER in a variety of tissues. These findings suggest that Yuri mediates F-actin function at cellular membranes through an interaction with Tm1 in many cellular contexts.

MATERIALS AND METHODS

Fly stocks and genetics

A fly line with the UAS-2xEGFP transgene [19] on the X chromosome was used to generate a *UAS-2xEGFP; yuri*^{c26}/*CyO* stock by standard genetics. For examination of EGFP expression, tissues were dissected in PBS, mounted in 50% glycerol containing 0.1% Hoechst 33342 (Invitrogen), and examined immediately by epifluorescence. Homozygous transgenic lines carrying *P{UAS-TAP-yuri-65}* (see below) in a *w*¹¹¹⁸ background were prepared by standard genetic procedures after germline transformation (Genetic Services, Inc., Sudbury, MA). Fly lines containing a GFP exon trap into *Tm2* (line ZCL2456) and an ER-EGFP marker construct (BL7195) were obtained from the Bloomington Drosophila Stock Center. GFP-exon traps for *Tm1* (line CC02057), *kettin* (lines G053 and ZCL2144) and *reticulon-like1* (G71 and G301) were obtained from W. Chia, Inst. Molecular and Cell Biology, Singapore [20] and the FlyTrap project [21]. A GFP exon trap line for Protein Disulfide Isomerase (G198) was obtained from G. Tear, King's College, London, UK [22]. The stock *w*⁻; *yuri*^{F64}/*CyO*; *Tm1*^{CC02057}/*TM3*, *Ser* was prepared by standard genetics.

Creation and assay of *P{UAS-TAP-yuri-65}* transgenes

The *pREP-NTAP* plasmid containing the tandem affinity tag sequence was obtained from K. Gould (Vanderbilt Medical Center, Nashville, TN). The tag coding sequence was PCR-amplified with *Pfu* Ultra II HS polymerase (Stratagene) and primers that add a 5' EcoRI site and a 3' BamHI site. The product was cloned into the *pCR4-Blunt* cloning vector (Invitrogen), sequenced, then excised and recloned as an EcoRI - BamHI fragment into the EcoRI and BglII sites of *pP{UAS-TAP}* [23] to create *pP{UAS-TAP}*. The Yuri-65 coding sequence from cDNA SD06513 (Drosophila Genomic Resource Center) was amplified and cloned as a 5' NotI/3' KpnI fragment by similar procedures. After sequence validation, this NotI-KpnI fragment was cloned into the equivalent sites in *pP{UAS-TAP}* to create

pP{UAS-TAP-yuri-65} (Figure 1). Final sequencing across the ligation sites ensured that no frame-shift mutation had been introduced.

After preparing transgenic lines (see above), each *P{UAS-TAP-yuri-65}* insertion was assayed for tagged Yuri-65 expression after crossing to a heat-shock GAL4 driver line (Bloomington Stock #1799). *P{UAS-TAP-yuri-65}/hs-GAL4* transheterozygous adults were heat-shocked at 37° for one hour, allowed one hour recovery, then homogenized in SDS-sample buffer for electrophoresis and immunoblot assay with anti-Yuri antibody as described previously [13]. The highest expressing line was used for the studies described here. This line showed only low levels of tagged Yuri-65 expression in the absence of a GAL4 driver (see Figure 3).

Purification of TAP-Yuri-65 binding proteins

For experimental samples, (*yuri^{c263}/UAS-TAP-yuri-65*), homozygous males of the most highly inducible *UAS-TAP-yuri-65* line were mass-mated to virgin female *yuri^{c263}* homozygotes in 20 fly bottles. For control samples (*yuri^{c263}/+* and *UAS-TAP-yuri-65/+*), homozygous *yuri^{c263}* or *UAS-TAP-yuri-65* animals were each mass-mated to *w¹¹¹⁸* flies. Adults were continuously passaged to new bottles and 0-to-3-day-old pupae were scraped from the insides of the bottles with a flat metal spatula and rapidly frozen at -80° C until sufficient material was amassed. For each experiment, 30 grams of pupae per sample were used.

All buffers, materials, and equipment were chilled to 4° C. Frozen pupae were crushed thoroughly in 3 mL lysis buffer per gram of tissue (25 or 150 mM NaCl, 25 mM Tris [pH 8], 2.5 mM MgCl₂, 5% glycerol, 0.2% Nonidet P-40 substitute [Fluka], 1 mM dithiothreitol [DTT], 1 mM EDTA, 1 mM EGTA, 0.5 µg/mL Leupeptin [Sigma], 0.5 mg/mL Pefabloc [Sigma], protease inhibitor cocktail powder [Sigma P2714; 1 bottle per 20 g tissue]) using a mortar and pestle and strained through a very fine sieve into a 250-mL flask. Solids captured by the sieve were re-crushed in the mortar, with additional lysis buffer, and then added to the flask. The homogenate was incubated on ice for 30 min with gentle rocking to allow complete cell lysis and protein solubilization. After centrifugation at 4° C for 15 min at 13,800 × g, floating lipids were removed from the supernatant and the supernatant was filtered through a fine-gauge tissue sieve into a clean 250-mL flask. Rabbit IgG-agarose beads (Sigma), pre-rinsed twice with lysis buffer, were added to the filtered solution, and samples were sealed and incubated for at least 4 hours at 4° C to allow binding of the Protein A moiety of the TAP-tag to the beads.

The IgG-agarose beads were harvested from the lysates by low-speed (2,000 × g) centrifugation. Beads were exhaustively washed by repeated resuspension/centrifugation cycles using gentle agitation in batches of 25 mL IPP (25 or 150 mM NaCl, 25 mM Tris (pH 8), 5% glycerol, 1 mM DTT, 1 mM EDTA, 1 mM EGTA) with leupeptin (0.5 mg/mL) + Pefabloc (0.5 mg/mL) + 0.1% Nonidet P-40 at 4° C for 2 min. Washing was continued until the wash solution was clear and then two more washes were performed. Final washes in IPP alone were performed to remove detergent, which inhibits TEV protease [24]. Beads were then resuspended in 10 mL IPP + 1 “Complete EDTA-free Mini” protease inhibitor tablet (Roche), which does not inhibit TEV protease. AcTEV protease (200 U, 20 µL; Invitrogen) was added to each sample to cleave bound Yuri complexes from the IgG beads. Proteolysis was performed overnight at 18° C with rotation.

IgG-agarose beads were removed from the samples by centrifugation for 10 min at 2,000 × g, 4° C. Supernatants, containing Yuri complexes cleaved from the beads, were filtered to remove any non-pelleted beads and brought to 10 mL with IPP. Ten ml of 2x CBB (25 mM or 150 mM NaCl, 25 mM Tris, pH 8.0, 2 mM DTT, 2 mM MgOAc, 2 mM imidazole, 10

mM CaCl₂) with 1 EDTA-free Mini protease-inhibitor tablet (Roche) per 10 mL, were then slowly mixed into the supernatants. Calmodulin-agarose beads were rinsed twice in 1x CBB, resuspended in 10 mL 1x CBB and 3.3 mL aliquots were added to each sample, followed by rotation at 4° C for 4 hours to allow the calmodulin binding peptide (CBP) moiety of the TAP-tag to bind to the beads. The calmodulin-agarose beads were harvested by low speed centrifugation and then washed three times with 1x CBB. Washed beads were resuspended in 2 mL CBB (25 or 150 mM NaCl, 25 mM Tris, pH 8.0, 1 mM DTT, 1 mM MgOAc, 1 mM imidazole, 10 mM EGTA) and boiled for 5 min to released CBP from the beads. The boiled samples were centrifuged at 14,000 × *g* for 1 min, 4° C, to pellet the beads, the supernatants were transferred to new centrifuge tubes, and the centrifugation was repeated. The final supernatants, which contained the TAP-Tag purified protein samples, were removed to fresh tubes.

Analysis of TAP-Tag purified proteins

Proteins in the final supernatants were concentrated by addition of ice-cold trichloroacetic acid to a final concentration of 20%. After mixing and incubation at –20° C overnight, precipitates were pelleted by centrifugation at 14,000 × *g* for 20' at 4° C. Pellets were washed twice with 0.5 mL ice-cold acetone then air dried and resuspended in 20 µL standard 3× SDS loading buffer. When necessary, 0.5 M Tris-HCl solution, pH 6.8, was used to adjust sample pH to the “blue” range of bromophenol blue in the loading buffer. After solubilization by boiling for 5 min, aliquots of samples were separated on 12.5% SDS-polyacrylamide resolving gels and stained with Silver Stain Plus kit (BioRad) or Coomassie Brilliant Blue. Bands of interest were excised from the gel, dried, and sent to the Stanford University School of Medicine PAN Facility (Stanford, CA) for analysis. At the PAN facility, proteins were subjected to tryptic digestion followed by mass mapping of resultant peptides using the AB 4700 proteomics analyzer MALDI mass spectrometer. The SwissProt and NCBItr databases were searched globally, without filters, to identify peptide origins.

Histochemistry and Immunocytochemistry

Tissues were dissected, fixed and stained with affinity-purified chicken anti-Yuri antibody, Alexa Fluor 647-conjugated phalloidin (Invitrogen) and Hoechst 33342 (Invitrogen) as described previously [13]. Nuclei were also stained with DAPI, added at 1mg/ml to the mounting medium. Dilutions for additional primary antibodies were as follows: rat anti-myofilin [25], 1:100; guinea pig anti-Boca [26], 1:500; rat anti-insect Tropomyosin 1 (Mac 141, Babraham Technologies), 1:200. Appropriate secondary antibodies labeled with Alexa Fluor 488, 555, 594 or 647 (Invitrogen) were used at 1:500 dilution. Preparations were imaged on a Zeiss Axioplan 2 microscope and processed using Metamorph software (MDS Analytical Technologies).

Immunoblots and Co-immunoprecipitations

Adult thoraces and internal tissues of third instar larvae were boiled briefly in SDS-PAGE sample buffer, using 5 ml per animal. Immunoblots were prepared as described previously [13] and probed with Mac 141 antibody at 1:350 dilution. Bands reacting with Mac 141 were detected using a horse radish peroxidase (HRP)-linked rabbit anti-rat secondary antibody (1:80,000) and the HRP Luminata Classico reagent from Millipore. Magic Mark XP molecular weight (MW) markers (Invitrogen), which give a signal with HRP antibody detection systems, were run on all gels.

For immunoprecipitations, pupal extracts were prepared as described above, in the 25 mM NaCl lysis buffer plus protease inhibitors, as for the TAP-tag experiments. Extracts were pre-incubated for 30 min with protein A-agarose beads (Roche Diagnostics) that had been blocked with 1% bovine serum albumin in lysis buffer. After removal of the beads by

centrifugation, 2.5 µg of affinity purified anti-Yuri antibody [13] was added to the experimental aliquots (100 µl) and a similar amount of pre-immune antibodies from the same animal was added to the control aliquots. Samples were incubated with gentle rotation for 60 min at 4° C before rabbit anti-chicken IgG (2.5 µg) was added to all samples and the incubation continued for a further 30 min. A second aliquot of the pre-incubated Protein A-agarose beads was added to bind the immune complexes and after 30 min of incubation/rotation at 4° C, the beads were pelleted, washed three times with lysis buffer and finally boiled in SDS sample buffer to release bound proteins for SDS-PAGE and immunoblotting with Mac 141 antibody as described above.

RESULTS

Expression of TAP-tagged Yuri-65 under the *yuri*^{c263} GAL4 driver

In order to use a TAP-tagged version of Yuri-65 to capture authentic *in vivo* interaction partners, we needed to express the tagged construct in the natural expression pattern of the *yuri* gene. In the absence of promoter analysis for *yuri*, we took advantage of the GAL4 expression associated with the *yuri*^{c263} “enhancer trap” transposon. This transposon responds to subcomponents of the regulatory sequences associated with the *yuri* gene by generating GAL4 in a subset of tissues. We thus prepared a UAS construct [23] of the TAP-tagged Yuri-65 transgene which could be driven by *yuri*^{c263}-derived GAL4 expression (Figure 1). In 3-to-5-day-old adult males, GAL4 expression from the *yuri*^{c263} transposon is limited to a small set of mechanosensory neurons within the antenna [12] and similar neurons in the leg femoral chordotonal organ. Expression of the UAS-TAP-tagged Yuri construct in this limited number of cells would not yield sufficient material for biochemical analysis. However, using a UAS-EYFP reporter construct (see Materials and Methods) we investigated *yuri*^{c263} GAL4 expression at other points in the life cycle and identified a stage when it provides widespread expression.

The *yuri*^{c263} GAL4 driver is not active in embryos or first-instar larvae although Yuri protein is detected in these stages [13]. In third-instar larvae a limited number of tissues – the fat body, the visceral mesoderm of the midgut, a subset of cells in the ring gland, and the stellate cells of the Malpighian tubules, which regulate anion movement into the tubule lumen [27] – showed EYFP expression (Figure 2A). After pupation, widespread EYFP fluorescence was detected in developing adult tissues (Figure 2B-D). In late pupal life this global GAL4 expression abated such that only the mechanosense organs of the legs, wings and halteres expressed EYFP (Figure 2E, F) and by day 5 of adult life only the highly specific pattern detected previously remained. Thus essentially ubiquitous expression of a *UAS-TAP-yuri* construct with the *yuri*^{c263} driver could be achieved during the early/mid-pupal stages. Transgenic fly lines carrying a UAS-TAP-tagged construct of the Yuri-65 isoform were prepared, and a line that showed good expression of the tagged protein at mid-pupal stages with limited “leaky” expression in the absence of the *yuri*^{c263} driver (Figure 3) was chosen for the studies. Extracts were prepared from 0-to-3 day-old pupae.

TAP-tag purification and Mass Spec. identify Tropomyosin 1 as a major interaction partner for Yuri-65

The two affinity purification tags fused to the N-terminus of Yuri-65 (Figure 1) were used to purify the tagged protein and its bound interaction partners from extracts. After initial binding to an IgG-agarose bead column through the Protein A IgG binding domain tag (IBD), tobacco etch virus (TEV) protease [17,18] was used to cleave off the IBD and release the bound complexes from the column. The second tag, a calmodulin binding peptide (CBP), was then used for binding to calmodulin-agarose beads in the presence of calcium. Complexes were released by boiling in the presence of EGTA. After SDS-PAGE, stained

protein bands were excised from gels and sent to the PAN Facility at Stanford University Medical Center for trypsin digestion and peptide identification via Mass Spec. (see Material and Methods).

Experimental extracts were prepared from pupae expressing the tagged Yuri-65 under the control of the *yuri*^{c263} driver (*w*¹¹¹⁸; *UAS-TAP-yuri-65/yuri*^{c263}). Two control extracts were prepared: a) from pupae carrying the UAS construct alone (*w*¹¹¹⁸; *UAS-TAP-yuri-65/+*) and b) from pupae carrying just the *yuri*^{c263} driver (*w*¹¹¹⁸; *yuri*^{c263/+}). Given that the solubilities and interactions of proteins vary strongly with ionic strength, we prepared and analyzed extracts at three different total salt concentrations (50 mM, 175 mM, and 1 M) in initial experiments. Use of 1 M salt was unsuccessful; the tagged protein was largely aggregated in these extracts and no interacting proteins were isolated. At 175 mM salt, the overall gel profiles of control and the experimental extracts were very similar, suggesting non-specific contamination. Mass Spec. analysis revealed that muscle Myosin II heavy chain and muscle-specific actin 57B were major components of these extracts.

Muscle tissue represents a substantial fraction of the total mass of the organism. To explore interactions in the absence of solubilized muscle fibers, we focused on proteins present in the 50-mM salt extracts since muscle myosin is insoluble at low salt concentration [28]. This approach produced much cleaner control extracts. After subjection to the two rounds of affinity purification, the control 50-mM extract prepared from pupae expressing just the *yuri*^{c263} driver (*w*¹¹¹⁸; *yuri*^{c263/+}) contained no visible bands on SDS-PAGE except contaminant calmodulin released from the calmodulin-agarose beads by the final boiling step (Figure 4). For control pupae carrying the *UAS-TAP-yuri-65* construct alone (*w*¹¹¹⁸; *UAS-TAP-yuri-65/+*), the finally purified extract contained some bands after SDS-PAGE. These bands were weaker than, and not identical to, the bands in the experimental sample. These bands presumably reflect leaky expression of the *UAS-TAP-yuri-65* construct driven by an endogenous genomic regulatory element close to the insertion site of the UAS construct. Thus differences between the experimental sample and this leaky expression sample may reflect Yuri-65 expression in a different set of tissues than that produced by the *yuri*^{c263} driver.

Five sets of bands that were unique to the experimental samples were subjected to Mass Spec. analysis. Three were major single bands in the gels (Bands 1-3, Figure 4), and two were pairs of minor bands (Bands 4 and 5, Figure 4). Band 1, the strong band at ~200 kDa (Figure 4), proved to be a multimer of the 65-kDa Yuri isoform derived from the UAS construct. These multimeric complexes resistant to dissociation by SDS provide evidence that the coiled-coil regions of Yuri-65 function as oligomerization sites, as predicted.

The major band at ~35 kDa, Band 3, is tropomyosin (Tm). Although tropomyosin is best known for its role in muscle, in mammalian species numerous isoforms that are expressed in other tissues have been identified [4,8]. *Drosophila* contains two tropomyosin genes, *Tropomyosin 1* (also known as *Tropomyosin II*) and *Tropomyosin 2* (also known as *Tropomyosin I* and *Troponin H*). Tropomyosin 2 (*Tm2*) is the Tm of the contractile apparatus and encodes two isoforms of the conventional 284-residue type that differ in their 27 C-terminal residues as a result of alternative splicing [29,30]. One isoform is expressed in the tubular contractile muscles of the larval body wall whereas the other is specific to the adult indirect flight and leg muscles. Tropomyosin 1 (*Tm1*) encodes a third 284-residue Tm that is expressed ubiquitously in both muscle and non-muscle cells. This gene also produces two further Tm species (termed proteins 33 and 34) with long C-terminal extensions that are limited to the indirect flight muscles [31]. In addition, a shorter (252-residue) form of Tm (comparable to the 248-residue mammalian isoforms) is derived from the *Tm1* gene by use of a second downstream promoter. Exon maps for *Tm1/TmII* and *Tm2/TmI* can be found in

[32]. More recently, Flybase (<http://flybase.org>) has identified further minor products from both Tm genes.

From the various repetitions of the experiment, a total of 10 Tm peptides were identified from the 35-kDa protein band. Of these, eight derived from protein products of the *Tm1* gene, one was common to all three of the 284-residue isoforms produced by both Tm genes, and one derived from the *Tm2* gene. This single *Tm2*-derived peptide originated in the C-terminus of the isoform expressed in the larval body wall muscle. Each of the many isoforms from *Tm1* contains unique stretches of sequence in addition to regions shared with the conventional 284-residue isoform from this gene. However, all eight peptides from *Tm1* gene products derived from the conventional 284-residue Tm protein. Although this result does not exclude the possibility that Yuri interacts with other Tm isoforms, it indicates that, at the stage examined (early to mid-pupal life), the conventional 284-residue product of the *Tm1* gene is a major interaction partner of the protein. Thus these data implicate Yuri-65 in Tm-related cytoskeletal functions, rather than in muscle contraction.

The third major component present in the Yuri TAP-TAG complexes (Band 2, Figure 4) is a 45-kDa proteolytic fragment of fat body protein 1 (FBP1). FBP1 is a large transmembrane transporter protein, specific to the fat body, that functions in the reabsorption of larval serum proteins 1 and 2 (LSP1, LSP2) during the pupal stages [33]. LSP1 and 2 are major constituents of the larval hemolymph and are synthesized and secreted by the fat body. They are reabsorbed as a result of binding to FBP1, to serve as major nutrients for growth during pupal life. FBP1 is synthesized as a large precursor that undergoes cleavage to produce fragments of ~50 and 19 kDa several hours after synthesis [33]. The two pairs of minor bands at ~50 kDa and ~20 kDa (Bands 4 and 5; see Figure 4) contain peptides corresponding to these fragments of FBP1. That the major band for FBP1 found here has an apparent mass of 45 kDa (Band 2, Figure 4) indicates further proteolysis of the 50 kDa component, perhaps during extract preparation.

In addition to FBP1 fragments, the pair of minor bands at ~50 kDa (Band 4, Figure 4) also contained troponin T (TnT), a regulatory protein that in muscle binds directly to Tm and plays a role in positioning Tm on actin filaments. TnT is encoded by the single gene *upheld* in *Drosophila* [34]. Various TnT isoforms are produced [34], but in the repetitions of our Mass Spec. analysis, none of the peptides identified was unique to a particular isoform. In one experiment the minor band at ~50 kDa also contained muscle actin 57B, as identified in the preliminary experiments (see above).

Peptides from a further muscle protein, myofilin, encoded by *zeelin1*, were recovered from minor bands of the 20-kDa region (Band 5, Figure 4) in one repeat of the experiment. Myofilin is a component of the thick filaments and is thought to play a role in their assembly [25]. Three of the recovered peptides for Myofilin derived from an abundant ~36-kDa form of the protein that is expressed in many muscle types [25]. Myofilin is known to be sensitive to proteolysis [25], which could explain the presence of fragments of this isoform in the 20-kDa region.

Thus, two major interaction targets for Yuri-65 emerged from this analysis: Tm1 and FBP1. Given the clear linkage of Tm1 to F-actin filament stabilization and our previous findings linking Yuri to F-actin formation in spermatogenesis, we focused further study on the interaction of Tm1 and Yuri.

Co-immunoprecipitation confirms the interaction between Yuri and Tm1

As further evidence for an interaction between Tm1 and Yuri, we investigated coimmunoprecipitation of Tm1 with Yuri antibody. Pupal extracts identical to those used for

the TAP-tag experiments were subjected to immunoprecipitation using our affinity purified anti-Yuri antibody described previously [13]. As a control, extracts were subjected to immunoprecipitation with a comparable amount of pre-immune antibodies from the same animal. Immunoblots were probed with a monoclonal antibody (see Material and Methods) against insect tropomyosin [35,36] that we have shown by immunoblot and immunolocalization specifically recognizes Tm1 isoforms in *Drosophila* (see Figure 5 and below). As shown in Figure 5, Tm1 coimmunoprecipitates with the Yuri antibody and not with the non-specific pre-immune antibodies. Although these experiments cannot establish a direct link between the two proteins, they confirm that they are closely associated in a protein complex.

Yuri and Tm1 co-localize with ER and F-actin components in diverse tissues

Tm1 is an F-actin binding protein that, like Yuri, shows widespread expression, suggesting that the interaction we have detected between them could be present in many different cell types. We therefore examined Yuri and Tm1 localization in several tissues to determine whether the two proteins showed extensive colocalization. This work established not only that Yuri and Tm1 do colocalize in many situations but also that the sites of colocalization frequently correspond to the sites of ER membranes and F-actin accumulation.

For immunolocalization of Tm1, we have used a Tm1-GFP fusion protein from an exon trap line in which GFP is inserted into the 284-residue, 35-kDa, Tm1 isoform (see Material and Methods) and a monoclonal antibody (Mac 141) generated against insect tropomyosin (see Material and Methods). Previous studies with this antibody [35,36], and our own immunoblots (see Figure 5) have established that, of the various *Drosophila* Tm1 isoforms, Mac 141 detects only the 35-kDa isoform and the longer Tm1 derivatives (proteins 33 and 34 – see earlier) present in flight muscle (Figure 5). We further established that Mac 141 does not recognize the 35-kDa isoforms of Tm2 by comparing the Mac 141 immunolocalization pattern in muscle to that of a Tm2-GFP exon trap fusion protein (see Material and Methods) in that tissue. The Mac 141 staining pattern is identical to that of Tm1-GFP fusion protein (see below) and does not overlap any components of the Tm2 pattern. Given that the staining pattern for the Tm1-GFP fusion protein was faint and more labile than that of the Mac 141 antibody, we mainly used the Mac 141 antibody, for the Tm1 localization described here.

The localization patterns for Tm1-GFP, Mac 141, and Tm2-GFP in indirect flight muscle fibers are shown in Figure 6. A GFP-exon trap for Kettin [20] (a component of the muscle Z discs) (Figure 7) and immunolocalization of myofilin, a thick filament component, were used to orient/register/align the patterns relative to the sarcomere structure. As seen, the Tm2-GFP pattern consists of a strong band of localization along the F-actin thin filaments with a broad unstained region at the M line, the center of the thick filament arrays, and a narrow stain-free stripe at the Z disc. The common staining pattern of the Tm1-GFP and the Mac141 antibody (Figure 6A) contrasts markedly with that of Tm2-GFP (Figure 6B), and consists of a distinctive pattern of alternating strong and weak stripes along the myofibrils that showed no overlap with the Tm2 staining (Figure 6B). The stronger bands correspond to the Z lines (see Figure 7), with the broader, much weaker, bands representing M bands.

The Yuri staining pattern correlated strongly with that of Tm1, forming the same alternating strong/weak stripe pattern (see Figure 7). For both Yuri and Tm1, the relative intensities of accumulation at these two bands varied even within a single muscle fiber, suggesting a dynamic pattern perhaps dependent upon local fiber stretching. These findings established that Yuri and Tm1 colocalize to some structure in the sarcomere that is distinct from the thin/thick filament system.

The exit sites of the ER of the sarcomere are concentrated at the Z band with an extensive network of ER tubules spanning the M line [37], and thus we co-stained with markers for the ER. An antibody against Boca, a resident luminal protein of the ER [26], was used together with GFP-exon traps for Protein Disulfide Isomerase (PDI; another luminal protein) and Reticulin-like-1 (Rtnl-1; an ER membrane protein [22]), and with an artificial ER-EYFP marker [38]. Anti-Boca yielded the most reliable staining and was used for most of our studies. Boca localized to the Z disc and the M line exactly as Yuri and Tm1 did (Figure 7). In terms of intensity of staining at the two positions, in most preparations, Boca was more similar to Yuri, showing strongest localization at the Z band and weaker staining at the M Line. We conclude that Yuri and Tm1 localize in close proximity to the major exit sites of the muscle ER. Given that it is very difficult to keep the ER intact in histological preparations [38], we suspect that the variable relative intensity of staining at the Z and M lines reflects lability of the ER in fixed preparations.

Interestingly, in mammalian systems, Tm isoforms that function in muscle outside the contractile apparatus and in association with membrane systems have recently been identified [39]. One of these isoforms, Tm4, localizes to terminal sarcoplasmic reticulum elements and other tubulovesicular structures near the Z line. The data presented above indicate an ER association for Tm1, as do our findings below for other tissues. However, the localization of mammalian Tm4 could also suggest an association of Tm1 with the more specialized sarcoplasmic reticulum of muscle.

Possible co-localization of Yuri, Tm1, and elements of the ER was investigated in three other tissues – the third instar larval salivary glands and fat body, and the developing adult ovarian germ-line follicles. These tissues all contain very large polyploid cells, which would make any co-localization easier to detect. In fat body and salivary glands (tissues with major secretory roles), striking colocalization of Yuri, Tm1, and the ER marker Boca was detected (Figures 8, 9). In salivary gland cells, strong colocalization of these three elements at the plasma membrane (Figure 8) was always seen. That major elements of the ER can lie very close to the plasma membrane has been established in other systems [40,41]. In fat body, strong colocalization at the plasma membrane was also seen in some preparations (Figure 9) but was not always present. The fat body performs secretory functions early in the third larval instar but later in the instar, it is engaged in massive endocytosis of hemolymph proteins. It is possible that the variable colocalization at the plasma membrane reflects different functional states of the tissue. In the ovarian follicles, the germ-line-derived nurse cells show a distinctive layer of ER around their giant polyploid nuclei [42]. Boca localization to this ER layer was most pronounced in early follicles (stage 3-6 - see [43]). Yuri could also be readily be detected in the perinuclear layer of ER during these stages. Although Tm1 was present in these young follicles, it was detected in perinuclear ER (Figure 9) less reproducibly than Boca and Yuri, perhaps indicating tighter stage specificity of its function at this site.

Given that all known roles of Tm involve regulation of F-actin function, we also investigated the frequency with which these sites of Yuri and Tm1 colocalization correspond to sites of F-actin accumulation. In flight muscle, despite the presence of the F-actin thin filaments, phalloidin staining clearly revealed a concentration of F-actin at the Z bands, where Yuri, Boca and Tm1 colocalize (Figure 7). Similarly, in salivary glands, accumulations of F-actin at the plasma membrane that colocalize with Yuri and Tm1 were identified (Figure 8). Some phalloidin staining, indicative of F-actin filaments was found in ovarian follicles but not at the location of the perinuclear ER in the ovarian nurse cells. No phalloidin staining at all was detectable in fat body cells (data not shown). It has been reported previously [9] that the short actin filaments associated with the membranes of the Golgi apparatus cannot be detected by phalloidin staining or some actin antibodies,

suggesting that these negative findings for nurse cell nuclei and fat body cells may not preclude an association of actin with Tm1 and Yuri in these cells.

A Yuri-dependent role for Tm1 in F-actin cone formation

Tropomyosin is strongly implicated in regulation of F-actin stability and function in non-muscle cells [5,7,44]. Identification of Tm1 as a direct interaction partner of Yuri thus immediately suggested that Tm1 could mediate Yuri's role in the assembly of F-actin-containing structures during spermatogenesis. We therefore used the Tm1-GFP exon trap construct and the Mac141 Tm1 antibody described above to determine whether Tm1 could be detected in the dense complex and the F-actin cones and to investigate the effects of the *yuri*^{F64} mutation on any Tm1 localization identified.

Tm1 was not detected in the dense complex using either the Tm1-GFP exon trap or the Tm1 antibody. In contrast, Tm1 was detected in the actin cones in control testes (*yuri*^{F64}/*CyO* heterozygotes – Figure 10). Recently, the actin cones have been recognized as containing two compartments [45]. The broad base of the cones, which forms the leading edge in movement up the testis, consists of a meshwork of actin fibers. In contrast, the posterior region trailing to the cone “point” is formed from parallel bundles of actin filaments. Tm1-GFP mimicked phalloidin staining throughout the moving cones (Figure 10), suggesting interaction with actin filaments of both types. Formation of the cones around the condensing nuclei proceeds in two phases, with parallel bundles accreting along the long axis of the nuclei first, followed by formation of the meshwork at the apical tip of the nuclei where the basal bodies are positioned. Typically, phalloidin binding to the F-actin bundles around the nuclei at this stage is weak, with stronger binding developing as the meshwork is laid down. Staining for F-actin thus appears as flares fanning out from the nuclear tips (Figure 11A). On these developing cones growing around the condensed nuclei, Tm1-GFP colocalized exactly with the flares of F-actin forming there (Figure 11A). In the *yuri*^{F64} mutant, cone formation aborts as F-actin fails to accumulate on the spermatid nuclei. As shown in Figure 11B, we found that this loss of F-actin accretion is associated with a parallel failure of Tm1-GFP to accumulate on the nuclei.

In *yuri*^{F64} mutant animals, the defective accumulation of dense complex components, failure in basal body positioning, and failure of actin cone formation are the only cellular phenotypes we have detected. Given this absence of developmental effects in most tissues, it seemed unlikely that the *yuri*^{F64} mutation would result in alterations to Tm1 localization in other cell types. Nevertheless, we examined the distribution of Tm1 in flight muscle, fat body, and salivary glands derived from *yuri*^{F64} mutant males. No consistent differences in Tm1 localization in these tissues were detected.

DISCUSSION

Is Tm1 a direct interaction partner of Yuri-65?

In our TAP-tag experiments, the 35-kDa isoform of Tm1 remained associated with Yuri-65 through two rounds of affinity purification and represented the major protein bound to Yuri at the end of the purification scheme. In contrast, the related and highly abundant corresponding isoform of Tm2 was only a minor component of the proteins found in the final complexes. A strong, and specific, interaction between Yuri-65 and Tm1 is thus indicated. Tm1 is an F-actin binding protein, but non-muscle actin was never identified in the Yuri-65 complexes, and muscle actin was only a minor contaminant in a single experiment. The association of Yuri and Tm1 is thus not likely to involve an indirect interaction mediated by direct binding of Yuri to actin. If the interaction of Yuri-65 and Tm1 were indirect, the intermediate protein would have to be present at the same levels as Tm1

itself in the final complexes and would probably be present at a higher level, given the indirect nature of the Tm1 interaction. As shown in Figure 4, the only protein present at levels comparable to that of Tm1 is FBP1, a tissue-specific protein which could not account for the close co-localization of Tm1 with Yuri in tissues such as muscle. Based on these considerations, we conclude that the most reasonable interpretation of our data is that Tm1 is a direct interaction partner of Yuri-65.

Yuri-65 contains domains shared with both the 30-kDa and 100-kDa Yuri isoforms, and thus Tm1 could also be an interaction partner for these other Yuri variants. Like Tm1, the longer Yuri isoforms contain coiled-coil regions, raising the possibility that Yuri could form heterodimers with Tm, in addition to homodimers with itself, as shown in Figure 4. However, for Tm, self-dimerization is an essential component of its interaction with F-actin, and although Tm isoforms do heterodimerize [46], to our knowledge there is no evidence that Tm can form heterodimers with other proteins.

Yuri mediation of F-actin accumulation through Tm1

The results presented here suggest a mechanism whereby Yuri mediates the formation of the F-actin cones associated with spermatid individualization. We show for the first time that growth of the cones is accompanied by accumulation of Tm1, and that Tm1 persists throughout the cones as they move up the axonemes during individualization. In the *yuri*^{F64} mutant, in which the Yuri-30 and Yuri-65 isoforms are absent in the testis, both Tm1 and F-actin fail to accumulate at the normal sites of cone formation. Given the interaction detected here between Yuri-65 and Tm1, we propose that the role of Yuri in formation of the actin cones is mediated by its interaction with Tm1.

The actin cones consist of two different F-actin structures that have parallels with the actin structures seen in cultured cells. The “base” of the cones, which forms the leading edge during movement, contains a branched F-actin meshwork and requires Arp2/3 for formation [45]. This structure is comparable to the Arp2/3-rich meshwork of the lamellipodium beneath the leading edge plasma membrane in moving cells [7]. In contrast, the rear of each cone is composed of parallel actin bundles that contain actin-bundling proteins and require Profilin (*Drosophila* “Chickadee”) for formation [45]. The cone rear is thus similar to the actin bundles of the lamella in moving cultured cells. For the actin cones, it is the rear bundles, not the front meshwork that provides the force for movement [45], just as migration in cultured cells requires the actin bundles of the lamella rather than the treadmilling actin meshwork of the lamellipodium. Given that the actin bundles of the lamella contain Tm whereas the meshwork of the lamellipodium does not [7], we predicted that Tm would be found only in the rear regions of the actin cones. However, Tm proved to be localized throughout the actin cones. The presence of Tm in the cone meshwork could reflect the unusual properties of this region as compared to other Arp2/3-containing, actin structures such as the lamellipodium or the “comet tails” of *Listeria monocytogenes* [47]. In contrast to these structures [48,49], there is no actin treadmilling in the cone meshwork, and actin turnover is 10-fold slower [50]. The presence of Tm on actin filaments in the meshwork is consistent with these altered properties.

Although Tm1 was detected in the actin cones, we could not detect Tm1 in the dense complex using either the Tm1-GFP exon trap or the Tm1 antibody. This failure to could reflect technical complications. The dense complex is highly electron-dense in EM, suggesting a tightly packed structure. The insertion of the GFP into Tm1 may prevent its packing into such a structure. Similarly, tight packing may prevent access to the epitope recognized by the antibody. Alternatively, a different Tm1 isoform might play a role in dense complex formation. The Tm1-GFP exon trap used here inserts GFP into the 284-

residue isoform of Tm1 but not into several others. With the Mac 141 Tm1 antibody, we can only detect three isoforms of Tm1 (Figure 5).

Yuri and Tm1 action at the ER and plasma membrane

Our finding that the sites of association of Yuri with Tm1 and F-actin correspond to sites of ER membranes suggests that this interaction of Yuri with Tm1 has roles in membrane trafficking events and secretion. The fat body and salivary glands are highly active in secretion. Mammalian studies indicate that in muscle, the export sites at which proteins leave the ER in vesicles for transfer to the Golgi are found at the Z bands [37]. We have also detected Yuri on cytoplasmic vesicles (data not shown) in the stellate cells of the Malpighian tubules, which further suggests a role in membrane trafficking.

Tm-containing elements of the actin cytoskeleton are already linked to directed transport of secretory vesicles in yeast [44] and to budding of vesicles from the Golgi system in mammals [9]. In addition, Tm is known to interact directly with transmembrane proteins of the ER and the plasma membrane. A mammalian tropomyosin isoform binds to the cytoplasmic tail of polycystin-2, a channel protein component of the ER [51]. Vesicles containing the channel aquaporin-2 (AQP2) are transported to the plasma membrane via an interaction of AQP2 with the tropomyosin isoform TM5b [52]. How Yuri might contribute to such tropomyosin functions remains to be determined. However, the limited sequence similarity (~25%) of the Yuri coiled-coil regions to several golgins could suggest a role in ER tethering for the longer Yuri isoforms.

Tm1 and FBP1 are the major proteins identified here as co-purifying with TAP-tagged Yuri-65. Data from previous studies argue that the presence of FBP1 in the final Yuri complexes is not a non-specific artifact. Thus in previous work using TAP-tagging to purify interaction partners from pupal extracts [53], LSP1, an abundant secreted protein of the fat body and the ligand for FBP1, was a major contaminant of the final complexes. The absence of LSP1 in our experiments and the lower organismal levels of FBP1 relative to LSP1 indicate that FBP1 binds specifically to a component in the final complexes. The direct interactions of tropomyosin with transmembrane proteins in other systems (see above), opens the possibility that FBP1 may copurify with Yuri-65 as result of binding to Tm1.

Muscle proteins in the Yuri-65 complexes

Several proteins normally described as muscle-specific were found in the Yuri-65 complexes isolated in our experiments. Troponin T (TnT) was consistently present in the extracts, and peptides derived from Tm2, myofilin and muscle actin 57B were each identified in one experimental run. In muscle, TnT is the troponin (Tn) component that binds directly to tropomyosin on the actin thin filaments [54]. However, postulating TnT presence in the Yuri complexes via an indirect interaction with tropomyosin is hard to reconcile with the fact that it is Tm2, not Tm1, that is present in the contractile apparatus. Recent findings help explain this paradox. Sahota et al. [55] have shown that, like Tm1, the Tn complex and Tm2 have roles outside muscle and that the Tn complex and Tm1 and Tm2 isoforms all act together in early embryogenesis to maintain chromosome stability. Thus, i) direct interactions between TnT and Tm1 and ii) heterodimer formation between Tm1 and Tm2 isoforms in non-muscle tissues might explain the low levels of TnT and Tm2 found in our Yuri-65 complexes. For Myofilin, we confirmed its previously reported localization [25] in the thick/thin filament overlap region and observed its absence at the Z and M bands where Yuri is detected. Its staining pattern is thus the inverse of the Yuri pattern suggesting no real interaction between these two proteins in muscle. However, given the recent findings for the Tn complex, interactions of Myofilin and Yuri outside muscle cannot be excluded.

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Abbreviations

GFP	Green Fluorescent Protein
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
MW	molecular weight
HRP	horse radish peroxidase
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol tetraacetic acid

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Figure 1. Generation of a UAS-TAP-tagged construct for Yuri-65

An N-terminal fusion of the TAP-tag cassette (Protein A-TEV-CBP) to the Yuri-65 sequences in cDNA SD06513 was created by serial ligation of the two DNA sequences into multiple cloning sites (MCS) of transposon pP{UAST} [23]. Sites inactivated by ligations are shown with an asterisk. The double asterisk marks a XhoI site that is no longer unique in the final construct.

Figure 2. Expression patterns of the *yuri*^{c263}P{GawB} enhancer trap element

Tissue expression patterns of UAS-EYFP driven by the *yuri*^{c263}P{GawB} GAL4 enhancer trap, are shown. The stellate cells of the Malpighian tubules (A) are one of the few cell types that show expression during larval life. In early/mid pupal stages, EYFP is expressed throughout developing appendages such as the wing (B), haltere (B and C), and legs (C), and in all muscle tissue including the body wall musculature (D). By adulthood, expression is limited to the chordotonal organs such as those of the wing hinge (E, arrow) and legs (F, arrows). Bar, 40 μm.

Figure 3. TAP-tagged Yuri-65 expression from *P{UAS-TAP-yuri-65}*

An insertion showing high GALA-inducibility of tagged Yuri-65 expression was selected as described in Material and Methods. A comparison of the levels of tagged-Yuri-65 produced from animals carrying this insertion and the *yuri^{c363} P{GawB}* driver (right lane) with individuals carrying just the insertion (center lane), or the driver (left lane), is shown. An immunoblot for Yuri protein in total SDS-solubilized proteins from three whole midstage pupae for each line is presented.

Figure 4. Proteins associated with Yuri-65 *in vivo*

The proteins present in final extracts after affinity purification on IgG-then calmodulin-agarose are shown. Stained SDS-PAGE gel patterns for aliquots representing identical tissue mass for the three samples are shown. The single protein present in the driver alone extract (*c263-GAL4*) is calmodulin leached from the calmodulin-agarose column (square bracket). Bands present in the *UAS-TAP-yuri-65* lane show similarities to those in the experimental lane (*c263-GAL4 + UAS-TAP-yuri-65*) but are not identical and probably represent “leaky” tagged Yuri expression in a different tissue pattern (see text). Three major bands (1, 2 and 3) and two minor doublets (4 and 5) were subjected to Mass. Spec.

Figure 5. The 35 kDa Tm1 isoform specifically co-immunoprecipitates with Yuri from pupal extracts

A. The Mac 141 antibody specifically recognizes the ubiquitous 35-kDa isoform of Tm1 and the flight-muscle-specific Tm1 isoforms, proteins 33 and 34. Proteins present in adult thoraces or the internal tissues of third instar larvae were solubilized by boiling in SDS-PAGE sample buffer and after electrophoresis were probed with Mac 141 tropomyosin antibody. Lanes 1, 3 and 4 = proteins from 0.02, 0.004 and 0.002 adult thoraces respectively; lane 2 = MW markers (in kDa); lanes 5, 6 = proteins from internal tissues of 0.1 and 0.02 third instar larvae, respectively. The two bands at ~ 80 kDa (arrows) in thoracic samples are proteins 33 and 34, flight muscle specific isoforms of Tm1. The band at ~35 kDa in both larval and thoracic samples is the 284 residue conventional isoform of Tm1.

B. Yuri antibody co-immunoprecipitates the 35 kDa form of Tm1. Pupal extracts prepared as for the TAP-tag experiments were treated with affinity purified Yuri antibody or pre-immune antibodies from the same animal. Immune complexes were captured on protein-A beads and subjected to SDS-PAGE followed by immunoblotting with Mac 141 (see Material and Methods). Lane 1a, 1b = initial pupal extract at two exposures to show Tm1 doublet in initial extract; lane 2a, 2b = MW markers at two exposures; lane 3 = immune complexes from Yuri immunoprecipitation; lane 4 = immune complexes from control immunoprecipitation. The doublet of Tm1 immunoprecipitated by the Yuri antibody probably reflects mild proteolysis in the pupal extract, given the single Tm1 band detected in samples immediately solubilized in SDS-PAGE sample buffer (see A).

Figure 6. Muscle immunolocalizations identify a distinctive distribution for Tm1 in the sarcomere and establish the specificity of the Mac141 antibody for Tm1 isoforms
 Images show individual muscle fiber teased from indirect flight muscle. **A.** The Tm1-GFP fusion protein and Mac 141 antibody show identical localization patterns. Additional experiments established that the strong bands of stain are at the Z band (see Figure 7). **B.** The staining pattern for Mac 141 (Tm1-Ab) does not overlap Tm2 distribution in the sarcomeres as visualized by a Tm2-GFP fusion protein. Z - Z band; M - M band. Bar, 5 μ m.

Figure 7. Yuri localizes with Tm1, ER markers, and F-actin at the Z band in muscle sarcomeres
 All images show indirect flight muscle fibers. **A.** Co-staining with anti-Yuri and anti-Tm1 antibodies reveals co-localization at both the Z band and the Mline. The stronger bands for Yuri correspond to the Z line (see **D.**) and the stronger bands for Tm1 correspond to the M line. **B.** Co-staining for Yuri and Boca, an ER resident protein, shows co-localization at the Z band (stronger bands) and the M line. **C.** Co-localization of Boca and Tm1. **D.** Co-staining for Yuri and GFP in a fly line carrying a GFP-exon trap into Kettin, which localizes to the Z band. **E.** Co-staining of Yuri and Phalloidin, to detect F-actin. **A.-C.** and **E.** – fly line *ew¹¹¹⁸*; **D.** – Fly Trapline ZCL214; Bars, 5 μ m.

Figure 8. Yuri localizes with Tm1, an ER marker, and F-actin in larval salivary gland cells A-A''. Co-staining with Yuri, Boca and Tm1 antibodies shows strong colocalization at the cell membrane and as a network throughout the cells. **B-B''.** Co-staining with Yuri and Tm1 antibodies and phalloidin reveals strong co-localization of F-actin with Yuri and Tm1. Bars, 150 μ m.

Figure 9. Yuri localizes with Tm1 and an ER marker in fat body cells and at the periphery of nurse cell nuclei

A-A''. Colocalization of Yuri, Boca and Tm1 in fat body cells. Staining for each protein in the same field of fat body cells is shown. Black arrows point to plasma membranes. Dark holes in the cells represent the original sites of fat globules. **B., C. and D.** Colocalization of Yuri (**B**) Boca (**C**) and Tm1(**D**) at the periphery of the giant poly ploid nurse cell nuclei in young ovarian follicles. Blackarrows in **C.** and **D.** point to Boca and Tm1 accumulation around the nuclei in the somatic follicle cells that surround the germ-line nurse cells in these follicles. Bars , A-A'' 30 μ m; B-D 20 μ m.

Figure 10. Tm1 is present throughout the F-actin cones of spermatid individualization

A set of mature F-actin cones in the process of moving up a spermatid cyst is shown in a control testis, heterozygous for the *yuri*^{F64} mutation and the Tm1-GFP exon trap CC02057. Movement is toward the upper left corner with the flat bases of the cones as their leading edges. **Left panel** F-actin as visualized by phalloidin staining; **center panel** - GFP fluorescence of Tm1-GFP; **right panel** - overlay. Note that GFP fluorescence is throughout the entire length of the cones and also shows low levels of accumulation both behind and in front of the cones. Bar, 20 μ m.

Figure 11. Tm1 does not accumulate on the spermatid nuclei in the *yuri*^{F64} mutant
 (A) Left panel shows F-actin cones (phalloidin staining) forming on sets of condensing spermatid nuclei (Hoechst stain, *e.g.*, at black arrow – see right panel) at the base of a control testis. After cone movement initiates, nuclei stripped of F-actin condense further (white arrow). Tm1-GFP fluorescence (center panel) exactly overlaps F-actin staining on the nuclei (see overlays in lower panels). Strong Tm1-GFP fluorescence is also seen in coiling spermatid bundles (black star) where there is little overlap with F-actin. (B) shows equivalent views of a similar region of a *yuri*^{F64} mutant testis. Loss of Yuri-65 and -30 results in failure of the condensing spermatid nuclei (black arrows) to remain in orderly “chevron” configuration and failure of both F-actin and Tm1-GFP to accumulate on the nuclei. Bar, 40 μ m.