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Characterization of the gravitaxis-related protein Yuri Gagarin through genetic and biochemical approaches

by

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ABSTRACT

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The *Drosophila melanogaster* gene *yuri gagarin* was first identified in a screen for aberrant behavioral responses to the mechanosensory stimulus provided by the force of gravity. This novel gene encodes Yuri isoforms of four sizes; the three larger isoforms are predicted to be composed largely of coil-forming domains, which are common protein interaction domains. The four isoforms are expressed in varying ratios throughout the animal, at all stages of development. Notably, Yuri is present in muscle tissue, localized to Actin-containing regions of the sarcomere.

A male-sterile deletion allele of *yuri*, *yuri<sup>F64</sup>*, was identified and sequenced. This deletion removes roughly 500 base pairs of sequence upstream of the Yuri coding region; in these animals, the mid-sized Yuri isoforms are not expressed in any tissue, and the smallest isoform is no longer expressed in the testis. In wild-type animals, Yuri protein is present throughout the testis, and it assumes a dynamic microtubule-dependent nuclear localization pattern during the process of nuclear condensation, appearing to be a component of the “dense complex” described in ultrastructural studies of spermatogenesis. A previously undescribed filamentous Actin network co-localizes with
Yuri on the nuclear surface; Yuri may act as a linker between the Actin and microtubule cytoskeletons.

In $yuri^{F64}$ homozygotes, no Yuri-staining or filamentous Actin structures are detected on the condensing sperm nuclei, and the basal body and centriolar adjunct are mis-localized or absent. Axonemal defects are apparent in heterozygotes and homozygotes, and the Actin-dependent sperm individualization does not take place, leaving immature sperm bound in bundles of 64.

Yuri physically interacts with components of the Actin cytoskeleton (Actin, Tropomyosin 1, and Troponin T) in an *in vivo* pull-down assay. Tropomyosin 1 (Tm1) is the interacting protein purified in greatest abundance in the assay, suggesting that it and Yuri interact directly; this coil-forming molecule wraps actin filaments, stabilizes them against enzymatic or physical degradation, and blocks the interaction of the filament with other proteins. Tm1 is present on the condensing sperm nucleus, and Yuri co-localizes with it in muscle tissue. Thus, the defects seen in $yuri^{F64}$ animals may reflect aberrant Actin dynamics arising from a lack of Yuri-Tm1 interaction.
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CHAPTER 1: BACKGROUND

Gravity has been ubiquitous throughout the development of life, and most multicellular animals possess a mechanism to detect its direction to aid in movement and balance. Most examined phyla of animals more complex than sponges, with the notable exception of most terrestrial arthropods, possess sensory structures thought to be specifically attuned to detection of the gravity vector (reviewed in Beckingham et al., 2005). The prototypical gravity-detection organ is called the statocyst (Figure 1-1). This structure comprises a fluid-filled cavity lined with ciliated mechanosensory neurons; a dense substance within the statocyst lumen is accelerated by gravity and deflects the neuronal cilia, activating them and providing a perception of "down."

The variations on this theme within the animal kingdom are many and diverse. The sensory cilia may be exposed within the statocyst and in direct contact with the stimulating material, as in vertebrates, or they may be enclosed within a cuticular hair or bristle, as in some crustaceans. In some organisms, including mollusks, lobsters, and fish, the dense substance within the organ takes the form of discrete particles ("statoliths") that are free to move about within the statocyst lumen and settle to the subjective "bottom"; in other systems, such as crustaceans, the dense material is attached to the sensory structures. In vertebrates, the neuronal cilia of the vestibular system are embedded in a gelatinous matrix, which is not free to move about but is instead deformed under the influence of accelerational forces, gravitational or otherwise.

Statocyst-type organs are located at many anatomical positions, varying between and within animal phyla. In vertebrates, of course, the statocyst organs are located in the head. In jellyfish, these structures are situated around the rim of the "bell" (body), and in
FIGURE 1-1: The prototypical statocyst. A fluid-filled chamber lined with ciliated mechanosensory neurons contains dense particles or material; these fall to the position within the statocyst lumen that is lowest at any given time and stimulate the neurons located there, thereby providing orientation information to drive righting reflexes and other behaviors.
bivalves, they are within the muscular "foot." Within the arthropods, different subgroups have different statocyst arrangements. In most decapod crustaceans, e.g., lobsters, crayfish, and crabs, the statocysts are located at the base of each antenna and are used for orientation (Hensen, 1863; Kreidl, 1893; Schöne, 1967; Schöne and Steinbrecht, 1968); in other decapods, such as mysis shrimp, the organs are located at the opposite end of the animal on the uropod endopodites (inner branches of modified abdominal biramous appendages) (Delage, 1887; Espeel, 1985; Neil, 1975), and in at least some marine isopods (the group that also includes terrestrial pill-bugs or "roly-polies"), they are located on the telson (the "tail") (Rose and Stokes, 1981).

Nest-building wasps and hornets have statocyst-like organs, although they are thought to be used only during nest construction in dark enclosed spaces (Ishay et al., 2007; Ishay et al., 1983). However, most insects lack statocyst-type organs altogether, perhaps in part because they cannot rely on capturing exogenous statolith particles during molting, as marine arthropods can, or because of evolutionary constraints on their sense-organ development programs; therefore, most insects must rely on input from other types of sense organs to perceive gravitational information. Fields of mechanosensory bristles between body or appendage segments have been described and associated with proprioception or gravity sensation in ants (Markl, 1962a), bees (Markl, 1962b; Markl, 1963; Markl, 1965; Thurm, 1964), locusts (Pflüger et al., 1981), roaches (Pringle, 1938), and stick insects (Wendler, 1964). Feedback from body-weight strain in the leg muscles is also a component of gravity perception, at least in stick insects (Wendler, 1971).

Drosophila possess a multitude of mechanosensory organs of three general types which perceive different modes of physical stimuli, any combination of which could play
a role in gravitational perception. These structures are classified according to the presence or absence of neuronal cilia and associated non-neuronal support cells into "type I" and "type II" mechanosensors, respectively. Type-II mechanosensors are highly branched non-ciliated neurons that innervate patches of cuticle and are thought to subserve the touch sense by detecting indentation of the cuticle; these cells are less closely related to the systems described above and will not be discussed further, although they may play a role in gravity sensation.

Type-I mechanosensory organs are further subdivided into external sensory organs ("ESOs") and internal chordotonal organs ("ChOs"), depicted in Figure 1-2. Each functional unit of these structures is called a sensillum (or, in the case of ChOs, a scolopidium) and comprises a ciliated neuron and several associated support cells, all of which are derived from a single sense-organ precursor cell. Despite the functional and structural differences between these sensory structure types, they develop through similar mechanisms and it is thought that they are evolutionary elaborations of a single ancestral sense organ type (reviewed in Lai and Orgogozo, 2004).

ESOs can be purely mechanosensory, or they can perform chemosensory functions as well. Mechanosensory ESOs comprise four cells — a ciliated neuron, a sheath cell around the neuron, a cuticular bristle cell, and a cuticular socket cell; the deflection of the bristle by wind or touch is thought to compress the neuronal cilium, opening ion channels and leading to neuronal depolarization. ESOs are found in isolation and in fields of closely spaced bristles called hair plates, present between the head and body and at some cuticular joints, where they are thought to perform proprioceptive functions.
FIGURE 1-2: The Drosophila type-I mechanosensory organs. A: A typical external sense organ. These mechanosense organs sense deflection of the external bristle, caused by touch or wind. It is thought that the cuticular bristle acts as a lever to compress the ciliary membrane against the tubular bundle at the tip of the cilium, opening ion channels and leading to membrane depolarization (Thurm, 1964). B: A typical chordotonal organ sensillum. The ends of the organ are attached to regions of cuticle that can move relative to one another, such as at appendage joints. The physical stretching of the neuronal cilium, thought to be modulated by the rigid Actin scolopale rods and the structure of the axoneme, leads to the opening of physically gated ion channels. Labels in both panels: bb, basal body; ci, cilium; cd, ciliary dilation; cr, ciliary rootlet; cu, cuticle; ne, neuron; sc, scolopale rods; tb, tubular bundle. Darker ovals represent nuclei. (Adapted from Eberl et al., 2000.)
The other type-I mechanosensory structures, the chordotonal organs, are internal stretch-responsive organs. Chordotonal sensilla are attached between cuticular segments that can move relative to one another, such as the cuticle of neighboring segments of the antenna or body (Moulins, 1976; Shanbhag et al., 1992), or between the cuticle and associated muscle membranes. One of the accessory cells, the so-called scolopale cell (the chordotonal homolog of the ESO bristle cell), surrounds the neuronal cilium and contains rigid Actin- and microtubule-rich structures, called “scolopale rods” (Uga and Kuwabara, 1965; Wolfrum, 1997); the rigidity of these rods and Actin dynamics within them, combined with motility of the axoneme itself (Crouau, 1980; Crouau, 1982; Crouau, 1997; Moran et al., 1977), are thought to maintain a slight tension across the neuronal cilium, and this tension is thought to be important for its stretch-receptive function (Todi et al., 2004).

Clustered and single ChOs are present in each larval segment, where they sense the peristaltic contraction of the animal and provide locomotory feedback. Clusters of ChOs are also present in several locations in the adult (Figure 1-3); the two largest are the femoral ChO and Johnston’s organ of the antennae. The femoral chordotonal organ is located within the first segment of each leg and senses the position of the appendage relative to the body; it is estimated to comprise 71-74 sensilla (Shanbhag et al., 1992). Johnston’s organ (“JO”), a cluster of ~227 (Kamikouchi et al., 2006) chordotonal sensilla filling the second segment of each antenna (“A2”), is the Drosophila analog of the ear (Eberl et al., 2000). The third antennal segment (“A3”) bears a hook-like cuticular projection that is anchored within a socket formed by the second segment; JO chordotonal sensilla line this socket and are attached apically to the hook (Figure 1-4). In
FIGURE 1-3: Locations of major chordotonal organs in Drosophila adults. ChOs are present at one or more locations in every adult appendage, as well as at certain body-segmental junctions. Stretch receptors at any joint that can be bent by the weight of a body part may be involved in a proprioceptive gravity sense, depending on how the proprioceptive input is interpreted. As described above, JO in particular may play a key role in gravity sensation (in addition to its role in hearing) by detecting the plumb angle of the dead-weight third antennal segment. In addition to these ChOs, fields of ESOs are present at several locations at which they could transduce proprioceptive information, such as between the head and thorax, and near certain leg joints. Background image from insects.eugenes.org.
FIGURE 1-4: A cross section through Johnston’s organ. This compound chordotonal organ comprises ~230 sensilla, distributed around the joint between the second and third antennal segments (A2 and A3), connected basally to the cuticle of A2 and apically to the A3 cuticular projection. Oscillation of A3 induced by air movement alternately stretches and relaxes sets of sensilla and allows the insect to detect certain types of near-field vibrations. It has been hypothesized that JO also participates in gravity detection, wherein the overall position, not the movement, of A3 (functioning as a dead weight) is interpreted to yield gravity-vector information. If this is the case, it may also be true that only certain JO sensilla, or only certain neurons within these sensilla, subserve this specialized sense; see main text for discussion. (Image adapted from Caldwell and Eberl, 2002.)
this arrangement, vibration-induced oscillation of A3 will alternately stretch and relax these sensilla, leading to a perception of certain types of sound.

It has also been hypothesized that JO plays a large role in the detection of the gravity vector. In the proposed model, certain JO sensilla, or certain specialized neurons within some sensilla, are adapted to detect the overall position of the hanging dead weight of A3, rather than its oscillation; this idea is consistent with the discovery that whereas most JO sensilla contain two neurons, a subset of them (in an undescribed distribution within JO) contain three (Todi et al., 2004). In addition, Kamikouchi et al. (2006) have mapped the entire set of JO neurons and their pattern of projection to the brain; they found that neurons from sensilla in different regions of JO innervate disparate locations in the central nervous system, suggesting that subsets of JO neurons are functionally distinct. They also proposed that certain JO sensilla are positioned within the A2 “socket” in such a way that their most efficient stimulus is likely to be the angle of A3 relative to A2, rather than its oscillation about the A2-A3 axis.

The statocyst-like gravity-detection organs of various phyla likely arose independently, judging from their radical diversity; however, much of their apparatus is present in non-statocyst-like organs as well, and these simpler subunits appear to be evolutionarily related in many aspects of their development and function. Since ciliated neurons are central to a large class of mechanosensory structures (except the type-II mechanosensors in Drosophila, for example), the general neurogenesis and ciliogenesis pathways involved in mechanosensory function are highly conserved. In addition, many factors involved in the specification, development, and function of mechanosensory structures in particular are highly conserved: for example, the Drosophila neural-fate-
specifying gene *atonal* and its mouse homolog *Math1*, and the Drosophila homeotic gene *spalt* and its human homolog *SALL1*, are required for the specification and development of hearing structures in both species, even though the hearing structures themselves are highly divergent; Myosin-VII motors are required for proper hearing-organ morphology in both flies and mammals, the morphological differences between them notwithstanding; and the Drosophila mechanosensitive ion-channel protein Inactive shares 55% and 45% sequence similarity with its closest sea-urchin and human homologs, respectively (Bermingham et al., 1999; Boekhoff-Falk, 2005; Caldwell and Eberl, 2002; Dong et al., 2003; Duggan et al., 2000; Kavlie et al., 2007; O'Neil and Heller, 2005; Todi et al., 2005; Wang et al., 2002, and BLAST data not shown).

Screens for touch-insensitive and uncoordinated (Kernan et al., 1994) or deaf (Eberl et al., 1997) Drosophila mutants have led to the identification of several conserved genes that are necessary for the development or function of Drosophila mechanosensory organs. However, before the work described below, no previous screen had specifically addressed gravity perception in Drosophila at a genetic level. We undertook a behavioral screen to identify mutant lines exhibiting aberrant behavioral responses to gravity in a defined assay. We sought answers to the following questions: What genes are involved in the development and function of gravity-sensing structures (and the neural systems that process the output of those structures), and how exactly do those genes contribute to gravity perception? Which class of mechanoreceptors is most important for the transduction of gravitational information in Drosophila, and are any particular organs more important than others? These questions are addressed in the chapters below.
CHAPTER 2: THE GRAVITAXIS SCREEN

2-1: The maze apparatus

The Drosophila behavioral response to gravity, namely directed movement with respect to the gravity vector, is commonly known as "geotaxis" (cf. "phototaxis" and "chemotaxis"). We in the Beckingham lab take a Copernican position and use the more precise term "gravitaxis" (since the animals’ attraction is to gravity, which happens to inhere in the Earth in this case, rather than to the Earth per se – one would imagine that "geotaxis" and "gravitaxis" would become uncoupled in our planned but canceled behavioral experiments on the International Space Station). Measurements of Drosophila gravitaxis have been performed by many groups using a "gravitaxis maze" apparatus. This assay device comprises a network of plastic tubing of an inner diameter just large enough for flies to walk through unhindered, but not large enough for them to fly. Groups of flies are introduced into the maze through a single entrance port, and, drawn by food odors and a light cue, they proceed through the maze, making a series of up-or-down choices along the way, until they emerge into one of nine collection tubes (Figure 2-1). (Inside the maze, a fly may wander forwards or backwards, circle, pause, fall, and so on; once a fly enters an exit tube, however, it becomes trapped so that it can be counted.) The distribution of flies among these exit tubes reflects the cumulative behavior of the flies within the maze.

One can show that this apparatus does in fact measure gravity-related behavior. The exit distribution that should result from a series of eight random choices (with no "backtracking" or other non-forward motion in the maze) is a binomial curve centered on the central exit, with 70 times as many flies exiting at this point as through either outer-
FIGURE 2-1: The gravitaxis maze. The entry point is at the left; the nine exit tubes, numbered 1-9 (bottom to top) are off the right side of the image. A fluorescent bar lamp is positioned beyond the exit tubes, providing a phototactic cue to draw the animals through the maze. The intensity of the light cue across the height of the maze was found to be uniform (not shown), thus avoiding any light-based skew in the exit distribution. Several possible paths through the maze are shown, reflecting a fly's range of wayfinding possibilities.
most exit (Figure 2-2, A). When control flies are run through mazes laid horizontally, which requires them animals to make left-\textit{vs}-right choices rather than up-\textit{vs}-down choices, the distribution across the exits has a shape broadly similar to the binomial curve, peaking at the central exit tube (Figure 2-2, B), indicating that the flies display no behavioral preference for one direction over the other. When the maze is set vertically, however, so that the two directional options presented at each choice point differ in their relation to the gravity vector, the distribution is radically different: it is skewed towards the upper exits, indicating firstly that the device can measure a behavioral response to gravity, and secondly that control flies display a tendency to choose the “up” option at vertical choice points, which is consistent with the generally observed climbing behavior of these organisms.

\textbf{2-2: Previous studies of Drosophila gravitaxis}

The first reported genetic studies of Drosophila gravitaxis were conducted by J. Hirsch (1959). He began his studies with a wild population of flies, which were run through a vertical maze apparatus to identify the most positively and negatively gravitaxic among them. Animals exhibiting each extreme of behavior were mated to produce offspring for the next iteration of the experiment; performing this selection over many generations, he generated a “high” line, JH1, whose maze distribution was skewed more dramatically towards the upper maze exits than that of wild-types (Ricker and Hirsch, 1985), and a positively gravitaxic “low” line named JHL (see Figure 2-3 for maze profiles of these lines in our apparatus). He and coworkers mapped a genetic factor correlated with positive gravitaxis (downwards movement) to the “\textit{X}” (first) chromosome
FIGURE 2-2: Horizontal maze exit distributions. A: The expected exit distribution for a maze in which flies make directional choices randomly, and can only move forwards within the device. B: A typical exit distribution from a horizontal maze run, in which flies make left vs. right decisions and in which gravity therefore plays no role in directional choice. Because of the design of the maze, animals may also move backwards along a path different from their immediately preceding forward path; numerical simulations show that this factor contributes to, but does not explain completely, the flattening and broadening of the distribution (not shown). (N=129 animals.)
FIGURE 2-3: Vertical maze distributions for Hirsch lines. A: Control flies (cn bw) exit the maze in a bimodal distribution, with an overall skew towards higher exits in the maze – assuming that forwards, upright walking and choosing are the only behaviors exhibited in the maze, the animals choose the upper path at ~62% of choice points. (N=495.) B: The Hirsch JH1 “high” line distribution exhibits a much greater skew towards the top exits, reflecting the larger proportion (~77%) of “up” choices made by these animals. (N=115.) C: The Hirsch JHL “low” line produces a dramatically different distribution, one weighted towards the bottom exits, indicating that these animals rarely (at ~15% of opportunities) make “up” choices in the assay. (N=266.)
of his JHL line (Ricker and Hirsch, 1988), and his laboratory was able to map a genetic factor partially responsible for the exaggerated negative gravitaxis behavior (strong preference for upwards movement) of the JH1 "high" line to somewhere in the vicinity of the Alcohol dehydrogenase (Adh) gene on the second chromosome (Stoltenberg and Hirsch, 1996).

Differences in gene expression between the JH1 and JHL lines have been examined in microarray experiments (Toma et al., 2002). Presumably, many of the identified expression-level differences are not causative of the behavioral differences between the lines but rather reflect downstream effects of the actual causative factors, as well as genetic drift and "bottlenecking" during the generation of the two lines; however, independent mutations in a few of the genes identified in the microarray data were shown to cause behavioral differences in the gravitaxis maze (ibid.).

2-3: A genetic screen for gravitaxis mutants

In an effort to identify genes involved in gravitaxis behavior, we conducted a behavioral mutant screen, led by Dr. J. D. Armstrong (Armstrong et al., 2006). He provided a collection of ~400 $P(GawB)$ (Figure 2-4) enhancer-trap transposon-insertion mutants that had been pre-screened for GAL4 reporter activity in the nervous system (see Chapter 4) (Brand and Perrimon, 1993; Gustafson and Boulianne, 1996; Yang et al., 1995); these lines, as well as ~1,000 lines from the Zuker point-mutant collection (Koundakjian et al., 2004), were screened for aberrant behavior in the gravitaxis maze by Armstrong and O. Bachilo, S. Baxley, E. Carter, D. Kesselman, E. Kuo, C. Nadorff, and J. Siqueira.
FIGURE 2-4: The P(\textit{GawB}) element. P-element transposons are mobile genetic elements comprising inverted-repeat "P ends" and intervening transferable sequences. Natural P elements (~3 kb in length) contain only the gene encoding P transposase, which recognizes the inverted-repeat ends and catalyzes element release and insertion of the element into other DNA sites; in artificial elements this gene is replaced with various marker genes and other sequences of interest (Rubin and Spradling, 1983; Spradling and Rubin, 1982). P(\textit{GawB}) is a ~11-kb base-pair P derivative that was designed for enhancer-trap studies (Chapter 4) and ease of cloning of insertion sites. It contains the yeast transcriptional activating factor gene \textit{GAL4} and the marker gene \textit{white}, which confers red eyes on transgenic animals, as well as a bacterial origin of replication and an ampicillin-resistance gene for use in mapping (Brand and Perrimon, 1993).
Mutant lines were pre-screened using a “bang” test that tests the locomotion and coordination of mutant animals by banging them to the bottom of an empty plastic vial and observing the startle and climbing responses (Guan et al., 2000; Nelson et al., 1997); mutant lines with grossly aberrant performance in this assay, indicating general neurological or physiological defects, were not analyzed further. Each mutant line that passed the bang test was tested in the maze apparatus in at least four “maze runs” of ~25 males between three and five days old. Each batch was run in a separate maze in a temperature- and humidity-controlled room; after three hours (a time determined empirically to be long enough for normal flies to complete the maze), the number of flies in each exit tube was recorded and entered into an online database system written by Armstrong.

Only lines in which at least 80% of the flies finished the maze in three hours, indicating proper phototaxis, locomotion, coordination, and general fitness, were considered further. Additionally, each line chosen for further analysis was post-screened for deficits in several control behavioral tests: a “flight test” in which flies are dropped into a sticky cylinder, and the distance they fall before they fly into the cylinder wall is measured (based on Benzer, 1973); a horizontal maze test, to measure their mobility, phototaxis, and other behaviors required for progress through the maze; and a courtship test, which measures coordination, vision, olfaction, and hearing (based on O’Dell, 2003). Using these criteria, we selected 23 $P\{GawB\}$ insertion lines with aberrant behavior for further study; the maze exit distributions for these lines is shown in Figure 2-5, and their control-assay results are shown in Figure 2-6. (None of the point-mutant lines have been studied in detail.)
FIGURE 2-5: Vertical-maze exit distributions of the lines identified in the screen. The lines are ordered by mean maze exit value. Mutant lines range from those with positive gravitaxis to lines with exaggerated negative gravitaxis. Mutant c263 insertion-line flies (boxed in red) make “up” choices at roughly 70% of opportunities. (Adapted from Armstrong et al., 2006.)
FIGURE 2-6: Control behaviors of lines identified in the gravitaxis screen. Flight index: The speed with which a fly dropped into a cylinder rights itself and begins to fly is measured by the vertical position on the cylinder wall at which it lands; higher is faster. Courtship index: The percentage of time that a mutant male courts a wild-type female. MMEV (H): The “mean maze exit value” at which flies exit a horizontally oriented maze device; exit “S” is at the middle of the maze, where distributions should peak if random choices are made within the maze. In all panels, the scores of control animals (“CS”; Canton-S wild-types) are shown as dotted intervals. Only lines with wild-type scores in at least two of these tests were retained. (Adapted from Armstrong et al., 2006.)
2-4: Cloning of transposon insertion sites

To identify the gene(s) affected in each mutant line, I used three techniques to map precisely the insertion point of the $P\{GawB\}$ transposon: plasmid rescue (Ashburner, 1989), inverse polymerase chain reaction (PCR), and “thermally asymmetric interleaved PCR” (“TAIL PCR”) (Liu et al., 1995). These three techniques take advantage of various features of the $P\{GawB\}$ transposon to allow molecular mapping of insertion sites that is far faster and easier than the mapping of a point mutation.

The plasmid-rescue technique (Figure 2-7) makes use of the bacterial origin of replication (ori) and antibiotic-resistance marker ($amp^R$, encoding beta-lactamase) located at the 3' end of the $P\{GawB\}$ element. These sequence features have no function in Drosophila, but they are very useful for mapping. DNA from each mutant line is digested with one of several selected restriction enzymes that cut the genomically integrated P-element DNA upstream of, but not within or downstream of, the two bacterial sequences. The enzyme will also cut the genomic DNA at thousands of sites, and the cut site downstream of and closest to the 3' end of the transposon will release a fragment comprising the bacterial ori and $amp^R$ sequences as well as transposon-flanking genomic DNA. The myriad linear genomic fragments are circularized by ligation and transformed into competent Escherichia coli. Only the fragment spanning the 3' transposon/genome boundary will contain the ori and $amp^R$ sequences, and therefore only this fragment will be replicated in the bacteria that take them up, conferring on them a resistance to ampicillin.
FIGURE 2-7: The plasmid-rescue technique for mapping of P\{GawB\} insertion sites. Genomic DNA is isolated from insertion lines, digested, and circularized; the fragments containing the P\{GawB\} ori and amp\(^R\) genes (and transposon-flanking Drosophila genome) are selected by transformation into bacteria, isolated, and sequenced.
Each resistance-conferring plasmid was sequenced from a primer ("PR3") located within P-element sequences near the 3' end of the transposon; the resulting sequence data thus represented a short stretch of known P-element DNA, which ends with the marker sequence "CATCATG," along with the genomic transposon-flanking DNA. This flanking genomic DNA was mapped to the sequenced Drosophila genome using BLAST tools located on the FlyBase system (www.flybase.net/blast), allowing the base-wise-precise localization of the inserted P-element within the genome.

This method worked well for roughly half of the mutant lines; in other cases, I was unable to propagate any rescued plasmid, perhaps because the distance to the closest downstream restriction site was too great, resulting in difficulty in plasmid ligation, transformation, or propagation. To avoid the requirement for transformation into E. coli, I made use of an inverse PCR technique (Figure 2-8). For this technique, the DNA of each mutant line is digested, and the fragments are circularized as for the plasmid-rescue technique. However, instead of relying on bacteria to select and amplify the hybrid transposon/genomic fragment, I made use of a modified PCR reaction; without the requirement that the ori and amp^R genes remain intact, I was able to use additional restriction enzymes – those that cleave the transposon within these sequences – in the algorithm.

I designed a pair of oligonucleotide primers located near the 3' end of the transposon, oriented with their 3' ends facing away from one another. Only when they were placed in the correct orientation by circularization would they be able to amplify the intervening DNA, which (as for the plasmid-rescue technique) contains both P-element and flanking genomic sequences. Successful reactions generally produced a linear PCR
FIGURE 2-8: The inverse-PCR technique. Genomic DNA is digested and recircularized, which places the IPF and IPR primers into a configuration that spans the 3′ transposon/genomic junction point. This segment is amplified by PCR and sequenced.
product of less than ~2,000 base pairs, which could be purified and sequenced with the PR3 primer. (Because this technique does not require the function of the ori and amp\textsuperscript{R} elements at the 3' end of the transposon, restriction sites and primer sites at the 5' end would have worked equally well, although I did not take advantage of this.)

This inverse PCR technique was successful for some lines for which the plasmid-rescue technique was not. However, some lines could not be mapped by either technique. In these cases, I took advantage of another PCR technique, “thermally asymmetric interleaved PCR” (“TAIL PCR”), originally designed for mapping of T-DNA insertions in plant genomes (Liu et al., 1995). This technique is performed entirely \textit{in vitro} and is purely sequence-based; it therefore does not require the presence of any particular arrangement of restriction sites or bacterial genes. It makes use of three nested sequence-specific primers matching sequences near the 3' end of the transposon (the 5' end would have worked as well) (Figure 2-9), oriented to extend beyond the P-element into adjacent genomic DNA, in conjunction with degenerate primers that anneal every few hundred bases along the genomic DNA. Alternating cycles of high- and low-stringency amplification, along with the use of the nested sequence-specific primers in serial reactions, results in specific amplification of a fragment spanning the transposon/genome boundary, which can be sequenced directly. Because no enzymatic digestion, ligation, transformation, or propagation of DNA is required, the entire process from animal to sequence data can be performed in one to two days.

\section*{2-5: Genes identified by insertion mapping}

Each of these insertional mutations may affect the sensation, processing, or motor
FIGURE 2-9: The thermally asymmetric interlaced PCR technique. A PCR fragment spanning the transposon/genomic junction point is selectively amplified in a series of reactions using nested transposon-specific and degenerate primers. The purely in vitro nature of the reactions allows the entire procedure to be conducted in one day.
responses to gravitational stimuli; these errors may arise from developmental defects in sensory organs, central processing structures, or neuronal connectivity patterns, or they may result from changes in the function of these structures after they are formed. To identify the gene or genes affected by each insertion, the insertion site of each transposon was mapped using one or more of the techniques outlined above; after the entire set of lines was mapped in one way or another, the mapping was repeated, mostly using the TAIL-PCR technique. The results from this mapping are presented in Table 2-1.

Among the genes identified in the screen were several previously characterized genes encoding transcription factors with known roles in nervous-system development, such as broad (Restifo and White, 1991) and escargot (Ashraf et al., 1999; Hayashi et al., 1993; Hekmat-Scafe et al., 2005; Whiteley et al., 1992). We also identified several genes encoding proteins with roles in signal transduction during development, including the receptor tyrosine kinase ("RTK") Off-track (Cafferty et al., 2004; Whitford and Ghosh, 2001; Winberg et al., 2001), involved in neural pathfinding and mammalian homologs of which are required for proper inner-ear innervation (Schimmang et al., 1995; Schimmang et al., 2003), and the RTK downstream adapter protein She (Lai et al., 1995; Luschnig et al., 2000). In addition to previously characterized genes, we also identified several previously unstudied genes encoding proteins with sequence similarity to known proteins, such as the RTK-downstream Grb signal transduction molecules, and other members of the Beckingham lab are studying selected genes of this type. We also identified many entirely novel genes with no studied homologs; one gene of this class is the subject of this thesis work.
<table>
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<th>Line</th>
<th>Location of (5'UTR)</th>
<th>Function of Psmoly (Affected Gene)</th>
<th>Protein Homologues</th>
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<td>1 get HTRPA2-domain and C2C5'-finger-containing transaminase before affecting sactopan (RNA) adult</td>
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</table>

**TABLE 2-1:** The results of an insertion-site mapping in the mutant lines identified in the screen. (Adapted from Armstrong et al., 2006.)
2-6: The c263 insertion affects a gene we named yuri gagarin

One of the previously uncharacterized genes, temporarily named CG31732 and disrupted in insertion line c263, is located only 650 kb (~1 centi-Morgan recombination map unit) away from the Adh gene on the second chromosome – that is, in the general region in which Hirsch had identified a “gene correlate of geotaxis.” We wondered if perhaps we had identified the gene that Hirsch had begun to map. In addition, the GAL4 reporter activity of the c263 insertion in older adult animals appeared limited to a subset of the chordotonal neurons within JO (see Chapter 4 below), consistent with functional partition of JO sensilla and suggesting that perhaps this uncharacterized gene was specifically important for the function or development of these gravity-sensing organs.

The c263 insertion is inserted 24 bases upstream of the transcriptional start site predicted by the Berkeley Drosophila Genome Project for the small gene CG31732, which has no identifiable homologs in higher organisms and no recognizable functional domains (Figure 2-10). At the time of its identification in the screen, it was represented in the genome annotation data by a single cDNA sequence, GH14032, which was predicted to encode a protein of 29 kilo-Daltons (kDa). We renamed the gene yuri gagarin, after the first human to orbit the Earth. The overall goal of my thesis work was to characterize this gene and its function within the animal; specifically, my aims were:

1. To define the structure of the yuri gene – that is, to identify the range of transcript types and protein isoforms produced from the locus;
2. To determine the sub-cellular localization of the Yuri protein using immunolocalization; and
3. To identify functional roles for the protein and gene through mutant analysis and the identification of interacting proteins and genes using physical and genetic-interaction assays.
FIGURE 2-10: The structure of the *yuri* locus as it was predicted when insertion c263 was first mapped. The c263 P(GawB) element (represented as a wedge, although it is co-linear with the genome, and not to scale) is inserted 24 bases upstream of the predicted start site of the uncharacterized gene *CG31732*, which we named "yuri gagarin" after the first human to orbit the earth. The transcript comprises two exons and is predicted to encode a novel protein with a mass of 29 kDa. The locations of the start and stop codons are indicated; the transcript contains a 5' untranslated region (UTR) of ~600 bases.
2-7: Materials and methods

Maze assay

To prevent any in-maze following or courtship behaviors that could override gravitational behaviors in the maze, only male flies were assayed. To check for interactions between males within the maze that could distort the assay, single control flies were run, and their exits were summed to create a distribution; this distribution was not significantly different from that exhibited by isogenic batched flies (not shown), so all mutant lines were batch-assayed in the screen. Batches of ~25 males aged 0-3 days were isolated with light CO₂ anesthesia and placed in a food vial. After two days’ recovery, flies were tapped into an opaque plastic transfer tube and were allowed to acclimate for two hours. Mazes were checked for the presence of dead flies or broken connections. A small amount of yeast paste was placed in glass test tubes, which were attached to the maze exits. Mazes were arranged within a light-proof enclosure, with the exit tubes protruding from a slit in the enclosure. A fluorescent bar lamp was placed three inches from the ends of the exit tubes. Transfer tubes were attached to the entrance point of mazes (horizontal or vertical) and lightly tapped to stimulate the flies to exit the transfer tube into the maze. After thirty minutes, the transfer tube was tapped again. After 2.5 hours more, the number of flies in each exit tube, as well as those remaining in the maze and in the transfer tube, were recorded.

Courtship assay

Plexiglas “mating wheels” based on those of O’Dell (2003) were constructed, to
allow the controlled meeting of male and female flies. Wild-type (Canton-S) virgin females were collected and aged in food vials for 2-3 days. Naïve mutant males were collected and aged separately. Animals were lightly anaesthetized with CO₂ and placed into separate chambers in the apparatus. After 30 minutes of recovery, the male and female chambers were joined. The percentage of time the mutant males spent in various courtship activities (orienting, “singing,” tapping, tasting, and attempting copulation) was recorded for 5 minutes.

**Flight assay**

The flight assay is based on one described by Benzer (1973). A 40-cm × 15-cm Plexiglas cylinder was marked into five equal lengths (numbered 1-5, bottom to top) and coated inside with a thin layer of sticky paraffin oil; a wide-mouthed funnel was placed in the top of the cylinder, and the cylinder was placed on end in a shallow container to catch excess oil. Flies were collected as above, and after recovery, batches were injected into the cylinder by tapping them through the funnel. The number of flies stuck in the oil within each cylinder segment was recorded, and the cylinder was cleaned for re-use.

**Bang test**

This qualitative assay is based on one described previously (Guan et al., 2000; Nelson et al., 1997), with some differences. An empty food vial was marked in 1-cm intervals from the bottom of the vial, up to 5 cm. Batches of male flies were sorted and allowed to recover as above. After the flies were banged to the bottom, the general level of coordination and climbing was noted.
Statistics

Maze exit profiles were compared using one-way ANOVA. Dunnett’s range test was used to compare flight test, courtship, and mean maze exit value data. Range-limited behavioral-test values were transformed to values in an infinite range using an arcsine function prior to statistical analysis. Computations were made using Statistica software (Statsoft, Tulsa, OK), by Dr. D. Baker (University of Edinburgh, UK).

DNA isolation

DNA from batches of flies was prepared using the method described in Ashburner (Ashburner, 1989) or using TriZol reagent (Invitrogen). In the Ashburner protocol, flies were placed in an Eppendorf tube, frozen at -20°C, and ground using a plastic pestle in cold extraction buffer (100 mM Tris, 100 mM EDTA, 1% SDS, pH 7.5). One one-seventh volume of 8-M KOAc was added, and tubes were chilled on ice for 30’. Aggregated proteins and debris was pelleted by a 10-minute centrifugation, and supernatants were transferred to a new tube. One one-half volume of cold isopropanol was added, tubes were mixed by inversion, and DNA was allowed to precipitated at -20°C for at least 5’. DNA was pelleted, rinsed with 70% ethanol, and redissolved in sterile water (10 μL per fly).

For Trizol preps, flies were placed in an Eppendorf tube and frozen. 1 mL Trizol reagent was added and flies were ground using a plastic pestle. Debris was pelleted; supernatant was transferred to a new tube and incubated for 30 minutes at room temperature. 200 μL of chloroform was added, and the tubes were shaken vigorously. Phases were separated by centrifugation, and the upper (aqueous) phase was moved to a
new tube. To remove any residual phenol, 500 μL ether was added, tubes were shaken, and the phases were allowed to separate. The ether phase (containing phenol) was removed. To precipitate DNA, 0.5 mL isopropanol was added to the aqueous phase, and tubes were mixed. DNA was allowed to precipitate at room temperature for at least 30’. DNA was pelleted by centrifugation and washed with 70% ethanol. For both protocols, DNA was redissolved in sterile water, 10 μL per fly.

**Plasmid rescue**

Eight fly-equivalents of DNA were digested with an appropriate restriction enzyme: 10 μL manufacturer’s 10× buffer and 10 μL 10× BSA solution were added to 80 μL of fly DNA extract. A restriction enzyme (1-5 μL; 10-50 U) that cuts within the P-element somewhere 5’ of the ori and ampR sequences, was added, and reactions were incubated at 37° overnight. Digestion was halted by denaturing the enzymes at 80° for 10’. Reactions were diluted to 1 mL each (to favor intramolecular ligation) by adding 100 μL 10× T4 ligase buffer (500 mM Tris-HCl, 100 mM MgCl2, 10 mM ATP, 10 mM DTT, 50% PEG-8000, pH 7.6) and 800 μL ddH2O. T4 ligase (Invitrogen) was added (1-5 μL, 3-15 U), and reactions were incubated overnight at 14°. Reactions were split into three 333-μL aliquots, and ligated DNA in each was precipitated by addition of 40 μL 3-M NaOAc (pH 5.2) and 1 mL ice-cold 100% ethanol; precipitation was allowed to occur overnight at -20°. DNA was pelleted by centrifugation (maximum speed for at least 30’) and rinsed with 70% ethanol. DNA in each of the three tubes was redissolved in TE (5 μL per aliquot), and the three 5-μL solutions were combined. Five microliters was used to transform Top-10 ultra-competent cells (Invitrogen) according to the manufacturer’s
protocol. Cultures were plated on LB-ampicillin plates and incubated overnight at 37°. Any colonies formed were spiked into 3-mL LB-ampicillin aliquots and grown overnight at 37°. Plasmid DNA was prepared using QiaQuik (Qiagen) columns and protocol or the TENS protocol. As a quality test, plasmids were re-digested using the enzyme used in their initial creation, and fragments were visualized in a 1% agarose gel with ethidium bromide; only plasmids that produced a single linear band after digestion were analyzed. Plasmids were sequenced at Lone Star Labs, the Rice sequencing facility, or the Baylor core facility, using the PR3 primer (CGCCTTATTGCAAGCATAACG), which anneals to P-element DNA ~80 bases from the 3' end of the transposon. In the returned sequence data, the end of P-element DNA was signaled by the sequence "CATCATG," and the P-element-flanking sequences were mapped to the genome using the FlyBase BLAST service.

**Inverse PCR**

Fly DNA was isolated, digested, circularized, and re-concentrated as for the plasmid-rescue protocol. One microliter of the circularized DNA was used in a standard PCR reaction using the primers IPF and IPR ("inverse PCR forward/reverse": GGGTAAATCAACAATCATATCGCTGTCAC and CGACTCACTATAGGGCGAATTGGA-GCTC, respectively). PCR reactions were visualized on a 1% agarose gel, and any bands (which should have arisen from circularized fragments containing P-element and fly-genomic DNA) were excised from the gel, purified using the QiaQuik gel-purification kit (Qiagen), and sequenced as above. In some cases, bands were cloned using "Topo"
cloning vectors *pCRII*, *pCR2.1*, or *pCR4* (Invitrogen) to create more material for sequencing.

**Thermally asymmetric interlaced PCR**

This protocol was designed for isolation of T-DNA insert junction DNA from Arabidopsis (Liu et al., 1995) and was adapted for use with Drosophila P-elements. The procedure makes use of a set of three nested *P(GawB)*-specific primers and a collection of degenerate primers, along with alternating high- and low-stringency amplification steps to selectively amplify DNA fragments with one end within the P-element and the other within the adjacent genomic DNA. The *P(GawB)*-specific primers were Tail1 (AGTTATTCAAACCCACGGACAT), Tail2 (CAATCATATCGCTGTCTCACTCAG), and Tail3 (CGCACTTTATTGCAAGCATACTGTTA); the T<sub>m</sub> of these primers is ~60°. The degenerate primers used were AD1 (nTCGA<sub>s</sub>wTswGTT) and AD2 (nGTCGA<sub>s</sub>wGAnA-wGAA), where \( n=A/T/C/G, s=C/G \) and \( w=A/T \); AD1 is 64-fold degenerate, and AD2 is 128-fold degenerate. T<sub>m</sub> for these degenerate primers averages ~45°.

Fly DNA was isolated as above. PCR reactions were set up with 5 µL fly DNA stock, 5 µL manufacturer’s 10× polymerase buffer, 4 µL dNTP mix (10 mM each), 3 µL 25-mM MgCl<sub>2</sub> (1.5 mM final), 1 µL Tail1 primer (100-µM stock), 10 µL AD1 or AD2 primer (100-µM stock), and 27 µL ddH<sub>2</sub>O. Finally, 2.5 units of Taq polymerase (various vendors) was added, and the following PCR program was run:

```
1  95°, 2'
2  94°, 30''
3  62°, 1'
4  72°, 2.5'  (Creates some sequence-specific forward strands)
5  Repeat from step 2, four more times
6  94°, 30''
```
25°, 3' (Allows degenerate primers to anneal and extend)
Ramp to 72° over 3' (~0.26°/sec)
72°, 2.5'
94°, 30"
68°, 1'
72°, 2.5' (Create sequence-specific forward strands)
94°, 30"
68°, 1'
72°, 2.5' (Create sequence-specific forward strands)
94°, 30"
44°, 1'
72°, 2.5' (Create second strands from AD primers)
Repeat from step 10, 14 more times
72°, 5'
16°, until stopped

The products of this reaction series were a mixture of undesired fragments initiated from AD primers at both ends, fragments with Tail1 at both ends, and a small amount of Tail1-AD fragment. To selectively amplify these latter products, the nested Tail2 primer was used in the next reaction, set up as follows: 5 µL from the reaction above, diluted 1:50 in ddH₂O; 5 µL manufacturer’s 10× polymerase buffer; 4 µL dNTP mix (10 mM each); 3 µL 25-mM MgCl₂ (1.5 mM final); 1 µL Tail2 primer (100-µM stock), 10 µL AD1 or AD2 primer (whichever was used above; 100-µM stock), and 27 µL ddH₂O. To this mixture, 2.5 U Taq polymerase was added, and PCR program #2 was run:

95°, 2'
95°, 30"
64°, 1'
72°, 2.5'
95°, 30"
64°, 1'
72°, 2.5'
95°, 30"
44°, 1'
72°, 2.5'
Repeat from step 2, 11 more times
72°, 5'
To further select for the desired products, the third nested Tail3 primer was used to amplify a dilution of the second reaction: 1 µL from the reaction above, diluted 1:10 in ddH₂O; 5 µL manufacturer’s 10× polymerase buffer; 4 µL dNTP mix (10 mM each); 3 µL 25-mM MgCl₂ (1.5 mM final); 1 µL Tail3 primer (100-µM stock), 10 µL AD1 or AD2 primer (whichever was used previously; 100-µM stock), and 31 µL ddH₂O. Taq polymerase (2.5 U) was added, and PCR program #3 was run:

1 95°, 2’
2 95°, 30”
3 44°, 1’
4 72°, 2.5’
5 Repeat from step 2, 19 more times
6 72°, 5’
7 16°, until stopped

The final PCR reactions were electrophoresed through a 1% agarose gel containing ethidium bromide. Any bands present were excised, purified, cloned as necessary, and sequenced using the Tail3 primer.

TENS plasmid DNA miniprep

_TENS buffer:_
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1 N NaOH
0.5% SDS

Cultures were pelleted by centrifugation in Eppendorf tubes, 1.5 mL at a time, at maximum speed for 1’. All but 100 µL of supernatant was poured away, and cells were completely resuspended in the remainder by pipetting. TENS buffer (300 µL) was added, and tubes were mixed by inversion. Bacterial lysis was performed for 5’ at room
temperature, after which 150 μL 3-M NaOAc (pH 5.2) was added to denature and aggregate proteins. Preps were mixed by inversion and incubated on ice for 5'. Insoluble material was pelleted by spinning for 10' at maximum speed, and supernatants were transferred to another Eppendorf tube containing 900 μL ice-cold 100% ethanol. Preps were mixed by inversion and incubated on ice for 15-30 minutes, after which precipitated DNA was pelleted by centrifugation at maximum speed for 10'. Pellets were washed with 1 mL 70% ethanol and dried at room temperature for 10'. DNA was resuspended in 50 μL ddH2O and stored at -20°.

**Bioinformatics**

Sequences obtained using the three methods described above were mapped to the Drosophila genome using the FlyBase BLAST service (www.flybase.net/blast). Gene function predictions were based on those on the FlyBase annotation page of each identified gene. Gene orthologs were identified using the NCBI BLAST service (www.ncbi.nlm.nih.gov/blast), referencing the identified gene's predicted nucleotide sequence against the "nr" non-redundant nucleotide database and the gene's predicted encoded protein against the "nr" non-redundant protein database. Default settings were used in searches.
CHAPTER 3: ENHANCER-TRAP ANALYSIS IN LINE c263

3-1: Background regarding “enhancer traps”

Transposon-mediated mutagenesis has several advantages over more traditional chemical or radiological mutagenesis. One advantage, described above, is the ability to rapidly and precisely map insertions within the genome. Another advantage of insertional mutagenesis has to do with the way that different types of transposons have different genomic milieux into which they transpose preferentially. P-elements most often transpose into the near-upstream regions of genes, where regulatory elements are generally located, or into intronic sequences, rather than into protein-coding regions of the genome. As such, P-element insertions usually do not completely destroy gene function; rather, they cause more subtle defects that arise from the alteration of gene expression. (This targeting propensity of P-elements presumably evolved through its lessened tendency to reduce the fitness of their hosts.)

Genes within natural transposons carry their own regulatory elements. However, certain artificial transposons, called “enhancer-trap” elements (Figure 3-1), contain reporter genes with no expression-regulatory sequences of their own; expression of the reporter is therefore dependent on regulatory “enhancer” elements in the genome near the transposon-insertion site (Wilson et al., 1989). Enhancer elements regulate the spatial and temporal expression pattern of associated genes, which may be close by or separated by tens of kilobases; most enhancer activity is thought to involve the recruitment of transcription machinery to enhancer sites in the DNA, followed by chromatin looping.
FIGURE 3-1: Enhancer-trap insertion. Top panel: Genomic enhancer elements regulate the spatiotemporal expression pattern of nearby genes (here, expressing endogenous gene XYZ at a level of "3"). Bottom panel: The insertion of an enhancer-trap P-element disrupts the expression of the endogenous gene by altering the relationship between the gene and its regulatory elements. Expression of the inserted GAL4 transcription-factor reporter comes under the control of at least a subset of these regulators, and the activity of the endogenous gene is correspondingly altered (here, reducing its expression to a level of "1"). The patterned GAL4 expression in turn allows patterned expression of UAS-regulated transgenes elsewhere in the genome.
that brings these proteins to the promoter of the regulated gene (reviewed in Bondarenko et al., 2003; West and Fraser, 2005).

When a transposon is inserted near a gene, reporter genes within that transposon will tend to come under the control of some or all of the endogenous regulatory enhancer elements of the disrupted gene. To the degree that the influence of an enhancer on a gene is related to the physical distance between them along the DNA molecule, the expression pattern of that gene may be altered by the insertion of several kilobases of transposon DNA. In addition, for genes whose expression is regulated by enhancers involving a DNA-looping mechanism, to the extent that chromatin looping carries enhancer-bound transcriptional machinery to the promoter of the inserted reporter gene rather than that of the endogenous gene, the expression of the latter must be reduced (i.e., expression is a zero-sum game).

The mutagenic P-element used during the gravitaxis screen was the $P\{GawB\}$ (Brand and Perrimon, 1993) enhancer-trapping transposon. This transposon carries the yeast $GAL4$ gene, which encodes a site-specific transcriptional activator, as a reporter. Because it has no expression-regulatory elements of its own, the inserted $GAL4$ is dependent on the enhancers in its genomic milieu for spatiotemporal regulation of its expression. With no endogenous Drosophila regulatory targets, GAL4 protein normally has little effect on the animal; however, if a transgene regulated by the GAL4 binding sequence (the upstream activating sequence or "UAS") is also present elsewhere in the genome, this transgene will in turn be expressed in the pattern of GAL4 expression. This two-part system allows for combinatorial efficiency, by allowing any UAS-regulated transgene to be expressed in any pattern for which a GAL4 "driver" is available, simply
by mating the lines carrying the GAL4 driver insertion and the UAS target insertion; genome-saturating mutagenesis projects have created tens of thousands of random-insertion and artificial-enhancer GAL4 driver lines that can be used to express transgenes in virtually any desired pattern within the animal.

This system allows for two types of disruption. The mere presence of the transposon, by virtue of its tendency to usurp and disrupt enhancer activity, may alter the expression of any genes in the vicinity; therefore, the insertion may alter the physiology or development of tissues in which the disrupted gene is normally required. In addition, because of the mechanism by which enhancer usurpation is thought to occur, the disrupted tissues tend to be marked by GAL4 expression; these tissues can therefore be identified by expressing UAS-regulated fluorescent-protein genes, for example, and they can be developmentally or physiologically altered through the expression of UAS-regulated transgenes encoding, say, toxins or modified signaling molecules.

The behavioral abnormalities of the lines identified in the genetic screen arise from only the gene-disrupting aspects of the P-element insertions, since no UAS-regulated transgenes were expressed. (Expression of toxins and neural-function-disrupting proteins in selected lines in later experiments generally led to much stronger phenotypic effects [not shown].) To identify the tissues in which gene function was likely to be disrupted in each behaviorally interesting line, we introduced a UAS-regulated marker transgene – either EYFP, encoding “Enhanced-brightness Yellow Fluorescent Protein”, or lacZ, encoding beta-galactosidase, which produces a blue compound when appropriate substrate is applied – and the fluorescent or colored tissues of adults were identified by microscopy. All the GAL4 lines examined gave reporter
expression in the central neural tissues of adults (since they had been pre-screened for this); in addition, reporter expression in the peripheral nervous system was used to identify sensory structures that may be important for maze performance. Enhancer-trap reporter expression was seen in the antennal second segment in larger fraction of behavioral mutants compared to randomly chosen GAL4 insertions, consistent with a role for antennal chordotonal organs in the screened behavior (Dr. J. D. Armstrong, N. Loo, and R. Liou, unpublished data).

In the c263 insertion line, enhancer-trap reporter expression in 3-to-5-day-old adults was initially observed only in neurons of Johnston’s organ (JO) (Figure 3-5, P), consistent with JO defects’ being at the root of the behavioral defect seen in this line. Indeed, expression was observed in only a subset of JO neurons, suggesting a functional partition of JO neurons as discussed in Chapter 1. This expression pattern was one of the primary reasons that the yuri gene was investigated further. However, as it became clear that Yuri was likely to play a more fundamental role in the organism than was previously suspected (e.g., the protein is present in embryos and testes), the c263 GAL4 expression pattern in other parts of the animal and at other developmental stages became important. To identify this pattern, and therefore to understand where and when yuri expression was most likely to be disrupted, I examined EYFP expression driven by c263-GAL4 in embryos; first- and third-instar larvae; 1-, 3-, and 5-day-old pupae; and 1- and 5-day-old adults.

3-2: Enhancer-trap expression results

No reporter expression was seen in embryos or first-instar larvae (not shown),
suggesting that though Yuri is expressed in these animals (Chapter 4), its expression at these developmental stages is not altered by the c263 insertion. However, in wandering third-instar larvae, EYFP was detected in several tissues: certain cells of the Malpighian tubules (the Drosophila kidney analog), the visceral mesoderm of the midgut, the fat body, and the ring gland (Figure 3-2). Interestingly in light of the neural expression previously observed in adults, reporter activity was not detectable in the larval imaginal discs, from which all adult appendages are derived, even though neurogenesis has already taken place in these tissues (Jan et al., 1985; Jarman et al., 1993; Stocker et al., 1976; Zipursky et al., 1984).

The marked cells of the Malpighian tubule were identified as the “stellate cells” (J. Dow, personal communication), epithelial cells which allow regulated anion diffusion into the tubule lumen (O'Donnell et al., 1996; O'Donnell et al., 1998). The fat body is the Drosophila liver analog; it secretes various proteins, lipids, and carbohydrates into the lymph, and it also re-absorbs serum proteins and stores them for use during pupal metamorphosis (reviewed in Hahn and Denlinger, 2007). The ring gland is a larval endocrine organ whose secretion of juvenile hormone and the molting hormone ecdysone regulates growth and metamorphosis (reviewed in Jones and Jones, 2007; Mirth et al., 2005). The limiting of reporter expression to these larval secretory tissues indicates that enhancer elements near yuri are activated by factors active in these tissues, and, inasmuch as reporter activity reflects the activity of the endogenous undisrupted gene, suggests that yuri is involved in a process common to these tissues.

EYFP reporter expression becomes much more widespread during early pupal development, appearing in all pupal tissues examined (Figure 3-3). In contrast to the lar-
FIGURE 3-2: UAS-EYFP expression driven by c263-GAL4 in third-instar larvae.

A: Stellate cells of the Malpighian tubules. B: Visceral mesoderm of the midgut. C: Malpighian tubules and fat body (right). D: No fluorescence is visible in the brain lobes, ventral ganglion, segmental nerves, or imaginal discs (orange, yellow, green, and teal in E). However, EYFP is visible in cells of the ring gland (red arrows; purple in E; it appears to be crushed and distended to the left).
FIGURE 3-3: Early pupal *UAS-EYFP* expression driven by *c263-GAL4*.  

A: Expression in head structures.  
B: Expression in developing legs.  
C: EYFP is strongly expressed in the developing wing.
val imaginal discs, the developing adult appendages that form from them are strongly fluorescent. As pupal development continues, c263 reporter activity begins to drop off in most tissues (Figure 3-4); by the fifth and last day of pupation, only certain tissues continue to express EYFP, especially the peripheral nervous system. After eclosion, adult reporter expression continues to fade (Figure 3-5). In particular, reporter fluorescence is intense in chordotonal-organ neurons of 1-day-old adults, but over the next four days, the number of neurons that express the reporter falls off until almost no fluorescence remains.

3-3: Conclusions from enhancer-trap expression analysis

This pattern of reporter-gene expression indicates that the elements regulating GAL4 reporter expression from the c263 enhancer-trap insertion are most active in pupal development, and, to the extent that reporter activity comes at the expense of yuri expression, it suggests that yuri function is disrupted most highly during pupation. This is consistent with the time-course of the expression of yuri itself (Arbeitman et al., 2002), which peaks 12 hours into the 120-hour pupal stage (Figure 4-6). Early in pupation, the flat larval imaginal discs migrate to their adult positions and evert in a "telescoping" movement to form the extended adult appendages; disc eversion occurs through changes in cell shape generated by the Actin/Myosin cytoskeleton during the first ~15 hours of pupal development, not by increases in cell number, since most disc cell proliferation occurs during larval growth (e.g., Bryant and Schmidt, 1990; Condic et al., 1991; Escudero et al., 2007; Fristrom and Chihara, 1978). Neural differentiation within the discs occurs during larval life and the very earliest stages of pupal metamorphosis, before
FIGURE 3-4: Mid-pupal *UAS-EYFP* expression driven by *c263-GAL4*. 

A: Expression throughout the developing wing. 

B: Expression throughout the leg and haltere. 

C: Head structures are highly fluorescent. 

D: The second antennal segment, site of JO, is especially bright. 

E: Muscle tissue (in this case, body-wall muscle) also expresses the c263 enhancer trap.
FIGURE 3-5: Adult c263 enhancer-trap expression. Complete legend on next page.
FIGURE 3-5 (previous page): Adult UAS-EYFP expression driven by c263-GAL4.
(A-N: 1 day post-eclosion; O, P: 5 days post-eclosion.)

A, B: The femoral ChO neurons strongly express EYFP; a low level of expression is also visible in the body. Note that the leg muscles no longer express detectable EYFP. C, D: Expression in head structures. E, F: Reporter activity is strong in the wing-blade radial chordotonal organs. In addition, in F, fluorescence can be seen in the neural cell bodies of some wing-margin chemosensory bristles. G: ChO neurons throughout the haltere. H: Chemosensory bristles and taste pegs of the maxillary palp and proboscis show reporter activity, as does the pharynx, the first segment of the foregut. I: The hindgut and rectum are visible; the four wedge-shaped structures in the rectum are the rectal papillae, sites of water re-uptake. J, K: Cells of the gut. L: The brightly fluorescent structure at left is the proventriculus; the cells at right are visceral mesoderm of the midgut. M: Male accessory-gland secondary secretory cells. N: Developing spermatocytes in the testis. 

O, P: Reporter activity in older adults appears to be limited to a subset of the femoral chordotonal neurons and JO neurons.
FIGURE 3-6: *yuri* transcript levels throughout development (data from Arbeitman et al., 2002). Transcript levels of *yuri* (and ~6,000 other genes) were analyzed in microarray experiments and scaled relative to a pan-genomic and pan-developmental average. Note that levels are charted on a log-2 scale, and that whereas embryonic, larval, and metamorphosing animals are of both sexes, adult data are separated according to sex. During early embryonic stages (cyan, 1-hour time points), *yuri* is expressed at a low level; by the end of embryogenesis, the level has quadrupled, and expression remains roughly constant during larval life (red, assayed at 2-to-12 hour intervals). Expression increases strongly during metamorphosis (blue), peaking 12 hours after puparium formation, before falling back to roughly larval levels. In adults, *yuri* is highly transcribed in males (blue) but not in females (yellow), the increment presumably arising in the testis and associated tissues. Because the expression levels during other time periods are derived from mixed-sex collections, and because sex-specific tissues are present from embryonic life onwards, it is possible that male-specific enhancement of transcription is present in all stages of development. (Image obtained from [www.flymine.org/query/portal.do?origin=flybase&class=gene&externalid=FBgn0045842](http://www.flymine.org/query/portal.do?origin=flybase&class=gene&externalid=FBgn0045842)).
the discs begin their eversion (e.g., Lienhard and Stocker, 1991).

Thus, reporter activity appears to correspond temporally with a phase of cellular migration and profound changes in cell morphology rather than with cell proliferation or with any particular eventual cell fate. In this light, it is interesting that neurons in particular retain reporter expression for the longest time after eclosion; the hypothesis above would imply that this reporter activity correlates with their extreme morphology rather than their neural identity. This would suggest that c263 reporter activity correlates with cytoskeletal dynamism, and would imply correspondingly that yuri is involved in this dynamism and that disruption of native yuri expression by the c263 insertion may be at a maximum in tissues which are cytoskeletally active.

3-4: Materials and methods

UAS-2×EYFP driven by c263-GAL4

To visualize reporter expression from the c263 insertion, I created fly lines carrying a homozygous UAS-2×EYFP insertion in conjunction with the c263-GAL4 insertion; to detect any anatomical changes arising from the effects of the c263 insertion, I created lines homozygous and heterozygous for the c263 insertion (the behavioral phenotype of the insertion is recessive). No obvious differences were observed between them (not shown). The UAS-2×EYFP transgene (Halfon et al., 2002) encodes two EYFP proteins within each mRNA, separated by an internal ribosomal entry site; this leads to increased fluorescence intensity; flies were obtained from the Bloomington Drosophila Stock Center, Bloomington, IN.
Epifluorescence microscopy of GFP-expressing tissues

Tissue was dissected in PBS and mounted immediately in 50% glycerol containing 0.1% Hoechst 33342 (Invitrogen), a membrane-permeant DNA stain. Tissues were observed on Leica MZ12, Zeiss AxioSkop, and Zeiss AxioPlan microscopes; images were captured using MetaMorph (Molecular Devices) and AxioVision (Zeiss) software.
CHAPTER 4: CHARACTERIZING yuri

TRANSCRIPTS AND PROTEINS

4-1: Background and preliminary transcript data from the BDGP

As described above, the $P_{GawB}$ insertion in line c263 was mapped to a position 24 bases upstream of a gene we have named "yuri gagarin." When the yuri project began, the only experimental evidence of transcription from the locus was a small cDNA clone called GH14032, derived from adult head tissue and sequenced by the Berkeley Drosophila Genome Project (BDGP). As described above, this cDNA contains a polyadenylylation signal and a poly-dA tail, indicating that it derives from a processed transcript, and comprises two exons. The open reading frame is predicted to encode a 29-kDa Yuri protein of novel sequence.

Further complete and partial sequencing by the BDGP of cDNA clones from various tissues (Figure 4-1) as part of its ongoing annotation process led to the prediction of additional, larger Yuri isoforms. Clones SD06513 and RE16347, from cultured Drosophila cells and embryos, respectively, were sequenced from end to end by BDGP (Figure 4-1) and predicted to encode proteins of 64 and 65 kDa, consisting of most or all, respectively, of the 29-kDa protein encoded by GH14032, plus a ~400-residue C-terminal extension.

A testis-derived cDNA, AT15149, was also sequenced by the Genome Project (Figure 4-1). This cDNA clone comprised roughly the 3' half of the SD06513 and RE16347 clones, plus several downstream exons. Because the genomic region surrounding the apparent 5' end of this transcript scored poorly in a computational prediction of promoter sites (data not shown), it was possible that the apparent 5' end of
FIGURE 4-1: BDGP yuri cDNAs. As part of its Drosophila genome annotation efforts, the Berkeley Drosophila Genome Project created several cDNA libraries from various tissue sources; some of these cDNA clones were sequenced completely, end to end, whereas others were sequenced only for short reads at one end of the molecule, to allow the clone to be associated with a particular gene. All of these cDNA clones were obtained from the Drosophila Genome Resource Center (Bloomington, IN). The sequences are displayed above, aligned with the genomic sequence; the unsequenced continuation of most clones is indicated with an arrow. Several sites where alternative splicing is exhibited are indicated, and the apparent start sites of all the cDNAs are marked. Spacing is accurate to within the resolution limit of the composition software; e.g., the 5' ends of the last seven transcripts are scattered within a small region, as illustrated.
*AT15149* was an artifact of incomplete reverse transcription, and that the actual transcript from which it was made was initiated at a more upstream position; it was not known, however, if any such “full-length” transcripts were actually produced from the locus.

Over time, more than a dozen additional cDNA clones were end-sequenced and mapped to the locus by the BDGP as well (Figure 4-1). None of these new *yuri* cDNA clones had been fully sequenced, however – only their 5' ends were known – so these new sequences did not reveal any new information regarding the length or exon make-up of possible *yuri* transcripts. Several positions which exhibited alternative splicing were apparent; given these sites, as well as several apparent transcription start sites, many transcript variants are mathematically possible, but which combinations were actually produced in the animal was unknown.

The available experimental data supported the existence of the 29-, 64-, and 65-kDa Yuri proteins; it also suggested an unusual ~70-kDa isoform lacking the N-terminal half of the 64/65-kDa proteins. If the transcript from which this protein was predicted actually began at the same site as the other transcripts, it would encode a protein of 102 kDa. One aim of this study was to determine if any transcripts encoding this 102-kDa isoform actually exist. In addition, I wanted to determine which of the many mathematically possible initiation/splice variants are actually produced. To accomplish these goals, I obtained all the available cDNA clones and sequenced them from both ends as well as at sites where other sequences indicated that alternative splicing was possible.

### 4-2: cDNA sequencing results

Sequencing of cDNA *SD11641* shows that it exactly matches cDNA *SD06513*;
clones \textit{RE12523} and \textit{RE13793} were also found to match \textit{RE16347} exactly. Together, these sequences provide further support for the existence of the 64- and 65-kDa Yuri isoforms (Figure 4-2). These cDNAs end in polyadenylation signals and poly-A tracts, indicating that their 3’ ends are intact. The 5’ ends of gerarium (ovary) transcripts \textit{GM26777} and \textit{GM26781} match those of the \textit{RE-} and \textit{SD-}library clones; they are more similar to the former in their retention of sequence that is excised as intronic from the latter.

The 3’ ends of these clones, however, map to the middle of exon #5 of the “medium” transcripts, a spot that is not a termination site for any of the other clones. No polyadenylation signal or poly-dA tract are present at the terminus of these cDNAs. A \textit{Xho I} digestion step was involved in the creation of the library; the sequence of the cDNAs at this position is a \textit{Xho I} restriction site, indicating that this apparent termination site is most likely an artifact. Therefore, it is likely that these \textit{GM-}library clones represent transcripts that extend to one of the other attested termination sites and encode either 65- or 102-kDa Yuri proteins. (No large Yuri protein other that the 65-kDa isoform is detected in the ovary [Figure 4-5], indicating that these transcripts are the same as the \textit{RE-}library transcripts.)

The testis-library clone \textit{AT19027} was found to span the entire predicted open reading frame of \textit{yuri} – that is, it begins in the same region as the other clones, includes the sequence contained in them, and extends a further five exons; it encodes a 102-kDa protein comprising the 29-kDa N-terminal region, the ~40-kDa segment present in the ~65-kDa isoforms, and the ~30-kDa C-terminal extension encoded by \textit{AT15149}. It also includes a poly-A signal and poly-A tract, indicating that this is a fully processed tran-
FIGURE 4-2: Sequenced yuri cDNAs. After sequencing all the BDGP cDNAs, it became clear that only two start sites are likely to be relevant and that yuri transcripts can be grouped into three classes: GH14032 represents a short class, initiated at the proximal start site; the GM, RE, and SD clones represent a medium-length class, initiated at the distal start site; and the AT clones represent a long class, initiated at the proximal site. The GM clones end at a Xho I restriction site, presumably reflecting cleavage during cloning; no proteins larger than the size predicted from “medium” transcripts are detectable in ovary blots (see below), so the transcripts represented by GM clones therefore probably resemble those of the RE class. The existence of yuri transcripts that span the full distance from the transcription start sites to the furthest exons is demonstrated. The 5’ ends of most AT clones are not predicted to be near promoters; the scattered apparent start sites of these clones likely are artifacts of incomplete reverse transcription. Spacing is accurately to scale as displayed.
script. This finding represents an experimental verification of the existence of the "long" transcript predicted from the combination of the other transcripts.

Each of the other three AT-library transcripts (AT03435, AT15480, and AT25733) begins at a different site that does not map near either of the sites at which the other cDNAs begin. These three apparent "start sites" are probably artifacts of incomplete reverse transcription during the library's creation; the genomic region surrounding these sites scores poorly for the presence of a promoter. These clones include polyadenylation signals and poly-A tracts, indicating complete 3' ends. Clone AT15149 begins at precisely the same base as AT15480, suggesting that they derive from the same incomplete first-strand cDNA.

The first exon (exon #4) of AT15149 and AT15480 was found in only those two clones. The disposition of this exon with relation to other intron-exon boundaries suggests that this exon is a discrete entity that is sometimes retained, at least in the testis, and sometimes is excised; alternatively, this exon may be a 3'-extended form of exon #3. On the other hand, the first (partial) exon of AT03435 is a 5'-extended version of exon #3; its presence in the cDNA could reflect the reverse transcription of a partially processed mRNA from which the intron between exons #2 and #3 had not yet been removed. Note that the intronic sequence between exons #2 and #3 contains a stop codon and a poly-A signal (see clone GH14032); any finished medium or long transcript containing this sequence would be destroyed through nonsense-mediated degradation (Shyu et al., 2008).

At least two transcription start sites are represented in the data – a proximal one around which the RE, SD, and GM clones cluster, and a distal one at which GH14032 and AT19027 begin. The proximal start site scores very well (0.98 on a 0.0 – 1.0 scale) in a
computational search for promoter elements. The \textit{RE} clones all begin at the same precise base, and these clones have the greatest 5' extent. The first bases of SD06513 and SD11641 are the 17\textsuperscript{th} and 20\textsuperscript{th} bases, respectively, of the \textit{RE} sequences, and the first base of both \textit{GM} clones is the 32\textsuperscript{nd} \textit{RE} base. The scatter in these start sites possibly reflects incomplete reverse transcription or "play" in the transcription initiation process, but the clustering of first bases from clones from different libraries made at different times indicates that this transcription initiation site is biologically real and significant, and computational models support its existence.

\textit{GH14032} and \textit{AT19027} start at the distal promoter, about 460 bases downstream of the proximal promoter sequences. The first base of \textit{GH14032} is base #464 of the \textit{RE} sequences, and \textit{AT19027} begins at \textit{RE} base #465. These clustered start sites seen in clones from different libraries support the biological existence of this second transcription initiation site; this start site is \(~150\) bases downstream of a large region that scores well (0.78 on a 0.0 - 1.0 scale) in computational promoter modeling. No coding sequence is contained within the 464 bases' difference between the two initiation sites. In my data set, initiation at the proximal site is correlated with production of transcripts encoding "medium-sized" 64-/65-kDa Yuri isoforms; the distal site gives rise to transcripts encoding the 29- and 102-kDa isoforms, although only one complete transcript of each type exists.

4-3: Conclusions from cDNA sequencing

The sequencing of these cDNA clones allows me to draw three main conclusions regarding the diversity of transcripts from the \textit{yuri} locus. The first of these is that the
locus produces three classes of transcripts — "short" ones predicted to encode a 29-kDa Yuri protein, "medium-length" transcripts predicted to encode 64- and 65-kDa proteins, and a "long" transcript predicted to encode 102-kDa isoform. Secondly, two main transcription start sites are represented in the data: transcripts encoding the 64- and 65-kDa isoforms begin at a proximal site ~460 base pairs upstream of the distal site at which transcripts encoding the 29- and 102-kDa isoforms are initiated. This may be important for the interpretation of protein-expression defects caused by mutations to the locus (see Chapter 5). (The c263 transposon is inserted 24 base pairs upstream of the proximal start site.) Lastly, only a subset of the many possible combinations of alternative splicings are present in the surveyed transcripts. The deduced structure of the yuri locus is shown in Figure 4-3. Some ambiguities remain, especially regarding the nature of exons #3 and #4.

4-4: Generation of specific anti-Yuri antibodies

Prior to our work, nothing was known regarding the proteins produced from the yuri locus, or indeed if any proteins at all were expressed. The analysis of yuri transcripts above suggests that four Yuri isoforms can potentially be expressed from the locus (Figure 4-4). To determine the existence of the predicted proteins encoded by the sequenced cDNA clones, members of the lab created a chicken anti-Yuri IgY reagent and used it to probe extracts of various tissues in immunoblots.

To verify the existence of the three predicted isoform classes and to verify that the anti-Yuri IgY could detect them specifically, unfertilized eggs and embryos homozygous for the wild-type yuri locus or for a large chromosomal yuri-region deletion were analyz-
**FIGURE 4-3:** Predicted *yuri* transcripts. Based on the sequenced cDNAs described above, I have predicted three major *yuri* transcript types (the medium-length class comprises two members, differentiated by alternative splicing and translation-initiation sites). These four transcripts are predicted to encode Yuri isoforms of 29, 64, 65, and 102 kDa. Other minor variants may exist, such as a long transcript including the incompletely characterized exon #4, but the four transcripts represented above are supported end to end by unambiguous physical evidence. The position of the c263 $P\{GawB\}$ insertion is indicated. Spacing is to scale as displayed. (From Texada et al., in press.)
FIGURE 4-4: Yuri protein isoforms predicted from the complete cDNAs. A poly-proline motif is present near the N terminus of all Yuri isoforms. Coiled-coil-forming regions were predicted using the method of Lupas et al. (1991). The C-terminal-most coil-forming region of the each smaller isoform is not present in the next larger isoform because of alternative exon usage. The coil-forming domain near the end of Yuri-29 is the most weakly predicted, scoring just above the threshold for significance. Spacing is accurate as displayed. (From Texada et al., in press.)
ed by Western blot (Figure 4-5, A). In the extracts from homozygous \textit{CyO-GFP (yuri\textsuperscript{+})} embryos, bands of the three predicted apparent sizes can be seen; in addition, a \textsim 50-kDa band, likely a degradation product of a larger predicted Yuri isoform, is visible. In contrast, in extracts from homozygous \textit{yuri}\textsuperscript{-} deficiency embryos and from unfertilized eggs, only the smallest 29-kDa Yuri isoform can be detected, indicating that the bands stained by our anti-Yuri antibody in fact represent Yuri proteins; this also shows that Yuri-29 protein is maternally deposited into the oocyte, whereas larger isoforms are only expressed zygotically. Later blots of \textit{yuri}\textsuperscript{F64} mutants (see chapters 5 and 6) indicate that this band does in fact represent the Yuri-29 protein; see Figure 4-5, C, testes lane.

\textbf{4-5: Time course of zygotic Yuri expression}

To examine the time course of zygotic Yuri expression, I collected developmentally staged \textit{w1118} embryos and analyzed their Yuri isoform complements by Western blotting (Figure 4-5, B). This blot verifies that very little of the larger-isoform Yuri proteins are maternally deposited into the embryo. The \textit{Drosophila} "mid-blastula transition," at which zygotic genes begin to be expressed, occurs roughly 2-3 hours after egg laying; soon after this time, Yuri-64/-65 begin to be expressed. (The largest Yuri-102 isoform is not visible in this blots, whereas it is visible in Figure 4-5, A and C; the detectability of this protein varies from blot to blot, perhaps reflecting differences in efficiency of large-protein transfer from the gel to the blotting membrane.)

\textbf{4-6: Yuri proteins are detected in all tissues examined}

The cDNAs sequenced above suggest that transcripts encoding Yuri proteins of
29, 64, 65, and 102 kDa are produced in the animal. Interestingly, all the cDNAs from each tissue source were of the same class, and each transcript class was found in only one tissue type, suggesting that each different Yuri protein isoform might be specific to certain tissues and thus have a function specific to that tissue. To determine if this is actually the case, extracts from wild-type larvae, heads, thoraces, testes, and ovaries were analyzed by anti-Yuri Western blotting (Figure 4-5, C). In contrast to the cDNA distribution, the 29- and 64-/65-kDa Yuri isoforms are present in all wild-type tissues examined, and the 102-kDa Yuri protein is present in all tissues examined except the ovaries. In the head, the mid-sized Yuri proteins appear to be the most abundant, whereas in other tissues, the Yuri isoforms appear to be expressed in similar abundance.

In summary, the predicted Yuri-29, -64/-65, and -102 isoforms are expressed in various combinations in all examined tissues of the animal, and these proteins are specifically detectable with the chicken anti-Yuri antibody. Only Yuri-29 is maternally loaded into the oocyte before fertilization; larger Yuri isoforms are zygotically expressed, starting very soon after the transition to zygotic gene expression early in embryonic life. Different tissues express different levels of the Yuri isoforms, but there is no simple one-to-one correspondence between tissues and isoforms.

4-7: Sequence analysis of Yuri proteins

Sequences of the four predicted and confirmed Yuri proteins are shown in Figure 4-6, and the residues shared by several isoforms are indicated. Sequence comparisons of Yuri isoforms against all public sequences (ranging from bacteria to the human genome)
FIGURE 4-5: Yuri protein expression appears to be ubiquitous. A: Whereas proteins of the three predicted size groups are detectable in anti-Yuri Western blots of yuri- embryo extracts ("CyO-GFP homozygotes"), only the smallest isoform is present in unfertilized eggs and in embryos homozygous for a yuri deficiency; this protein is likely to be maternally deposited into the embryo. B: In offspring of yuri+ parents, Yuri-29 is maternally deposited, but Yuri-64/65 is only zygotically expressed beginning 4-6 hours after fertilization. C: Yuri proteins of all three size groups are detectable in all tissues examined wild-type animals, except for the ovary, in which no Yuri-102 is detectable. Homozygous yuriF64 animals (Chapter 5) do not detectably express Yuri-64/-65 in any tissue, and Yuri-29 is not detected in testes from these animals. Probable degradation products are marked with an asterisk. (From Texada et al., in press.)
Yuri-29:
1 MASRQAGFVL DAKSMGNNES RPASSPPPP SSASQMCROV OPTGTSASHP HOESTLKASS
61 PDRTCASTEA MSTEHYRCI QKLRTNGDGA GMSFELNSI FLIKRLPEIDC LDHEGDGDMH
121 TCQLRLVTFO EIVWFDLLVHN SVILGNSVDL KEKAYSKIVA CCOSQVRORQ HTLEENBRLR
181 EDICAIERYV QHPSCHTDFD FNGISLETLT VNQLRNGVKG PAHAECESEK VGHRSRLKKG
241 GGSNVDKWDP KSSHKRESNKR 260

Yuri-64:
1 MGLNPSRPAS SPPPPPSASS OMQCROQVPTG TAYASHPQEQS TLKSSPDDRC TASTEFMSTE
61 HYRCGLQIKLR QTNAGDAGDMSE FELNSIELKR LDEIDCLDEH EGDOMHTCQIL ALITFOEWD
121 FALHYNVLIL GNVSDELKEA YSKKVCQCSS QVRRQHTLIE ENRRLREDIC AIIEVQHISP
181 HCTDFDENGJ SLETLTVQNL RGVGKPDAPA ECESEKMSM ESMSLVEGIAA KHDEIGELKS
241 QTNADDEVVH TARKLLLKQD QCIAQLNQOL HQEITCIESR DQAKMEEPSN DTLLTDAITS
301 DMLNLSHTHD TQQESMALRL NAELNDLDDL HNKKQFQTIE IRRKRVSFCFI EKLHVERDNT
361 LVKLDSSLH ESILQDSLLQ SQLSVDPESS EPDELADADAQ MEALRRLRL NLSQNLRLH
421 GKYQRDLDES KIKISELEAE IESQSVQLQ NSVNLREDIS IEILSGKSKF SYEINYEDESS
481 KENPFTITIA DIFARKFEEE QNQVAIECDT KTFQTSRKTPT KTTKIPGATA KTNKQQPTTK
541 PNSNKPTTTKT IPTKNTTTTF RTSK 564

Yuri-65:
1 MASPAGFVL DAKSNMGNES RPASSPPPP SSASQMCROV OPTGTSASHP HOESTLKASS
61 PDRTCASTEA MSTEHYRCI QKLRTNGDGA GMSFELNSI FLIKRLPEIDC LDHEGDGDMH
121 TCQLRLVTFO EIVWFDLLVHN SVILGNSVDL KEKAYSKIVA CCOSQVRORQ HTLEENBRLR
181 EDICAIERYV QHPSCHTDFD FNGISLETLT VNQLRNGVKG PAHAECESEK MSESMSKSLV
241 ETAAHKEIETG ELSQPOINLQ EVVITARQKL LKKQCIACQL NQOLLHEITRC IESRODAQKME
301 ESPNDTTLAD AITSMDLENL SIHOTQESEM LRLNNEALNL LDLHNNKQEF QTIERRKVRK
361 SCFIEVLKVE SREDTLKLES IRSHLTILQS DLQOQLSILS DPESECELDD ADAQMELEAR
421 RLRNLNQLQN RELHGYOLRQ DTSESKEISET LEARIESSESS VIOQNSVIRI EIAELICSLG
481 SKEFSNYEITI DESSKENPFC TTIADIFARK FEEFQONQVAI ECDKTFPSQT RKPRTKTIKF
541 GATAKTNQQQ PTKTKSNKPT TTKTPTKNT TTKFTSRTS 578

Yuri-102:
1 MASPAGFVL DAKSNMGNES RPASSPPPP SSASQMCROV OPTGTSASHP HOESTLKASS
61 PDRTCASTEA MSTEHYRCI QKLRTNGDGA GMSFELNSI FLIKRLPEIDC LDHEGDGDMH
121 TCQLRLVTFO EIVWFDLLVHN SVILGNSVDL KEKAYSKIVA CCOSQVRORQ HTLEENBRLR
181 EDICAIERYV QHPSCHTDFD FNGISLETLT VNQLRNGVKG PAHAECESEK MSESMSKSLV
241 ETAAHKEIETG ELSQPOINLQ EVVITARQKL LKKQCIACQL NQOLLHEITRC IESRODAQKME
301 ESPNDTTLAD AITSMDLENL SIHOTQESEM LRLNNEALNL LDLHNNKQEF QTIERRKVRK
361 SCFIEVLKVE SREDTLKLES IRSHLTILQS DLQOQLSILS DPESECELDD ADAQMELEAR
421 RLRNLNQLQN RELHGYOLRQ DTSESKEISET LEARIESSESS VIOQNSVIRI EIAELICSLG
481 SKEFSNYEITI DESSKENPFC TTIADIFARK FEEFQONQVAI NQQLSCQIKG LQSNLKDORDN
541 QTSQILOSMIN SYSDFSENRR LKEEMWHIKQ KNCDSLQRQQL DLPSREKNEQ NQVSCTEKY
601 ESLNASFEDQ CAILPDAKRR AQSILTRLQVE VEQLOQDELAT ERLKIREEVE ALKEKEAVSS
661 GRELKQQQ KSAQLEMEKH RTLVKMQSHE LQOLDIHHRE SIQMVINEET EREELRTIS
721 ENCQOMQIRL NQQTEVNOQQ EQIIDSFRKWD KDAQVRDIAE RRLCAKRAEIE H1HMLDLENR
781 TLAEVRNLNF RDIYKILLETI KRVRKVAVNGCA SSAAVSCPNN KSGGLTNPEA GNMARLQDN
841 LTSTSQRIFN QNQTMNDQYQN SLSISGSPAG IQGTPQASIL RRRRSSQQDS 890

FIGURE 4-6: The Yuri isoforms encoded by the predicted yuri transcripts.

Residues common to all Yuri isoforms are heavily underlined. Residues common only to the 64-, 65-, and 105-kDa isoforms are lightly underlined.
failed to detect any high-quality matches, except among the twelve sequenced Drosophila species. Whereas Yuri is strongly conserved among the Drosophilids, with 50-83% sequence similarity in the Yuri-29 “head” region and with 52-93% sequence similarity across the entire 102-kDa isoform, no Yuri orthologs were identified in the next most closely related sequenced organisms, the mosquitoes (Figure 4-7); thus, Yuri arose at or soon after the time these two insect groups diverged. It presumably evolved to perform some Drosophilid-specific function, and may have since become integrated into more-general, non-Drosophilid-specific processes as well.

Database queries also failed to detect any specific conserved functional domains in any Yuri isoform; however, a structure-prediction algorithm predicts that most Yuri protein sequence apart from the 29-kDa “head” region is likely to take part in “coiled coil” interactions (Figure 4-4). Coiled-coil-forming domains are a fairly common structural motif, present in an estimated 10% of all eukaryotic proteins, compared with, for example, ~20% that contain transmembrane helices (Rost, 2002). These domains comprise long alpha-helices that exhibit a heptad pattern of hydrophobic residues, occupying the fourth and seventh position of each repeat; these residues form a hydrophobic surface along one face of the helix, and the burial of these surfaces drives homo- or hetero-oligomerization between helices, which wrap around one another in a parallel or anti-parallel relationship to form a (most frequently) left-handed helix of helices – the coiled coil. (Therefore, a single domain cannot be said to be a coiled coil itself, but simply an alpha-helix with an interesting sequence feature.) The “leucine zipper” domains of certain dimerizing transcription factors form a prototypical coiled coil. Coiled-coil-forming domains are common in proteins of the Actin and microtubule
FIGURE 4-7: Yuri protein sequence similarity among insects. A dozen Drosophila genomes have now been sequenced, and a consensus set of predicted proteins, the GLEANR set, has been produced from analysis of these genomes. Amino-acid sequence comparisons of the 29-kDa and 102-kDa Yuri isoforms against this data set indicates that between 48 and 82 percent of the residues present in the D. melanogaster Yuri-29 protein are represented by similar residues in other Drosophila species, and between 52 and 91 per cent of the residues of the melanogaster Yuri-102 are represented in other species. No protein-prediction data set exists for the other eight sequenced insects, so a comparison of Yuri-29 protein sequence against all possible translations of these genomes was performed; no significant similarities were identified. Tree image adapted from FlyBase, with permission. Estimated times since divergence from the D. melanogaster lineage are shown to the right of similarity values, in millions of years (MY). Times for splits within the Drosophilidae are from Tamura et al. (2004); times outside this group are from Porcelli et al. (2007) Adapted from Texada et al. (in press).
cytoskeletons, including α-Actinin, α-Spectrin, Tropomyosin, α-, β-, and γ-Tubulin, and various Dynein-, Kinesin-, and Myosin-like motor proteins; in proteins of the centrosome, centriole, and basal body; and in vesicle-trafficking systems (reviewed in Rose and Meier, 2004. Structural predictions by COILS server, data not shown).

Sequence alignment of the Drosophilid Yuri orthologs reveals regions in which the spaced hydrophobic residues forming the putative inter-helix binding surface are more strongly conserved than their neighbors (not shown), suggesting that these regions comprise interaction domains and that the precise shape of the interaction surface is evolutionarily constrained, indicating specificity in its interactions. The predicted presence of coiled-coil-forming domains in the longer Yuri isoforms suggests that these proteins may function as oligomers, in combination with other Yuri molecules or with other coiled-coil proteins. The absence of this structural motif in Yuri-29 indicates that it cannot participate in this type of interaction; therefore, to the extent that this small isoform/domain interacts with other proteins, its expression may serve to sequester those proteins away from longer Yuri isoforms.

4-8: Yuri immunolocalization in larval tissue

Because anti-Yuri Western blotting showed that Yuri is present in third-instar larvae, and enhancer-trap analysis indicated that regulatory elements near the yuri locus are active in certain tissues at this developmental stage, I wanted to determine the subcellular localization of Yuri in larval tissues. Wild-type larval tissues were dissected and stained by C. McGee with the affinity-purified antibody described above. She examined the stained tissues using epifluorescence microscopy, and I followed later with
confocal imaging. Anti-Yuri staining was present in all larval tissues examined. Although no c263 enhancer-trap activity was detected in the larval imaginal discs, brain lobes, or ventral ganglion, these tissues were strongly stained for Yuri (Figure 4-8, A-D). In addition, the larval ring gland, which did display enhancer-trap activity, was very strongly stained (Figure 4-8, A and B). Confocal images of imaginal discs showed that Yuri is present at the cell cortex in these tissues (Figure 4-8, C and D). This pattern is consistent with a cytoskeletal function for Yuri, as suggested by the enhancer-trap and protein-structural analyses described above. Protein-interaction assays (Chapter 7) show that Yuri physically interacts with components of the Actin cytoskeleton in the pupa; this localization pattern is also consistent with that result.

4-9: Yuri immunolocalization in adult muscle tissue

Because of interaction between Yuri and the Actin cytoskeleton shown in Chapter 7, I examined the localization of Yuri within muscle tissue, in conjunction with some of the cytoskeletal proteins that were identified in the interaction assay. (See Figure 4-9 for a schematic of muscle-tissue zones.) In non-fibrillar muscle tissue, Yuri appears to be present at the Z line and in the Actin-containing regions (Figure 4-10, A), but appears to be absent from the M line. Tropomyosin 1, the interactor purified in the greatest abundance in Chapter 7, is also present in the same regions (although it is not especially concentrated at the Z line). These patterns are consistent with the physical interaction between the two proteins identified below.

In fibrillar indirect flight muscle, in contrast, Yuri appears to be localized most strongly to the Z disc and the M line (Figure 4-10, B). The muscle-specific regulator of
FIGURE 4-8: Anti-Yuri stains most larval tissues; it stains a cortical meshwork in larval imaginal discs. A: Anti-Yuri immunoreactivity is visible in most larval tissues; shown here are the brain lobes and ventral ganglion, along with several imaginal discs. B: In this shot, the larval ring gland is more strongly stained than other tissues. C: In confocal micrographs, anti-Yuri staining appears as a meshwork at the cortex of imaginal-disc cells. D: An enlarged shot of the central part of panel C. Abbreviations: AD, antennal disc; BL, brain lobes; ED, eye disc; FB, fat body; LD, leg discs; ON, optic nerve; RG, ring gland; VG, ventral ganglion. Scale Bars: A: 500 μm; B: 250 μm; C: 20 μm; D: 10 μm.
FIGURE 4-9: Schematic of Drosophila muscle-fiber zones. Muscle tissue is composed of repeating elements called sarcomeres, containing two main types of cytoskeletal fibers: Actin "thin" filaments and Myosin "thick" filaments. Thin filaments are wrapped by Tropomyosin molecules, which regulate muscle activity; the thick filaments contain Myofilin, which is proposed to regulate the spacing of Myosin subunits. Sarcomeres are divided into zones named for their appearance under polarized light. The Z discs form the sarcomeric ends; these regions are rich in Actin cross-linking proteins such as alpha-Actinin, Spectrin, and Dystrophin, and these proteins anchor the thin filaments (Byers et al., 1989; Dhermy, 1991; Dubreuil et al., 1990; Roberts and Bobrow, 1998; Saide et al., 1989). The I bands are regions into which thick filaments move when the muscle is stimulated, and they also contain elastic or connecting filaments made up of, e.g., Sallimus (Bullard et al., 2002; Burkart et al., 2007), which anchor the thick filaments to the Z disc. The A band comprises the bulk of the sarcomere and contains interdigitated thick and thin filaments. At the midpoint of the A zone lies the H band, into which the thin filaments move upon stimulation; bisecting this band is the M line, composed of proteins that crosslink the thick filaments. (A large number of additional sarcomeric proteins have been identified, in addition to the representative proteins above.)
FIGURE 4-10: Yuri localization within muscle tissue. A: In non-fibrillar muscle tissue (body-wall muscle and “skeletal” muscle within appendages), Yuri is abundant at the Z line (white arrowheads) and present in the I and A bands. Tm1 is present at these locations as well, although it is not as concentrated at the Z line. Both are absent from the H band/M line (black arrowheads). B and C: In fibrillar tissues such as indirect flight muscle, Yuri is abundant at the Z and M lines, but appears to be reduced in the I and A bands. Yuri, Tm2, and Myofilin are all present in the Z disc. (Tm1, Tm2, and Myofilin are also present in the not-shown tissue type. Muscle zones were assigned by reference to the published patterns of Tm1, Tm2, and Myofilin localization. Actin is present at the same sites as the Tm1 and Tm2.) Scale bar in all panels: 10 μm.
Actin dynamics Tropomyosin 2 is present at the Z disc and in the Actin-containing A and I zones, but not the H zone; thus, the two could possibly interact at the Z disc. One of the other proteins identified in the interaction assay, Myofilin, is present at high levels in the A band and the Z disc and at somewhat lower levels in the I bands; it is absent from the H zone and M line (Figure 4-10, C, and Qiu et al., 2005). Thus, Myofilin and Yuri could conceivably interact at the Z disc.

4-10: Conclusions

Sequencing of cDNAs from the yuri locus support the existence of at least four types of yuri transcript; anti-Yuri Western blotting confirms the existence of the proteins encoded by these transcripts. These proteins are expressed in varying combinations in every examined tissue, indicating that they take part in a common cellular process. Tissues that do not express the c263-GAL4 enhancer trap nevertheless do express Yuri protein itself, indicating that c263-GAL4 does not capture the entire Yuri expression pattern; this is consistent with the idea that yuri expression is disrupted by the transposon in only a subset of tissues. The localization of Yuri to the cell cortex of imaginal discs and its co-localization with Actin, Tropomyosin 1, Tropomyosin 2, and Myofilin in muscle tissue are consistent with its functioning as part of the Actin cytoskeleton and with the physical interactions detected in Chapter 7.

The concentration of Yuri at the Z line, and its appearance at the M line of some muscle tissues, where no Actin filaments are present, suggests that Yuri may interact with proteins present at these locations, in addition to or instead of Tropomyosin/Actin. The Z disc and M line contain several proteins that cross-link Actin and Myosin filaments,
respectively. Intriguingly, the Actin cross-linkers alpha-Actinin and Spectrin, present at the Z line, are rich in coil-forming sequence (COILS server, data not shown), suggesting that a coiled-coil interaction with extended Yuri isoforms may be possible.

4-11: Materials and methods

cDNA clone analysis

CDNA clones were obtained from the Drosophila Genetics Resource Center (DGRC, Bloomington, IN) as plasmid DNA adsorbed onto a small (~1-mm) disc of proprietary blotter paper. Each disc was rinsed very briefly in TE to remove a transformation-inhibiting residue, per the instructions received with the DNA, and then each disc was incubated in 10 μL of TE for ten minutes at room temperature to elute the bound plasmid DNA. “Top10” competent cells (Invitrogen) were transformed with 5 μL of the eluate, using the manufacturer’s protocol, and were plated on LB+ampicillin or LB+chloramphenicol plates, as appropriate. Plates were incubated at 37° overnight; colonies were picked from each plate and grown in 2-mL LB+antibiotic cultures overnight at 37° with shaking. Each culture was miniprepped using QiaQuick spin columns and protocol (Qiagen), and DNA was eluted in 30 μL sterile water. The cDNA clones were sent to Lone Star Labs (Houston, TX) for sequencing from both ends, using primers within the vector near the cloning site as indicated in the list below, and primers within the yuri gene.

Cloning vectors used by the BDGP:

pOTB7: Chloramphenicol-res.; SP6 - EcoR I - 5' cDNA 3' - Xho I - T7
pOT2: Chloramphenicol-res.; T7 - EcoRI I - 5' cDNA 3' - Xho I - SP6

pFLC-I: Ampicillin-res.; T7 - BamHI 1 - 5' cDNA 3' - Xho I - T3

Clones obtained:

AT03435, AT15480, AT19027, and AT25733 – Adult testis library in pOTB7;

GH14032 – Adult head library in pOT2;

GM25777 and GM26781 – Adult ovary library in pOT2;

SD06513 and SD11641 – S2 cell library in pOT2; and

RE12523 and RE13793 – Embryo library in pFLC-I.

Preparation of anti-Yuri antibodies

To express recombinant Yuri-29 protein for the creation of antibodies, the Yuri-29 coding region was amplified from the GH14032 cDNA using primers that added a 5' EcoRI I and a 3' Xho I, and the resulting fragment was cloned into the pET-28a(+) lactose-inducible HIS-fusion expression vector (Novagen) to create pET-28a(+)–yuri-29 (Dr. R. Munjaal). This vector was transformed into BL21(DE3) competent cells (Novagen), and the HIS-tagged protein was purified in the following way by R. Simonette. Cells from overnight LB+ampicillin cultures (10 mL) were pelleted and resuspended in fresh LB+ampicillin (to remove beta-lactamase secreted into the medium) and diluted 1:100 into 1 L fresh LB+ampicillin. Cultures were incubated at 37° with shaking, and culture growth was monitored by taking periodic optical-density readings at 650 nm. When an absorbance of 0.5-0.8 was reached, expression of the fusion protein was induced by adding isopropyl-beta-d-thiogalactopyranoside (IPTG) to a concentration of 1 mM, and protein was expressed for 4 hours at 37°.
Cells were pelleted by centrifugation and resuspended in a small amount of PBS. The cells were lysed using the Emulsiflex system, and 6×His-tagged Yuri-29 protein was harvested by passage through a nickel-NTA agarose resin (Novagen). The resin was rinsed with three column volumes of PBS and suspended in 1× thrombin cleavage buffer (Novagen). Yuri-29 was released from the resin by overnight cleavage with thrombin protease (Novagen). The eluted protein was concentrated using a Centricon columns (Millipore), and the protein concentrate was sent to Aves Labs, Inc. (San Diego, CA) for generation of anti-Yuri-29 IgY in two hens.

**Affinity purification of anti-Yuri antibody**

The two IgY pools received from Aves Labs were separately affinity purified by R. Simonette in the following way. Untagged recombinant Yuri-29 was immobilized on NHS-activated Sepharose 4 Fast Flow beads (Amersham) using the manufacturer's protocol. Anti-Yuri IgY solution was passed through this resin, and the resin was washed with PBS. IgY reactive to Yuri-29 was eluted with elution buffer (100 mM glycine, pH 2.8), and the eluted fractions were immediately brought to neutral pH by addition of 0.1 volume neutralization buffer (1 M Tris, pH 8.5). Purified anti-Yuri IgY from the two hens was tested against head extracts and recombinant Yuri-29 by Western blotting, and the more specific and sensitive batch was used for all later experiments. The affinity purification resulted in a roughly 1:10 dilution of antibody from its initial concentration; the diluted antibody was used at 1:100, for a 1:1,000 final dilution.
Bioinformatics

Coil-forming domains in the four predicted Yuri isoforms and in various other proteins were predicted using the “COILS” server at www.ch.embnet.org/software/COILS_form.html, using default settings; this server automates the algorithm of Lupas et al. (1991). Sequence comparisons against sequenced Drosophila genomes (Celniker et al., 2002; Consortium, 2007; Crosby et al., 2007; Stark et al., 2007) and the genomes of other sequenced insects (Mita et al., 2004; Xia et al., 2004) were conducted using the FlyBase BLAST service using default settings, except that the “low complexity filter” was disabled. Searches against all published nucleotide sequences were conducted using the NCBI BLAST service at www.ncbi.nlm.nih.gov/blast, using default settings. Searches for conserved protein domains were conducted using the NCBI Conserved Domain Detector service at www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi, using default settings. Potential yuri promoter regions were predicted using the Neural Network Promoter Prediction server at www.fruitfly.org/seg_tools/promoter.html (Reese, 2000; Reese, 2001; Reese et al., 2000).

Western blots

The yuri locus lies at chromosomal position 35C5 on the left arm of the second chromosome (2L). The homozygous-lethal yuri-deficiency chromosome Df(2L)do1 lacks a ~1.2-megabase region (34F5-35D2) containing 118 genes (Ashburner et al., 1981) and was obtained from the Bloomington stock center (stock #3212) as “Df(2L)do1, pr1 cn1/In(2LR)Gla, wgGla-1 DNApol-γ352". The In(2LR) inversion chromosome was
replaced with the CyO-GFP balancer by crossing the stock to “w; noc<sup>Sc</sup>/CyO, P<sup>GAL4-HSP70.PB</sup>TRI, P<sup>UAS-GFP.Y</sup>TRI” (Bloomington stock 5702) (Rudolph et al., 1999) and collecting and mating fluorescent offspring.

For Figure 4-5, A (R. Simonette), embryos were collected from Df(2L)do1/CyO-GFP parents on grape plates. Collected embryos were dechorionated in bleach and incubated in moist chambers for 24-48 hours at room temperature to allow Df(2L)do1/CyO-GFP heterozygotes to hatch. Unhatched embryos were sorted into deficiency homozygotes (not fluorescent), CyO-GFP homozygotes (green fluorescence), and unfertilized eggs (which have a distinctive appearance) using a Leica MZ12 epifluorescence dissecting scope. Sorted embryos were ground in 3× SDS loading buffer in Eppendorf tubes using plastic pestles (Fisher). The extract was boiled, spun to pellet insoluble debris, and electrophoresed on a 12.5% polyacrylamide gel at 150 V for ~2 hours. Proteins were blotted onto Immobilon PVDF membrane (Millipore) at 400 mA for 3 hours, after which the membrane was dried in open air overnight to reduce nonspecific background staining. Membranes were blocked in Blotto (TBS + 0.05% Tween-20 + 5% Carnation powdered milk) for 2 hours at room temperature, and incubated with affinity-purified anti-Yuri (1:100 in Blotto) for 2 hours. Membranes were washed 3×5’ in TTBS (TBS + 0.05% Tween-20) and incubated with horseradish peroxidase-conjugated goat anti-chicken antibody (Sigma; 1:100,000 in TTBS). Membranes were again washed 3×5’ in TTBS, followed by 3×5’ in TBS (to remove Tween, which inhibits the chemoluminescence reaction). Membranes were incubated for 5’ with chemoluminescence reagent (Pierce Dura or West Pico), blotted dry, and exposed to film.
For Figure 4-5, B (M. Texada), w^{1118} embryos were collected on grape plates over 2- or 6-hour periods, as appropriate, and aged at room temperature as necessary. Embryos were dechorionated in bleach and analyzed as above; each lane represents thirty embryos. For Figure 4-5, C (C. Johnson and M. Texada), w^{1118} and yuri^{F64} heads, thoraces, testes, ovaries, and third-instar larvae were collected and analyzed as described above. Extracts equivalent to 15 testes (7.5 pairs), 3 heads, 0.5 third-instar larva, 3 thoraces, and 15 ovaries (7.5 pairs) were loaded per lane.

**Embryo collections**

Flies were lightly anaesthetized with CO₂ and placed into a perforated three-cornered plastic beaker. A "grape plate" of egg-laying substrate in a 50-mm Petri dish was smeared with yeast paste and placed over the opening, and the junction was sealed with Parafilm to prevent escape or infiltration of flies. The egg-collection setup was left in this inverted state until the flies became active again, to prevent their becoming mired in the yeast paste. The container was righted and incubated at room temperature for the desired period of time, after which the grape plate was replaced with a new one by lightly banging the flies to the bottom of the beaker and rapidly swapping the plates. The used plates were incubated for a desired time before eggs were harvested to allow development to occur.

Eggs were collected from plates by washing the plates with a solution of 50% bleach in dH₂O (plates were not re-used). Eggs and yeast paste were suspended by agitating with a paint brush, and the suspension was sieved through a square of Nitex fabric held in a cut-off 50-mL Falcon tube by the screw top, which was hollowed to allow
passage of fluid. The filter was placed in a small glass dish and was incubated in 50% bleach for 30 seconds to 1 minute to dissolve the protective chorion layer. Dissolved material and bleach were washed away with distilled water, and the embryos were collected either in bulk with a paint brush, or individually by placing the filter on a moist grape plate and selecting individual embryos with a metal dissecting probe.

**Embryo fixation**

Dechorionated embryos were collected from the Nitex filter using a paint brush and placed in an Eppendorf tube containing 0.6 mL hexane (to permeabilize the vitelline membrane) and 0.6 mL of 4% paraformaldehyde in PBS. The tube was rotated at room temperature for 10 minutes. Damaged embryos and other materials sink to the bottom of the tube (in the aqueous phase), and fixed embryos float at the aqueous/organic interface. The aqueous phase was removed, along with the materials within, and 0.6 mL of methanol was added to the tube. The tube was shaken vigorously for 30 seconds to disrupt the embryos' vitelline membranes; after shaking, de-vitellinized embryos sank to the bottom of the lower (methanol) phase. If many embryos remained at the hexane/methanol interface, the shaking was repeated. Both phases of liquid were removed, and 1 mL of 100% methanol was added; the tubes were rotated for a few seconds, and the embryos were allowed to settle. The methanol was replaced, and the process was repeated several times to remove all remaining hexane and formaldehyde. Embryos were then stored in methanol at -20°.
Embryo Staining

Embryos were rehydrated through a stepped gradient of 75%, 50%, 25%, and 0% methanol in water and blocked in PBTN (PBS + 0.2% Tween-20 + 5% normal goat serum) for 30 minutes at room temperature with rotation. (Because embryos were de-vitellinized and stored in methanol, which alters the structure of Actin filaments, phalloidin cannot be used to stain them; monoclonal anti-Actin and a labeled anti-mouse secondary were used instead.) Blocked embryos were stained overnight at 4° with primary antibodies diluted in PBTN. Unbound primary antibody was removed with three 5-minute washes of 1 mL PBTN, and secondary antibodies were diluted in PBTN and incubated with the embryos in the dark at room temperature for 1-2 hours with rotation. Unbound antibody was removed with three 5-minute washes of 1 mL PBTN, and stained embryos were mounted in 50% glycerol/water containing 0.1% Hoechst 33342 (Molecular Probes/Invitrogen) DNA stain, if desired.

Fixation of larval and adult tissues

Larval and adult tissues were dissected in PBS and collected in a PBS-filled Eppendorf tube on ice. The PBS was replaced by PBS + 4% formaldehyde, and fixation was allowed to proceed for 15 minutes at room temperature with rotation. The fixative was removed, and the tissues were washed three times for 1 minute with BBX (PBS + 0.3% Triton-X-100 + 0.1% BSA). Tissues were blocked in BBGS (BBX + 2% goat serum) for 1 hour at room temperature with rotation; primary antibodies were diluted in BBGS and incubated with the tissues overnight at 4° with rotation. Unbound antibody was removed with three 1-mL washes of BBGS. Secondary antibodies were diluted in
BBGS and incubated with tissues at room temperature for 1-2 hours with rotation, in the dark; if desired, fluorophore-labeled phalloidin was added with the secondary antibodies. Unbound label was removed with three 1-mL washes of BBX. Tissues were mounted in 50% glycerol (with 0.1% Hoechst 33342 DNA stain, if desired).

**Muscle-protein GFP fusion lines**

To demarcate different muscle zones and to localize Tm1 and Tm2, for which suitable antibodies are unavailable, several transgenic “exon-trap” fly lines expressing GFP-tagged Drosophila fusion proteins from their endogenous loci were obtained from various sources. GFP-Kettin line G53 and GFP-MSP-300 line G129 (Morin, 2003; Morin et al., 2001) were obtained from W. Chia (King’s College, London) and used to mark the Z-disc (Hakeda et al., 2000; Volk, 1992). Tm1-GFP lines CC01710, CC02057, and YC0096 were obtained from the FlyTrap project (flytrap.med.yale.edu) (Buszczak et al., 2007; Cooley et al., 2002; Kelso et al., 2004; Quiñones-Coello et al., 2007). The Tm2-GFP insertion line Tm2\textsuperscript{ZCL2456} (Cooley et al., 2002) was obtained from the Bloomington stock center. GFP retains its fluorescence after formaldehyde fixation, but to enhance the GFP signal, fixed GFP-expressing tissues were stained with mouse anti-GFP (Zymed) and Alexa Fluor-488-labeled goat anti-mouse.

**Antibody dilutions**

Affinity-purified chicken 4012 anti-Yuri: 1:100 (1:1,000 final dilution; the affinity purification resulted in a 1:10 dilution factor). Mouse monoclonal anti-Futsch 22c10 (Fujita et al., 1982, from DSHB): 1:50. Mouse monoclonal anti-gamma-Tubulin

**Microscopy**

Images were obtained using a Zeiss AxioPlan instrument and MetaMorph software (Molecular Devices), a Zeiss LSM-410 confocal microscope and LSM software, or a Zeiss AxioSkop and AxioVision software.
CHAPTER 5: GENERATION AND CHARACTERIZATION
OF STRONGER *yuri* ALLELES

5-1: Background

The phenotype of the original *yuri* mutation, the *P(GawB)* transposon insertion c263, is limited to a subtle behavioral defect. However, widespread c263-*GAL4* enhancer-trap reporter activity (Chapter 3) and the ubiquity of Yuri expression as shown by Western blotting (Chapter 4) and immunostaining (Chapters 4 and 6) suggest that Yuri isoforms perform a function in the animal that is more fundamental than the modulation of gravity-related behavior. To investigate this hypothesized fundamental role, we sought to create stronger *yuri* mutant alleles that might cause more severe phenotypes.

To make these new alleles, we took advantage of the imperfect repair of double-stranded DNA breaks that can arise when a P-element is transposed away from its site of insertion. In attempting to resolve these breaks, cells sometimes create deletions or other lesions at the breakage site; this type of event is called an "imprecise excision," even though the error arises not in the excision event *per se* but in the DNA repair afterwards. Through a series of crosses (Figure 5-1), Z. Ali, Dr. J. D. Armstrong, S. Bell, J. Jordan, I. Husain, Dr. R. Munjaal, and F. Sultan generated potential mutant chromosomes through transposase-mediated excision of two different P-elements inserted into the *yuri* locus in different lines: the c263 *P(GawB)* transposon identified in our screen, as well as a *P(SUPor-P)* insertion called KG03019, generated by the Drosophila Gene Disruption Project (Bellen et al., 2004; Roseman et al., 1995) and obtained from the Bloomington
\[
\begin{align*}
\frac{w}{w} & \cdot \frac{c263(w^*)}{c263(w^*)} + \times \frac{w}{w} \cdot \frac{Sco}{Y} \cdot \frac{Dr \Delta 2-3}{CyO} \cdot \frac{TM3, Sb}{+} \rightarrow \frac{w}{w} \cdot \frac{c263(w^*)}{Y} \cdot \frac{Dr \Delta 2-3}{Sco} \cdot + \\
\frac{w}{w} \cdot \frac{c263(w^*)}{Y} \cdot \frac{Dr \Delta 2-3}{Sco} \cdot + \times \frac{w}{w} \cdot \frac{Sco}{CyO} \cdot + \rightarrow \frac{w}{w} \cdot \frac{c263^*}{w/Y} \cdot \frac{+}{CyO} ; + (\text{Single animals}) \\
\frac{w}{w} \cdot \frac{c263^*}{w/Y} \cdot \frac{+}{CyO} ; + \times \frac{w}{w} \cdot \frac{Sco}{Y/w} \cdot \frac{+}{CyO} ; + \rightarrow \frac{w}{w} \cdot \frac{c263^*}{Y} \cdot \frac{+}{CyO} ; + \text{ and } \frac{w}{w} \cdot \frac{c263^*}{w} \cdot \frac{+}{CyO} ; + \\
\frac{w}{Y} \cdot \frac{c263^*}{Y} \cdot \frac{+}{CyO} ; + \times \frac{w}{w} \cdot \frac{c263^*}{w} \cdot \frac{+}{CyO} ; + \rightarrow \text{Potentially mutant stock}
\end{align*}
\]

FIGURE 5-1: The crossing scheme by which potential \textit{yuri} mutant chromosomes were created. Homozygous insertion animals were mated to a line carrying P-element transposase. Male offspring from this cross, in whose germ line the transposon-mobilization event takes place, are mated to a marked stock to create potentially mutant offspring; an alteration of the P-element is marked by a reversion to white eyes. Individual white-eyed animals are mated again to the \textit{Sco/CyO} balancer-chromosome stock to create multiple animals bearing the same potentially mutant chromosome; curly-winged sibling offspring are then mated together, to create homozygous animals (along with curly-winged siblings). If all offspring are curly-winged — that is, they are all heterozygous, this is an indication that a homozygous-lethal mutation has arisen somewhere on the altered chromosome. (These are not necessarily at the \textit{yuri} locus — see text for discussion.)
stock center. Roughly 100 homozygous-viable excision chromosomes and 11 homozygous-lethal chromosomes were generated from these two insertions.

The initial excision chromosomes created by transposon excision are carried in a heterozygous state with a non-recombining homologous chromosome carrying a dominant marker mutation (CyO, which causes curly wings). Heterozygous excision animals are mated together to generate animals homozygous for the excision chromosome, along with CyO heterozygous offspring. For ~90% of the excision chromosomes, these homozygotes were present in the expected ratio to heterozygotes, and a fertile, viable homozygous stock was created. However, in 11 cases, no homozygous offspring were created, indicating the presence of a homozygous-lethal mutation somewhere on the excision chromosome (not necessarily at the yuri locus, since the excised transposon can be reinserted at a new location and then re-removed, creating second-site mutations). Different characterization algorithms were used for these two types of chromosome.

5-2: Characterization of homozygous-viable excision lines

In cases in which a homozygous stock of a given excision chromosome was created, molecular characterization of any alterations at the yuri locus could be conducted in a straightforward way using the homozygous DNA. I conducted the analysis of these lines through a PCR-based algorithm, shown in Figure 5-2, with C. Johnson, R. Munjaal, A. Shankar, and F. Sultan. The first reaction performed on any homozygous excision line was an attempt to PCR across the former transposon insertion site with a pair of primers located ~400 bases upstream and downstream of this location. A successful reaction
FIGURE 5-2: The basic excision-characterization algorithm. A “walking” method is used to map genomic and intra-P-element deletions.
would indicate the removal of all or most of the P-element sequence; a product of a
different size from the wild-type control product would indicate the presence of a
genomic deletion or a small remnant of the excised transposon. Products of successful
reactions were cloned into the pCR2.1 or pCR4 (Invitrogen) or pGEM-T (Stratagene)
PCR cloning vectors and sequenced (by Lone Star Labs, Houston, TX). Although no
deletion chromosomes were identified by this first reaction, several “precise” excision
lines were identified in this way to be used as controls in various behavioral experiments.

A failed reaction indicated that a fragment of transposon sequence too large to
span in the reaction remained at the insertion site, or that a genomic deletion of one or
both primer sites had taken place. To differentiate between these two situations, a pair of
reactions to ascertain the presence of one or both ends of the P element were performed,
using each primer used previously with a primer that anneals to the nearby P-element
end. If both end-reactions were successful, indicating that the changes to the
chromosome were most likely internal to the P-element, the line was discarded. If one of
the reactions failed, however, suggesting a deletion in that region, a “PCR walking”
algorithm was followed until undisturbed genomic DNA was reached, and then a
fragment was generated spanning the disturbed region, cloned, and sequenced.

5-3: Characterization of homozygous-lethal excision chromosomes

In eleven cases, homozygous stocks could not be generated from a given excision
animal, indicating a homozygous-lethal defect on the second chromosome, not
necessarily at the yuri locus. To map the lethality to the yuri locus, I conducted a series
of deficiency crosses, in which the putative mutants were mated to animals carrying a
chromosome lacking a very large region containing the *yuri* gene (and 117 others). If the lethality of an excision chromosome was due to a loss-of-function defect at the *yuri* locus, no offspring carrying the excision and the deficiency chromosome would be expected to survive, having no functional *yuri* gene. However, for 10 of the 11 homozygous-lethal excision lines, these offspring were present and able to survive to adulthood (data not shown), indicating that the lethal mutation on the excision chromosome was not within the region "uncovered" by the deficiency chromosome. In the eleventh line, excision LE1, characterized by C. Johnson, the lethality was associated with the uncovered region, although not necessarily with *yuri* itself (see Results below).

Even though the homozygous lethality of most lethal excision chromosomes was not due to defects at the *yuri* locus, it was still possible that a non-lethal *yuri* defect was present on the chromosome, in addition to the lethal second-site mutation; if this was found to be the case, the second-site lethal mutation could be removed by recombination, leaving a "clean" *yuri*-mutant chromosome. To characterize the *yuri* gene in these lines, a source of DNA not containing the *CyO* chromosome (required for keeping the excision chromosome heterozygous), which presumably carries a wild-type *yuri* allele, was needed. The *CyO* balancer of some of the lethal lines was replaced with a *CyO-GFP* balancer chromosome (Rudolph et al., 1999); this enabled me to identify homozygous excision-chromosome embryos (by their lack of GFP fluorescence), which were harvested and used in the PCR-based algorithm described above. In the other cases, animals from the deficiency crosses described above carrying only one copy of the *yuri* locus – the one on the excision chromosome – were harvested for analysis.
5-4: Results

Roughly 100 potential mutant chromosomes were created by excision of the c263 or KG03019 P-elements; the excision chromosomes were analyzed by the PCR algorithm outlined above. Several "precise" excisions were identified for use as control lines in behavioral experiments. The lethality of 10 of the 11 homozygous-lethal chromosomes was shown to arise from defects located far from the yuri locus (in fact, several of these "lethal" lines were found to carry wild-type yuri alleles). Most of the homozygous-viable excision chromosomes harbored deletions only within the P-element itself, and those without an obvious phenotype were discarded. However, four excision lines were identified that exhibit interesting phenotypes or carry deletions within the yuri locus: R7, L5, LE1, and F64.

The R7 allele of yuri, created by Dr. R. Munjaal, is a deletion of sequences within the c263 P-element itself, with no alteration of the genomic DNA at the yuri locus, suggesting an alteration of yuri expression caused by the changes within the transposon. Homozygous yuri<sup>R7</sup> animals were identified by Munjaal as being "sluggish" and exhibiting a low frequency of antennal-segment duplications. Sluggishness or ataxia is commonly seen in mutants with mechanosensory defects – feedback from mechanosensory systems is necessary for the animal to move (Caldwell and Eberl, 2002; Eberl et al., 2000; Kernan et al., 1994), suggesting that yuri<sup>R7</sup> animals may exhibit anatomical or functional defects in mechanosensory organs. Because of the low penetrance of these phenotypes, however, we have not analyzed these animals in great detail.
The L5 deletion chromosome was created by Dr. R. Munjaal. This chromosome is homozygous-lethal, but deficiency tests showed that its lethality does not arise from defects near the yuri locus. Nevertheless, I found that this chromosome also carries a 346-base-pair deletion of sequences upstream of the predicted yuri transcription start site (Figure 5-3). Anti-Yuri Western blots of yuri<sup>L5</sup>/Df(2L)do1 animals showed that expression of all Yuri isoforms from this allele is attenuated by approximately 90% (C. Johnson, not shown); however, this reduction in Yuri abundance does not cause any obvious defect in survival, fertility, or activity level (C. Johnson, not shown). Because of the second-site homozygous-lethal mutation and the lack of any obvious phenotype, we have not studied this allele further.

The yuri<sup>LE1</sup> homozygous-lethal allele was created by S. Bell. Deficiency crosses show that the lethality of this chromosome does arise from the chromosomal region near the yuri locus (C. Johnson); Johnson also showed that this allele is a large deletion extending upstream of the yuri gene for at least 2 kilobases, reaching into the adjacent upstream gene, guftag (gft). Mutant guftag alleles are homozygous-lethal (Mistry et al., 2004), and this gene is also within the deletion carried by the deficiency chromosome used to map the line's lethality; therefore, the observed lethality may arise from gft defects rather than yuri defects. Because of the confounding gft mutation, we have not analyzed this mutation further.

Meanwhile, the F64 deletion (created by F. Sultan) has been studied intensely because homozygous males were observed to be completely sterile (Munjaal, Beckingham, and Johnson). The deletion was sequenced by Dr. R. Munjaal and shown to
FIGURE 5-3: The yuri-locus deletions, to scale. The first exons of yuri are shown in blue on the right of the image; the transcripts that encode 29- and 102-kDa Yuri isoforms are represented by the upper row, and the transcripts that encode the 64- and 64-kDa isoforms are represented by the lower two rows. The 3'-most exon of guftagu, the adjacent gene upstream of yuri, is shown on the left. The site of insertion of the c263 and KG03019 transposons is indicated by a vertical black bar. The yuriF6d allele is a deletion of 578 base pairs between the former transposon-insertion site to a point just 5' of the translation-initiating ATG of the various yuri transcripts. The transcriptional start sites and most of the 5' untranslated region are deleted; however, much more of the transcripts that encode the 64- and 65-kDa isoforms is removed, which may explain why these isoforms are most severely affected by the deletion. The yuriL5 allele is an upstream deletion of 346 base pairs, presumably containing regulatory sequences. The lethal yuriLE1 deletion spans at least 2 kilobases of sequence extending upstream from the site of transposon insertion, removing at least the last exon of the upstream gene, guftagu. Small tick marks on scale mark 200-base-pair intervals.
be a deletion of 578 base pairs extending from the former KG03019 insertion site into the yuri transcribed region, deleting the predicted initiation sites of all Yuri transcripts as well as most of their 5' untranslated sequence but leaving unaffected the Yuri open reading frame (Figure 5-3). Anti-Yuri Western blotting showed that the 64- and 65-kDa Yuri isoforms are not detectably expressed in any tissue in yuri^{F64} homozygotes, and the smallest 29-kDa isoform is not detectable in mutant testes (Figure 3-5, C). However, the 102-kDa isoform is still expressed at normal levels, and the 29-kDa isoform is still present in most tissues, indicating the presence of a cryptic transcription-initiation site somewhere in the upstream region. Most of the deleted region is transcribed into the 5' UTR of the mid-length transcripts; since there is no obvious reason that these transcripts could not be initiated from the same cryptic start site as the long and short transcripts, there may be functional elements within the deleted 5' UTR sequences that are required for the splicing together of exons #8 and #9, rather than #8 and #10 (Figure 3-3). The lack of Yuri-29 expression in the testis suggests the existence of a testis-specific mechanism of regulating the splicing of exon #2 as well.

Fertility is not rescued in male yuri^{F64}/Df(2L)do1 hemizygotes, indicating that their sterility is most likely due to the yuri-locus defects. Heterozygous yuri^{F64}/CyO animals show no reduction in fecundity in offspring-counting assays (data not shown), indicating that 50%-reduced levels of Yuri-29/-64/-65 expression in the testis is sufficient for the production of at least some viable sperm. However, as will be seen later (Chapter 6), the normal fertility of yuri^{F64}/CyO males is actually associated with some defects in spermatogenesis.
5-5: Materials and methods

Excision crosses

Transposons were excised using a stable genomically integrated P-transposase source, called Δ2-3 (Robertson et al., 1988). The crossing scheme for c263 excisions is laid out above in Figure 5-1. Because few deletions were obtained from c263 excisions, another transposon, KG03019 (Bloomington stock #13563), was also mobilized in the hope that the spectrum of deletions would be different; the crossing scheme is analogous to that for c263 excisions. Several series of excision crosses were carried out by Dr. J. D. Armstrong, Summer Bell, Inna Hussain, Zahra Ali, Dr. R. P. Munjaal, and Faraz Sultan.

DNA isolation

Flies of interest were gathered under CO₂ and placed into an Eppendorf tube. Tubes were placed at -20° for 10' to kill the animals. These flies were then used in one of two DNA isolation protocols:

**DNA isolation from many flies using DNAzol**

Roughly 10 μL DNAzol (Invitrogen) per fly was added to the frozen flies (200 μL min.; 1 mL max.). Flies were thoroughly ground in the Eppendorf tube using a clean, sterile plastic pestle (Fisher), and the mashate was incubated at 85° for 10 minutes to denature most proteins. The tubes were spun in a microcentrifuge at maximum speed for 5 minutes to pellet insoluble material (cuticle, polysaccharides, denatured protein) and to float lipids. The clean phase was removed to a new Eppendorf tube, and 0.5 volumes of ice-cold 100% ethanol was added. Tubes were mixed by inversion and incubated at -20°
for at least 30' (up to overnight). DNA was pelleted by spinning at maximum speed for 30'; the supernatant was removed, and the pellet was washed with 1 mL 70% ethanol. After another 5' spin, the wash was removed, and the tubes were left open for at least 10' at room temperature to allow any remaining ethanol to evaporate. Dried DNA was resuspended in sterile water, at a concentration of 10 µL per fly, and was stored at -20° until use.

**Proteinase-K-based DNA isolation from single flies**

*Squishing Buffer (SB):*
- 10 mM Tris, pH 8.2
- 1 mM EDTA
- 25 mM NaCl
- 200 µg/ml Proteinase K
- Stored at -20° until use.

The single fly was thoroughly mashed in an Eppendorf tube using a pipette tip containing 50 µL SB, without expelling the liquid. After grinding, the remaining SB is expelled into the tube. Proteinase K was allowed to degrade fly proteins for 20' at 37°, after which it was inactivated by a 5' incubation at 95°. Insoluble materials were pelleted by centrifugation, and the supernatant was moved to a new tube and stored at -20°.

**PCR reaction setup**

Each PCR reaction contained the following:

- 2 µL DNA
- 5 µL 10× Taq reaction buffer
- 0-4 µL 25-mM MgCl₂ solution, as needed
- 1 µL forward primer (stored at 100 µM)
- 1 µL reverse primer (stored at 100 µM)
- 1 µL dNTP stock (10 mM each dNTP)
- 1 µL forward/reverse Actin primers, if desired (DNA quality control)
- N µL ddH₂O to a total of 49.5 µL
- 0.5 µL Taq polymerase (1 U) (added last)
PCR programs

PCR was conducted on the departmental Perkin-Elmer machine or the Beckingham lab’s Eppendorf MasterCycler machine. The general program used was the following:

1: 95° for 2’
2: 95° for 30’’
3: (lower Tm of primer pair – 5°) for 30’’
4: 72° for 1’ per kb of expected product, + 30’’
5: Repeat from step 2, 34x
6: 72° for 10’
7: 16° until retrieved

Cloning

PCR products were electrophoresed directly after generation with the addition of 6x loading dye, in 1% agarose/TAE gels, with ethidium bromide DNA stain at 0.5-2 μg/mL in the gel itself and in the 1x TAE running buffer. Bands representing PCR products to be sequenced were visualized with the departmental UV transilluminator and excised from the gel using a clean scalpel blade; DNA was extracted from the agarose block using the Qiagen PCR Purification Kit and accompanying protocol, and eluted in 20-50 μL ddH2O, depending on the concentration desired.

Purified PCR products were ligated into cloning vectors for amplification in E. coli using one of two kits: the pCR2.1 and pCR4 topoisomerase-based cloning kits from Invitrogen, or the Stratagene pGEM-T system.
Invitrogen pCR2.1/4-TOPO cloning system

This system makes use of Topoisomerase-I covalently linked to dT overhangs on the pCR2.1 or pCR4 vector. When dA-tailed PCR product (such as those generated by Taq polymerase) base-pair with these overhangs, the topoisomerase ligates the ends together and is released from the molecule. Four microliters of purified PCR product were added to 1 μL 6x buffer (provided) and 1 μL pCR vector; the mixture was incubated at room temperature for 30 minutes, and was used in a standard transformation reaction. pCR2.1 makes use of blue/white screening for identification of correct clones; IPTG (to induce expression of beta-galactosidase) and X-Gal (a color-forming beta-galactosidase substrate) were spread onto ampicillin-containing plates several hours before use (to allow for evaporation of solvents). pCR4 colonies are viable only if they contain a cloned PCR product, so no IPTG or X-Gal was added.

Stratagene pGEM-T cloning system

This system also makes use of the dA overhangs produced by Taq polymerase but relies on standard ligation techniques instead of topoisomerase. Reactions comprised 7 μL purified PCR product, 1 μL 10x T4 ligase buffer, 1 μL pGEM-T vector (Stratagene), and 1 μL T4 ligase (3 U) (New England Biolabs) and were incubated overnight at 16° before being used in standard transformation reactions. pGEM-T makes use of blue/white screening, so ampicillin plates were prepared as for use with pCR2.1 above.

TENS Plasmid DNA Miniprep

TENS buffer:
10 mM Tris-HCl, pH 7.5
1 mM EDTA
0.1 N NaOH
0.5% SDS

Cultures were pelleted by centrifugation in Eppendorf tubes, 1.5 mL at a time, at maximum speed for 1'. All but 100 μL supernatant was poured away; cells were completely resuspended in the remainder. 300 μL TENS was added and tubes were mixed by several inversions. Tubes were incubated for 5' at root temperature, after which 150 μL 3-M NaOAc (pH 5.2) was added. Preps were mixed by inversion and incubated on ice for 5'. Insoluble material was pelleted by spinning for 10' at maximum speed, and supernatants were transferred to another Eppendorf tube containing 900 μL ice-cold 100% ethanol. Preps were mixed by inversion and incubated on ice for 15-30 minutes, after which precipitated DNA was pelleted by centrifugation at maximum speed for 10'. Pellets were washed with 1 mL 70% ethanol and dried at room temperature for 10'. DNA was resuspended in 50 μL ddH₂O and stored at -20°.

**Sequencing at Lone Star Labs**

Colonies were picked from plates and grown in 3-mL LB + 100 μg/mL ampicillin overnight at 37° with shaking. Cultures were miniprepped using Qiagen QiaQuik columns and protocol or using the “TENS” protocol described above. Miniprep DNA from pGEM-T and pCR2.1 was sometimes restriction-digested to ensure that DNA of the correct size had been cloned. DNA was then sent to be sequenced at Lone Star Labs, Inc., (Houston, TX), using either primers located within the cloning vector itself (e.g., T7) or primers within the cloned product; if custom primers were used, they were sent at a concentration of 3.2 μM, as recommended by Lone Star Labs (generally, 3.2 μL of 100-
μM primer stock was diluted with 97 μL ddH₂O). The returned sequences were mapped against the Drosophila genome using the FlyBase BLAST server.
CHAPTER 6: SPERMATOGENESIS PHENOTYPES OF THE \textit{yuri}^{F64} DELETION

6-1: Background

The male-sterility phenotype of \textit{yuri}^{F64} homozygotes is intriguing, in light of the fact that Type-I mechanosensory neurons and sperm cells are the only ciliated cells in Drosophila (e.g., Drosophila cells do not possess primary cilia). Several mutations have been found that cause the syndrome of mechanosensory deficits and male sterility, due to axonemal defects: \textit{touch-insensitive larvae B (tilB)} (Eberl et al., 2000; Kernan et al., 1994), \textit{uncoordinated (unc)} (Baker et al., 2004; Kernan et al., 1994), \textit{Pericentrin-like protein (cp309)} (Martinez-Campos et al., 2004), and \textit{smetana} (Caldwell and Eberl, 2002). I conducted a series of immunostaining experiments to identify the defects underlying the sterility seen in \textit{yuri}^{F64} males, and thereby to gain insight into the function of Yuri in mechanosensation, as well.

6-2: Outline of \textit{Drosophila melanogaster} spermatogenesis

The testis is a coiled tubular organ, with spermatogenic stem cells at one tip and the seminal vesicle for storage of mature sperm at the other (Figure 6-1). Through a series of mitotic and meiotic divisions, each stem cell gives rise to a cluster of 64 haploid nuclei partially separated by plasma membranes, but linked by cytoplasmic bridges; the cluster is called a cyst, and each cyst of germ-line nuclei is wrapped by a pair of somatic cyst cells. As the nuclear divisions occur, the cyst is pushed away from the stem-cell tip down the length of the testis by continued cyst formation.
**FIGURE 6-1: Overview of Drosophila spermatogenesis.** 1: A germ-line stem cell gives rise to a cluster of 64 haploid sperm nuclei (blue), linked by cytoplasmic bridges within a single plasma membrane (brown); somatic stem cells give rise to "cyst cells" that enclose the bundle of nuclei. The single centriole associated with each nucleus becomes linked to the nuclear membrane and transitions into a basal body. 2: The nuclei condense, each basal body extends a microtubular axoneme (black), and the cyst becomes elongated as the ends spread apart. 3: After elongation and condensation are complete, individualization of the syncytial sperm takes place through the action of the Actin-rich "investment cones" (red). Excess cytoplasm is jettisoned from the distal end of the sperm in a membrane-bound waste bag. 4: Individual mature sperm are coiled and (5) stored in the seminal vesicle. Multiple cysts at all of these stages of development exist within each testis.
After the final meiotic divisions within a cyst, the single centriole accompanying each nucleus attaches to the nuclear membrane and undergoes a transition into a basal body, which gives rise to the axonemal sperm tail (Figure 6-2). One face of the nuclear membrane becomes associated with a microtubule-rich complex (the "dense complex"), which stretches from the basal-body pole of the nucleus to the opposite pole (where the acrosome will eventually form) (Tates, 1971; Tokuyasu, 1974). As the cyst moves towards the seminal-vesicle end of the testis, it elongates to accommodate the lengthening axonemes, and concurrently each of the 64 nuclei undergoes a shape change, condensing from a large spherical structure via an intermediate spindle shape to a final tightly bundled needle-like nucleus, with the basal body at one end (Figure 6-2). The microtubule-rich surface undergoes a corresponding narrowing during the condensation process, forming a stripe along the acrosome–basal-body axis; in addition, all the mitochondria within the cell fuse to form two very large mitochondrial derivatives ("major" and "minor") that extend the length of the axoneme.

By the time the nuclei mature and the axonemes have fully elongated, the nuclear end of the cyst has descended to the seminal-vesicle end of the testis. The 64 syncytial sperm must now be separated from one another and "invested" each with its own plasma membrane (Figure 6-1). This process relies on Actin-rich "investment cones" that form over the basal-body tip of each nucleus and travel by Actin treadmilling in unison down the length of the axoneme, stripping away excess cytoplasm and organelles and reforming the plasma membrane to remove cytoplasmic linkages between cells (Fabrizio et al., 1998; Noguchi et al., 2006; Noguchi and Miller, 2003; Tokuyasu and Lindsley, 1980; Tokuyasu et al., 1972a). The stripped-away waste materials are jettisoned as a bag
FIGURE 6-2: Spermatid nuclear development. Complete legend on next page.
FIGURE 6-2 (previous page): Spermatid development after the final nuclear division. One hemisphere of the nuclear membrane becomes associated with a microtubule-rich layer called the dense complex. The centriole becomes bound to the nuclear membrane at the center of the dense-complex hemisphere and transitions into a basal body, which begins to extend an axoneme. At this stage of development, the centriolar adjunct resembles a tubular sleeve around the length of the basal body. The mitochondria of the cell fuse to form the Nebenkern, which will become associated with the axoneme and eventually split into the major and minor mitochondrial derivatives. As the nucleus begins to condense, the dense complex transitions to cover one lateral aspect of the nucleus, with the basal body at its distal boundary. The centriolar adjunct begins to shorten and broaden. As the axoneme is extended, the mitochondrial derivatives lengthen alongside it. Towards the end of condensation, the dense complex remains associated with the nucleus, occupying an indentation along one side, and the centriolar adjunct has transitioned into a doughnut-shaped structure around the basal body. By the completion of nuclear condensation, the dense complex and the gamma-Tubulin centriolar adjunct have disappeared completely; the cytoplasmic, non-axonemal microtubules are also degraded at this time, and the Actin-based investment cones begin to form.

Abbreviations: Ax, axoneme; BB, basal body; CA, centriolar adjunct; DC, dense complex; MD, mitochondrial derivatives; Nk, Nebenkern; Nu, nucleus. The cytoplasmic bridges between sperm are not indicated. (Images adapted from Tates, 1971.)
of waste products at the stem-cell end of the testis. Once this process is complete, the
now-individualized sperm are coiled and stored in the seminal vesicle (Tokuyasu et al.,
1972b).

6-3: Individualization defects seen in yuri\textsuperscript{F64} animals

Spermatogenesis defects in yuri\textsuperscript{F64} animals were examined with the use of a don-
juan-GFP fusion transgene (Civetta, 1999; Santel et al., 1998; Santel et al., 1997). Don-
Juan-GFP is imported into the sperm-tail mitochondrial derivatives, thereby endowing
each sperm with a fluorescent tail. In control yuri\textsuperscript{F64}/CyO animals carrying this construct,
fluorescent immature sperm tails could be seen in the testis proper, and mature, coiled
sperm were seen to fill the seminal vesicle (Figure 6-3, A; R. Simonette). In yuri\textsuperscript{F64}
hemizygotes, however, no fluorescence was visible in the seminal vesicle, and sperm tails
were coiled improperly in the testis itself (Figure 6-3, B; R. Simonette), indicating that
the sterility of these animals arises from a failure to produce mature individualized sperm.

To identify any defects in individualization, the Actin-rich investment cones of
control and yuri\textsuperscript{F64} animals were stained using fluorophore-labeled phalloidin, which
binds specifically to filamentous Actin. In yuri\textsuperscript{F64}/CyO heterozygotes, nascent
investment cones were visible on the sperm nuclei (Figure 6-4, E; R. Simonette), and sets
of cones in all stages of progression down the length of the axoneme and into the waste
bags were seen (Figure 6-3, A', and A''; R. Simonette). However, little Actin
accumulation was seen at the tips of yuri\textsuperscript{F64} nuclei (Figure 6-4, F; R. Simonette), and no
well-formed investment complexes were visible at any stage of progression in yuri\textsuperscript{F64}
testes (Figure 6-3, B'; R. Simonette), although a few scattered individual cones were de-
FIGURE 6-3: Sperm-maturation defects seen in yuri$^{F64}$ animals. A: In control animals, sperm tails (visualized by Don-Juan-GFP expression) are coiled and stored within the seminal vesicle (arrow). A': Staining of the filamentous Actin muscle sheath of the testis outlines the seminal vesicle (black arrow). Progressing investment cones are also present (red arrow; magnified in A''). B: In yuri$^{F64}$ hemizygotes, fluorescently marked sperm tails are aberrantly coiled within the testis itself (arrowhead), and the seminal vesicle (arrow) is devoid of mature sperm. B': Filamentous Actin outlines the seminal vesicle (black arrow), which is empty; unusual Actin structures are also visible within the testis (red arrow). B'': At higher magnification, these structures appear as featureless tubes or blocks. Scale bars: 200 µm. (From Texada et al., in press.)
FIGURE 6-4: Filamentous Actin and nuclear-morphological defects in $yur{i^{F64}}$
testes. A-A'': The unusual Actin structures seen in $yur{i^{F64}}$ testes are contained within
the enclosing cyst cells. A: GFP expressed in the cyst cells; A': Actin structures; A'':
Overlay showing that the Actin is within the cyst cell. In orthogonal section, the Actin
structures are hollow tubes (not shown). B: These previously undescribed structures are
also visible in control ($yur{i^t}$) animals. C: In control animals, condensed sperm nuclei
are straight and tightly bundled (arrow). D: In $yur{i^{F64}}$ mutants, condensed nuclei are
loosely gathered together and are aberrantly curled into hooked or helical shapes
(arrows). E: In control animals, filamentous Actin of nascent investment cones is visible
on condensed nuclei (arrow). F: In $yur{i^{F64}}$ animals, this Actin is much less abundant
(arrow). G: Very infrequently, a few investment cones form and travel in a scattered
manner down the cyst in $yur{i^{F64}}$ mutants. **Scale bars:** A, C, D: 10 μm; B, E, F: 100
μm; G: 50 μm. (From Texada et al., in press.)
ected (Figure 6-4, G). Instead, collar-like structures were visible within the testis (Figure 6-3, B”; R. Simonette); genetic marker expression and optical cross-sectioning established that these structures were actually contained within the enveloping cyst cells rather than in the sperm-cell syncytium (Figure 6-4, A; Dr. W. Deery). Therefore, in yuri^{F64} animals, sperm never mature because investment cones never form properly; instead, the 64 sperm remain grouped within a single plasma membrane. This phenotype reflects a defect in Actin dynamics and suggests that Yuri may play a role in regulating these dynamics.

In addition to this defect in individualization, yuri^{F64} sperm nuclei also exhibit a morphological defect. Whereas the nuclei of wild-type sperm are straight and tightly bundled together before individualization begins (Figure 6-4, C), the condensed nuclei of yuri^{F64} mutants are often curved or helical and much less tightly bundled together (Figure 6-4, D), suggesting that Yuri may have a role in maintaining the shape of the condensed nuclei.

6-4: Yuri is localized to the sperm nucleus during cyst elongation and nuclear condensation

As described in Chapter 4, no Yuri-64/-65 is detectable by Western blot in any tissue of yuri^{F64} homozygotes, and in the testis, no Yuri-29 is detectable. The sterility phenotype of yuri^{F64} remains when this allele is in the hemizygous condition (that is, with a deficiency for the yuri region on the homologous chromosome), indicating that this phenotype is most likely due to the lack of one or both of these proteins in the testis.
Therefore, I examined the localization of Yuri protein in the testes of wild-type and yuri^{F64} animals using the pan-isofrom antibody described previously.

In wild-type testes, Yuri (of unknown isoform makeup) is distributed throughout the testis with a cytoplasmic and cortical distribution, especially in early-stage cysts (Figure 6-5, A). After the last nuclear division, however, Yuri protein becomes localized to the condensing sperm nuclei. Starting as a hemisphere over one surface of non-condensed round nuclei (Figure 6-5, C), the staining narrows as the nuclei condense, forming a stripe along one side of the spindle-shaped intermediate nuclei and a dot at the tip where the basal body is located (Figure 6-5, D). As nuclear condensation continues, the stripe continues to narrow and eventually disappears, leaving the dot structure (Figure 5, E and F). Just before the investment cones begin to form over the tips of the nuclei, the dot structure disappears as well (Figure 6-5, F); Actin cones and the Yuri “dot” have never been seen together. In yuri^{F64} mutants, however, no Yuri-staining structures are seen on the nuclei at any stage of development (Figure 6-7, A). Therefore, since Yuri-29 and -64/-65 are not detectably expressed in these animals, at least one of these isoforms is necessary for the formation these structures.

6-5: Localization of the centriolar adjunct to sperm nuclei requires Yuri

Because the Yuri “dot” is positioned at the tip of the nucleus where the basal body is located, I wanted to determine if Yuri is a component of the basal body itself. As a proxy for the basal body, I examined the localization of the centriolar adjunct (“CA”), a gamma-Tubulin-rich structure surrounding the basal body prior to individualization (Figure 6-2) (Tokuyasu, 1975; Wilson et al., 1997). In control animals, the CA, and
FIGURE 6-5: Yuri localization within control testes. A: Anti-Yuri broadly stains the testis, especially in the cytoplasm of dividing cysts. B: Stages of nuclear condensation corresponding to those in panels C-E (adapted from Tates, 1971). C: At the start of condensation, Yuri is present in a hemispherical pattern over the nuclear membrane. D: By the middle of condensation, the hemispherical Yuri staining has converged to a longitudinal stripe and a “dot” at the basal-body tip of the nucleus. E: The stripe fades as condensation progresses. F: The nuclear-tip dot remains after the stripe has disappeared, but it too eventually disappears before the nascent investment cones (red) appear. Scale bars: B: 100 μm; C-F: 10 μm. Inset in F: 2 μm wide. (From Texada et al., in press.)
therefore the basal body within, and Yuri “dot” do not overlap (Figure 6-6, A-C), indicating that Yuri is not a component of either structure, but the two structures are closely associated with one another, suggesting that perhaps Yuri plays a part in maintaining the attachment of the basal body to the nucleus. The basal body is anchored in a socket-like indentation in the nuclear membrane (Figure 6-6, C) (Tokuyasu, 1975), and Yuri may be a component of the dense complex that is important for this linkage.

In heterozygous yuri\(^{F64}/CyO\) testes, the anti-Yuri-staining structures appear to be properly formed, but gamma-Tubulin appears to be distributed along the Yuri “stripe” rather than adjacent to the “dot” (not shown), indicating either that the lack of one gene-dosage of Yuri-29 or Yuri-64/65 causes a haplo-insufficiency defect or that the ratio between Yuri isoforms is important for their proper function. In homozygous yuri\(^{F64}\) mutants, which do not exhibit visible Yuri structures, no gamma-Tubulin stain is detectable in association with the nuclei (Figure 6-7, B), indicating that Yuri is required for the recruitment of gamma-Tubulin to the CA or for the attachment of the CA to the nucleus or basal body.

6-6: Previously unreported Actin filaments are visible on the nucleus in a Yuri-dependent fashion

While examining the distribution of filamentous Actin in wild-type testes, I noticed that Actin filaments were present on the surface of condensing sperm nuclei (Figure 6-6, D and E). Co-staining with anti-Yuri revealed that the two proteins are localized to the same part of the nuclear membrane; in yuri\(^{F64}\) animals, no nuclear Actin filaments were detectable (Figure 6-7, C), demonstrating that these filaments require
FIGURE 6-6: Yuri, the centriolar adjunct, and Actin.  A: In pre-condensation nuclei, the gamma-Tubulin centriolar adjunct (CA; red) is visible at the center of the Yuri-staining region.  B: As condensation progresses, the CA becomes closely apposed to the Yuri “dot” structure.  C: A model for the relationship between Yuri and the CA: Yuri may line or be a component of the indentation of the nuclear membrane into which the basal body is anchored and against which the CA is juxtaposed.  (Line art adapted from Tokuyasu and Lindsley, 1980).  D: Filamentous Actin is visible over one surface of pre-condensation sperm nuclei, also forming a projection that presumably encompasses the basal body.  E: These Actin filaments are present on sperm nuclei during condensation, overlying both the “stripe” and “dot” components of the Yuri pattern.  F: Yuri is a component of the investment cones (seen here both head-on and from the side).

Scale bars: A-E: 10 μm; F: 20 μm. (From Texada et al., in press.)
FIGURE 6-7: Protein mis-localization in mutants.  

A: In yurif64 homozygotes, no Yuri staining is present on sperm nuclei at any stage of development (here, mid-condensation).  

B: The gamma-Tubulin-rich CA is not detected in association with yurif64 sperm nuclei.  

C: The Actin filaments visible on sperm nuclei in controls are not detectable at any stage of development in yurif64 mutants.  

D: In testes from Dlc90F65/90 dynemin light-chain mutants, the Yuri staining pattern is altered: the hemispherical pattern is reduced in intensity (arrowhead), the “dot” structure appears prematurely (arrows), and a nascent second “dot” structure is visible (asterisks) in addition to the normal structure.  

E: At later stages of nuclear condensation in these Dlc90F mutants, the Yuri “dot” appears normal (arrow), but the Yuri “stripe” structure is not detectable (arrowhead); the novel “dots” (asterisks) appear more discrete.  

F: The CA fills the space between the normal and novel Yuri dot structures.  Scale bars:  A-C: 20 μm; D-F: 10 μm.  (From Texada et al., in press.)
some combination of Yuri-29, -64, and -65 for their formation, maintenance, or localization. Thus, since Yuri is dependent on the microtubule network for its localization, it appears that Yuri may act as a bridge connecting the Actin and microtubule cytoskeletons during spermatogenesis.

6-7: **Proper Yuri localization depends on the microtubule cytoskeleton**

The Yuri staining pattern on the nuclear membrane is similar to the known pattern of enrichment of microtubules. I was unable to visualize these tubules specifically (the available reagents are not specific for dense-complex tubules, detecting instead all microtubules as well as free beta-Tubulin monomers), so to determine the relationship between the Yuri pattern and these tubules, I made use of a mutation in \textit{Dlc90F^{05090}}, a dynein light chain mutant that perturbs microtubule dynamics during spermatogenesis (Caggese et al., 2001; Li et al., 2004). In \textit{Dlc90F^{05090}} mutant testes, the hemispherical Yuri staining over early-stage nuclei is lost or severely reduced in intensity. In more-condensed nuclei, the apical Yuri punctum is visible, but a second Yuri-staining structure appears distal to it with the centriolar adjunct in between and the longitudinal Yuri stripe is not present (Figure 6-7, D-F). The precise nature of the mutant Yuri punctum is not known, but its presence may reflect an inappropriate interaction with the apical end of the basal body. In any case, the correct nuclear localization of Yuri during spermatogenesis appears to depend on the proper functioning of the microtubule nucleoskeleton.
6-8: The basal body itself is present but sometimes mis-localized in yuri$^{F64}$ sperm, and defects in localization of a centrosomal protein are apparent in these animals

Since the CA was missing from yuri$^{F64}$ sperm, I wanted to determine whether the basal body itself was present. I obtained antibodies against Centrosomin (Cnn), a component of the centrosome (Heuer et al., 1995; Li and Kaufman, 1996; Li et al., 1998), from Dr. T. Kaufman (Bloomington, Indiana) and stained wild-type testes to determine the relationship between Yuri and Cnn. Unfortunately, it appears that Cnn is lost from the centrosome at the time of its association with the sperm nuclear membrane (not shown), so it could not be used to trace basal-body dynamics in yuri$^{F64}$ animals.

Other antibodies against centriolar or basal-body components are not readily available. To determine if the basal body itself is missing or mis-localized in yuri$^{F64}$ animals, a transgene encoding a centriole-targeted GFP fusion protein was crossed into the yuri$^{F64}$ mutant background by Dr. K. Beckingham. This transgene encodes a green fluorescent protein (GFP) tagged with a PACT ("pericentrin-associated centriolar targeting") domain, a fragment from the Drosophila centrosomal protein Cps09 that is necessary for the localization of that protein to the centriole and is sufficient to localize fusion proteins similarly (Martinez-Campos et al., 2004). In wild-type and yuri$^{F64}$/CyO animals, the tagged GFP is clearly visible, localized in a punctate pattern at the pole of each nucleus, from the division stages until the latest stages of nuclear condensation, at which time the staining is lost (Figure 6-8, A, B, D). In yuri$^{F64}$ homozygotes, however, instead of being localized to the centrosomes during the early stages of spermatogenesis, the GFP signal is spread across the surface of the nuclei (Figure 6-8, F), indicating that
FIGURE 6-8: **Nuclear orientation defects.** A: In controls, sperm nuclei at the seminal vesicle end of the testis are properly bundled, and the basal body (marked by GFP-PACT) is properly associated with the caudal nuclear tip. GFP-PACT association with the nucleus is lost after the completion of nuclear condensation (arrowhead). B: In *yurt* P64 mutants, sperm nuclei are scattered throughout the cyst (arrowheads), and the basal bodies are frequently mis-positioned at the wrong end of the nucleus, even in bundles of nuclei at the proper location within the cyst (arrows). C and D: Bundles of
(Figure 6-8, continued) condensed nuclei are present at the stem-cell end of the testis (indicated by an asterisk) in yuri$^{F64}$ heterozygotes and homozygotes (arrows), with their basal-body ends oriented towards the seminal vesicle end; the axonemes associated with these nuclei are therefore “backwards” with respect to normal axonemes, giving rise to the reversed-chirality axonemes in the TEM figures below. **E:** In control animals, GFP-PACT forms puncta in primary spermatocytes (arrow). **F:** In yuri$^{F64}$ mutants, GFP-PACT appears as a diffuse layer over part of the nuclear membrane. **Scale bars:** **A-D:** 20 µm; **E, F:** 100 µm. (A-D from Texada et al., in press.)
Yuri is required for proper localization of the PACT domain at these stages. At later stages, GFP-PACT appears to have condensed into puncta, indicating that Yuri is not strictly necessary for eventual centriolar/basal-body protein localization of this protein. Interestingly, this first mis-localization of GFP-PACT in yuri<sup>F64</sup> homozygotes occurs at a stage in spermatogenesis at which Yuri has not yet localized to the nucleus, indicating defects in a Yuri-dependent cytoplasmic process.

Homozygous yuri<sup>F64</sup> sperm frequently exhibit GFP-PACT puncta mis-localized to the wrong tip of condensed nuclei (Figure 6-8). Intriguingly, condensed nuclei were also observed at the stem-cell end of the testis in both heterozygotes and homozygotes (Figure 6, C and D), indicating a loss of the normal mechanism that keeps the nuclei of a cyst grouped together. Each basal body associated with these each mis-localized nuclei is directed towards the seminal vesicle; therefore, the axonemes associated with these nuclei are anti-parallel to those of normal sperm, giving rise to the “reversed” appearance of some axonemes seen in TEM images (below).

6-9: **Axonemal ultrastructural defects are present in homozygous and heterozygous yuri<sup>F64</sup> mutants**

Since defects in localization of components of the centriole and centriolar adjunct are apparent in yuri<sup>F54</sup> animals, we examined the axonemal ultrastructure of yuri<sup>F64</sup> sperm tails. R. Simonette prepared testes of wild-type and heterozygous and homozygous yuri<sup>F64</sup> animals for transmission electron microscopy (TEM), which were sectioned and imaged by Dr. Wenhua Guo of the Rice TEM facility and Dr. Kenneth Dunner, Jr., of the M. D. Anderson Cancer Center microscopy core facility.
In cross-sections of wild-type testes, the axoneme and mitochondrial derivatives of each sperm tail were clearly visible (Figure 6-9, A); each axoneme is associated with one major and one minor mitochondrial derivative (MMD and mMD, respectively; Figure 6-9, A), and cysts of a range of maturities, from early elongating cysts to mature cysts of fully individualized sperm, were present (not shown). In testes from \textit{yuri}^{F64} homozygotes, since the investment cones do not form in these testes, no mature cysts of individualized sperm were visible. Unusual associations between axonemes and mitochondrial derivatives are apparent, with some axonemes associated with multiple MMD’s and some MD’s shared between axonemes (Figure 6-9, C-G). It may be the case that these aberrant associations are not \textit{caused} by the \textit{yuri} defect \textit{per se}, but are merely \textit{allowed} by the lack of individualization in these mutant cysts; however, since all wild-type cysts also go through a syncytial phase but do not show such associations, these defects may reflect a direct effect of Yuri loss. In the mutant cysts, a fraction of the axonemes also exhibit ultrastructural defects: some are split into two parts, others are open on one side (Figure 6-9, F and I), and others are missing one or both of the internal singlet microtubules or the linker arms that connect them to the outer tubule doublets (Figure 6-9, G and H). In addition, some axonemes were observed to have the appearance of reversed chirality (Figure 6-9, D-F); this is presumably due to the “backwards” sperm described above.

Strikingly, the same types of defects seen in \textit{yuri}^{F64} homozygotes were also seen in \textit{yuri}^{F64}/\textit{CyO} heterozygotes (Figure 6-9, B). To rule out the possibility that the defects arose from aberrations in the \textit{CyO} chromosome, testes from +/\textit{CyO} and \textit{yuri}^{F64}+/ animals were also examined; only the \textit{yuri}^{F64}+ animals displayed defects, indicating that they
FIGURE 6-9: Transmission electron micrographs of testis cross-sections showing ultrastructural defects. A: In w¹¹¹⁸ animals, each mature sperm cell comprises a single axoneme ("Ax," of "9×2+2" microtubular structure) and a pair of mitochondrial derivatives (major, "M," and minor, "m"), enclosed by a plasma membrane. B: Cysts from yuri¹⁶⁴/CyO animals contain mixtures of mature (upper half) and malformed (lower half) sperm. C: Cysts from yuri¹⁶⁴ homozygotes contain no individualized sperm; in
(Figure 6-9, continued) addition, the mitochondrial derivatives are not properly formed (line arrows). **D-I**: Mutant cysts exhibit several types of defect. Axonemes sometimes exhibit split or missing outer microtubule rings (arrows), or are lacking the internal singlet microtubules or the linker arms that attach them to the outer doublet ring (arrowheads). Abnormal associations of mitochondrial derivatives with axonemes are also apparent: axonemes contact multiple derivatives of the same type, derivatives appear to be shared between two or more axonemes, and derivatives are sometimes not associated with any axoneme. Axonemes of opposite apparent helicity are present, indicated by colored arrows, reflecting the fact that some nuclei are at the opposite end of the cyst and thus extend their axonemes in the opposite direction. **Scale bars**: **A-F**: 500 nm; **G-I**: 250 nm. (From Texada et al., in press.)
arise from the $yuri^{F64}$ mutation. Therefore, the proper gene-dosage of, or ratio between, Yuri isoforms is required for wild-type function. (Recall that the localization of gamma-Tubulin is also disrupted in the $yuri^{F64}$ heterozygotes.) Although some heterozygous cysts contained a mixture of normal and aberrant sperm (Figure 6-9, B), the presence of abnormal sperm apparently does not hinder the ability of the properly formed sperm to mature, coil, and be stored in the seminal vesicle: heterozygous $yuri^{F64}$ males and wild-types produce similar numbers of offspring when mated with wild-type females. (The number of females and the rate at which eggs could be produced, rather than the number of functional sperm, was likely the limiting factor in these experiments.)

6-10: Tropomyosin 1 is present on condensing sperm nuclei

Because the Actin-dynamics regulator Tropomyosin 1 was identified in an assay for proteins that interact with Yuri-64 in vivo, and I hypothesize that Yuri interacts with this protein directly, I wanted to determine if this molecule is present on the sperm nuclei during the same developmental stages as Yuri. I dissected testes from animals expressing Tm1-GFP from the endogenous Tm1 locus, stained them with anti-GFP, and observed them by fluorescence microscopy. Anti-GFP stained the sperm nuclei during condensation (Figure 6-10, A and B), but not after the completion of the process (arrowhead, Figure 6-10, A). The presence of Tm1 on the nuclei during condensation is consistent with the presence of Actin and Yuri, and its loss afterwards is consistent with the loss of Yuri.
FIGURE 6-10: Tropomyosin-1 is present on condensing sperm nuclei. **A:** Nuclei undergoing the condensation process (arrows) exhibit anti-Tropomyosin-1-GFP staining. Fully condensed nuclei (arrowhead) do not. **B:** A closer view of spindle-shaped condensing nuclei, stained for Tm1-GFP. **Scale bars:** **A:** 20 μm; **B:** 10 μm.
6-11: Conclusions

Yuri is normally present throughout the testis. Through the mitotic and meiotic divisions, the protein appears to be primarily cytoplasmic, but once nuclear condensation begins, Yuri assumes a dynamic nuclear localization pattern like that of the dense complex. This localization appears to require the proper function of the microtubule cytoskeleton. Actin filaments are present on the nucleus, co-localizing with Yuri. Tropomyosin 1 is also present on condensing nuclei; it disappears after the condensation process is complete. Near the end of condensation, Yuri appears to form a socket-like shape around the proximal tip of the basal body, juxtaposed against the centriolar adjunct. Prior to individualization, Yuri localization to the nucleus is lost. Yuri is a component of the Actin-rich investment cones that individualize the syncytial sperm.

In yuri^{64} homozygotes, Yuri-29, -64, and -65 are not expressed in the testis. This defect leads to several types of cytological defects. The basal-body-targeted GFP-PACT fusion protein appears diffuse, rather than punctate, at early stages of spermatogenesis. The centriolar adjunct is not detected on condensing nuclei in these animals, and it appears spread down the length of the nucleus in heterozygotes. Filamentous Actin that is normally co-localized with Yuri is no longer present on the nuclei; Tml1 is presumably lost as well. At later stages, the basal body is often localized to the wrong end of the nucleus. Condensed nuclei appear to be less rigid than wild-types, since they often assume helical or twisted shapes, and they also are frequently mis-localized at the wrong end of the cyst. Properly formed sets of investment cones never form on condensed nuclei, so the sperm consequently never are individualized. Axonemal defects, such as
splits in the outer doublet-microtubule ring and missing internal singlet tubules or linker arms, are sometimes present in both heterozygotes and homozygotes.

These defects suggest that Yuri is involved in Actin dynamics and basal-body and axonemal function during spermatogenesis. Since Yuri localization depends on the function of the microtubule cytoskeleton, Yuri may act as a linker between the two cell-structural systems. The basal-body, centriolar-adjunct, and axonemal defects may arise indirectly as a result of mis-regulation of Actin dynamics in the absence of Yuri; alternatively, Yuri may be required directly to localize one or more proteins critical to the function of the centriole/basal body.

Like Yuri, the Uncoordinated protein (Unc) is Drosophila-specific and rich in coiled-forming sequence (Baker et al., 2004; Kernan et al., 1994). Like Yuri, Unc is localized to the basal-body tip of the nucleus and is required for the formation or retention of the centriolar adjunct; all these similarities to Yuri raise the intriguing possibility that the two proteins physically interact to address some insect-specific peculiarity of basal-body function. Like Yuri and Unc, TilB (Kavlie et al., 2007), Cnn (Heuer et al., 1995), and Cp309 (Kawaguchi and Zheng, 2004; Martinez-Campos et al., 2004) are predicted to be highly coiled-coil-forming (COILS server, data not shown) and are required for mechanosensation and male fertility, but they are evolutionarily conserved through the vertebrates; Yuri or Unc could conceivably interact with one or more of them as a connection between conserved and insect-specific protein networks. A ~30-residue stretch near the N terminus of the PACT domain is also predicted to form a short coil (data not shown).
6-12: Materials and methods

Fixation and staining of testes

Testes were dissected in PBS and collected in a PBS-filled Eppendorf tube on ice. The PBS was replaced by PBS containing 3% formaldehyde, and tissues were fixed for 10 minutes at room temperature with rotation. The fixative was removed, and the tissues were washed three times for 1 minute with BBX (PBS + 0.3% Triton-X-100 + 0.1% BSA). Tissues were blocked in BBGS (BBX + 2% normal goat serum) for 1 hour at room temperature with rotation; primary antibodies were diluted in BBGS and incubated with the tissues overnight at 4° with rotation. Unbound antibody was removed with three 1-mL washes of BBGS. Secondary antibodies were diluted in BBGS and incubated with tissues at room temperature for 1-2 hours with rotation, in the dark. If desired, fluorophore-labeled phalloidin was added with the secondary antibodies. Unbound label was removed with three 1-mL washes of BBX. Tissues were mounted in 50% glycerol + 0.1% Hoechst 33342 DNA stain. An easy way to ensure that all tissue was mounted was to wash the tissue from the Eppendorf tube into a 50-mm Petri dish with two aliquots of BBX, and to transfer the stained tissues to the droplet of mounting medium with dissecting tweezers.

Tropomyosin-1-GFP lines

To localize Tm1, for which suitable antibodies are unavailable, several transgenic "exon-trap" fly lines expressing GFP-tagged Drosophila Tm1 fusion protein from its endogenous locus were stained with anti-GFP. Tm1-GFP lines CC01710, CC02057, and
YC0096 were obtained from the FlyTrap project (flytrap.med.yale.edu) (Buszczak et al., 2007; Cooley et al., 2002; Kelso et al., 2004; Quiñones-Coello et al., 2007).

**Antibody and phalloidin dilutions**

Affinity-purified chicken 4012 anti-Yuri: 1:100 (1:1,000 final dilution; the affinity purification resulted in a 1:10 dilution factor). Mouse monoclonal anti-gamma-Tubulin GTU-88(Sigma): 1:100. Mouse monoclonal anti-beta-Tubulin E7 (DSHB): 1:100. Rabbit anti-Centrosomin R19 (Heuer et al., 1995): 1:200. Mouse anti-GFP (Zymed): 1:50. All fluorophore-labeled goat secondary antibodies (Invitrogen and Vector Labs) were diluted 1:500. Fluorophore-labeled phalloidin was used at 1:50.
CHAPTER 7: TANDEM AFFINITY PURIFICATION
OF YURI-INTERACTING PROTEINS

7-1: Background

Most proteins function by interacting with other proteins. Few clues about Yuri function can be gleaned from its sequence, except that it may form a coiled-coil domain, a motif that is often present in proteins associated with membrane trafficking or cytoskeletal function. The identification of proteins that physically interact with Yuri will therefore be of great help in identifying its physiological role. To do this, I chose to use a technique called tandem affinity purification ("TAP"), which involves the use of two affinity tags with different binding modes, separated by a protease cleavage site, to reduce non-specific co-purification of contaminant proteins (Gould et al., 2004; Puig et al., 2001; Rigaut et al., 1999). The serial use of two tags is analogous to the use of a size-exclusion column followed by an ion-exchange column during protein purifications, but is much more selective.

Many different combinations of tags and proteases have been tested (e.g., Burckstummer et al., 2006). The TAP tag in the following experiments comprises a Protein-A moiety, which is very tightly bound by IgG-agarose beads; a tobacco etch virus (TEV) protease site, to release bound complexes from the IgG matrix; and a calmodulin-binding peptide (CBP), which will be bound by calmodulin-agarose beads in the presence of Ca\(^{2+}\) (thus, purified complexes can be released from the calmodulin-agarose matrix by the addition of EGTA, or by boiling to denature the calmodulin). A schematic of the fusion protein is shown in Figure 7-1, and an outline of the procedure is shown in Figure 7-2. After purification of Yuri-nucleated complexes, their protein components are con-
FIGURE 7-1: Schematic of TAP-Yuri-64 fusion protein. An N-terminal IgG-binding Protein-A moiety is the first affinity tag; this region binds extremely tightly to IgG-agarose beads, so washed complexes must be cleaved from them with tobacco etch virus (TEV) protease. A Calmodulin-binding peptide then is used in a second affinity purification over Calmodulin-agarose beads. Calmodulin and the target peptide bind only in the presence of Ca$^{2+}$; bound complexes can therefore be eluted from the beads by the addition of the Ca$^{2+}$ chelator EGTA (or by boiling, which is more efficient).
Extracts are passed over an IgG-agarose column, to which the Protein A moiety binds (along with, perhaps, several contaminant proteins).

Unbound contaminant proteins are washed away, and TEV protease is added to cleave tagged Yuri complexes from the beads.

After cleavage with TEV protease, tagged Yuri complexes are eluted from the beads (along with the protease and some contaminants, perhaps).

These eluates are incubated with CaM-agarose beads in the presence of calcium, allowing Ca\(^{2+}\)-loaded CaM to bind to the Calmodulin-binding peptide component of the tag.

TEV protease and remaining contaminants are removed by washing with a buffer containing Ca\(^{2+}\), which prevents the release of tagged Yuri complexes from the beads.

Complexes are released from the beads by the addition of EGTA, which chelates Ca\(^{2+}\) and causes Calmodulin to release the tagged protein, or by boiling, which denatures the Calmodulin.

FIGURE 7-2: Schematic of tandem affinity purification procedure.
centrated by trichloroacetic acid (TCA) precipitation, separated on an SDS-PAGE gel, and stained with silver or Coomassie Brilliant Blue. Selected bands can then be excised with a razor blade, and the proteins they contain can be identified by mass spectrometry, a very sensitive technique that requires only ~5 ng of protein.

This method has great advantages over techniques such as yeast two-hybrid or co-immunoprecipitation. The use of two tags separated by a protease cleavage site requires a protein to contain three specific components in the correct relationship to be carried through the purification, greatly reducing the number of non-specific contaminant species. (Suppose that 10% of endogenous proteins non-specifically bind IgG, 10% have a sequence cleavable by TEV protease, and 10% bind calmodulin; one protein in a thousand will have all three elements, and in only one sixth of these – about two of the ~14,000 distinct Drosophila proteins – will they be in the correct order to allow passage through the procedure.) By placing expression of the tagged protein under the control of the GAL4/UAS system, the tagged protein can be expressed in any tissue of interest in whole Drosophila by driving its expression with an appropriate GAL4 driver, allowing isolation of different “suites” of interacting proteins from different tissues or developmental stages. The use of inducible TAP-tagged proteins to identify interactions in whole Drosophila has been reported only twice previously (Veraksa et al., 2005; Yang et al., 2006). The purification as described here is thus also a native in vivo technique, meaning that normal post-translational modifications will be made to the tagged protein, which is generally not the case in heterologous expression systems such as in a yeast-two-hybrid set-up.
7.2: Experimental design

Because, as described in Chapter 3, we hypothesize that the c263 GAL4 expression pattern broadly reflects where the behavioral phenotype of c263 arises, I expressed my UAS-TAP-yuri-64 construct under the control of this GAL4 driver; and because c263-GAL4 is most highly expressed during pupal development, I used mid-stage pupae as starting material. I found that 30 grams of tissue per preparation yielded enough purified material for mass-spectrometric protein identification. Two negative-control extracts, prepared from animals carrying heterozygous c263-GAL4 alone or heterozygous UAS-TAP-yuri-64 alone, were analyzed in addition to the experimental extracts, prepared from pupae heterozygous for both c263-GAL4 and the most highly inducible P[UAS-TAP-yuri-64] insertion.

To verify that the tagged protein was expressed at usable levels under c263-GAL4 control, I performed an anti-Yuri Western blot of samples of the three starting materials; the resulting autoradiogram is shown in Figure 7-3. Only native Yuri-64 was detected in the absence of the UAS construct (first lane). A small amount of tagged Yuri-64 (with a total mass of ~85 kDa) was detected in extracts from animals carrying the UAS construct alone (second lane); however, low levels of "leaky" expression of UAS-regulated transgenes, induced by nearby active endogenous enhancer elements, is a common phenomenon. As seen in the third lane, the presence of c263-GAL4 greatly enhances expression from the UAS construct.

Protein solubility and binding can be modulated by adjusting the salt concentration; some proteins are insoluble in low-salt conditions, whereas higher salt content weakens ionic interactions between proteins and strengthens hydrophobic inter-
FIGURE 7-3: Expression of tagged Yuri-64 in starting materials for tandem affinity purification. In this anti-Yuri Western blot, three whole pupae were loaded per lane, from the two negative controls and the experimental material. The first lane represents animals carrying $c263$-$GAL4$ alone (three whole pupae); the dark band is endogenous Yuri-64, and no higher-mass tagged protein is detectable. The second lane contains extracts from animals carrying $UAS$-$TAP$-$yuri$-$64$ alone; the endogenous 64-/65-kDa Yuri band is present, as well as a small amount of tagged Yuri (the higher-mass band), which presumably is caused by active enhancers near the $P_{UAS}$-$TAP$-$yuri$-$64$ insertion site. The last lane is from animals carrying both $c263$-$GAL4$ and $UAS$-$TAP$-$yuri$-$64$; the presence of GAL4 causes a great increase in the expression of tagged Yuri-64, overwhelming the endogenous Yuri-64 band.
actions, causing some proteins (which could be either contaminants or proteins of interest) to denature, aggregate, or precipitate. To avoid losing information because of these effects, I performed the purification at three different salt concentrations – at physiological salt levels (175 mM total salts) and at low and high salt levels (50 mM and 1 M total salts).

7.3: Results

The purification performed at 1-M total salts (not shown) did not result in the isolation of any interacting proteins; analysis of fractions from different time points suggest that the tagged protein forms denatured aggregates under this condition. Purification at 175 mM total salt was more successful; a silver-stained gel of the final purified products is shown in Figure 7-4. The protein bands boxed in green were excised and identified at the Stanford Protein and Nucleic Acid Biotechnology Facility. The ~200-kDa band contained muscle Myosin II heavy chain (MHC, encoded by MHC), and the ~41-kDa band contained muscle Actin 57B (Act57b). These results are consistent with immunostaining data (Chapter 4) that suggest that Yuri and Actin colocalize in vivo.

The ~40-kDa band contained the protein encoded by CG16886, an uncharacterized gene which has orthologs throughout the eukaryotes: e.g., a putative fungal microtubule-binding protein (Loftus et al., 2005); protozoan articulins, which are constituents of their cortical cytoskeleton (Marrs and Bouck, 1992); and putative mouse proteins (data not shown). Thus, CG16886 may encode a component of the Drosophila cytoskeleton. In the two smallest bands, mass mapping identified the 21-kDa “Conserved Cuticle Protein at 84Ae” (Karouzou et al., 2007) and the 13-kDa protein encoded by “Ec-
**FIGURE 7-4:** Silver-stained gel containing eluates from the tandem affinity purification performed at 175 mM total salt, with original protocol. The left-most lane contains an extract from pupae carrying the c263 transposon alone; two contaminant bands are faintly visible in all three lanes. The second lane contains extracts from animals carrying only the UAS-TAP-yuri-64 construct. The third lane contains extracts from animals carrying both c263-GAL4 and UAS-TAP-yuri-64. Bands unique to or enhanced in this lane were excised (green boxes) and identified by mass spectrometry.
*dysone-dependent gene at 78e*" (Fechtel et al., 1988), both of which are putative larval and pupal cuticle proteins with many invertebrate orthologs (BLAST data not shown).

The yield from the protocol was very low, and contaminants are evident in the GAL4-alone lane, so I reworked the protocol to make it cleaner and more efficient. We also decided to perform the next preparation under conditions that would not solubilize muscle Myosin, which comprises ~60% of the fly’s protein, to reduce the possibility of contamination with muscle proteins. After revamping the protocol, I performed the purification at 50 mM total salts, a condition under which muscle Myosin is known to be insoluble (it precipitates below ~100 mM salt). The concentrated final eluates from the procedure were run on an SDS-PAGE gel and stained with Coomassie Brilliant Blue; the gel is shown below in Figure 7-5. This purification was much more successful than the previous one. Lane #1 contains pre-stained mass standards. Lane #2 (GAL4 alone) shows only one band, which is present in all three extracts; its mass suggests that it is calmodulin from the second affinity step, disassociated from its agarose matrix in the final elution step. The preparation is otherwise clean, with no visible bands, indicating that no Drosophila proteins are retained through the purification in the absence of tagged “seed” protein.

Some banding is visible in the “UAS alone” negative-control lane; these bands likely represent proteins bound to tagged Yuri expressed “leakily” from the inserted construct (Figure 7-3). These purified proteins are not background contaminants, because the genetic background of the animals in the three samples is the same. These proteins are most probably bound to the Yuri moiety of the fusion protein, not the attached tags – any proteins bound to the protein-A moiety have been eliminated by pro-
FIGURE 7-5: Coomassie-stained gel of eluates from tandem affinity purification performed at 50 mM total salt, with revised protocol. The extract containing no tagged protein is clear of visible contaminant Drosophila proteins (a strong Calmodulin band from one of the purification steps is present in all three extracts). The purified extract from animals carrying only UAS-TAP-yuri-64 contains proteins presumably bound to “leaked” tagged Yuri-64 (Figure 7-3). The last lane represents purified extract from animals driving expression of the tagged Yuri-64 with c263-GAL4. Bands unique to or enhanced in the experimental lane were excised (green and yellow boxes), and the strongest bands (green) were identified by mass spectrometry.
teolytic removal of this domain from the tagged protein, and endogenous calmodulin-like proteins should not be bound to the Calmodulin binding peptide moiety, because all the steps up to the calmodulin-agarose binding step contain agents to chelate bivalent cations, which these proteins require for target binding. The identity of these purified interacting proteins could possibly shed light on Yuri function, but since we have not identified the tissues in which the tagged construct is "leaking," we do not know where in the animal these interactions take place; therefore, no effort was made to identify the interacting proteins.

The lane containing the experimental extracts contains bands similar in mass to those of the "UAS alone" lane, as well as additional bands and bands of increased intensity, representing proteins that interact with Yuri-64 in tissues where c263-GAL4 is expressed. The bands boxed in green and yellow in the figure were excised from the gel, and the proteins within the bands boxed in green were identified by mass spectroscopy at the Stanford Protein and Nucleic Acid Biotechnology Facility.

The ∼200-kDa band contained an aggregate of Yuri, identified as the same isoform expressed as tagged protein. This aggregation, present after boiling in SDS, indicates that CBP-tagged Yuri-64 can trimerize through very tight interactions, perhaps at the coiled-coil domains; however, since we do not see such aggregates in blots of wild-type Drosophila extracts, the aggregation may be mediated by the CaM-binding peptide tag or may be caused by the high concentration of Yuri in the samples. In any case, the presence of Yuri in the final extract is a necessary indication that Yuri-containing complexes were successfully purified. The bands at ∼45 and ∼20 kDa contained fragments of Fat-body protein 1 (Fbp1), a transmembrane protein transporter found in the
fat body, the fly’s equivalent of the liver. Native Fbp1 is a 118-kDa protein, which is cleaved immediately after translation into fragments of 69 (N terminus) and 49 (C terminus) kDa; after some hours’ delay, the larger fragment is re-cleaved into fragments of 50 (N terminus) and 19 (internal) kDa (Burmester et al., 1999). Thus the fragmentation of the isolated protein reflects normal endogenous processes rather than degradation of the samples.

In addition to the small Fbp1 fragment, the band at ~20 kDa contained the muscle protein Myofilin (encoded by Zeelin1) (Qiu et al., 2005). The ~50-kDa band contained the muscle-activity regulator Troponin T (TnT, encoded by upheld), and the 35-kDa band contained the Actin-binding proteins Tropomyosin 1 (Tm1) and Tropomyosin 2 (Tm2). *(N.b.: Tropomyosin 1 is also sometimes called Tropomyosin II and cytoplasmic Tropomyosin; Tropomyosin 2 is also known as Tropomyosin I, Troponin H [TnH or TpnH], and muscle Tropomyosin.)*

I repeated this purification, and sent the putative Myofilin, Tropomyosin, and Troponin-T bands for re-identification. (To be certain that I sent the correct band representing TnT, I actually sent two bands of roughly 55 kDa.) The isolation of Tm1 and TnT were confirmed by this repetition. In addition, Actin57B, the same Actin isoform that was detected in the 175-mM salt purification, was identified in the TnT “backup” band. The re-isolation of Actin57B in the absence of MHC indicates that the interaction of Yuri-64 and Actin does not occur indirectly via MHC but is either via Tropomyosin/Troponin, or direct. Myofilin was not re-identified in the 20-kDa band.
7-4: Conclusions

Actin, MHC, Tm1, Tm2, TnT, and Myofilin are all components of muscle fibers. Actin fibers ("thin filaments") and Myosin fibers ("thick filaments") are the main structural and mechanical components. Tm1 and Tm2 proteins wrap the Actin filaments, preventing access to them at rest by the Myosin motor heads; after an increase in sarcoplasmic $[\text{Ca}^{2+}]$, a complex containing TnT causes Tropomyosin to move to a different site on the Actin filament, allowing Myosin access to the thin filament, thereby causing contraction. Myofilin is thought to be present within the Myosin fibers, perhaps functioning to regulate the spacing of Myosin monomers (Qiu et al., 2005).

Tropomyosin 2 is the prototypical Tropomyosin and is present only in muscle, with different isoforms expressed in different muscle types (Gremke et al., 1993). Tropomyosin 1, on the other hand, exists in muscle-specific and cytoplasmic isoforms (Hanke and Storti, 1988; Karlik and Fyrberg, 1986). Its cytoplasmic isoforms are thought to provide cytoskeletal Actin filaments with structural rigidity and resistance to mechanical and enzymatic disassembly or breakage. Interestingly, Tm1 is known to be a component of the scolopale rods of insect chordotonal organs (Wolfrum, 1992).

Based on their relative intensities in the extract gels, the most abundant putative Yuri interactors in the final eluates are the Tropomyosins. Troponin T binds very tightly to the Tropomyosins, and both of these proteins bind to Actin; thus the three may be co-purified as a complex. The co-staining of muscle tissue for Tm1 and Yuri is consistent with this interaction. Likewise, Myofilin and muscle Myosin are also thought to be interlinked (Qiu et al., 2005). The extraction buffers contain excess EGTA (i.e., no free $\text{Ca}^{2+}$), which should lead to the blocking of the Actin/Myosin interaction by Tropomyosin.
and Troponin; therefore, it is possible that there are two main complexes, one comprising Actin, Troponin, and Tropomyosin, and the other, Myosin and Myofilin, that both separately bind Yuri or are bound to each other through Yuri. The presence of Yuri and Myofilin staining at the Z disc in indirect flight muscle (Chapter 4) is consistent with this.

7-5: Materials and methods

Creation of pP[UAS-TAP-yuri-64]

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' EcoR I site and a 3' BamH I site,. The product was gel-purified and cloned into the Topoisomerase-based pCR4-Blunt cloning vector (Invitrogen). Clones were sequenced to avoid PCR-induced errors. The tag sequence was excised from the cloning vector by digestion with EcoR I and BamH I and cloned into EcoR I/Bgl II-cut pP[UAST] (Brand and Perrimon, 1993) to create pP[UAS-TAP], into which any appropriate open reading frame can be cloned to for GAL4-inducible expression of TAP-tagged fusion protein.

The Yuri-64 coding sequence was amplified from the SD06513 cDNA using Pfu Ultra II HS polymerase and primers that add a 5' Not I site and a 3' Kpn I site. The fragment was ligated into pCR4-Blunt, and clones were sequenced to avoid mutations.
The Not I-Kpn I fragment was ligated into Not I/Kpn I-digested pP\{UAS-TAP\} to create pP\{UAS-TAP-yuri-64\} (Figure 7-6). Ligation clones were sequenced to ensure that no

FIGURE 7-6: Schematic of pP\{UAS-TAP-yuri-64\}. TAP-tag coding sequence were ligated into pP\{UAST\} to create pP\{UAS-TAP\}. Yuri-64 coding sequences were ligated into this vector to create pP\{UAS-TAP-yuri-64\}. The ends of the linear map above are joined in the actual circular construct. (*Note that the BamH I/Bgl II hybrid site is no longer cleavable.*)
frame-shift mutation had been introduced in the cleavage and ligation steps. The plasmid was co-injected with a helper plasmid encoding P transposase into w118 embryonic germ line tissue by Genetic Services, Inc. (Sudbury, MA), and through a series of matings multiple independent homozygous transgenic fly lines were established. The fusion-protein expression level of each line was tested by mating to Heat-shock-GAL4 animals, heat-shocking adult offspring at 37° for one hour, followed by one hour of recovery, and assaying Yuri levels by Western blotting after a 1-hour recovery time.

Collection of experimental material

To create animals carrying both the UAS-TAP-yuri-64 transgene and the c263-GAL4 insertion, homozygous males of the highest-expressing UAS-TAP-yuri-64 line were mated to virgin female c263-GAL4 homozygotes in mass matings (~20 bottles of flies). To create the two negative-control samples, c263-GAL4 and UAS-TAP-yuri-64 animals were separately mated to w118 in mass matings. Every 9 days, the parental animals were moved to new food bottles. On the third, sixth, and ninth days after pupae first appeared in each bottle, the 0-to-3-day-old pupae were scraped from the inside of the bottle with a flat metal spatula and frozen at -80° until sufficient material was amassed (~30 grams of pupae per sample per experiment). Bottles were discarded after the third collection.

Tandem affinity purification
Lysis buffer:
25 or 150 mM NaCl
25 mM Tris (pH 8)
2.5 mM MgCl₂
5% glycerol
0.2% Nonidet P-40 Substitute non-ionic detergent (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)

IPP:
25 or 150 mM NaCl
25 mM Tris (pH 8)
5% glycerol
1 mM DTT
1 mM EDTA
1 mM EGTA

2× Calmodulin binding buffer (CBB):
25 mM or 150 mM NaCl
25 mM Tris (pH 8.0)
1 mM DTT
2 mM MgOAc
2 mM imidazole
10 mM CaCl₂
1 Complete EDTA-free Mini protease-inhibitor tablet (Roche) per 10 mL

1× Calmodulin binding buffer (CBB):
25 or 150 mM NaCl
25 mM Tris (pH 8)
1 mM DTT
1 mM MgOAc
1 mM imidazole
3 mM CaCl₂

Calmodulin elution buffer (CEB):
25 or 150 mM NaCl
25 mM Tris (pH 8.0)
1 mM DTT
1 mM MgOAc
1 mM imidazole
10 mM EGTA
All buffers, materials, and equipment were chilled to 4°C. Frozen samples were thoroughly pestled in lysis buffer (3 mL per gram of tissue) in mortar, and mashate was strained through a very fine sieve into a 250-mL flask. The solids captured by the sieve were re-crushed in the mortar, with the addition of lysis buffer as necessary. This material was added to the flask of filtrate, and the mashate was incubated on ice for 30 minutes with gentle rocking to allow complete cell lysis. The lysate was centrifuged for 15' at 13,800 × g at 4°C to pellet tissue and cell debris, cuticle fragments, and polysaccharides and to float lipids to the top of the tube. During this time, rabbit IgG-agarose beads were pre-rinsed twice with lysis buffer. After the tissue centrifugation, floating lipids and debris were removed with a metal spatula and by pipetting, and supernatants were filtered through a fine-gauge tissue sieve (to prevent carry-over of loose debris) into a clean 250-mL flask. The collected solids in the sieve were pressed to release remaining liquid. Washed rabbit IgG-agarose was added to the filtered solution, and samples were sealed and incubated for at least 4 hours at 4°C with agitation.

The IgG-agarose beads were pelleted from the samples in 50-mL batches by low-speed (2,000 × g) centrifugation in 50-mL polypropylene tubes at 4°C; after each batch was pelleted, the supernatant was discarded and another sample batch was added. Pooled pelleted beads were resuspended in 25 mL IPP + leupeptin (0.5 μg/mL) + Pefabloc (0.5 mg/mL) + 0.1% Nonidet P-40 Substitute and rocked at 4°C for 2 minutes. The washed beads were pelleted by low-speed centrifugation, and the supernatant was discarded. This wash step was repeated until the discarded wash buffer appeared clear, and then twice more. To remove detergent, which inhibits TEV protease (Mohanty et al., 2003), the beads were washed with IPP. Washed beads were resuspended in 10 mL IPP + 1
“Complete EDTA-free Mini” protease inhibitor tablet (Roche; does not inhibit TEV protease) and transferred to a 15-mL polypropylene tube. AcTEV protease (200 U, 20 μL; Invitrogen) was added to each sample to cleave bound complexes from the IgG beads, and proteolysis was performed overnight at 18° with rotation.

The proteolyzed samples were centrifuged for 10' at 2,000 × g at 4° to pellet IgG-agarose beads. Supernatants (containing Yuri complexes cleaved from the beads) were transferred into a 50-mL polypropylene tube by passing them through a PolyPrep column (BioRad) to capture any suspended IgG-agarose beads. The pelleted beads and PolyPrep columns were discarded. Samples were brought to 10 mL with IPP.

Calmodulin-agarose beads were rinsed twice with 1× CBB and resuspended in 1× CBB. The proteolyzed samples were brought to 20 mL 1× CBB by slowly adding 10 mL 2× CBB while mixing. Washed Calmodulin-agarose beads were added to each sample, and the tubes were rotated at 4° for 4 hours to allow Ca^{2+}-loaded Calmodulin to bind to the Calmodulin binding peptide (CBP) moiety of the TAP tag. Tubes were centrifuged at low speed to pellet the Calmodulin-agarose beads, and the supernatant was discarded. The pelleted beads were washed three times with 1× CBB. Washed beads were resuspended in 2 mL CEB and boiled for 5' to disrupt the Calmodulin/CBP binding. The boiled samples were spun at low speed for 1 minute at 4° to pellet the beads, and the supernatant was transferred to Eppendorf tubes, 1 mL apiece. These were spun at 14,000 × g to pellet any transferred beads, and the supernatant was removed to a new Eppendorf tube.

Proteins in these supernatants were concentrated by a trichloroacetic acid (TCA) precipitation step. Ice-cold 100% TCA (200 μL) was added to each 1-mL supernatant
aliquot, and the tubes were mixed by inversion and incubated at \(-20^\circ\) overnight. Proteins were pelleted by centrifugation at 14,000 \(\times\) \(g\) for 20' at 4\(^\circ\), and supernatants were carefully drawn off and discarded. Residual TCA was removed from the pellets by washing them with 500 \(\mu\)L ice-cold acetone. Samples were mixed by inversion and centrifuged again. The supernatant was discarded, and this wash step was repeated. Residual acetone was allowed to evaporate from the washed pellets by leaving the tubes open on the benchtop for 30'. Pelleted proteins were resuspended in 20 \(\mu\)L standard 3\(\times\) SDS loading buffer. If the buffer turned yellow, indicating the presence of residual TCA, Tris-HCl solution (0.5 M, pH 6.8) was added, 1 \(\mu\)L at a time, until the sample became blue again.

Tubes were boiled for 5', placed on ice, and pulse-spun to pellet any insoluble material. Samples were electrophoresed through an SDS-polyacrylamide gel (5% acrylamide stacking gel, 12.5% acrylamide resolving gel) at 100 volts for 1-2 hours (until the dye front reached the bottom of the gel). The resolving gel was silver-stained using the Silver Stain Plus kit (BioRad) or with standard Coomassie staining solution.

Each band of interest was excised from the gel with a fresh scalpel blade and placed into an Eppendorf tube, with great care taken to prevent contamination with keratin from microscopic skin flakes (by wearing full lab coat and face shield). Each excised gel sliver was rinsed several times with filtered sterile water to remove any skin flakes or other minute contaminants, and the tubes were left open but protected by a crimped aluminum-foil cover to allow the rinsed slivers to dry overnight. The proteins within each gel slice were identified by mass mapping at the Stanford University School of Medicine Protein and Nucleic Acid Facility (Stanford, CA).
CHAPTER 8: SUMMARY, DISCUSSION, AND
POTENTIAL FUTURE DIRECTIONS

8-1: Summary of results

I identified *yuri gagarin* in a behavioral screen for mechanosensory defects specifically related to gravity perception. The c263 GAL4 transposon insertion into the locus drives enhancer-trap reporter expression in a variety of tissues, beginning in larval life and peaking during early pupal metamorphosis, when it is expressed in all tissues examined; after adult eclosion, reporter expression declines, being expressed in chordotonal neurons for the longest time before dissipating completely. This GAL4 expression is likely to come about at the expense of endogenous *yuri* expression; to the extent that this is true, the tissues in which reporter expression is visible are therefore the tissues in which *yuri* function is disrupted. Therefore, among these tissues is the anatomical seat of the mutant behavioral phenotype; of the tissues described above, the chordotonal organs appear to be the most likely focus of the mechanosensory defects caused by the mutation.

The *yuri* gene encodes at least four Yuri isoforms, of 29, 64, 65, and 102 kDa. These proteins are expressed in varying ratios in all tissues examined, including embryos, larvae, and adult heads, thoraces, ovaries, and testes. Yuri protein forms a cortical mesh in the larval imaginal discs, and in muscle tissue, Yuri is present in the Actin-containing regions of each sarcomere. Whereas Yuri is novel in sequence, the larger isoforms are predicted to be composed primarily of coiled-coil-forming domains, which are a common protein interaction-surface motif. Yuri is well-conserved within the Drosophila
genus, but there are no recognizable Yuri homologs outside this group, suggesting that Yuri has evolved to perform a Drosophila-specific function.

We have created several deletion alleles of *yuri*. In one of these, *yuri*<sup>F64</sup>, most of the 5' untranslated region of all *yuri* transcripts is removed; because the transcripts that encode the 64- and 65-kDa Yuri isoforms have more extensive UTR's, the deletion removes more of their sequence. This results in the complete abolition of expression of Yuri-64/-65, as well as the loss of Yuri-29 in the testis, while leaving Yuri-102 levels unchanged, perhaps reflecting the loss of splicing-regulatory elements. Homozygous *yuri*<sup>F64</sup> animals exhibit no reduction in viability, and females are fertile; however, *yuri*<sup>F64</sup> males are completely sterile, a phenotype that is intriguing because type-I sensory neurons and sperm are the only ciliated cells in Drosophila.

In control animals, Yuri is present on sperm nuclei during their maturation, and this localization is dependent on underlying microtubule cytoskeletal structures. Filamentous Actin is present at the nucleus, co-localizing with Yuri, during nuclear condensation; Tropomyosin 1, a regulator of Actin dynamics (reviewed in Gunning et al., 2008), is present as well. The localization of Yuri suggests that it is a component of a structure involved in the anchoring of the basal body to the nucleus. In addition, Yuri is a component of the Actin-rich "investment cone" structures that are required for sperm individualization.

The sterility of *yuri*<sup>F64</sup> males arises from a lack of proper sperm maturation. In developing *yuri*<sup>F64</sup> sperm, the nuclear localization of Yuri is lost. In addition, the co-localized nuclear Actin filaments are no longer detectable. An accessory structure to the basal body, the gamma-Tubulin centriolar adjunct, is no longer associated with the basal
body in \(yuri^{F64}\) homozygotes; in heterozygotes, gamma-Tubulin is spread along the length of the nucleus rather than concentrated at the tip, indicating that alteration of the ratio between Yuri isoforms can disrupt their function. The basal body itself appears to be mis-localized on the nucleus from time to time, and nuclei are frequently “reversed” within the cyst; some nuclei are retained at the distal end of the cyst, sending their axonemes in the opposite direction from most; in addition, many nuclei are morphologically aberrant, assuming bent or helical shapes rather than the normal needle-straight structures. In both \(yuri^{F64}\) homozygotes and heterozygotes, ultrastructural defects are seen in the sperm-tail axonemes, such as the lack of internal components or disruption of the outer ring of microtubules; aberrant dynamics of the mitochondrial derivatives and unusual relationships between them and the axoneme are also apparent. Axonemes of apparent reversed symmetry are visible, reflecting the presence of the inverted sperm described above. Finally, the investment cones of the individualization complex fail to form properly, halting sperm development at the bundle-of-64 stage.

I have shown that Yuri physically interacts with components of the Actin cytoskeleton in an \textit{in vivo} assay; in particular, Yuri appears to interact with the Actindynamics regulator Tropomyosin 1, a coiled-coil-forming molecule that wraps Actin filaments and modulates their stability and rigidity. This is consistent with the colocalization of Yuri, Actin, and Tropomyosin in muscle tissue, and with the presence of Tropomyosin on condensing sperm nuclei; this would also suggest that the cortical localization of Yuri in imaginal discs reflects its association with the cortical Actin cytoskeleton.
8-2: Discussion

The data described above indicate that Yuri is required for proper cytoskeletal structure or function in spermatogenesis, and that it is a component of Actin structures in other tissues as well. The lack of any obvious non-spermatogenesis phenotype of \( yuri^{F64} \) suggests that the 64-/65-kDa Yuri isoforms are not required for most cytoskeletal functions, although these proteins are present in most tissues. (Yuri immunoreactivity remains in muscle and imaginal disc tissue of \( yuri^{F64} \) homozygotes [data not shown], indicating that there are other Yuri isoforms present in these tissues.) The 29- and 102-kDa isoforms may be more important for other functions, such as muscle function or general Actin-cytoskeletal processes, or Yuri-102 may be at least partially redundant with Yuri-64/65 in most tissues.

Yuri interacts directly with the Actin cytoskeleton, perhaps via a coiled-coil interaction with Tropomyosin. Its function during some aspects of sperm maturation also appears to depend, at least indirectly, on the microtubular cytoskeleton; perhaps Yuri functions as a link between the two systems. Yuri is also required for proper basal-body and axonemal development and function. As such, Yuri may function to recruit components of these structures to their proper locations; or, if the recruitment of these component requires the Actin cytoskeleton, the effect of the \( yuri \) mutation may be indirect, via its effect on Actin dynamics.

Whatever their origin, the ciliary defects observed in \( yuri^{F64} \) spermatogenesis provide insight into the mechanosensory defect that led to the initial identification of the gene. Because both sperm and mechanosensory neurons are ciliated, both cell types may require a precise combination of Yuri isoforms to form well-structured axonemes. As
described in Chapter 1, the cilium of mechanosensory neurons is the stimulus-receptive organelle. In chordotonal organs, tension on the axoneme is thought to be required for efficient mechanoreception; this tension is produced by Actin dynamics in the scolopale cells sheathing the cilium and by the axoneme itself, which is motile for part of its length.

Thus, several possible mechanisms link the mechanosensory and spermatogenesis defects seen in yuri mutants. The mis-positioning of the basal body on yuri\textsuperscript{F64} sperm nuclei suggests that a similar defect may occur in the chordotonal neuron cilium; if the axoneme is not properly attached at its basal end, the proper tension for efficient signaling cannot be maintained. Alternatively, the appearance of split axonemes or axonemes lacking their internal components (which are required for motility) in sperm of yuri\textsuperscript{F64} animals could indicate that neuronal axonemes sometimes might be defective — either structurally unstable or non-motile — in other yuri mutants. A third possibility involves the function of Yuri in the regulation of Actin dynamics. Because the scolopale cells produce rigid Actin rods, templated on microtubules, and because the dynamics or rigidity of these rods is likely to be regulated by Tropomyosin (Wolfrum, 1992; Wolfrum, 1997), a Yuri-based defect in Actin dynamics in these cells (as seen in the lack of the investment apparatus in yuri\textsuperscript{F64} spermatogenesis), perhaps arising from a defect in modulation of Tropomyosin function, could lead to structural defects in chordotonal sensilla.

The lack of obvious Yuri orthologs outside the Drosophilids suggests that Yuri has evolved to perform some Drosophila-specific function. One such possibility involves the mechanism by which Drosophila sperm axonemes are constructed; in most organisms, and in the ciliated neurons of Drosophila, the axoneme must be built by an
intra-flagellar transport (IFT) process, because a tightly apposed membrane prevents the addition of axonemal subunits via any cytoplasmic route (Han et al., 2003; Sarpal et al., 2003; Scholey and Anderson, 2006, for review). However, the growth of Drosophila sperm axonemes within a larger cytoplasm allows them to grow by the addition of monomers diffusing within the cytosol, and the unique individualization mechanism removes the excess cytoplasm after the growth of the axoneme is complete. These features allow the formation of, and have presumably co-evolved with, the very long sperm tails of Drosophila species (up to 6 cm long in some species), which contribute to reproductive fitness via sperm-competition mechanisms (Joly et al., 2003; Luck and Joly, 2005; Pitnick and Markow, 1994; Pizzari, 2006). Although the cilia of Drosophila mechanosensory neurons grow via an IFT mechanism, the central processes of basal-body function or ciliary growth common to both cell types may have adapted to allow the modifications seen in Drosophila spermatogenesis, and in so doing become dependent on Yuri (although not the 64-/65-kDa isoforms) themselves. Alternatively, ciliated neurons may have no need for Yuri, the aberrant expression of which in these cells may disrupt the axonemal development process.

The presence of Yuri in all or most cells indicates that its role is not limited to ciliary function, since Drosophila cells, unlike those of some other organisms, lack "primary cilia," which are the focus of many cell signaling pathways (e.g., Marshall and Nonaka, 2006, for review). No association of Yuri with somatic centrosomes has been observed, suggesting that the presence of Yuri in most cells serves primarily to regulate Actin dynamics; it would follow that the ciliary phenotypes seen in yuri^{F64} arise from defects in this system. The microfilament cytoskeleton is known to participate in
dynamics of the centrosome/basal body in other systems (reviewed in Bornens and Azimzadeh, 2007; Gavin, 1997); in mice respiratory epithelia, for example, cell-apical Actin enrichment is necessary for the recruitment and anchoring of the ciliary basal body (Pan et al., 2007), and in Drosophila, during early embryogenesis, the centrosomes are linked to a special “Actin cap” structure that participates in syncytial nuclear divisions (Karr and Alberts, 1986; Stevenson et al., 2001).

8-3: Potential molecular models

Yuri appears to be present in three isoforms; the bulk of Yuri-29 is predicted to comprise a mixture of alpha helices and beta sheets (not shown), and as such may form a globular “head” domain; the coil-forming helices present in longer isoforms could perhaps be thought of as tail domains (Figure 8-1, A). These coiled-coil-forming tail domains may be sites of interaction with other coil-forming domains – tailed Yuri isoforms may form parallel or anti-parallel dimers, and Yuri may interact with other coil-forming proteins, such as Tropomyosin (Figure 8-1, B). Yuri dimers may cross-link other structures, and Yuri-containing heterodimers may function to link disparate systems (Figure 8-1, C). Yuri may also serve to recruit proteins to their proper cellular location, or it may function to sequester them away from sites where their presence would be deleterious.

The binding between Yuri and Tropomyosin may occur through a coiled-coil interaction with the Yuri “tail” domain. In this situation, the Yuri “head” domain may bind to other proteins or complexes, serving either to recruit those complexes to sites of Tropomyosin concentration (such as Actin filaments), or to link these structures (e.g.,
Tropomyosin-stabilized Actin filaments) to another system (such as, say, the microtubular cytoskeleton). Because Tropomyosin is a stabilizer of Actin filaments, the interpretation of $yuri^{F64}$ phenotypes depends on the wild-type nature of the affected Actin structures – that is, whether their normal function requires dynamism or rigidity and stability. Since Actin filaments appear to be lost from the surface of condensing sperm nuclei of $yuri^{F64}$ animals (i.e., in the absence of nuclear-localized Yuri-64), the normal function of Yuri and Tropomyosin in this situation may be to promote their formation and stabilization at the dense complex by preventing the degradation or dynamics of these filaments.

In other contexts, Yuri may function to sequester Tropomyosin away from regions of Actin dynamics. In these contexts, loss of Yuri activity would lead to the presence of ectopic Tropomyosin; therefore, filamentous Actin structures may form properly but be over-stabilized, leading to a loss of their normal dynamism or the prevention of normal degradation. For example, the Actin-rich investment cones translate down the length of the sperm axoneme by “treadmilling,” or the addition of Actin protomers at the leading edge and their removal at the training edge, rather than being pulled along as a unit by motor proteins, say; if Tropomyosin were allowed to bind the investment-cone filaments, this treadmilling could not occur, and the non-motile cones therefore could not perform their individualization function.

8-4: Possible future directions

To determine which protein(s) identified in Chapter 8 interacts directly with Yuri, I am in the process of performing *in vitro* pull-down assays between recombinant Tropo-
FIGURE 8-1: Molecular models of Yuri. Sequencing of cDNAs and anti-Yuri blotting indicates that the protein exists in three main sizes (A). Models predict that the larger isoforms contain a helical tail that may participate in coiled-coil interactions. These interactions can take place in a parallel or anti-parallel fashion, between two Yuri molecules or between Yuri and other proteins, such as Tropomyosin (B). The function of the “head” domain is unknown; it could conceivably form non-coil interactions with other proteins. A Yuri protein with these features could fill many roles in the cell (C). A dimer of Yuri proteins, joined either by the tail or the head, could cross-link networks of other proteins; a heterodimer containing Yuri might join two disparate networks, such as the microtubule and Actin cytoskeletons. Alternatively, Yuri may serve to sequester proteins at sites of storage, or to recruit proteins to sites of activity. In addition, Yuri could conceivably form filaments, perhaps serving a structural or scaffolding role.
myosin 1 and Yuri-64. Various *Tm1* mutant alleles are available, including the GFP fusion "exon trap" alleles described above; these alleles could be to create, for example, mosaic animals in which Tm1 is absent from the germ-line stem cells, or from imaginal-disc cells, to study the cytoskeletal defects that arise in its absence. The study of Yuri-knockout mosaics or mutants would also shed light on its function in various tissues; mutants or "RNA interference" lines that specifically affect Yuri-29 and Yuri-102 expression would be of particular interest. Expression of *UAS-yuri-29* and *UAS-yuri-64* constructs as "dominant negatives" could also address some aspects of Yuri-102 function; expression of UAS-regulated dominant-negative proteins comprising only the coil-forming "tail" sequences of longer isoforms may also shed light on the function of the 29-kDa "head" region.

Since muscle function appears to be unperturbed in *yuri*<sup>64</sup> animals (e.g., these animals can fly as well as wild-types; data not shown), it may be the case that Yuri-29 and Yuri-102, or both, are the main functional isoforms in muscle tissues, or can compensate for the loss of Yuri-64/65 through partial or complete redundancy. As shown in Figure 4-5, C, Yuri-102 and -29 are much more abundant than Yuri-64/65 in the thorax, which contains primarily muscle tissue (along with nervous tissue, such as the thoracic ganglia, and a small length of gut, which merely passes through); this is consistent with a muscle-tissue Yuri-isoform composition comprising only the smallest and largest isoforms, with the mid-sized Yuri isoforms detected in the thorax being derived from the other tissue types, which are present in smaller amounts. Muscle tissue is also present in larvae, as a sheath around the testis, and in the head, consistent with the presence of Yuri-102 in these tissues. However, the lack of Yuri-102 in the ovary is not
consistent with this model, since this organ is also sheathed in muscle tissue, unless these muscle types are functionally distinct from other muscle tissues in the animal. Western blotting of isolated muscle tissue of different types — from the larval body wall, the gut, the adult thorax, and adult abdominal body wall — would address this question, as would muscle-specific RNA interference experiments or the investigation of mosaic animals bearing yurĩ clones in the musculature; the localization of Tropomyosin and other coil-forming proteins within these mutant tissues would be of particular interest.

The delay of expression of larger Yuri isoforms until the beginning of zygotic expression, and the maternal contribution of Yuri-29 only, indicates that the Yuri-64/65 present in the ovaries is not a component of the oocytes themselves, but is instead a component of the maternal accessory structures of the organ, such as muscles, nerves, and tracheae. In addition, if Yuri is required for the earliest stages of embryonic development, it can only be Yuri-29; since this isoform lacks most of the predicted coil-forming “tail” region, it most likely cannot dimerize with itself or with other proteins (e.g., Tropomyosin) through coiled-coil interactions; instead, the function of the “head” region would appear to be primary in this tissue. Mosaic animals carrying yurĩ germ-line clones (i.e., producing eggs lacking any Yuri) would allow this function to be investigated.

The interpretation of the Actin-related phenotypes of yuri mutants depends on the function of the various affected Actin structures — are they normally stable and rigid, performing a structural or scaffolding role, or are they dynamic or transient? To determine this, tissues of interest, such as the testis, can be cultured in vitro (Noguchi et al., 2006); cytoskeleton-disrupting drugs can then be applied, and defects in the
progression of nuclear condensation, for example, can be identified. For example, phalloidin, which stabilizes Actin filaments, or cytochalasin D, which blocks Actin polymerization, can be applied to cultured testes; if nuclear condensation requires Actin dynamics, this process should be halted. On the other hand, if the microtubule cytoskeleton is required for condensation, then the addition of phalloidin should have no effect on this process, whereas incubation with colchicine (a tubule destabilizing agent that blocks Tubulin polymerization) or taxol (a tubule stabilizing agent) should be disruptive. The presence of many cysts within each testis, each at a different stage of development, would enable the effects of these manipulations on each step in spermatogenesis to be investigated; sperm development would be expected to arrest at the first step after drug addition that requires cytoskeletal stability or dynamism, depending on the compound.

As an adjunct to these experiments, over-expression experiments could also provide information regarding the activity level of the cytoskeleton in processes of interest. For example, over-expression of Tropomyosin in tissues of interest where Actin dynamism is thought to be important would be expected to stabilize Actin filaments in these regions, thereby attenuating any processes that rely on Actin dynamics. The co-expression of Yuri in these same tissues would be expected to suppress the phenotype of Tropomyosin overexpression if the main function of Yuri in the tissue is to sequester Tropomyosin away from sites of Actin dynamics; on the other hand, if the function of Yuri is to potentiate the association of Actin filaments and Tropomyosin, co-expression of Yuri would be expected to exacerbate the defects caused by Tropomyosin expression. Conversely, overexpression of Yuri in tissues where Actin-filament stability is important
would cause destabilization of these filaments, and perhaps a defect in dependent processes, if Yuri functions to sequester Tropomyosin away from sites of activity.

As described above, the coil-forming protein Unc is Drosophila-specific and required for centriolar-adjunct formation and ciliary function (Baker et al., 2004; Kernan et al., 1994). These characteristics shared with Yuri raise the possibility that the two proteins may physically interact. Other coiled-coil-forming proteins described above are required for mechanosensation and male fertility, but are evolutionarily conserved; Yuri or Unc or both might connect the basal, conserved protein network with the insect-specific adaptations seen in Drosophila. To address this matter, these proteins could be visualized (either by immunofluorescence or, in the case of Unc and Centrosomin, with available GFP fusion proteins), and Yuri localization in mutants for these proteins could be examined as well. In addition, many other coil-forming proteins, such as alpha-Actinin and alpha-Spectrin, involved in various other processes (here, cross-linking of Actin filaments), are present at the same locations as Yuri (for these examples, at the Z discs of muscle tissue and at the cortex of epithelial cells). Any interaction between these proteins and Yuri could be detected by directed interaction assays, or by observing localization disruption caused by mutations in yuri and the other proteins in question.
LITERATURE CITED


