AN ANALYSIS OF PROPOSED MITOTIC DEFECTS IN THE TOYS ARE US MUTANT AND DROSOPHILA HYBRIDS

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By
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AN ANALYSIS OF PROPOSED MITOTIC DEFECTS IN THE *TOYS ARE US* MUTANT AND *DROSOPHILA* HYBRIDS

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Animals homozygous for mutations in the *toys are us* (*trus*) gene are developmentally stunted and third instar lethal. The phenotype was initially proposed to stem from a mitotic defect, but has since been associated with a defect in the ecdysone pathway. In addition *trus*¹ mutants lack optic lobes, have malformed imaginal discs, and are often missing the imaginal cells of the salivary glands, all of which are known hallmarks of ecdysone deficient mutants.

Trus is primarily expressed in the prothoracic cells of the ring gland, which is the main location of ecdysone synthesis. To test if Trus might play a role in ecdysone synthesis, I fed larvae 20HE precursors, all of which are able to overcome the developmental delay. I have tested the effects of the *trus*¹ mutant on expression levels of ecdysone pathway genes by constructing double mutants with *trus*¹ and members of the ecdysone pathway.

Recent results of TAP-tagging experiments indicate that Trus is in a tight complex with the products of the *string of pearls* gene, which is the S2 subunit of the small ribosomal subunit, and Elongation Factor 1-α. My findings are consistent with a model that Trus acts downstream of PTTH but upstream of the *ecdysoneless* gene to promote the translation in the ring gland of mRNAs for genes in the ecdysone pathway.
Matings between *D. melanogaster* females and males of sibling species in the *D. melanogaster* complex yield males that are also developmentally devalyed and die at a stage similar to the trus¹ mutation (prior to pupal differentiation). I have re-examined a previous report suggesting that the developmental defects in hybrid males may be the consequence of problems in mitotic chromosome condensation. I found in contrast that the frequencies of mitotic figures, as well as cells in S phase in the brains of hybrid male larvae are extremely low. The cells in hybrid male brains appear to be particularly sensitive to environmental stress; my results indicate that certain in vitro incubation conditions induce widespread cellular necrosis in these brains, causing an abnormal nuclear morphology noticed by previous investigators.
BIOGRAPHICAL SKETCH

Bonnie Jean Bolkan started “life” as a ball of undifferentiated cells. After rounds of mitosis, cell differentiation, and growth she entered the world showing her sense of humor, self-determination, and spunk from the moment she decided to be born in 1979. To this day she claims that she chose to be nearly a month premature because the night before her brother claimed, “…at least she won’t be an April Fool’s baby…” In actuality being an April Fool sounded good to her and really, who can pass up making a liar out of their older brother?

In 1997 she graduated as valedictorian from Hilo High School in Hilo, Hawaii. She then attended Willamette University in Salem, Oregon where she received a BS in biology. During the summers she returned home and worked as a teacher and tutor for the Upward Bound Program as well as spending a summer doing research on Hawaiian “Picture Wing” Drosophila in the lab of Dr. Donald Price at the University of Hawaii at Hilo. During her semester abroad she attended the University of Oulu in Northern Finland and completed her senior research project on chromosomal inversions between isolated populations of Drosophila virilis, in the laboratory of Dr. Janna Liimatainen.

In 2001 she entered the Genetics and Development PhD program at Cornell University where she joined the lab of Dr. Michael Goldberg, and completed the research described in this thesis.
ACKNOWLEDGEMENTS

A Ph.D. is not a solitary achievement and I have received support from many that deserve my thanks and acknowledgement.

I would first like to thank my advisor, Dr. Michael Goldberg, for advice, financial support, and allowing me to follow where my projects took me, even though it was outside the scope of the lab. Mike is also a talented scientific writer and his comments have greatly helped me in my writing.

I would also like to acknowledge my committee members. I collaborated with Dr. Daniel Barbash on the hybrid incompatibility project that composes chapter 3 of this thesis. His scientific expertise and enthusiasm for the research and results were instrumental to that project and a great advantage to me.

My additional committee members: Dr. Anthony Bretscher and Dr. Timothy Huffaker provided me with valuable advice both during and outside of committee meetings though out my Ph.D. career. I greatly appreciated Dr. David Lin and Dr. Kathleen Whitlock who served as special committee members for my A-exam. Dr. Whitlock and Dr. John Ewer also deserve a big thanks for their assistance in finding protocols, reagents, and understanding when my project took me outside the scope of our lab. I also thank them both deeply for their friendship.

I believe that those who provided me with emotional support to be as important to my success as a graduate student as those who provided the intellectual support. First and foremost I thank my parents whom have always supported and believed in me. To my brother Raymond who often called to remind me that, “there is more to life than maggot brains,” such as sci-fi TV.
and books. And to all of the other family members who have expressed their support of my endeavors, thank you.

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The extensive support staff of the Biotech Building and MBG department also deserve acknowledgement as they filled the roles of custodian, glassware washer, fly food maker (and cleaner of dirty fly vials), filer of paperwork, building care manager, and the multiple others behind the scenes. Kevin Tadock and Mick LoPinto deserve an extensive thank you in the role of “computer guys” with my multiple computer “mishaps”, and for rescuing the completed figures of this thesis from a “dead” computer.

Finally I would like to thank the sources that funded me and my research; NIH training grant (Issued to the field of Genetics and Development, Cornell University), NIH grant,# 5R01GM048430-14 (Issued to Michael Goldberg) and The Cornell Graduate School Travel Grant.
Dedicated to those who believed...
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LIST OF ABBREVIATIONS

- Deficient
°C degrees Celsius
+ wild type
µg micrograms
20HE 20-Hydroxyecdysone
7dC 7,7-dehydrocholesterol
ABB annexin binding buffer
AMP adenosine monophosphate
AMPK AMP-activated kinase
asl asterless
Baz Bazooka
B- ftz beta-fushi tarazu
BrC Broad complex
BrDU 5-bromo-2-deoxyuridine
cAMP cyclic AMP
cdc # cell division cycle protein number __
cDNA complimentary DNA
Cnn Centrisomin
Cy Curly (dominant marker on the 2nd chromosome)
Cy Cyano (Fluorocene used in Cy2 and Cy3 conjugated antibodies)
D. mel Drosophila melanogaster
D. sim Drosophila simulans
DAPI 4',6-Diamidino-2-Phenylindole (double stranded DNA staining)
DaPKC Drosophila atypical protein kinase C
Df Deficiency
dlkb1 Drosophila lkb1
DNA Deoxyribose nucleic acid
DNApol-δ DNA polymerase-delta
Dpn Deadpan
E74 Ecdysone-induced protein 74
E75 Ecdysone-induced protein 75
ecd ecdysoneless
ECL Enhanced chemiluminescence
(HRP Western blot detection Solution)
Ecr Ecdysone Receptor
EF-1a Elongation Factor 1 alpha
EMS Ethyl methanesulfonate
F1 First generation descendants
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>FITC</td>
<td>Fluorescein Isothiocyanate</td>
</tr>
<tr>
<td>FM7i</td>
<td>X-Chromosome balancer</td>
</tr>
<tr>
<td>FucTA</td>
<td>Fucosylation Protein TA</td>
</tr>
<tr>
<td>g</td>
<td>grams</td>
</tr>
<tr>
<td>G1</td>
<td>Gap 1 stage of the cell cycle</td>
</tr>
<tr>
<td>G2</td>
<td>Gap 2 stage</td>
</tr>
<tr>
<td>Gαi</td>
<td>subunit αi of the G protein</td>
</tr>
<tr>
<td>GAL4</td>
<td>galactose-induced protein 4</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>gmc</td>
<td>ganglion mother cell</td>
</tr>
<tr>
<td>hmr</td>
<td>hybrid male rescue</td>
</tr>
<tr>
<td>HP1</td>
<td>Heterochromatic protein 1</td>
</tr>
<tr>
<td>hr</td>
<td>hour</td>
</tr>
<tr>
<td>Hu</td>
<td>Humeral (Dominant marker on the 3rd chromosome)</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>Insc</td>
<td>Inscuteable</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-beta-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>Kc</td>
<td>Embryonically derived Drosophila cell culture line</td>
</tr>
<tr>
<td>kD</td>
<td>kilodalton</td>
</tr>
<tr>
<td>l</td>
<td>lethal</td>
</tr>
<tr>
<td>LB</td>
<td>Lutrient Broth (1% w/v Bactotryptone, 0.5%w/v yeast, 0.17M NaCl)</td>
</tr>
<tr>
<td>LC</td>
<td>loading control</td>
</tr>
<tr>
<td>Ihr</td>
<td>lethal hybrid rescue</td>
</tr>
<tr>
<td>M</td>
<td>molar</td>
</tr>
<tr>
<td>MALDI</td>
<td>matrix assisted laser adsorption ionization</td>
</tr>
<tr>
<td>MARK</td>
<td>Microtubule affinity regulating kinase</td>
</tr>
<tr>
<td>MCM</td>
<td>mini chromosome maintenance</td>
</tr>
<tr>
<td>MI</td>
<td>Mitotic Indices</td>
</tr>
<tr>
<td>Mira</td>
<td>Miranda</td>
</tr>
<tr>
<td>ml</td>
<td>milliliters</td>
</tr>
<tr>
<td>mM</td>
<td>millimolar</td>
</tr>
<tr>
<td>mRNAs</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MTs</td>
<td>microtubules</td>
</tr>
<tr>
<td>Mud</td>
<td>Mushroom body defect</td>
</tr>
<tr>
<td>MYB</td>
<td>myeloblastosis</td>
</tr>
<tr>
<td>NaCl</td>
<td>Sodium Chloride</td>
</tr>
<tr>
<td>NBs</td>
<td>neuroblasts</td>
</tr>
<tr>
<td>NTAP</td>
<td>(see Tap) with tag on N-terminus of protein</td>
</tr>
<tr>
<td>ORC</td>
<td>Origin recognition complex</td>
</tr>
<tr>
<td>PAGE</td>
<td>PolyAcrylamide Gel Electrophoresis</td>
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</tbody>
</table>
PBS  Phosphate Buffered Saline
PBT  PBS + 1% Triton X
PBX  PBS + 3% Triton X
PDCD2  Putative Domain Cell Death 2
PDCD2L  Putative Domain Cell Death 2 Like
Pfk  Phosphofructo Kinase
PH3  Phosphohistone H3
pins  partner of inscuteable
Pon  Partner of Numb
Pros  Prospero
PTTH  Prothoracicotropic hormone
RNA  Ribonucleic acid
RNAi  RNA interference
Rps6  Ribosomal protein subunit 6
RXR  Retinoic acid receptor
S  DNA Synthesis stage of the cell cycle
S6  Subunit 6
SDS  sodium dodecyl sulfate
Ser  Serine
sgs3  salivary gland secretion 3
Sop  String of pearls
TAP  Tandem Affinity Purification
Tb  Tubby (Dominant marker on the 3rd chromosome)
TBS  Tris Buffed Saline (20mM Tris-HCl, 137mM NaCl)
TBST  TBS + 0.02% Tween 20
TCA  Trichloroacetic acid
TM6B  3rd Chromosome Drosophila balancer
TRITC  Tetarhodamineisothiocyanate
trus  toys are us
Tub  Tubulin
USP  Ultraspirical
v  vermilion
w  white
w/v  weight to volume
wt  wild type
X  fold (increase or decrease)
Y  Y chromosome
y  yellow
Zfrp8  Zinc finger protein RP-8
CHAPTER 1
INTRODUCTION

I. Review of Drosophila development, mitosis, and classical mitotic mutants

II. Ecdysone signaling

III. Hybrid incompatibility in the Drosophila melanogaster clade

IV. Organization of thesis

I. Review of Drosophila development, mitosis, and classical mitotic mutants

Development of all multi-cellular eukaryotic organisms requires a complex and regulated series of events controlling cell differentiation, shape, movement, and division. Scientists often focus their studies on these topics by obtaining mutations in a gene encoding a particular protein product that controls the division or development of a single cell type.

Developmental stages of Drosophila melanogaster

Development of Drosophila melanogaster embryos begins when oocytes are fertilized internally in the female, and then laid immediately thereafter. During their 26 hours as embryos, the organisms undergo multiple rounds of replication and cell differentiation. After hatching, larvae undergo additional rounds of replication, cell differentiation and growth to prepare to undergo metamorphosis after the third instar larval molt.

In order for Drosophila to enter successive stages of development, they must complete essential tasks at each stage, including achieving a minimum
size and weight, and producing certain tissues needed for the next stage of
development (Demerec, 1950). After these tasks have been completed,
Prothoracicotropic hormone (PTTH) is synthesized, which triggers a protein
cascade allowing the synthesis of ecdysone, which then triggers the molt to
the next stage of development. The transition of embryos into first instar
larvae by hatching is still considered a molt because of its dependence on
ecdysone.

After hatching, the first instar larva begins to feed, allowing the larval
tissues and the imaginal tissues (those that will subsequently metamorphose
into adult tissues) to increase in size and further differentiate. Although cells in
tissues such as the salivary glands are all the same size at the beginning of
the first instar they show characteristic size and shape differentiation by
second instar. During the second instar stage the larva continues to feed and
grow. The imaginal discs also continue to grow, differentiate, and begin to
move and evaginate themselves into tissue shapes that will aid in them
becoming adult structures.

The beginning of the third instar stage is similar to the earlier two larval
stages, with a continuation of feeding and growth. However, at 24 hours post
molt a small ecdysone peak is produced, which commits the animal to
undergo the molt to pupariation (Richards, 1981; Riddiford, 1993). After this
peak the larvae stop feeding and enter the wandering stage, during which they
undergo the physical and chemical changes need to pupariate and undergo
metamorphosis.

At approximately 96 hours post hatching the larvae will pupariate
(Demerec, 1950). During this 4-hour stage the larvae become immobile and
form a pupal case that tans and hardens. A small ecdysone peak at 100
hours post hatch allows the puparia to undergo the prepupal molt to become pupae. During pupation the larval tissues are broken down by apoptosis or rearranged to form adult tissues. The imaginal discs and other imaginal tissues also now begin to quickly differentiate, move, divide, and develop into adult structures. By 192 hours the pupa is ready for a final ecdysone pulse that will allow it to eclose (emerge) as an adult fly a few hours later.

**Mitosis**

Cell division is an essential, highly regulated process that in multicellular organisms allows cell numbers to increase, providing the raw material for differentiation. The cell cycle is usually described as containing four stages: G1 (gap 1 phase), S (DNA synthesis), G2 (gap 2), and M (mitosis). In total, the cell cycle allows the DNA replicated during S phase as well as other cellular components within the parental cell to become equally divided among the two daughter cells that are produced at the end of mitosis. The three stages prior to mitosis (G1, S, and G2) are often grouped together under the term interphase when discussing mitosis.

Mitosis itself can be subdivided into more specific stages. Cells exit interphase and first enter **prometaphase**, which is characterized by nuclear envelope breakdown, condensation of the chromosomes and the attachment of microtubules to the kinetochores of the chromatids (the two newly replicated copies of each chromosome). Of interest later to this thesis, prometaphase is also the time at which histone H3 is phosphorylated at serine 10 by aurora A kinase (Prigent and Giet, 2003). The phosphorylation state of Ser10 also regulates proper condensation of chromatin in during the cell cycle (Prigent and Dimitrov, 2003). **Metaphase** is considered to have begun when the
chromosomes align along the metaphase plate due to the attachment of sister kinetochores to spindle microtubules emanating from opposite poles. A checkpoint exists which monitors the tension arising from these bioriented attachments, so as to hold a cell in metaphase until the kinetochores of all chromatids are properly connected to the spindle, ensuring that all chromosomes are aligned properly and kinetochore microtubules have a proper amount of tension. During **anaphase**, the sister chromatids separate and move along the kinetochore microtubules to opposite poles of the cell. When the chromatids are fully separated, the cell enters **telophase**. The nuclear envelopes are reformed during this stage, while the chromosomes decondense. The two daughter cells are physically separated during **cytokinesis**, which occurs by the formation of a cleavage furrow at a position between the asters of the daughter cells. With mitosis complete the cells then re-enter interphase. The length of time spent in interphase depends on the cell type and developmental stage of the organism, but it can range from a couple of minutes as is the case in the rapid divisions of *Drosophila* embryogenesis (Foe and Alberts, 1983), or alternatively, the cell may in some extreme cases remain quiescent for the rest of the life of the organism.

**Drosophila and mitosis**

*Drosophila* is an ideal model organism for studying mitosis because mutants in genes encoding proteins important for many aspects of mitosis die at characteristic stages, allowing cell division to be observed at high cytological resolution. By comparing mitosis and the development of tissues containing these cells in mutants and wild type flies, the role of individual proteins can be elucidated.
Drosophila melanogaster mitotic mutants characteristically die at one of two developmental stages, although there are some exceptions. Several rounds of syncytial mitoses occur in early embryos immediately after fertilization. Since these mitoses are extremely rapid and occur before the zygotic genome is transcribed, they depend on maternally supplied mRNAs and proteins. Mutations in such mitotic genes will therefore act as maternal effect lethal mutations: mothers homozygous for the mutations will produce embryos that die soon after fertilization (Foe, 1989). Since the maternal stores of many proteins are sufficient to allow development to the third instar larval stage, zygotes homozygous for mutants in the corresponding genes will develop to this stage, but will then die as third instar larvae or during the larval-to-pupal transition (Baker et al., 1982). These larval lethal mutants show defects in the three tissue types mitotically active in larvae: the imaginal discs, the abdominal histoblasts and the neuroblasts (Gatti and Goldberg, 1991). Cytologically, the most advantageous tissue in which to view these defects is the larval brain. Neuroblasts in various mutants can show a wide variety of mitotic abnormalities, including an increase or decrease in the mitotic index, unusual patterns in chromosome condensation, and aneuploidy or polyploidy (Gatti and Goldberg, 1991).

Hypomorphic mutations that are less severe or mutations in genes that are less essential for mitosis can result in the sterility of homozygous mutant flies. These homozygous mutants are able to complete cell division for their own development, but are unable to provide the needed proteins for meiosis and/or the mitotic proliferation of their germline. As a result, these animals cannot produce viable gametes. As mentioned previously, some female sterile mutants can produce apparently normal gametes, but if these females
are unable to provide sufficient maternal contributions of proteins needed for early mitotic divisions of the zygote, early embryonic lethality will result.

II. *Toys are us* and ecdysone signaling

Ecdysone signaling

Ecdysone signaling can be thought of in five major steps: (1) physical/developmental cues that the animal is ready to progress to the next stage of development; (2) synthesis of ecdysone; (3) the ecdysone early response; (4) secondary responses (ecdysone transcription factors providing competence for target tissues through the activation/repression of ecdysone late genes and the repression of ecdysone early genes); (5) and finally ecdysis. Each of these steps contains multiple components and levels of regulation. These components can differ depending on developmental stage, and in later steps, the components and targets are often tissue specific (Riddiford et al., 2003).

Physical and developmental cues to trigger ecdysone synthesis

In *Drosophila* each larval stage produces an increase in the size of the animal; in fact, the main function of the larval molts is to allow for these increases of size. In other holometabolous insects it has been shown that a minimum or critical weight must be achieved to undergo a molt (Nijhout, 1994). In *O. fasciatus* for example, larvae continue to eat until an optimum weight is achieved; however, this signal can be bypassed by injecting saline into the abdomen, causing an equal expansion of the animal which transmits a "ready to molt" message to the brain. This critical mass requirement may explain why wild type larvae raised on ecdysone-augmented food do not undergo
premature molts, since the animals are not competent to respond to the ecdysone until they have reached the critical weight. It has been shown that the critical mass “checkpoint” is not absolute and can be overcome by time, such that even small larvae can continue to the next molt. In *Tineola bisselliella* researchers have even been able to get starved larvae to molt time and time again while losing weight each time (Titschack, 1926).

**Synthesis of ecdysone**

When a particular stage of development is complete, some unknown mechanism triggers Prothoracicotrophic Hormone (PTTH) to be synthesized in the dorsal lateral region of the developing brain hemispheres (Mizoguchi et al., 2001). Much of what is understood of PTTH is derived from studies in the silk worm, *Bombyx mori*. After its synthesis, a further cue causes PTTH to travel to the prothoracic cells of the ring gland, via neurons that connect the two cell types. This system allows for quick delivery of PTTH from a site of neural activity to the prothoracic cells, where ecdysone is synthesized (Riddiford, 1993). Ecdysone is then released and modified from alpha-ecdysone to the chemically active 20-Hydroxyecdysones (Thummel, 2001). The direct target(s) of PTTH which trigger this cascade of events are unknown, but PTTH causes an increase in extracellular calcium and cAMP (Smith et al., 1984; Birkenbeil, 1998; Birkenbeil, 2000; Gilbert et al., 2000; Birkenbeil and Dedos, 2002). This increase in cAMP activates S6 Kinase, which phosphorylates and activates Ribosomal Protein Subunit 6 (Rps6) (Gilbert et al., 2000; Smith et al., 2003), a subunit of the 40s ribosome subunit. What proteins are translated by this presumed up-regulation is also currently unknown. One likely target, however, is the Halloween family of cytochrome p450 proteins, which are involved in
various chemical conversions in ecdysteroid production. As shown in Figure 1.1, these Halloween proteins and the steps they govern include: 7,7-Dehydrogenase (Cholesterol to 7-Dehydrocholesterol); 5ß-Reductase (Δ4-Diketol to Diketol); 3-Dehydroecdysteroid-3ß-Reductase (Diketol to Ketodiol); Phantom (Ketodiol to Ketotriol); Disembodied (Ketotriol to 2-Deoxyecdysone); Shadow (2-Deoxyecdysone to Ecdysone); and finally Shade, which converts Ecdysone to 20-Hydroxyecdysone (20HE) after the Ecdysone has been released from the prothoracic cells into the hemolymph.

The phenotype of a mutant in the aptly named ecdysoneless (ecd) gene defines the effects on the organism of a lack of ecdysone. The ecdysoneless¹ phenotype was described in 1977 after researchers discovered that the ring gland of these larvae failed to make ecdysone (Garen et al., 1977). The ecd³ third instar larval lethal phenotype is rescued by feeding 20HE to the larvae, which are then able to pupate. ecd³ is a conditional temperature sensitive allele, so that phenotypes including larval lethality and female sterility are exhibited only when the animals are shifted to a restrictive temperature of 29°C. The female sterility can be rescued by shifting back down from 29°C to a permissive 20°C. While these phenotypes have been known for a number of decades, the gene responsible was not identified until 2004 (Gaziova et al.); the role of the gene product in the ecdysone synthesis pathways is still unknown.
Figure 1.1 Synthesis of Ecdysone

Modified from K.F Rewitz et al. (Rewitz et al.). Ecdysone is synthesized from cholesterol in multiple steps by cytochrome proteins. The majority of these steps occur in the prothoracic cells of the ring gland. The final step of modifying ecdysone to the active form 20-hydroxyecdysone occurs in the hemolymph and tissues where the ecdysone will be utilized.
**Ecdysone early response**

The ecdysone early response (also known as the ecdysone immediate response) is defined as the transcription of genes that are immediately up-regulated by the presence of ecdysone. The early genes belong to a family of thirteen nuclear receptors with both ligand and DNA binding domains (Thummel, 1995). This allows them to function both as hormone receptors and transcriptional activators. The first acting, and arguably most important, of these early ecdysone genes is *ecdysone receptor*, whose protein product, when it is coupled with Ultraspriical, the fly homologue of the vertebrate Retinoid-X-receptor (RXR), directly binds ecdysone. This trimeric complex then up-regulates the Broad Complex (BrC, which is composed of the four isoforms of the *broad* gene), *E74*, and *E75*. These genes, first studied in the salivary glands, were also the first studied downstream responses of ecdysone. The polytene chromosomes of *Drosophila* salivary glands are highly endoreplicated and so are much larger than chromosomes in other cell types. When ecdysone pulses reach the salivary gland cells, these genes (BrC, E74, E75, and others) are transcribed so quickly and at such a high level that puffs can be visualized on the chromosomes at the bands corresponding to their genetic location (Ashburner, 1974; Becker, 1959).

**Ecdysone Receptor** and Ultraspriical effect this control by forming a heterodimeric complex that is capable of binding ecdysone that up-regulates the ecdysone early transcription factors. EcR contains both a DNA-binding-domain and ligand-binding domain, and is the only of the seventeen known nuclear receptors in *Drosophila* where the ligand is known (Bender et al., 1997). Three functionally separable isoforms of EcR have been identified, which result from differential splicing of a single gene (King-Jones and
Thummel, 2005; Thummel, 1995). The isoforms differ from each other by the tissue type in which they are expressed. Isoform EcR-A is the predominant form in imaginal discs, imaginal rings, prothoracic cells, and the neurons innervating from the brain into the prothoracic cells. EcR-B1 is found in the abdominal epithelium and in the imaginal histoblasts that will form the adult gut. Because of the subset of tissues in which EcR-B1 is expressed, null mutants in this isoform are third instar lethal. The localization of EcR-B2 is restricted mainly to the larval epithelium and fat bodies (Cherbas et al., 2003). Null mutants of EcR that fail to express all three isoforms are embryonic lethal, and homozygous mutant female germline clones are incapable of progressing past mid-oogenesis (Buszczak et al., 1999; Carney and Bender, 2000). This same phenotype is also seen in animals homozygous for mutant alleles of genes encoding the ecdysone early proteins BrC, E74 and E75 (Bayer et al., 1997; Bialecki et al., 2002).

**Ultraspirical** (USP) is a single polypeptide and a member of the nuclear hormone binding superfamily. In structure and function it is homologous to RXR, the vertebrate receptor for the vitamin A derivative, retinoic acid. Like RXR, USP in conjunction with various cofactors can bind numerous hormones. In addition to ecdysone, it binds the receptor for Juvenile Hormone (JH), which is also involved in molting and developmental signaling. Other functions of Usp include dimerizing with hormone receptor 38 to bind an unknown hormone, and binding with Seven-up inhibits USP’s function in the orphan R7 photoreceptor pathway (Zelhof et al., 1995).

The **Broad Complex** (BrC) is made up of four different isoforms of Broad that are transcribed from one of two ecdysone inducible promoters. All four isoforms share an N-terminal BTB domain (Bric-a-brac,
Tramtrack, Broad Complex Domain), while the C-terminal regions formed by differential splicing result in C-terminal regions with one of four zinc finger domains. Most tissues express all isoforms of the BrC, however, relative levels of each isoform are tissue and stage dependant. Mutants disrupting isoform specific regions are not complemented by mutations affecting other isoforms (Bayer et al., 1997), suggesting that each isoform has unique functions. The products of the Broad Complex are transcription factors that not only up-regulate ecdysone late genes but are also involved in the rapid up-regulation of the early genes $E74$ and $E75$.

$E74$ and $E75$ are both considered orphan nuclear receptors. Similar to EcR and BrC, they encode multiple isoforms that share an N-terminal region. Despite being identified early as essential ecdysone immediate genes, little is known about the function of either protein. $E74$ functions primarily in the salivary glands and triggers autophagic cell death when the animal enters metamorphosis (Bayer et al., 1996). Mutants in $E75$ uncouple molting from metamorphosis and are pupal-to-adult lethal depending on the isoform (Bialecki et al., 2002).

**Secondary ecdysone responses: tissue competence and late ecdysone gene regulation**

It is interesting to note that each ecdysone pulse signals a major developmental transition, yet each of those transitions is different. The mid-embryonic ecdysone pulse is the least well understood, but Chavez and colleagues (Chavez et al.) proposed that it plays a role in morphogenesis and cuticle deposition. The first and second instar pulses trigger molts of epithelial tissue (Riddiford, 1993), which allow larvae to grow despite their chitinous
cuticle. The mid-third instar pulse of ecdysone commits the animal to undergo metamorphosis. The animal stops feeding, begins to “wander” in search of a place to pupate, and up-regulates genes such as salivary gland secretion 3 (sgs3), which produce proteins needed early in the pre-pupal stage (Warren et al., 2006). The late third instar ecdysone peak triggers massive cell and tissue breakdown and rearrangement, which continue through pupation. Strictly larval tissues such as the salivary glands now undergo histolysis even though they turned on gene expression in response to ecdysone peaks at earlier molts (Bodenstein, 1943). Imaginal cells (discs, rings, and histoblasts) undergo equally dramatic changes and begin to form adult structures including eyes, wings, and internal tissues (Fristrom et al., 1993). Even larval tissues that remain and retain their general function in adults undergo cellular and morphological rearrangements during metamorphosis. The pupal peak triggers eclosion from the pupal case, and finally the early adult peak completes the remaining transitions to adulthood, including extension of the wings through control of βftz1 (Fortier et al., 2003). In adult females the role of ecdysone seems to be mainly limited to the ovaries and the control of oogenesis (Garen et al., 1977; Carney and Bender, 2000; Gaziova et al., 2004).

**Phenotype of mutants in the ecdysone pathway**

The phenotypes of mutants in the ecdysone pathway are variable depending on the tissues and developmental stages at which the individual genes are expressed, and the penetrance of any given mutations. Mutations in the EcR for instance vary from EcR10–2, which is embryonic lethal (Bender et al., 1997), to EcR-B1dsRNA.B1 which is viable but developmentally delayed
Developmental delay is a consistent hallmark of ecdysone pathway mutants. This makes sense since ecdysone and a response to ecdysone are required for the progression from each stage to the next. Also, a majority of the lethal mutants die during the third instar larval stage. Lethality at this time is consistent both with the large titer of ecdysone needed to progress to pupation and with depletion of the supply of maternally provided gene products by this point in development.

In *Manduca sexta* it has been shown that ecdysteroid control is essential for the proliferation of the optic lobes in the brain (Champlin and Truman, 1998). When ecdysteroid levels drop below a critical threshold, the optic lobe neuroblasts arrest in G2 of the cell cycle. In *Drosophila* cell culture, the addition of ecdysone increases the rate of cell proliferation (Kirschenbaum et al., 1995). This increase in proliferation has also been observed in transplanted ecdysoneless imaginal disc cells (Gaziova et al., 2004).

### III. Hybrid incompatibility in *Drosophila*

Successful matings between closely related species are prevented by either prezygotic or postzygotic mechanisms. Prezygotic mechanisms include mate discrimination, gametic incompatibility, and temporal isolation. While inter-mating in the *Drosophila melanogaster* clade does exhibit some prezygotic mate discrimination, the work described in this thesis focuses on postzygotic reproductive isolation. In particular, I have investigated the inviability of hybrids, since the causes of this type of incompatibility are poorly understood. Dobzhansky pioneered the study of *Drosophila* hybrid sterility by looking specifically at male sterility in hybrids from what he described as races A and B of *Drosophila pseudoobscura* but which were later determined to be
*D. pseudoobscura* and *D. persimilis* [see (Coyne and Orr, 1998; Ortiz-Barrientos et al., 2007) for more comprehensive review]. Work by additional researchers has shown that many pair-wise matings between *Drosophila* species result in postzygotic hybrid inviability, with the phenotype generally increasing in severity with the evolutionary divergence between the parental species.

**Hybrids in the *Drosophila melanogaster* clade**

The *Drosophila melanogaster* clade is comprised of *D. melanogaster*, *D. sechelia*, *D. simulans*, and *D. mauritiana* (Figure 1.2A). Hybrids between *D. melanogaster* and *D. simulans* were first described by Sturtevant (1920; 1929) and have been extensively studied since then. Matings between *D. melanogaster* females and males from any of its sibling species occur at a low frequency; female progeny are viable but sterile while male progeny die at the third instar stage of development or as pseudopupae [Figure 1.2 B, (Sturtevant, 1920; Sturtevant, 1929; Hadorn, 1961; Sánchez, 1983)]. Inverse crosses (with *D. melanogaster* males and sibling species females) are also possible but result in significantly fewer progeny with males being viable but sterile, and females which are embryo lethal. This latter result shows that hybrid incompatibility, as a phenomenon, is not sex specific.
Figure 1.2 Evolution in the *Drosophila melanogaster* clade

(A) The *D. melanogaster* and *D. obscura* branches of the *Drosophila* phylogenetic tree, modified from [http://rana.lbl.gov/drosophila/](http://rana.lbl.gov/drosophila/) (07 March 2007). *Drosophila* species have diverged over millions of years: even *D. melanogaster* and its closely related sibling species are separated by over 4 million years. (B) The Dobzhansky-Muller model fitted with *Hmr* diverging in *D. melanogaster* from the common ancestor of the two species and *Lhr* diverging in *D. simulans* to explain the observed hybrid lethality. This scheme is modified from Brideau et al., 2006. (C) A cross scheme showing the phenotypes of the various classes of F1 progeny obtained from matings between *D. melanogaster* females with a single copy of *Hmr* deleted, and male *D. simulans* with vermilion eyes.
Genetic basis of *Drosophila* hybrid incompatibility

The incompatibility between *D. melanogaster* and its siblings seems to be due to at least one *D. melanogaster* gene on the X chromosome and a minimum of one autosomal gene in the genome of the sibling species (Sturtevant, 1929; Pontecorvo, 1943; Hadorn, 1961; Hutter and Ashburner, 1987; Orr, 1991; Yamamoto, 1992). The Dobzhansky-Muller model proposes that the loss of fitness between two recently diverged species is due to an independently derived mutation in each of the species in genes that have a required interaction (Dobzhansky, 1936; Muller, 1942; Orr, 1995) [See (Johnson, 2002; Welch, 2004) for an in-depth review]. Since both mutations are in different lineages and thus have not existed in the same background, the genes are no longer compatible and cause inviability in the resulting hybrid (Figure 1.2C).

In *D. melanogaster* the X-linked *Hybrid male rescue* (*Hmr*) gene encodes a rapidly evolving protein (Barbash et al., 2003); the null mutation of this gene is able to rescue the lethality of the resulting males (Figure 1.2B), (Watanabe, 1979; Hutter and Ashburner, 1987; Barbash and Ashburner, 2003). *Lethal hybrid rescue* (*Lhr*) is also a rapidly evolving gene, but it is autosomally located on the second chromosome in both species. Consistent with the Dobzhansky-Muller model, mutations in the *D. simulans* copy but not the *D. melanogaster* contributed copy of *Lhr* are also able to rescue the lethality of the hybrid males. The functions of the proteins coded by *Hmr* and *Lhr* have been to date only minimally addressed.

*Hmr* shares homology with the MYB-related family of DNA binding transcriptional regulators, and contains two MADF domains (Barbash et al., 2003) and a putative BESS Domain (Brideau et al., 2006). BESS domains
frequently interact with MADF domains to facilitate protein-protein interactions. Lhr also contains a Bess domain. While there is no data supporting a direct physical interaction between Hmr and Lhr, it still remains possible that they interact directly or though other proteins; the Bess domains in both proteins suggests a possible method of interaction.

Lhr has been shown to interact physically with Heterochromatic Protein 1 (HP1) (Giot et al., 2003; Brideau et al., 2006; Greil et al., 2007) in yeast two hybrid studies. In addition Lhr and HP1 co-localize on polytene chromosomes, specifically at centromeres, telomeres and on the fourth chromosome. All of these regions are marked by high levels of heterochromatin, a state that HP1 has been proposed to help maintain (Eissenberg and Elgin, 2000). These data taken together suggest that the lethality seen in hybrids may be due to an improper maintenance of heterochromatic DNA.

**Mitotic defect proposed as a cause of hybrid lethality**

In 1997 Orr and colleagues (Orr et al., 1997) proposed that a mitotic defect is the proximate cause of the lethality of hybrid males. These authors reported that the brains of hybrid third instar male larvae contain many unusual cells, which they classified as under-condensed mitotic cells (Figure 1.3). The frequency of these cells dramatically increases with time in culture: in uncultured brains fixed immediately after dissection from the larvae, such cells constitute only 0.25% of the identifiable cells; however, with culturing for 90 minutes in 0.7% NaCl, this frequency increases one hundred fold to 25%. The same increase occurs in the presence or absence of the spindle poison colchicine while culturing.
Figure 1.3 Hybrid male mitotic phenotype as previously described

Modified from Orr *et al* 1997. (A) Colchicine arrested hybrid male brain showing mitotic figures. Arrowheads point to what were described as nuclei containing uncondensed mitotic chromosomes. (B,C) Wild-type *D. mauritiana* mitotic brains showing normal chromosome morphology including mitotic figures.
In males of either single species an increase in mitotic index is seen only when the brains are cultured in colchicine and even this increase is only a modest four-fold after 90 minutes. When we examined the cells in question using the mitotic marker Phosphohistone H3, we discovered that these aberrant cells are not mitotic. In addition we showed that this aberrant phenotype does not occur to the same degree when the brains are cultured in the more physiological buffer of Grace’s media. We therefore propose that what Orr et al. (1997) described as mitotic arrest is in fact an artifact of culturing in NaCl.

IV. Organization of thesis

This thesis describes projects that were all initiated because they were originally believed to reflect difficulties in mitosis. However, my work subsequently demonstrated that for the phenomena described in Chapters 2 and 3 of this thesis, the presumptive mitotic phenotype was either an artifact or secondary to a different defect.

Chapter 2 focuses on characterization of the Trus protein and the trus1 mutant. The visible phenotypes associated with homozygosity for this mutation are a low amount of cellular proliferation, developmental delays, and eventual lethality as third instar larvae. I will show that all of these phenotypes are related to a deficiency in ecdysone signaling. I will also show that Trus most likely acts downstream of PTTH but upstream of EcR and so is somehow involved in Ecdysone synthesis. I have cloned the trus gene and have shown that it is expressed at particularly high levels in the ring gland, one of the major locations of ecdysone production in the animal. In collaboration with Dr. Byron Williams, I have identified proteins that interact with Trus; the nature of these
interactors suggests that Trus acts to control the translation of mRNAs for proteins involved in ecdysone synthesis.

**Chapter 3** is a modified version of a paper on hybrid incompatibility that has recently been accepted by the journal *Genetics*. In this chapter, I will show that mitotic arrest is not the cause of the hybrid lethality. To study further the supposed mitotic defect causing this phenotype in hybrid male larvae, I initially attempted to replicate the results of Orr *et al.* (1997). But although I obtained mitotic indices similar to those reported by the previous investigators and also observed an increase of cells with abnormally condensed chromatin, when I probed these same brains with an antibody to Phosphohistone H3 (which stains chromatin starting in early prophase), I found that the frequency of mitotic brain cells in hybrid males remained low, even after culturing. Thus, the large numbers of cells with abnormal chromatin appear not to be in mitosis, in conflict with the interpretation proposed by Orr *et al.* (1997).

I hypothesized instead that these “mitotic” cells are actually undergoing apoptosis, since the initial stages of programmed cell death involve chromosome condensation (Thummel, 2001); and could thus be mistaken for uncondensed mitotic cells. I will show that although the levels of apoptosis are not high there is a massive amount of cell death occurring in the brains of hybrid males upon culturing in 0.7% NaCl.

**Chapter 4** discusses possible future avenues of research on both of these aforementioned projects.

**Appendix 1** contains a modified form of a paper published in the journal *Development* on dLKB1, which is the Drosophila homologue of the human LKB1 (STK11) that is mutated in patients with Peutz-Jeghers
syndrome. In collaboration with the laboratory of Professor Maurizio Gatti (University of Rome, Italy) we show that dLKB1 is essential for proper formation and stability of mitotic spindles and for asymmetrical neuroblast divisions. The mitotic phenotype seen in \textit{dlkb1} mutants is also exaggerated when combined with mutations in \textit{pins}, whose protein product has been also shown to form a sub-complex with G\(_\alpha\)i that is essential for asymmetrically dividing cells.

\textbf{Appendix 2} presents an overview of my work mapping and identifying genes responsible for mitotic mutants identified in the lab. Specifically these mutants were identified as having defects in chromosome condensation. I was able to determine the identity of the genes responsible for four of the mutants. The genes responsible were \textit{cdc6}, \textit{DNApol-delta}, and 2 alleles of polo kinase. Two of the mutants were not mapped to a small enough region to determine their identity. However, the phenotypes associated with these genes are still quite interesting and so I have included them in this section to guide future efforts in their study.
**LITERATURE CITED**


INTRODUCTION

Development is a highly regulated process. In holometabolous insects, metamorphosis and molting are controlled by hormone-dependent pathways. In *Drosophila melanogaster*, this control is regulated by ecdysone and juvenile hormone titers (Riddiford, 1993). The synthesis of ecdysone in the ring gland is triggered by prothoracicotrophic hormone (PTTH) (Gilbert et al., 2002). Part of the pathway downstream of PTTH has already been characterized, but there are still a number of unknown components and regulatory controls.

Although peaks in ecdysone concentration are observed at every developmental transition, the most studied are the transitions beginning in third instar larvae and continuing through metamorphosis. Commitment to enter metamorphosis occurs in response to a small ecdysone peak during the mid-third instar stage (Korge, 1977; Andres and Thummel, 1992). We have identified a mutation in a gene we call *toys are us* (*trus*) that causes a developmental delay throughout development and causes the animals to arrest after this critical stage, so mutants remain as “wandering” third instar larvae for up to 10 days before their death. We believe that the Trus protein is required for the ecdysone pulses that regulate metamorphosis from the mid-third larval instar onward.

In this chapter, I describe the initial identification of the *trus*" mutant, molecular characterizations of the *trus* gene and its protein product, and data indicating that *trus* is involved in the ecdysone pathway. I found that *trus*
corresponds to the transcriptional unit CG5333, and that it encodes a novel but highly conserved cytoplasmic protein with a putative cell death domain (PDCD2L). CG5333 is highly expressed in cells that produce ecdysone, particularly the prothoracic cells of the ring gland. The phenotypic effects of trus\(^1\) mutations are partially rescued by ecdysone feeding. I also report the results of an experiment performed in collaboration with Dr. Byron Williams indicating that the Trus protein is complexed with at least one ribosomal protein, and perhaps also with a translation factor. These results considered together suggest a model in which the Trus gene product is involved in regulating the translation of mRNAs for genes that participate in the ecdysone pathway.

**MATERIALS AND METHODS**

**Flies**

Flies were reared on standard yeast glucose media unless otherwise noted and raised at room temperature of approximately 23°C. Fly stocks were received from the following sources. The trus\(^1\) mutant was isolated from the Zucker EMS mutant collection (Koundakjian et al., 2004) as described in the text. Enhancer trap lines P0206 and P0163 expressing GAL4 in the prothoracic cells of the ring gland were a gift from Dr W. Janning (http://FlyView.uni-muenster.de). All other stocks were received from the Drosophila stock center (Bloomington, IN).

When testing double heterozygotes for abnormal phenotypes, crosses were set up by crossing trus\(^1\) / TM6B Tb,Hu males with virgin females carrying the mutant of interest over a balancer chromosome.
Mapping of the trus' mutation

Our collaborator Fiammetta Verni at the University of Rome first roughly localized the trus' mutation to region 87B by recombination mapping against a multiply marked third chromosome. I then determined this location more precisely by complementation tests for lethality with deletions containing known breakpoints (Figure 2.1A). This analysis delimited trus to a genomic region of 21 Kb, containing 10 transcriptional units. The coding regions of these ten genes on the mutant chromosome were then amplified by PCR from wild type and homozygous mutants, and the PCR products were sequenced by the Biotechnology Resource Center (BRC, Cornell University, Ithaca, NY).

Transgenic rescue of the trus' mutation

To confirm the identity of the gene responsible for the trus phenotype, an ~5 kb fragment of genomic DNA was PCR amplified using the following primers (forward: CGGGGTACCCCAGAATTCCGTGTGGACTCAGAGGA, reverse: CGCGGATCCCGAGAATTAGAATGGATTTCTGCAAGTTGG) from wild type larval DNA; this fragment contained the entire coding region of CG5333 as well as 2 kb of DNA upstream of the start of the cDNA and 1 kb of DNA beyond the 3' end of the transcriptional unit (Figure 2.1A). This fragment was cloned into the PW8 vector (Klemenz et al., 1987) using the KpnI and BamHI restriction sites. The construct was injected into embryos by Genetic Services Inc (Sudbury, MA). Resulting transgenic lines were screened for integration into the genome by presence or absence of w+ eye color. The transgene was mapped to a given chromosome by looking at segregation with known markers on each chromosome. The transgene was then crossed into a trus' homozygote background to check for complementation.
Figure 2.1 Identification of CG5333 as the gene responsible for the trus phenotype

(A) Modified from Flybase 2007. A gene map of the cytological region 87B9 to 87C3 of chromosome 3 containing the trus gene. Lines in green represent deletions that complemented the trus mutant, while lines in red represent deletions that failed to complement. The blue vertical lines delineate the region containing the trus gene. The purple fragment represents the area cloned for a genomic rescue of the trus mutation. CG5333, which has since been renamed trus in Flybase, is highlighted in pink. Genes in black represent known lethals in the region that complemented the trus mutant. (B) Western blot of whole larvae against the Trus protein with α-Tubulin as a loading control. There is no detectable Trus protein in trus\(^1\) mutants. (C) Sequence homology between Trus and its homologues in other organisms (Numbers represent % similarity to Trus). Also shown is most closely related protein in Drosophila, Zfrp8. Trus and Zfrp8 proteins are the only two to contain the putative domain cell death 2 (PDCD2) shown in green. The N-terminal regions, in blue, share a high level of unidentified sequence similarity. Zfrp8 also contains a zinc finger (pink).
Antibody production and purification

A 1.5 kb fragment of the full-length trus cDNA clone RE69372 containing the coding region for amino acids 1 to 485 was cloned into the pDONR201 vector using the BP clonase system (Invitrogen) as described in the instruction manual. The reaction mix was transformed into DH5α cells; individual colonies were grown up and purified using the QIAprep Spin Miniprep Kit (Qiagen, Germantown, MD). Clones were test-digested with BsrGI, and candidate clones with the predicted restriction pattern were sequenced (BRC). The trus cDNA insert from a verified clone was cloned into the pMAL-GW vector using the LR clonase system (Invitrogen). A single clone with the correct sequence was grown in LB broth (1% w/v BactoTryptone, 0.5% w/v yeast extract, 0.17 M NaCl), and expression of Trus was induced with 2 mM IPTG. Maltose purification was done as described in the manual (New England Biolabs) and described briefly here. Cells were spun down and resuspended in Column Buffer (20mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, 1 mM DTT). Samples were then frozen o/n at -20°C and thawed in an ice water bath the following day, followed by 2 min of sonication to break cells open. Samples were then centrifuged for 30 min to removed cell debris and the supernatant was diluted 1:5 in Column Buffer. The crude protein solution was then loaded over an amylose resin column (New England Biolabs) and washed 12 times with Column Buffer. Finally the protein was eluted in Column Buffer + 10 mM maltose. Protein concentration was measured using the Bradford Assay and high concentration fractions were pooled and loaded on an SDS-PAGE gel. A gel band of the expected size for the Trus-MBP (maltose binding protein) fusion protein was isolated and sent to Cocalico
Biologicals Inc (Reamstown, PA) for injection into rabbits for antibody production.

Antibodies were purified from serum using the Econo-Pac Serum IgG Purification Kit (BioRad, Hercules, CA) as described in the instruction manual. For some experiments, antibodies were further affinity purified against the Trus-fusion protein as described by Starr et al (2000), with the exception that the column was made using cyanogen bromide-activated Sepharose 4B beads (Sigma, St. Louis, MO).

**Cytology**

Mitotic indices (MIs) were determined as described in Williams *et al.* (1992); briefly, the MI is presented as the number of mitotic figures per microscope field, and is a measure of the amount of mitosis in a tissue sample.

Whole tissue immunofluorescence was done by dissecting the tissue from third instar larvae in Phosphate Buffered Saline (PBS), permeabilizing the brains for 10 min in PBX (PBS + 0.3% Triton X100), fixing the samples in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA), and then briefly rinsing the samples 3X in PBS before antibody staining. Antibodies were diluted in PBS as described below, and tissues were incubated in primary antibodies overnight at 4°C. Tissues were rinsed 3X in PBX before being incubated in secondary antibody for 2-3 hours at room temperature. Tissues were again rinsed with PBS before being counterstained with ToproIII (Invitrogen, Carlsbad, CA) at a concentration of 1:10,000 in PBS. Tissues were imaged on a Leica TCS SP2 system. Primary antibodies were used at the following concentrations: rabbit anti-Phosphohistone H3 (Upstate,
Charlottesville, VA) a 1:500 dilution, rabbit anti-Trus at 1:1000, and anti-Daschund (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA) at 1:20. Secondary antibodies were all used at a concentration of 1:1000 and included: TRITC (tetrarhodamineisothiocyanate)-conjugated donkey anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA), TRITC-conjugated anti-mouse (Jackson Laboratories) and Cy2-conjugated anti-mouse (Jackson Laboratories); the latter anti-mouse reagent was used in conjunction with the TRITC anti-rabbit for double staining.

**Western Blots**

Whole larvae or specific tissues dissected in ice-cold PBS were homogenized in Laemmli Sample Buffer (BioRad, Hercules, CA) containing 5% 2-mercaptoethanol. Samples on the same gel were equalized to correspond to the same number of animals or to yield the same sample mass as indicated on each figure. After homogenization, samples were boiled for 10 min, cooled on ice for 1 min, and centrifuged briefly to remove non-soluble debris. Proteins were separated on a standard 10% SDS-polyacrylamide gel, and then transferred from the gel to Immobilon-P Transfer Membranes (Millipore, Beford, MA). Blots were blocked in 5% milk in Tween Tris Buffered Saline (TBST; 20mM Tris-HCl, 137mM NaCl, 0.02% Tween-20, pH=7.6) for 1 hour before incubation in primary antibody overnight at 4°C and secondary antibody for 2 hrs at room temperature. Purified Anti-Trus was used at a dilution of 1:5000, while mouse anti-EcR and anti-BrC were both from the Developmental Studies Hybridoma Bank and used at dilutions of 1:100. Secondary antibodies were HRP-coupled anti-rabbit IgG (Sigma) and HRP-coupled anti-mouse IgG (Sigma). Both secondary antibodies were used at a
1:10000 dilution. After three 10 min washes in TBST, blots were processed with ECL as described in the instruction manual (Amersham Corp, Arlington Height, IL).

**Purification of protein complexes containing Trus**¹

The entire coding sequence of *trus* from cDNA clone RE69372 was cloned into pMK33-NTAP (Veraksa et al., 2005), and the resulting constructs were stably transfected into *Drosophila* Kc tissue cells using Cellfectin (Invitrogen). The TAP-Trus fusion protein was assayed on Western blots using HRP-conjugated anti-Protein A antibody (Rockland, Gilbertsville PA) and by immunofluorescence of fixed cells using goat anti-Protein A antibody at 1:1000 followed by TRITC-conjugated anti-goat antibody (Jackson Laboratories) at a dilution of 1:500. Protein complexes from one liter of TAP-Trus-expressing cells were isolated following (Puig et al., 2001), using a lysis buffer for making *Drosophila* extracts (Veraksa et al., 2005). After purification using IgG-Sepharose and Calmodulin-Sepharose beads (Invitrogen), the final eluate was precipitated with trichloroacetic acid (TCA), resolubilized in Laemmli sample buffer (Bio-Rad, Hercules CA) and subjected to SDS-PAGE. Bands were excised, trypsinized and analyzed by MALDI (Cornell Bioresource Center.) Further details on these procedures can be found in (Williams et al., 2007).

**Ecdysone feeding**

Ecdysone was fed to larvae as described in (Riddiford, 1993). Briefly, 20-Hydroxyecdysone (20HE, Sigma) was diluted to 1 mM in 5% ethanol and

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¹ All post-cloning work on Tap-tagging was done by Dr. Byron Williams
mixed with 0.5 g dry yeast to make a yeast paste. Second- and third-instar larvae were transferred to plates containing the yeast paste and observed over time. Rescue of the developmental block was defined as a significant increase in the percentage of animals undergoing pupation. The following precursors were also fed to larvae in exactly the same way: cholesterol (Alfa Aesar, Ward Hill, MA), 7,7-dehydrocholesterol (7dC, MP Biomedicals, Solon, OH), and alpha-ecdysone (E, Axxora, San Diego, CA).

**Sucrose gradient fractionation of S2 cell extracts**

S2 cells were treated with 100 µg/ml cycloheximide (Sigma) for 10 min and lysed in 0.6 ml of 50 mM Hepes-KOH, pH=7.2, 100 mM KCl, 10 mM MgCl₂, 0.1% Triton X-100, 1 mg/ml heparin, 2 mM dithiothreitol, 100 µg/ml cycloheximide, and 0.1% RNAguardTM (Amersham Biosciences). Cell debris was removed via centrifugation for 10 min at 13,000 rpm, and extracts were resolved on 7.5–60% sucrose density gradients by centrifugation for 4.5 hours at 39,000 rpm at 4 °C using an SW41Ti rotor (Beckman). 600 µl fractions were collected while recording the A254 profile using a single path UV monitor (Amersham Biosciences).

**RESULTS**

**Identification of the trus gene as CG5333**

The trus¹ mutation was originally identified by Dr. Fiammetta Verní of the University of Rome in a screen of the Zuker collection of *Drosophila*

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¹ This protocol was received as personal communication from Ditte Andersen (Cancer Research UK London Research Institute) who performed the work, and is modified from Andersen et al. (2007).
mutations induced by high concentrations of the mutagen EMS (Koundakjian et al., 2004). The screen selected for mutant stocks in which homozygotes died as third instar larvae or pupae, since many mitotic mutants die at that stage due to the depletion of the maternally supplied gene product (Gatti and Goldberg, 1991). Initial observations classified $trus^i$ as a mitotic mutant since homozygotes exhibited only a low number of proliferating cells in the larval brain and modest abnormalities in chromosome morphology. However, as will be explained below, these effects are subsidiary to primary defects in the ecdysone pathway. For the genetic mapping of $trus$, we scored the phenotypes of developmental delays and third instar larval/pupal lethality that will be discussed in more detail below.

Dr. Verní roughly mapped the original $trus^i$ mutation (stock MA27) to the right arm of chromosome 3 by recombination relative to visible markers. By testing the $trus^i$ chromosome for complementation with a variety of third chromosome deletions, I was able to narrow down the $trus$-containing region to polytene chromosome bands 87B10-12. This region contains 16 known genes (Figure 2.1A); by complementation testing of $trus^i$ with known lethal mutations in the region, I was able to eliminate 6 genes from consideration, leaving 10 transcriptional units as $trus^i$ candidates (Figure 2.1A). Sequencing of the coding regions of these 10 genes identified only one non-synonymous mutation, which was located in the ATG start codon of gene CG5333. The ATG $\rightarrow$ ACG mutation should disrupt the initiation of the translation of this gene, leading us to believe that CG5333 could be the gene responsible for the $trus$ phenotype. To verify this conclusion, I cloned a wild type copy of CG5333 including its endogenous promoter (Figure 2.1A; see also Materials and Methods) and introduced this construct into the Drosophila germline. This
transgene was sufficient to rescue the lethality and developmental defects observed, supporting the correspondence of CG5333 with \textit{trus}.

Additional support for this conclusion was gained using an antibody generated against a fusion protein expressed in bacteria that contained CG5333 epitopes (Materials and Methods). On Western blots, a prominent band of approximately 70 kD is observed in larval brains and attached tissues of wild type third instar larvae that is missing in \textit{trus}^{1} mutant extracts (Figure 2.1B). Although the presumptive Trus protein runs slower on this gel than its predicted size of 53165 Daltons, this was also a characteristic of the bacterial fusion protein used to make the anti-Trus antibody. Other experiments described below provide additional assurance that this retardation of electrophoretic mobility is indeed a property of Trus proteins.

The \textit{toys are us} (CG5333) gene encodes a novel protein with a putative cell death domain (PDCD2). In addition to \textit{trus}, one other \textit{Drosophila melanogaster} gene called \textit{Zfrp8} contains this domain. The Zfrp8 protein also includes a single Zinc Finger domain that Trus lacks (Figure 2.1C). The genomes of all eukaryotic organisms from plants to humans contain genes homologous to both \textit{trus} and \textit{zfrp8}; the homologs of \textit{Zfrp8} in several other organisms are called PDCD2. The function of Trus has not to our knowledge been investigated in any organism, but PDCD2 has been proposed to have a function in negative regulation of cell proliferation in human cell culture and lymphomas (Scarr and Sharp, 2002; Chen et al., 2005) and a recent article suggests that \textit{Drosophila} Zfrp8 plays a role in control of cell proliferation and lymph gland development (Minakhina et al., 2007). It seems likely that Trus and Zfrp8 are functionally unrelated since mutations in the two genes complement each other (data not shown). Moreover, \textit{zfrp8} mutations are
associated with an increase in cell proliferation, while (as shown below) trus’ mutants have decreased proliferation. Interestingly, no published study has found a role of the PDCD2-containing proteins in cell death (Scarr and Sharp, 2002; Chen et al., 2005; Minakhina et al., 2007), so the original name for this domain may be a misnomer.

**Trus is expressed cytoplasmically in relevant ecdysis tissues**

The amino acid sequence of Trus lacks a nuclear localization signal and so it is predicted that its expression would be cytoplasmic. We used immunofluorescence with the antibody we generated to visualize Trus’ localization in various tissues at different developmental stages and found in all cases that Trus was indeed cytoplasmically localized (Figure 2.2). In third instar larvae, the highest apparent expression of this protein was observed in the ring gland, particularly in the prothoracic cells in which ecdysone is synthesized (Gilbert et al., 2000). Other significant sites of Trus protein accumulation include the salivary glands and the midgut, which while they do not make ecdysone, are tissues undergoing ecdysone regulated reorganization during this stage. In adults expression was seen in the ovaries, which also make and require ecdysone.

**Defective proliferation of trus’ neuroblasts**

trus’ was initially identified by Dr. Verní as a mitotic mutant, mostly because she detected a mild chromosome condensation defect in the brains of third instar larvae homozygous for the third chromosome bearing the trus’ mutation.
Figure 2.2 Expression of the Trus protein

(A) Trus is expressed in all stages of development; however, the strongest expression is in the third larval instar. This is also the stage when ecdysone peaks are the strongest. Adult female expression is also higher than in adult males, which may correspond to the higher levels of ecdysone needed in the ovaries. (B-E) Immunofluorescence analysis of Trus localization and expression; Trus is in red, DNA is in blue. (B) Trus in oocytes remains cytoplasmic and seems to be highly concentrated around the developing nucleus and around the outside of the oocytes. (C) In whole embryos, Trus remains highly localized on the cortex of the embryo. (D) In third instar larvae the expression is highly concentrated in the prothoracic cells of the ring gland. (E) The Trus is also detectable in the salivary glands of the third instar larvae. (F,G) Higher magnification of ring gland and salivary glands, Trus is in red overlaid on the corresponding DIC image. (F) In the ring gland Trus is strictly cytoplasmic, where it stains in a punctate pattern. (G) In salivary glands, the Trus pattern is also punctate. The protein appears to accumulate as well at the border between cells.
<table>
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**Figure B**

**Figure C**

**Figure D**

**Figure E**

**Figure F**

**Figure G**
When I examined the mitotic index (the number of mitotic figures per field of view) of *trus* \(^1\) mutant brains, I measured a value of 0.63, which is roughly half of the value of 1.29 seen in wild type brains. These observations point to problems in cell proliferation; however, I was unable to detect any obvious cytological defects in the mitotic cells themselves such as those reported by Dr. Verní.

Other experiments verify the lack of cell proliferation in *trus* \(^1\) mutants. When whole brains were examined with 5-Bromodeoxyuridine, used to mark replicating cells, a complete lack of staining in the optic lobes was observed (Figure 2.3 A-D). Staining against Dachshund, which is expressed in undifferentiated neuroblasts, showed a similar staining pattern to PH3 with near-normal populations of non-differentiated scattered neuroblasts (Figure 2.3 E-H) but a lack of non-differentiated optic lobe neuroblasts. At least some neuroblasts were, however, able to differentiate as shown by staining for Elav, which is only expressed in differentiated neuroblasts (Yao and White, 1994; Robinow et al., 1988) (Figure 2.3 A-D). Thus the low mitotic index observed is due mostly to a lack of proliferating optic lobe neuroblasts and not a general problem in entering mitosis or in cell differentiation after division.

**Defects in some *trus* \(^1\) mutant salivary glands**

In normal development, cells in the salivary gland are all the same size when the animal hatches to become a first instar larva. In wild type and in 75% of *trus* \(^1\) mutant individuals, these salivary gland cells are then able to differentiate and propagate different cell types, including the imaginal ring, which will develop into the adult salivary tissue (Figure 2.4A).
Figure 2.3 *trus*¹ larvae fail to develop optic lobe neuroblasts

(A, C, E,G) BrDU (red) is only incorporated into actively dividing cells, while Elav (green) stains neuroblasts that have differentiated into neurons. (A,C) Wild type (WT) brains show rings of dividing cells marking the optic lobes as well as scattered neuroblasts. (E,G) The *trus*¹ animals have scattered neuroblasts that are dividing but lack dividing optic lobe cells. Neuroblasts differentiation appears to be unaffected in *trus*¹ mutant brains. (B, D, F, H) Dachshund (Dasc, red; DNA counter-stain, blue) is expressed in non-differentiated neuroblasts. (B,D) WTt brains show Dachshund expression in both the rings of optic lobe cells similar to that seen with BrDU and also scattered neuroblasts. (F,H) Optic lobe staining is considerably reduced in *trus*¹ mutant brains.
Figure 2.4 Development of imaginal ring cells of the salivary glands.

Immunofluorescence staining of DNA by ToproIII of third instar larval salivary glands. (A) In wild type animals the salivary glands are composed of large polyploidy cells involved in making larval secretions and a small ring of imaginal cells (arrow) that will comprise the adult salivary tissues after metamorphosis. (B) In 25% of trus mutants the salivary glands are missing this imaginal tissue. However, 75% of trus¹ animals have apparently normal rings of imaginal cells, suggesting that differentiation of this cell type is not absolutely dependant on ecdysone or Trus.
However, in 25% of the \textit{trus} \textsuperscript{i} larvae, all of the cells remain the same size and the imaginal ring cells are missing (Figure 2.4B). It is unclear if this latter cell type is missing because of a defect in proliferation or differentiation.

\textbf{Trus mutants exhibit developmental delay and arrest}

A wild type \textit{Drosophila} raised at 25°C will take nine days to progress from being laid to eclosing from its pupal case as an adult (Demerec, 1950). However, in a bottle containing a balanced stock for the \textit{trus} \textsuperscript{i} mutation, the developmental delay of homozygous \textit{trus} \textsuperscript{i}/ \textit{trus} \textsuperscript{i} individuals is obvious relative to the progress of their heterozygous siblings (\textit{trus} \textsuperscript{i}/Balancer). By the time half of the homozygotes reach the third instar stage at day 12, 95% of their siblings have already formed pupae (Figure 2.5 A-C). Not only do the homozygous \textit{trus} mutants take 8.5 days longer to reach the third instar than wild type flies, but they also remain at this stage of development for an extended period (Figure 2.6). Homozygotes for \textit{trus} \textsuperscript{i} spend an average of 12.5 days as third instar larvae, with 88% of these larvae dying before pupariating. The remaining 12% of larvae that do manage to pupariate subsequently die within 24 hours, without reaching the point of pupal cutical tanning, which occurs normally at 12 hours post-pupariation (Demerec, 1950).

\textbf{Heterozygotes for \textit{trus}\textsuperscript{i} are partially temperature-sensitive for eclosion}

When pupae that are heterozygous for the \textit{trus} \textsuperscript{i} mutation are shifted from 25°C to 30°C, they exhibit a defect in eclosion as well as a significant increase in pupal lethality (Table 2.1).
Figure 2.5 Development in *trus* mutants is delayed.

(A) It takes an average of 12.5 days for *trus* homozygous mutant larvae to reach the third instar stage of development, whereas wild type larvae only take an average of 3.5 days post egg deposition to reach this same stage. Feeding of 20-hydroxyecdysone (20HE) to homozygotes is able to partially rescue this delay. (B) Feeding of 20HE is able to lower the third instar lethality in mutant animals from 89.1% to nearly 7.5%. (C) After 20HE feeding, 91.5% of homozygote larvae are able to pupariate, compared with only 13% raised in food without 20HE.
Figure 2.6 *trus*<sup>1</sup> mutants spend an extended period as third instar larvae

Homozygous mutant larvae that are unable to pupariate do not die immediately, but instead spend a considerable amount of time (up to 16 days) as third instar larvae. The feeding of 20HE is also able to rescue this phenotype, as was seen with the developmental delays (see Figure 2.5)
Normal eclosion is occurs quickly with the fly emerging from the top of the pupal case in approximately 30 seconds. Pupal lethals often have a characteristic air bubble showing their inability to invert their head (which is an early step of metamorphosis), as well as mass cell death visible with the naked eye. Some pharate adults are able to begin the eclosion process but unable to complete eclosion and die stuck in their pupal case. ecdysoneless is known to be a temperature sensitive allele, however, I was surprised to discover that at 30°C trus heterozygotes also exhibit a temperature sensitive pupal and eclosion phenotype. When animals carrying the temperature-sensitive mutation ecd are raised at restrictive
temperature, they exhibit similar phenotypes, suggesting that the developmental issues associated with trus\textsuperscript{i} mutations might be related to decreases in the ecdysone titer.

The larval lethality of trus\textsuperscript{i} mutants can be rescued by an ecdysone-enriched diet

Several of our observations suggested the possibility that the phenotypes associated with trus\textsuperscript{i} mutations might be due to defects in ecdysone-related pathways. First, several mutants in the genes involved in these pathways exhibit developmental defects similar to those we describe above for trus\textsuperscript{i} mutants. Second, we saw low proliferation of optic lobe neuroblasts; in Manduca sexta, this specific phenotype is seen in animals with low ecdysteroid levels (Champlin and Truman, 1998). Finally, the Trus protein accumulates to its highest levels in tissues that are involved in ecdysone synthesis.

I used a feeding strategy to determine whether the trus\textsuperscript{i} phenotypes described above are caused by a lack of ecdysone. Larvae that are competent to undergo molts to the next stage but are unable to do so because of a deficiency in ecdysone can be rescued to the next stage by the addition of ecdysone to their food (Garen et al., 1977; Riddiford, 1993). When 0.5 mg/ml of 20-Hydroxyecdysone (20HE) was fed to second instar trus\textsuperscript{i} larvae, it increased the rate at which they reached the third instar stage. 20HE-fed animals reached the third instar an average of 7.8 days post hatching (Figure 2.5 A), significantly later than the 3.9 days required for wild type animals, but significantly earlier than the 12.5 days needed by mutant siblings that are not fed ecdysone. Even more dramatic is the increase in pupariation, from 12.5%
in unfed populations to 92% with an ecdysone-enriched diet (Figure 2.5 B). This ability to pupariate represents rescue of the phenotype even though the animals die at this stage (Ono et al., 2006), since they have stopped feeding at the wandering third instar stage of development and so are no longer aided by the ecdysone enriched food.

If Trus was acting in the pathway that converts cholesterol to 20HE, we could in theory narrow down the step of its action by testing the ability of intermediates in the pathway to rescue the developmental delay and ability to pupariate. Trus does not have any sequence similarity with cytochrome p450 or other known enzyme in this pathway, however, it remains possible that Trus plays an unknown direct or indirect role in a specific part of the pathway. I found that Cholesterol, 7dC, Ecdysone, and 20HE were all able to rescue the pupariation defect of *trus*¹ mutants (Table 2.2). The rescue effected by earlier precursors such as Cholesterol was weaker than that produced by 20HE feeding, but this same effect has previously been noted in the rescue of genes known to function early in the pathway (Ono et al., 2006). This decrease in efficiency possibly is due to the need to coordinate the timing of multiple pathways involved in ecdysone synthesis and signaling that is not fulfilled when animals are presented with any form of exogenous ecdysone or its precursors.
Table 2.2 Percentage eclosion based on sterol additions to media

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* sterols provided on plates from time of egg laying

disembodied (dis) is the gene that codes for CYP302A1, which converts Ketotriol to 2-deoxyecdysone in the ecdysone synthesis pathway (see Figure 1.1)
Second-site noncomplementation of \textit{trus}$^1$ with mutations in known ecdysone pathway genes

Many animals that are simultaneously heterozygous for mutations in two different genes functioning in the edysone pathway (double heterozygotes) die as larvae or pupae, or display other developmental phenotypes including sterility (Bialecki et al., 2002). Many heterozygous ecdysone mutants appeared to have a normal phenotype in a \textit{trus}$^1$/+ background (Table 2.3). However, animals simultaneously heterozygous for \textit{trus} and mutations in any of several genes operating earlier in the pathway, including \textit{ecd} and the ecdysone immediate genes \textit{BrC}, \textit{E74}, and \textit{E75}, showed substantial decreases in fitness measured by their eclosion as adults (Table 2.3). In these latter cases, survivors that were able to eclose were then sterile. Sterility resulting from double heterozygosity was also seen in cases involving other mutations that at least partially complemented \textit{trus}$^1$ for viability, including \textit{broad}$^1$, \textit{ecd}$^1$, and \textit{EcR}$^{Q50st}$. These genetic interactions with ecdysone-related genes provides yet more assurance that Trus also acts somewhere in the ecdysone pathway.

Expression levels of proteins up-regulated by ecdysone are low in \textit{trus}$^1$ mutants

Since ecdysone directly up-regulates EcR, the expression level of EcR in the larvae can be used as a crude measurement of ecdysone titer (Bender et al., 1997). I determined EcR protein levels by Western blot analysis, and found that in \textit{trus}$^1$ third instar larvae, the concentration of EcR is 19 times lower than in their wild type counterparts (Figure 2.7 A).
Table 2.3 double heterozygote phenotype with *trus*¹

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<td>n/a</td>
</tr>
<tr>
<td>sop⁰⁰₁²⁷³</td>
<td>No</td>
<td>Female</td>
</tr>
<tr>
<td>spo¹</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>trof³</td>
<td>Partial</td>
<td>n/a</td>
</tr>
<tr>
<td>Usp⁴</td>
<td>No</td>
<td>Maleᵈ</td>
</tr>
<tr>
<td>Zfrp8</td>
<td>Noᶜ</td>
<td>No</td>
</tr>
</tbody>
</table>

a: not lethal but has dorsal appendage phenotype and slow development  
b: partial lethality is defined as less than 50% of expected double heterozygotes  
c: double homozygotes are embryonic lethal  
d: *Usp* males are hemizygous sterile without *trus*  
n/a: not assayed
Figure 2.7 Expression of EcR and BrC is significantly decreased in trus\textsuperscript{i} mutants

Wild type and mutant larvae were normalized by weight (rather than by number, since trus\textsuperscript{i} mutants are smaller than wild type. Wild type extracts were loaded in amounts corresponding to approximately 1/4, 1/2, and 1 larvae. (A) The trus\textsuperscript{i} mutants appear to have significant decreased expression of EcR. (B) Not surprisingly (since Broad is up-regulated by EcR), the concentration of BrC is also significantly reduced in trus mutant animals; the effect seems to be consistent for all of the isoforms of Broad. The antibody used is against the Broad core that is expressed in all of the isoforms. Size markers delineated by grey lines between the panels are 100 and 70 kD, respectively. The loading control is $\alpha$-Tubulin.
Expression of the Broad Complex is also significantly decreased in *trus*’ mutant larvae (Figure 2.7 B), which is expected since the BrC is up-regulated by the ecdysone, USP, EcR heterotrimer (Bayer et al., 1997).

The expression of all isoforms of EcR and BrC appears to be equally affected. Since some of these isoforms are tissue specific (Bayer et al., 1997; Bender et al., 1997), this finding suggests that the effect of the *trus*’ mutant is not tissue specific but is instead more general. This further suggests that Trus acts to regulate ecdysone synthesis and thus systemic levels of this hormone, rather than participating in an early response to ecdysone or the ability of the organism to respond to ecdysone.

**Trus forms one or more complexes with String of Pearls and perhaps with Elongation Factor 1 alpha**

To search for possible Trus interactors, we used the Tap-tagging system described by (Veraksa et al., 2005), which identifies interactions that form at least transiently stable complexes. Drosophila S2 tissue culture cells expressing a Tap-Trus fusion were lysed, and interacting proteins were isolated by affinity chromatography against the tag. The final eluate from the affinity column had two major bands and one band of somewhat lower intensity that were not found in controls (Figure 2.8). One of these bands was (as expected) Trus itself; this band assignment was verified both by Western blotting with anti-Trus and by mass spectrometry. It should be noted that the Trus band migrated on gels at ~70 kD, and thus had the same mobility as the Western blot band assigned to Trus in larval extracts (see Figure 2.2 above). The other major band (band 3 on Figure 2.8) is String of Pearls (Sop), which is the S2 subunit of the 40S ribosomal subunit (Cramton and Laski, 1994).
Figure 2.8 Tap-tagging reveals stable binding of Trus with Sop and Ef1-α

The three strongest bands visualized with Coomassie Blue staining on a SDS-Page gel after Tap-tagging with Trus are (1) Trus (2) Ef1-α and (3) Sop. Identifications were made using MALDI-Mass spectrometry. To limit false positives, bands selected for mass spectrometry were unique when compared against proteins pulled down with a tag only construct and also other Tap-tagged proteins in the lab.
Mass spectrometry determined that the minor band (band 2 on Figure 2.8) is Elongation Factor 1-alpha (EF1-α-48D), a factor that plays a role in shuttling tRNAs to the ribosome during translation (Negrutskii and El'skaya, 1998).

From a global yeast two hybrid interaction screen with Drosophila proteins, Giot and colleagues (2003) obtained additional data indicating that Trus can associate with Sop. We are less confident that Trus truly forms a complex with EF-1α, since this latter protein is very highly abundant. However, we have never detected EF-1α as in the final eluate of Tap-tagging experiments with several other baits, and the fact that both Sop and EF-1α are involved in translation further suggests that this interaction may be real.

**Trus fails to co-sediment with ribosomes or polysomes**

Based on the results in the previous section, we hypothesized that Trus would interact with Sop and/or EF1-α within the context of a ribosomal subunit or intact ribosome. To test this possibility, we fractionated cytoplasmic extracts of tissue culture cells on sucrose gradients and examined the sedimentation of Trus relative to that of the ribosomal subunits and ribosomes (which were detected with antibodies against components of the two ribosomal subunits). Surprisingly, Trus did not co-sediment with ribosomal units, ribosomes, or polysomes. Instead, Trus migrated at a position corresponding with lower molecular weight components (Figure 2.9).
Figure 2.9 Sucrose gradients show Trus does not associate with ribosomal subunits or intact ribosomes.

(A) Fractions of a 7.5-60% sucrose gradient were run on an SDS-page gel and then subjected to Western blot analysis with antibodies against translation factors and ribosomal proteins (HRPA is a component of the 60S subunit; while RpS25 is a component of the 40S subunit). Trus does not fractionate with ribosomal subunits, intact ribosomes, or polysomes. However, Trus is present in the same fractions as Ef1-α, consistent with their possible interaction. It also remains possible that Trus interacts with Sop in the early (lower molecular weight) fractions, but this hypothesis cannot currently be tested due to the lack of an antibody against Drosophila Sop. All work in panel A was completed by Ditte Andersen (see materials and methods for details).

(B) Further sucrose gradients and western blots verify that Trus migrates in a low molecular weight fraction much smaller than ribosomes. RNA concentration in each fraction was determined by UV spectroscopy.
The mouse homologue of *trus* (*pdcd2l*) is expressed in discrete areas of the brain in developing embryos\(^3\)

Trus is conserved in all eukaryotic organisms from plants to humans. While ecdysone does not exist outside of holometabolous insects and molting arthropods, many of the proteins involved in ecdysone signaling are used for signaling by other hormones in higher organisms (Gilbert et al., 2002). We therefore were interested in examining the expression pattern of *pdcd2l* (*trus*) in the mouse; in particular to determine whether it is specifically expressed in tissues involved in hormone manufacture that might be analogous to the fly’s ring gland.

Two stages of early mouse development were examined by in situ hybridization for *pdcd2l* mRNAs. At embryo stage e8.5 (e8.5 is 8.5 days post-partum), staining was restricted to neural tissue and epithelial layers (Figure 2.10A). The neural tissue staining was the strongest in the cephalic neural fold (developing forebrain) and in the neural epithelium. The anterior-most section of the developing head showed the highest level of staining (Figure 2.10E,G,I). The staining seen in the epithelial layers was non-mesenchymal in nature. It is also possible that the inside of the yolk sac and the amnion stain weakly for Trus, but further *in situ* would be needed to confirm this result. Of interest to hormone signaling at this age of development, there was no staining in the outer yolk sac, the trophoblast (developing placenta), or even any other neural tissues including the neural tube.

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\(^3\) This section was done in collaboration with the Garcia Lab, MBG, Cornell University. Mouse cDNA was isolated and prepared by Dr. Garcia, and all *in situ* work was done by Dr. Maegan Harden. For protocols on the techniques used see Nagy, A. (2003).
Figure 2.10 Mouse Trus (*pdcd2l*) is expressed in specific regions of the brain in 8.5e and 10.5e embryos.

(A,B) Whole mount *in situ* hybridizations. (A) Embryos 8.5 days post partum (e8.5) are devoid of staining in the trophoblast. Scale bar = 0.5 mm. (B) By e10.5, staining of *trus* is isolated to the head region of the animal. Scale bar = 1 mm. (C,D) Diagrams depicting essential regions of the e8.5 and e10.5 embryos. Red lines show the region and the angle of dissected sections for panels E-J. (E,G) Sections through the brain region of e8.5 embryos show Trus expression within neural and epithelial layers including the yolk sac. Scale bar = 7 mm. (F) Section of the developing head of an e10.5 embryo. Staining is epithelial, on the interior of the telencephalic vesicles “T”, developing mid brain “M” and hindbrain “H”. Scale bar = 1.5 mm. (H) Higher magnification of F showing a strongly stained region of cells of unidentified lineage (arrowhead) as well as the epithelial staining. Scale bar = 7 mm. (I,J) Higher magnifications showing the cells staining within the epithelial layers of the developing brain. Scale bar = 5 mm.
The yolk sac controls signaling from the outside environment, the trophoblast controls the passage of signals, nutrients, and hormones between the mother and the embryo, and the neural tube is a site of signaling within the developing embryo.

At e10.5, the staining pattern was similar to that at e8.5 in that signal was only seen in the developing head (Figure 2.10B,F,H,J). However, at this later time, trus expression was observed in the midbrain and hindbrain as well as the forebrain. This staining was not diffuse, and seemed to be restricted to certain areas of these broader regions.

DISCUSSION

We have identified a novel protein with a critical function in ecdysone synthesis in D. melanogaster. This protein, Trus, is the Drosophila homolog of mammalian PDCD2L, contains a conserved putative Programmed Cell Death Domain. Trus and its mouse homolog are expressed in the cytoplasm of specific cells and tissues.

We have shown that Trus interacts genetically with multiple members of the ecdysone pathway. Trus also decreases expression of EcR and BrC, which are ecdysone immediate genes that are up-regulated by ecdysone. Further, the larval lethality exhibited by trus mutants is rescued by augmenting their food with 20HE. All of these results indicate that Trus plays a role in the synthesis of ecdysone. This idea is also consistent with the high level of Trus expression in the prothoracic cells of the ring gland, since these are the cells most involved in ecdysone synthesis in developing larvae.

Tap-tagging experiments have identified the small ribosomal protein Sop and the translation factor Ef1-α as potential Trus interactors, suggesting
that Trus is involved in a mechanism that activates the translation of mRNAs encoding proteins involved in ecdysone synthesis. There are several precedents for translational controls in hormone biogenesis. In mice, Ef1-α and RpS6 (a component of the small ribosomal subunit) are both targets of S6 kinase during insulin signaling (Chang and Traugh, 1997). In the moth Manduca, RpS6 has been proposed to be essential for PTTH-sensitive gated translation in the ring gland. Although Sop is also a part of the 40S ribosome in Drosophila, we have found no evidence that Trus associates with the intact 40S subunit. It is possible that Trus became dissociated from ribosomes during the sucrose gradient fractionation shown in Figure 2.9; for example, since Ef1-α is a GTP-binding protein, Trus binding to ribosomes might be conditional upon the addition of a non-hydrolyzable GTP analog to the buffers in which the cell extract was made and in which the gradient was run. If this is not the case, it remains possible that Trus might nonetheless associate with Sop and/or Ef1-α in small complexes that regulate the ability of these proteins to interact with the intact ribosome, thus allowing Trus to regulate the translation of proteins needed for ecdysone synthesis.

The mouse homolog of Trus is expressed early in development and is restricted to certain neural tissues and certain undefined cell populations within these tissues. However, our limited data provide no support for the hypothesis that PDCD2L plays a function in mice analogous to its role in Drosophila ecdysteroid synthesis, since we failed to detect PDC2L expression in tissues involved in hormone synthesis. It remains possible that we simply looked at mouse embryos that were too young to have initiated certain kinds of hormonal signaling, but it is also possible that PDC2L plays other roles in
mouse development. These questions can only be answered by examining mice “knocked-out” for this gene.
LITERATURE CITED


CHAPTER 3

DEVELOPMENTAL AND CELL CYCLE PROGRESSION DEFFECTS IN DROSOPHILA HYBRID MALES

ABSTRACT

Matings between D. melanogaster females and males of sibling species in the D. melanogaster complex yield males that die prior to pupal differentiation. We have re-examined a previous report suggesting that the developmental defects in hybrid males may be the consequence of problems in mitotic chromosome condensation. We find in contrast that the frequencies of mitotic figures and of nuclei staining for the mitotic marker Phosphohistone H3 in the brains of hybrid male larvae are extremely low. We also find that very few of these brain cells in hybrids are in S phase, as determined by BrDU incorporation. The cells in hybrid male brains appear to be particularly sensitive to environmental stress; our results indicate that certain in vitro incubation conditions induce widespread cellular necrosis in these brains, causing an abnormal nuclear morphology noticed by previous investigators. We also document that hybrid larvae develop very slowly, particularly during the second larval instar.

INTRODUCTION

Speciation requires reproductive isolation between diverging populations (Mayr, 1942). One type of barrier that can isolate emerging species is the inviability or infertility of hybrids. For example, although the

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4 This chapter is modified from a paper submitted to Genetics. 2007. Bonnie J. Bolk, Ronald Booker, Michael L. Goldberg and Daniel A. Barbash. Daniel Barbash is corresponding author.
species in the *Drosophila melanogaster* complex (*D. melanogaster* and its sibling species *D. simulans*, *D. mauritiana*, and *D. sechellia*) are very closely related and morphologically almost indistinguishable, matings between *D. melanogaster* females and males of any of the sibling species yield F1 females that are semi-viable but sterile, as well as males that die as developmentally delayed larvae or pseudopupae with small or nonexistent imaginal disks (Sturtevant, 1920; Sturtevant, 1929; Hadorn, 1961; Sánchez, 1983).

The lethality of hybrid males appears to be due to an incompatibility between one or more genes on the *D. melanogaster* X chromosome and one or more autosomal genes in the other species (Sturtevant, 1920; Sturtevant, 1929; Pontecorvo, 1943; Hadorn, 1961; Hutter et al., 1990; Yamamoto, 1992; Brideau et al., 2006). Hybrid lethality is not sex-specific, since hybrid females homozygous for the *D. melanogaster* X chromosome also die at the same stage of development (Hutter et al., 1990; Orr, 1993). The X-linked *Hybrid male rescue* (*Hmr*) gene in *D. melanogaster* and the *Lethal hybrid rescue* (*Lhr*) gene in *D. simulans* are major players in causing this hybrid lethality because loss-of-function alleles in these genes suppress the lethality (Watanabe, 1979; Hutter and Ashburner, 1987; Barbash et al., 2000; Barbash et al., 2003; Brideau et al., 2006). Despite this progress in studying the genetic basis of hybrid male inviability, the developmental causes of the phenomenon remain poorly understood. One study proposed that hybrid males suffer from limited cell proliferation due to a failure in mitotic chromosome condensation (Orr et al., 1997). The investigators observed that the brains of hybrid male larvae incubated in 0.7% NaCl and in both the presence and absence of colchicine (a drug used to arrest cells in metaphase) contained very few normal mitotic
figures, but many cells displayed masses of diffuse chromatin. These latter cells were interpreted to have entered a defective mitosis in which the chromatin remains under-condensed relative to that normally seen in the prometaphase/metaphase mitotic figures in neuroblasts from each pure species.

To better understand the nature of the apparent chromosome condensation defects in hybrid male larval brains, we wished to characterize this phenotype with techniques developed since the publication of the earlier report. To our surprise, we found that the frequency of mitosis in these brains is extremely low. Although we observed in 0.7% NaCl-incubated brains the same high frequency of cells with masses of diffuse chromatin seen by the previous investigators, we established that these cells were not in mitosis but were instead dying. The very low mitotic index in the brains of male hybrid larvae is reflected in the extremely slow progression of these animals through the second larval instar stage of development. Our results suggest that the cells of hybrid male larvae display physiological defects that prevent mitotic entry and that render them particularly susceptible to cell death when environmentally stressed.

**MATERIALS AND METHODS**

**Fly strains and cultures**

Flies were reared on standard yeast glucose media and raised at 23°C on a 12 hr light / 12 hr dark cycle. All hybrid crosses were initiated with one-day-old *D. melanogaster* virgin females and 5-day-old *D. simulans* males. *D. melanogaster* females were either *y¹ w¹* received from the Drosophila stock
center (Bloomington, IN) or Df(1)Hmr, y w v / FM7i, P(w^{mc}=ActGFP)JMR3 (Barbash and Lorigan, 2007). All D. simulans males were vermilion / Y.

**Cytology for Analysis of Mitosis**

Larval brains were dissected, fixed, and squashed as described in Williams *et al.* (1992) except that incubation in 3.7% formaldehyde was decreased to 20 min. Incubations with antibodies were done overnight at 4°C in phosphate buffered saline (PBS) containing 0.1% Triton X (PBT). Rabbit anti-Phosphohistone H3 (Upstate, Charlottesville, VA) was used at a 1:500 dilution followed by incubation with TRITC (tetrarhodamineisothiocyanate)-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA), and DNA was detected by staining with 0.05 µg/ml Hoechst 33742 (Sigma, St. Louis, MO) in PBS for 5 min. Imaging was done on an Olympus BX-50 microscope with a Qimaging Retiga Exi CCD camera (Burnaby, BC, Canada) and MetaMorph 6.1 software (Universal Imaging, Downingtown, PA).

For aceto-orcein staining, larval brains were dissected in 0.7% NaCl and transferred immediately to a drop of aceto-orcein stain (2% orcein in 45% acetic acid) on a cover slip and squashed onto a glass slide (Gatti and Goldberg, 1991). In some experiments, brains were incubated in 0.5x10^{-5} M colchicine in 0.7% NaCl for 0.5, 1, or 2 hrs after dissection prior to transfer to aceto-orcein. Mitotic indices were determined as previously described (Williams et al., 1992); briefly, the mitotic index of a sample was defined as the number of PH3-staining mitotic cells per standard field of view at 1000X magnification. Individual stages of mitosis were scored in PH3-positive cells. Cells in prophase and prometaphase were characterized by their relatively low levels of chromosome condensation. Metaphase figures were scored on the
basis of their more highly condensed chromosomes. Anaphase and telophase were identified by the separation of chromatids into distinct populations, but these two stages could not be separately distinguished.

Determination of S Phase using Bromodeoxyuridine

Whole dissected brains were incubated in Grace’s Media plus 1mg/ml 5-Bromo 2-Deoxyuridine (BrDU; Sigma) for 1 hr, and then fixed, permeabilized, and antibody stained as described in (Truman and Bate, 1988). Mouse anti-BrDU and rat anti-Elav were used at a dilution of 1:150 in PBT overnight at 4°C. These antibodies were received from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242. After five 10-min washes in PBT, brains were incubated overnight at 4°C with the following secondary antibodies: AlexaFluor 488-conjugated donkey anti-mouse (Molecular Probes, Eugene, OR) and Cy3-conjugated donkey anti-rat (Jackson Immunoresearch) diluted to 1:1000 in PBT + 1% donkey serum. Brains were imaged on a Leica TCS SP2 confocal microscope system.

Cell Death Assays

Cell death was assayed using Trypan Blue 0.4% (Gibco, Carlsbad, CA) at a 1:100 dilution in PBS. Brains were stained for 10 min and then rinsed for 10 min in PBS. As a positive control, cell death was induced in D. melanogaster y¹ w¹ brains by treatment for 1 hr in 100 mM cycloheximide immediately before Trypan Blue staining.
Apoptosis was detected using the Vybrant Apoptosis Assay Kit #2 (Molecular Probes) following the manufacturer’s instructions. Whole brains were incubated for 20 min in a 1:4 dilution of Alexa Fluor 488-conjugated Annexin V stock solution in Annexin Binding Buffer (ABB). After a brief rinse in ABB, DNA was additionally counterstained for 5 min using To-Pro-3 (Molecular Probes) at 1x10^{-3} dilution in the same buffer. The brains were then mounted in 80% glycerol and imaged immediately on a Leica TCS SP2 confocal microscope. As a positive control, apoptosis was induced in *D. melanogaster* y¹ w¹ larvae by incubating dissected brains in 10 mM cycloheximide in 0.7% NaCl for 1 hr followed by recovery in Grace’s media (Invitrogen, Carlsbad, CA) at 25°C for 30 min before treating as above to detect apoptosis.

Autophagic cell death was detected using LysoTracker Green DND-26 (Molecular Probes) as described in the product manual. Briefly, LysoTracker Green was diluted to 75 nM in Grace’s media and warmed to 37°C. Dissected brains were incubated in the diluted LysoTracker at 37°C for 1 hr before washing with PBS, counterstaining DNA with To-Pro-3, and imaging as above.

### Ecdysone Feeding

20-Hydroxyecdysone (20HE; Sigma) was diluted to 1 mg/ml in 5% ethanol and mixed with 0.5 g dry yeast to make a yeast paste. Second- and third-instar larvae were transferred to plates containing the yeast paste and observed over time. Rescue of the developmental block was defined as a significant increase in the percentage of animals undergoing pupariation.
Larval staging and brain-size estimation

Females were allowed to lay eggs for a 24 hr period, and progeny were assayed for stage of development every 24 hrs after removal of adults. Larvae were staged by characterizing either mouth hook morphology or tracheal development. Mouth hooks were dissected from larvae and imaged at 400X magnification. Second-instar larvae have only 5-15 teeth per mouth hook, while third-instar larvae have 20-30 teeth per mouth hook (Demerec, 1950). For staging of live animals, larvae with unextended, balled trachea were classified as second instars, while larvae with extended trachea and inflated spiracles were classified as third instars (Park et al., 2002).

Brain sizes were measured as length of the ventral ganglion and width of the distance between the center of the larval brain hemispheres. Size was also estimated in squashes by the number of fields of view each brain occupied at 100X magnification.

RESULTS
Developmental Delays in Hybrid Males

It has long been known that the F1 males resulting from crosses between *D. melanogaster* mothers and *D. simulans* fathers develop more slowly than their sister F1 females or animals from either pure species (Sánchez, 1983). Since these developmental delays have never been described in detail, we took a closer look at the progression of hybrid males through the three larval instars and the pupal stage. Using the morphologies of both the mouth hooks and tracheal spiracles as markers to stage larvae (see Materials and Methods), we found that in comparison with controls, the hybrid males progress more slowly through each post-embryonic stage (Table 3.1), and died with high
frequencies at each step (Table 3.2). Controls included males and females of 
each pure species, hybrid females, and hybrid males rescued by a deletion of 
the $Hmr$ gene, $Df(1)Hmr^{-}$. Developmental delay was particularly noticeable in 
the period preceding the molt into the third instar. While wild-type animals of 
either species spend approximately only 1 day as second-instar larvae, $F_1$ 
hybrid males remained in the second instar for more than 7 days on average. 
There was considerable variation in the length of the second-instar period. 
The slowest-developing hybrid-male second instars remained in that stage for 
11-12 days (that is, until roughly 15 days after egg deposition). These animals 
became very large and could thus be mistaken for small third instars (Figure 
3.1A), but the mouth hook and tracheal markers confirmed they were indeed in 
the second instar. All of these large second instars failed to molt and were 
thus responsible for the elevated lethality observed during the second-instar 
phase (Table 3.2). The majority (75.3%) of hybrid males eventually became 
third instars (Figure 3.1B). This third instar stage is also where the size 
difference in the brain became most distinct (Figure 3.1C-D, Table 3.3). Most of 
these third instars failed to pupariate, and died within 2-3 days of the second- 
to-third instar molt. A small number of hybrid males (6.7% of the total) 
pupariated; these tended to be the individuals that progressed most quickly 
through the second-instar phase. In all cases, pupariating animals died within 
24 hours. Less than 1% of hybrid males were still alive at 16 days post egg 
deposition.
### TABLE 3.1. Days post egg deposition taken to reach indicated stage of development

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1st instar</th>
<th>2nd instar</th>
<th>3rd instar</th>
<th>Pupa</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Hmr^{+}/FM7$</td>
<td>1.3 ± 0.45</td>
<td>2.6 ± 0.62</td>
<td>3.7 ± 0.42</td>
<td>6.8 ± 0.35</td>
<td>903</td>
</tr>
<tr>
<td>$D. mel.$ f</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$Hmr^{+}/Y D. mel.$ m</td>
<td>1.2 ± 0.24</td>
<td>2.4 ± 0.48</td>
<td>3.6 ± 0.36</td>
<td>6.7 ± 0.41</td>
<td>576</td>
</tr>
<tr>
<td>$y^{+}w^{+} D. mel.$ b</td>
<td>1.2 ± 0.29</td>
<td>2.2 ± 0.62</td>
<td>3.5 ± 0.53</td>
<td>6.8 ± 0.48</td>
<td>862</td>
</tr>
<tr>
<td>$v_{sim} D. sim.$ b</td>
<td>1.3 ± 0.26</td>
<td>2.1 ± 0.47</td>
<td>3.4 ± 0.40</td>
<td>6.9 ± 0.43</td>
<td>625</td>
</tr>
<tr>
<td>$FM7/X_{sim}$ hybrid f</td>
<td>1.2 ± 0.21</td>
<td>2.0 ± 0.53</td>
<td>3.0 ± 0.38</td>
<td>6.3 ± 0.30</td>
<td>398</td>
</tr>
<tr>
<td>$FM7/Y_{sim}$ hybrid m</td>
<td>2.1 ± 0.56</td>
<td>4.1 ± 2.61</td>
<td>11.5 ± 4.8</td>
<td>12.1 ± 1.17</td>
<td>472</td>
</tr>
<tr>
<td>$Hmr^{+}/X_{sim}$ hybrid f</td>
<td>1.3 ± 0.26</td>
<td>2.4 ± 0.46</td>
<td>3.6 ± 0.55</td>
<td>6.9 ± 0.69</td>
<td>637</td>
</tr>
<tr>
<td>$Hmr^{+}/Y_{sim}$ hybrid m</td>
<td>1.5 ± 0.54</td>
<td>3.0 ± 0.81</td>
<td>4.4 ± 0.91</td>
<td>7.9 ± 1.90</td>
<td>452</td>
</tr>
</tbody>
</table>

---

| a $D. simulans$ chromosomes are indicated with the subscript “sim”.
| b Males and females were counted together. |
### TABLE 3.2. Lethality during indicated developmental stage

<table>
<thead>
<tr>
<th>Genotype</th>
<th>1st instar</th>
<th>2nd instar</th>
<th>3rd instar</th>
<th>pupal</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Hmr</em> / FM7 <em>D. mel.</em> females</td>
<td>2.1</td>
<td>1.1</td>
<td>&lt;1%</td>
<td>1.0</td>
</tr>
<tr>
<td><em>Hmr</em> / Y <em>D. mel.</em> males</td>
<td>2.0</td>
<td>0.9</td>
<td>&lt;1%</td>
<td>1.1</td>
</tr>
<tr>
<td><em>y’w’</em> <em>D. mel.</em> b</td>
<td>1.0</td>
<td>&lt;1%</td>
<td>&lt;1%</td>
<td>1.2</td>
</tr>
<tr>
<td><em>v</em>&lt;sub&gt;sim&lt;/sub&gt; <em>D. sim.</em> b</td>
<td>1.1</td>
<td>1.2</td>
<td>1.2</td>
<td>2.1</td>
</tr>
<tr>
<td><em>FM7/X</em>&lt;sub&gt;sim&lt;/sub&gt; hybrid females</td>
<td>2.3</td>
<td>1.5</td>
<td>2.3</td>
<td>1.4</td>
</tr>
<tr>
<td><em>FM7/Y</em>&lt;sub&gt;sim&lt;/sub&gt; hybrid males</td>
<td>5.3</td>
<td>19.4</td>
<td>68.6</td>
<td>6.8</td>
</tr>
<tr>
<td><em>Hmr</em> / <em>X</em>&lt;sub&gt;sim&lt;/sub&gt; hybrid females</td>
<td>n/a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.2</td>
<td>1.8</td>
<td>1.2</td>
</tr>
<tr>
<td><em>Hmr</em> / <em>Y</em>&lt;sub&gt;sim&lt;/sub&gt; hybrid males</td>
<td>n/a&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2.8</td>
<td>3.8</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Larvae were raised at 23°C and all *n’s* are > 100.

<sup>a</sup> *D. simulans* chromosomes are indicated with the subscript “*sim*”.

<sup>b</sup> Males and females were counted together.

<sup>c</sup> Rescued hybrids were not scored at 1st instar.
Figure 3.1. Developmental progress of hybrid males. (A) Second instar hybrid males are smaller than pure species of the same age, but they can continue to grow and become much larger than wild type second instars. The smaller and larger hybrid male second instar larvae at the bottom are 6 days and 12 days post egg deposition, respectively. (B) Third instar male larvae are smaller than those of either pure species, but considerably larger than second instar hybrids. The hybrid third instar is 12 days post egg deposition. Scale bar = 1 mm for A and B. (C) Brains from hybrid second instar larvae are slightly smaller than those of the corresponding pure species stage (2.4 versus 2.6 mm in length on average, n>20). (D) By the third instar, the discrepancy in brain size becomes more obvious (3.2 versus 5.3 mm in length on average, n>20). Hybrid male third instar larvae also lack the imaginal discs seen in the pure species (arrows). Scale bar = 0.5 mm for C and D.
### TABLE 3.3 Brain sizes

<table>
<thead>
<tr>
<th>Instar</th>
<th>Days Old</th>
<th>Brain length (A)</th>
<th>Lobe width (B)</th>
<th>Ganglion width (C)</th>
<th># of Brains scored</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>D. melanogaster</em></td>
<td>2nd</td>
<td>4</td>
<td>0.26</td>
<td>0.23</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>6</td>
<td>0.53</td>
<td>0.39</td>
<td>0.1</td>
</tr>
<tr>
<td>Hybrid males</td>
<td>2nd</td>
<td>4</td>
<td>0.24</td>
<td>0.21</td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>2nd</td>
<td>11</td>
<td>0.31</td>
<td>0.24</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>3rd</td>
<td>14</td>
<td>0.32</td>
<td>0.28</td>
<td>0.15</td>
</tr>
</tbody>
</table>
Defects in Ecdysone-Induced Behavior

Normal-sized hybrid second-instar larvae initiated the normal pre-molting behaviors of moving their bodies in an anterior-posterior fashion and biting with their mouth hooks to free the cuticle (Park et al., 2002). However, these animals often failed to molt, even after 30 minutes of trying, and returned to a wandering pattern of behavior.

As molting behaviors are induced by ecdysone (Park et al., 2002), we attempted to rescue these phenotypes by feeding the larvae 20-hydroxyecdysone at different stages of larval development (see Materials and Methods). This feeding, regardless of the protocol used, failed to rescue either the slow rate of development or the extended attempts at molting exhibited by hybrid males (data not shown). 20-hydroxyecdysone was however able to rescue the molting ability of control second- or third-instar larvae homozygous for mutations in the Eip75B gene, a gene that responds early within the ecdysone-induction pathway [(Segraves and Hogness, 1990); data not shown]. These results suggest that the developmental and behavioral abnormalities encountered by hybrid male larvae are unlikely to reflect defects in pathways mediated by ecdysone.

Low Mitotic Frequencies in Hybrid Male Larvae

It was previously proposed (Orr et al., 1997) that many cells in the brains of hybrid male larvae enter mitosis, but fail to condense their chromosomes properly and thus arrest in an aberrant prophase-like stage. To characterize these apparent mitotic defects in more detail, we examined preparations of larval brains stained either with orcein or with a combination of Hoechst 33742 (for DNA) and an antibody to phosphohistone H3 (anti-PH3) to
track mitotic chromatin (Hendzel et al., 1997). We were surprised to find a nearly complete absence of mitotic figures in second-instar hybrid male brains: usually only 0-1 cells per brain stained positive with anti-PH3. Third-instar hybrid male larval brains displayed more, but still very few mitotic figures (<10), as compared with several hundred PH3-staining cells in control single species brains from the same developmental stage (data not shown).

Of the few mitotic figures that were seen in the brains of hybrid male larvae, almost all were in prophase/prometaphase (79%) or metaphase (20%) (Figure 3.2), with less than 1% being in anaphase/telophase (Table 3.4). In contrast, about 20% of mitotic figures from pure species males are in anaphase/telophase. These data suggest that the spindle checkpoint may be activated in the very small number of hybrid male cells that do in fact enter mitosis. The deletion of Hmr in male hybrids returned the proportion of mitotic figures in anaphase/telophase to near single-species ratios, but the mitotic index remained somewhat lower than the controls (Table 3.4). Chromosome morphology in the rescued hybrid male larvae was often unusually elongated and spindly (Figure 3.3A,B). Thus, although the deletion of Hmr rescues the lethality of hybrid males, cell cycle progression in these animals is not completely normal.
TABLE 3.4. Percentage of mitotic figures in pure species and hybrid 3\textsuperscript{rd} instar larvae

<table>
<thead>
<tr>
<th>Genotype (^a)</th>
<th>Prophase / prometaphase</th>
<th>Metaphase</th>
<th>Anaphase / telophase</th>
<th>Mitotic index</th>
<th>Avg. no. fields / brain</th>
<th>Number of fields examined</th>
</tr>
</thead>
<tbody>
<tr>
<td>D. melanogaster males</td>
<td>70</td>
<td>10</td>
<td>20</td>
<td>0.56</td>
<td>103</td>
<td>947</td>
</tr>
<tr>
<td>D. melanogaster Hmr– males</td>
<td>71</td>
<td>10</td>
<td>19</td>
<td>0.60</td>
<td>109</td>
<td>526</td>
</tr>
<tr>
<td>D. melanogaster females</td>
<td>69</td>
<td>11</td>
<td>20</td>
<td>0.57</td>
<td>111</td>
<td>511</td>
</tr>
<tr>
<td>D. simulans males</td>
<td>70</td>
<td>11</td>
<td>19</td>
<td>0.59</td>
<td>106</td>
<td>932</td>
</tr>
<tr>
<td>D. simulans females</td>
<td>68</td>
<td>10</td>
<td>22</td>
<td>0.58</td>
<td>114</td>
<td>496</td>
</tr>
<tr>
<td>FM7/Y\textsubscript{sim} hybrid males</td>
<td>79</td>
<td>20</td>
<td>&lt;1</td>
<td>0.13</td>
<td>20</td>
<td>532</td>
</tr>
<tr>
<td>Hmr–/Y\textsubscript{sim} hybrid males</td>
<td>70</td>
<td>13</td>
<td>17</td>
<td>0.33</td>
<td>81</td>
<td>776</td>
</tr>
<tr>
<td>FM7/X\textsubscript{sim} hybrid females</td>
<td>52</td>
<td>3</td>
<td>45</td>
<td>1.04</td>
<td>92</td>
<td>404</td>
</tr>
<tr>
<td>Hmr–/X\textsubscript{sim} hybrid females</td>
<td>69</td>
<td>12</td>
<td>18</td>
<td>0.80</td>
<td>104</td>
<td>628</td>
</tr>
</tbody>
</table>

\(^a\) D. simulans chromosomes are indicated with the subscript “sim”.
Figure 3.2 Progress of mitosis based on the age of larvae. All mitotic figures shown are at the same magnification and are the only mitotic figures observed in the entire brain. (A) Second instar larvae rarely show any mitotic figures. (B) In brains where there are cells dividing the chromosomes usually appear highly under-condensed. (C) Young third instars show mitotic figures that have chromosomes that are individually distinguishable. (D) Larvae that reach the third instar stage and continue to develop during this stage can be seen with four, eight, or even rarely sixteen mitotic figures. These larvae are also the “escapers” that are capable of pupating.
Figure 3.3  Chromosome morphology in hybrid brains.

In all panels, DNA is shown in blue and PH3 in red.  (A)  *D. simulans* mitotic figure with condensed and clearly defined chromosomes.  (B) Many (>50%) mitotic figures in *Df(1)Hmr* hybrid male larval brains show a diffuse thread-like chromosome morphology.  (C) In *FM7/X*<sub>sim</sub> hybrid females, mitotic chromosomes are generally under-condensed, despite their positive PH3 signal.  (D) *Df(1)Hmr/X*<sub>sim</sub> hybrid female with normal appearing mitotic chromosomes. Scale bar = 5 µm.
TABLE 3.5  Mitotic indices of larval brain cells before and after colchicine treatment

<table>
<thead>
<tr>
<th></th>
<th>0 min&lt;sup&gt;a&lt;/sup&gt;</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D. melanogaster</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd instar</td>
<td>0.7% NaCl</td>
<td>0.45</td>
<td>0.43</td>
</tr>
<tr>
<td></td>
<td>+Colchicine</td>
<td>n.d.</td>
<td>0.91</td>
</tr>
<tr>
<td>3rd instar</td>
<td>0.7% NaCl</td>
<td>0.68</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>+Colchicine</td>
<td>n.d.</td>
<td>1.59</td>
</tr>
<tr>
<td><strong>X&lt;sub&gt;mel&lt;/sub&gt;/Y&lt;sub&gt;sim&lt;/sub&gt; hybrid</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd instar</td>
<td>0.7% NaCl</td>
<td>0.078</td>
<td>0.057</td>
</tr>
<tr>
<td></td>
<td>+Colchicine</td>
<td>n.d.</td>
<td>0.063</td>
</tr>
<tr>
<td>3rd instar</td>
<td>0.7% NaCl</td>
<td>0.15</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>+Colchicine</td>
<td>n.d.</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Mitotic indices were calculated as the number of PH3-positive cells per microscope field.

<sup>a</sup>Amount of time cultured after dissection but pre-fixation.

<sup>n</sup>’s are ≥ 15 brains.

n.d. = not determined.
Consistent with the low levels of mitosis seen in the larval brains of the male hybrids, these brains were smaller in size than those of control second or third instar larvae (Figure 3.1C,D; see also the “Number of fields per brain” column in Table 3.4), as noted previously by Orr et al. (1997). This difference was particularly stark for third instars, where the total volume of hybrid male brains was only about 1/5 that of control brains (Figure 3.1D, Table 3.4). We measured the mitotic index (defined as the number of mitotic figures per field of view) to normalize the amount of mitosis relative to the brain volume, and found that the mitotic index of third-instar hybrid brains was decreased by roughly four-fold relative to the single species at the same developmental stage (Tables 3.4 and 3.5).

A similar reduction in the mitotic index was also observed in second instar larvae. The absence of cell division in hybrid larvae was not restricted to the brains; these larvae also showed a near complete absence of imaginal disc tissue (Figure 3.1D), as previously reported (Seiler and Nothiger, 1974; Sánchez, 1983). In a further attempt to gauge the frequency of mitosis in hybrid males, we cultured larval brains in colchicine, a drug that inhibits microtubule polymerization and thus arrests cells in mitosis. When the brains of control pure species larvae were treated with colchicine in 0.7% NaCl [the solution normally employed for cytological analysis in *Drosophila* (Gatti and Goldberg 1991), mitotic indices increased roughly 2-fold after one hour and 4-fold after two hours incubation (Table 3.5). In contrast, the frequency of mitosis in hybrid brains did not increase even after two hours of colchicine treatment.
Figure 3.4 Hybrid male larval brains lack cells in S phase.

(A,B) Wild type *D. melanogaster* brains incorporate substantial BrDU in the developing optic lobe (arrows; antibody against BrDU is in green). Some scattered neuroblasts are also observed to undergo DNA replication (arrowheads). As a counterstain, differentiated neurons are marked by anti-Elav (red). (C,D) Brains from third instar hybrid larvae have no (C) or few (arrowheads in D) BrDU-incorporating cells. Because hybrid male brains have poorly-developed optic lobes, the outline of the brain tissue in (D) is shown with white dots. Scale bar = 0.5 mm.
This latter result is consistent with the idea that the brains of hybrid larvae have very few cells competent to enter mitosis. Interestingly, cells in hybrid females also appear to encounter difficulties in cell cycle progression, though these problems may be of a different nature. First, few of the PH3-positive mitotic figures in hybrid female larval brains display the degree of chromosome condensation characteristic of metaphase (Table 3.4; Figure 3.3C). Second, the mitotic index of female hybrid brains is ~1.8X higher than that of males or females in the single species, while at the same time an unexpectedly high proportion of cells in female hybrid brains are in anaphase/telophase (Table 3.4). Such problems in cell cycle progression may explain the sterility of adult hybrid females, since extensive cell division is required for amplification of the female germline and for oogenesis. This hypothesis is supported by findings that deletion of *D. melanogaster* Hmr in hybrid females normalizes both mitotic progression (Table 3.4) and chromosome condensation (Figure 3.3D), while at the same time Hmr deletion partially suppresses the sterility of these animals (Barbash and Ashburner, 2003).

**Low Frequency of S Phase in the Brains of Hybrid Male Larvae**

Since few cells in hybrid male larval brains are competent to enter mitosis, we also wanted to determine by BrDU incorporation whether any of these cells were in S phase. In the brains of third instar larvae from the pure species, the majority of DNA replication occurs in two bands in each optic lobe, but several neuroblasts scattered throughout the remainder of the brain also replicate their DNA (Figure 3.4A,B). In contrast, smaller third instar hybrid male brains displayed no BrDU incorporation (Figure 3.4C), while only a small number of cells were replicating their DNA in larger hybrid brains (Figure
3.4D). The low levels of DNA replication and mitosis seen in hybrid larval brains suggest that the large majority of cells in this tissue are unable to enter either S or \( M \) phases, but these data do not discriminate between arrest in \( G_1 \) or \( G_2 \) phases.

Orr et al. (1997) reported that when the brains of hybrid (\textit{D. melanogaster} / \textit{D. mauritiana}) male larvae were cultured in the presence or absence of colchicine in 0.7% NaCl, there was a dramatic increase in the number of cells displaying dense but diffuse chromatin. These authors interpreted such cells as being arrested in an early stage of mitosis, with chromatin more condensed than interphase chromatin but less condensed than the chromatin seen in metaphase mitotic figures. Even though we examined hybrids between \textit{D. melanogaster} and a different sibling species (\textit{D. simulans}), we were able to reproduce these results: cultured brains from hybrids but not from \textit{D. melanogaster} rapidly fill with cells showing this aberrant chromosome morphology (Figure 3.5A-D; Table 3.6). However, the fact that the PH3-positive mitotic index does not increase when the brains of hybrid males are exposed to 0.7% NaCl in the presence or absence of colchicine indicates that these cells are not actually in mitosis (Table 3.4; Figure 3.5E-H).

When male hybrid brains were incubated in Grace’s Media instead of 0.7% NaCl, the frequency of cells showing aberrant nuclear morphology decreased by 85% (Table 3.6). This result suggests that the changes in the appearance of chromatin are mostly an artifact of incubation in 0.7% NaCl.
Figure 3.5  Cells with aberrant nuclear morphology seen in the cultured brains of hybrid males are not in mitosis. (A, B, E, F) Control *D. melanogaster* third instar larval brains. (C, D, G, H) Hybrid male third instar larval brains. The brains in panel A, C, E and G were squashed immediately after dissection, while those in B, D, F and H were examined after a 1 hr incubation in colchicine. (A-D) Orcein stained brain squashes. The mitotic index in pure species brains increases after colchicine treatment (A, B). ~25% of the cells in hybrid male larval brains develop an abnormally dense chromatin morphology after the same treatment (C, D). Scale bar = 5 µm for A-D. (E-G) Brain squashes stained for DNA (blue) and phosphohistone H3 (PH3; red) to visualize mitotic chromosomes. The mitotic index as measured by PH3 staining increases in pure species with colchicine incubation (E, F), but few cells in hybrids stain with PH3 in the presence or absence of colchicine (G, H). Scale bar = 15 µm for E-H.
TABLE 3.6 Percentage of aberrant cells in larval brains

<table>
<thead>
<tr>
<th></th>
<th>0 min(^a)</th>
<th>60 min</th>
<th>120 min</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>D. melanogaster</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd instar 0.7% NaCl</td>
<td>0</td>
<td>0</td>
<td>2.8</td>
</tr>
<tr>
<td>Grace’s Media</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3rd instar 0.7% NaCl</td>
<td>0</td>
<td>0</td>
<td>3.1</td>
</tr>
<tr>
<td>Grace’s Media</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Hybrid male</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd instar 0.7% NaCl</td>
<td>3</td>
<td>23.8</td>
<td>48.6</td>
</tr>
<tr>
<td>Grace’s Media</td>
<td>2.8</td>
<td>3.7</td>
<td>4.8</td>
</tr>
<tr>
<td>3rd instar 0.7% NaCl</td>
<td>3.5</td>
<td>27.9</td>
<td>47.2</td>
</tr>
<tr>
<td>Grace’s Media</td>
<td>3.6</td>
<td>4.2</td>
<td>5.1</td>
</tr>
</tbody>
</table>

Percentage of total cells showing aberrant chromatin morphology.

\(^a\)Amount of time cultured after dissection pre-fixation.

\(n\)’s ≥10 brains
However, freshly dissected hybrid male larval brains that were either placed into Grace’s Media or immediately prepared for cytological analysis without any incubation showed a low frequency (2-4%) of aberrant cells (Table 3.6). It thus appears that 0.7% NaCl hastens a process that had already begun in hybrid larval brains prior to exposure to the salt solution.

We suspected that the cells with aberrant nuclear morphology were either dead or dying, so we stained larval brains with the vital dye Trypan Blue which labels degenerating cells (Krebs and Feder, 1997). Though control brains incubated in 0.7% NaCl for 1 hour had few if any cells that stained with this dye, a very high percentage of cells in identically-treated hybrid male larval brains were positive for Trypan Blue (Figure 3.6A,B). The levels of cell death were roughly equivalent to those achieved by exposing larval brains from either pure species to cycloheximide for 1 hour to induce apoptosis (Figure 3.6C). Cell death was much reduced (though still visible in a few cells) in hybrid male brains incubated in Grace’s Media (Figure 3.6D).

Further characterization of the dead or dying cells in the brains of hybrid male larvae after salt treatment suggests that this phenotype is the result of necrosis rather than apoptosis or autophagy. Although there was a reproducible, slight elevation of apoptosis in these brains as determined by detection of the apoptotic marker Annexin V (van den Eijnde et al., 1998), the frequency of apoptotic cells was far too low to explain the high prevalence of cell death (Figure 3.6E-H). When hybrid larval brains in 0.7% NaCl were stained with Lysotracker, which measures pH changes in lysosomes indicative of autophagic cell death (Anderson and Orci, 1988), little if no staining was observed (data not shown).
Figure 3.6 Cell death is increased in 0.7% NaCl cultured third instar hybrid male brains. (A-D) Trypan Blue staining of dead cells. (A) *D. melanogaster* brain showing no cell death after incubation in 0.7% NaCl for 1 hour. (B) Hybrid male brains incubated under the same conditions show massive cell death. (C) As a positive control, *D. melanogaster* brains cultured in 10 mM cycloheximide for 1 hour and allowed to recover for 30 minutes to induce cell death similarly show intensive staining with Trypan Blue. (D) Hybrid male brains incubated in Grace’s media for one hour instead of 0.7% NaCl. The number of dead cells is greatly diminished. Single species brains incubated in Grace’s media had virtually no Trypan Blue staining (data not shown). Scale bar =50 µm for A-D (E-H) Annexin V staining to visualize apoptosis. Neither *D. melanogaster* (E) nor hybrid brains incubated in 0.7% NaCl (F) or Grace’s media (G) display many apoptotic cells. (H) A positive control in which apoptosis was induced in *D. melanogaster* brains by cycloheximide. Scale bar = 1 mm for E-H.
Hybrid male larvae also contained obvious melanotic masses, primarily in gut tissues but also occasionally in salivary glands (Figure 3.7). These melanotic masses might be caused by cell death, but they could in theory also result from an aberrant immune response generated in these unhealthy larvae (Minakhina and Steward, 2006).

**DISCUSSION**

Although hybrid incompatibility between *D. melanogaster* and its sibling species was first studied in the 1920s (Sturtevant, 1920; Sturtevant, 1929), the cellular and developmental basis of hybrid male lethality remains unknown. To our knowledge, the first direct examination of this issue was performed by Orr *et al.* (1997), who proposed that hybrid lethality was caused by mitotic defects associated with failure of proper chromosome condensation. The results reported here verify that hybrid male larvae do in fact suffer from problems in cell proliferation. However, we find that these defects largely reflect the inability of cells to enter mitosis; cells in hybrid male larval brains are not, as described in the previous study, subject to cell-cycle arrest that occurs after M phase has initiated. The failure of most cells to enter mitosis would explain not only the almost total absence of brain cells that stain positive for phosphohistone H3 (Figure 3.5E-H), but also the small brain size and the absence of imaginal disks in hybrid male larvae [Figure 3.1; see also (Sánchez, 1983; Seiler and Nothiger, 1974)]. Since there is virtually no incorporation of BrdU in hybrid male brains (Figure 3.4), it is clear that very few cells are in S phase at any particular time, so the majority of cells must instead be arrested either in G1 or G2 phases.
Figure 3.7 Naturally forming melanotic tumors from third instar hybrid males. These tumors form primarily in gut tissue (A-D), although they also develop in salivary glands (not shown). Scale bar = 100 µm for E,F and 250 µm for G. (D) Lower magnification of several gut tumors in a single larvae.
Our results further suggest that cells in the brains of hybrid males are particularly sensitive to environmental stress. Incubation of brain tissue from hybrid male larvae in 0.7% NaCl, a solution thought to be of physiological osmolarity and widely used for *Drosophila* cytology (Gatti and Goldberg, 1991), induces considerable cell death (Figure 3.5). We believe that the aberrant nuclear morphology reported by Orr *et al.* (1997) and interpreted as incomplete mitotic chromosome condensation is mostly the result of artifactual cell death resulting from exposure to 0.7% NaCl (Table 3.6). With the benefit of hindsight, the results of these previous investigators do not comfortably fit their hypothesis of a mitotic arrest. The proportion of cells with aberrant nuclear morphology after 90 min of incubation was reported to be more than 25%, a frequency more than 5 times higher than the mitotic index of the strongest known metaphase arrest mutants in *Drosophila* (Gatti and Goldberg, 1991). Past experience thus suggests that such a high percentage of cells are highly unlikely to enter mitosis within a few hours of incubation. Moreover, the interpretation of Orr *et al.* (1997) fails to explain the much lower frequency of such cells (0.25%) they observed in freshly dissected hybrid brains. If 0.7% NaCl is truly physiological and the cells with aberrant nuclear morphology represent mitotic arrest, one would expect their frequency to be high prior to incubation as well.

The most straightforward interpretation of our results is that cellular physiology in hybrid male larvae is substantially altered, leading in vivo to a dramatic decline in the rate of cell proliferation and a modest increase in the level of cell death. Many of the surviving cells are sensitized to environmental conditions, and die readily by necrosis after exposure to 0.7% NaCl. We found
that the induction of cell death by 0.7% NaCl occurs in dissected tissue, and
that the slow development of hybrid male larvae is not rescued by treatment
with 20-hydroxyecdysone. More direct evidence that the cellular defects are
cell-autonomous comes from Orr et al. (1997), who showed that X/O somatic
clones induced in hybrid females are extremely small and thus proliferation-
deficient; the growth defects of the clones are not rescued by adjacent X/X
tissue.

While our results provide little direct guidance about the molecular basis
of hybrid lethality, they do affirm that hybrids are defective in cellular
physiology and proliferation. Since we have shown that the lethality of hybrid
larvae does not reflect arrest in M phase, what alternative mechanisms are
plausible? A recent microarray-based transcriptional profiling comparison of
lethal and viable [Df(1)Hmr-rescued] hybrids found a surprisingly small
magnitude of transcriptional differences between these genotypes, suggesting
that hybrid lethality is also not caused by large-scale transcriptional
misregulation (Barbash and Lorigan, 2007). Recent studies instead hint that
hybrids may have defects in chromatin structure. Pal-Bhadra et al. (2006)
found that dosage compensation proteins fail to localize to the hybrid male X
chromosome. Dosage compensation defects are unlikely to be the sole
explanation of hybrid lethality because this lethality is fully penetrant in both
male and females that are hemizygous or homozygous for the D. melanogaster X chromosome. These results do however suggest that the
hybrid male X chromosome has an aberrant chromatin structure, since proper
localization of dosage compensation proteins requires a unique X
chromosome chromatin state (reviewed in Akhtar, 2003). Brideau et al. (2006)
recently found that the hybrid lethal gene Lhr encodes a protein that interacts
and colocalizes with Heterochromatin Protein 1 (HP1). Greil et al. (2007) also independently found that LHR (alternatively referred to as HP3) colocalizes with HP1 and depends on HP1 for its correct localization in cultured cells. The requirement of HP1 for the maintenance of heterochromatin states (Eissenberg and Elgin, 2000) suggests the possibility that hybrid lethality may result from an altered structure of heterochromatin. Mechanistic studies of Lhr and Hmr will be required to test these speculations and to understand why cells do not proliferate properly in hybrids.

ACKNOWLEDGEMENTS
This work was supported by NIH grants to D.A.B. (5R01GM074737-02) and to M.L.G. (5R01GM048430-14). Antibodies were received from the Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA. I would also like to thank W Dr. H. Allen Orr and Dr. Patrick Ferree for helpful comments on the manuscript.
LITERATURE CITED


CHAPTER 4
CONCLUSIONS AND FUTURE DIRECTIONS

I. TOYS ARE US (TRUS) AND ECDYSONE SYNTHESIS

The intricacies of the ecdysone-signaling pathway in Drosophila have been extensively studied (Riddiford, 1993), but many components and processes are still unknown. Based on my research, it is highly likely that Trus is involved in this pathway by regulating the production of ecdysone.

A model for ecdysis including a potential role for Trus

When a juvenile stage of development (that is, all stages before eclosion as adults) is complete, Prothoracicotrophic hormone (PTTH) is synthesized in the dorsal lateral region of the brain and then travels to the prothoracic cells of the ring gland, where it triggers a cascade that culminates in the synthesis of ecdysone (Mizoguchi et al., 2001). Based on the high levels of Trus detected in the prothoracic cells, it is unlikely that Trus acts upstream of PTTH. Instead, I suggest that Trus acts downstream of PTTH to regulate the translation of mRNAs whose products are involved in ecdysone synthesis in the ring gland. This hypothesis is based first on our finding that Trus interacts with String of Pearls (Sop), a subunit of the 40S ribosome. Our evidence that Trus does not interact with the intact ribosome would require Trus to act on/with Sop and Ef1-α prior to their association with the intact ribosome, but does not exclude Trus from having an indirect, yet essential, function in translation control. Furthermore, the literature contains precedents for the translational regulation of hormone production and action. In Manduca
(and likely in *Drosophila* and other insects), the S6 kinase, which phosphorylates the 40S ribosomal protein RpS6, is required for PTTH stimulated gated translation in the ring gland (Gilbert et al., 2000). RpS6 in the mouse is again the target of S6 kinase, but during insulin signaling (Mizoguchi et al., 2001). In this light, our data indicative of a possible interaction between Trus and Ef1-α acquires some plausibility since mouse Ef1-α is also part of the S6 kinase cascade.

One confusing aspect of our results is the finding that Trus does not stably bind the 40S subunit of the ribosome. Assuming that this result is not artifactual, this suggests that Trus up-regulates Sop and/or Ef1-α activity by a currently unknown mechanism that occurs outside of the context of the ribosome (Figure 4.1). These activated proteins and others are then involved in the rapid translation of certain mRNAs needed to obtain precisely timed peaks of ecdysone. Positive feedback loops provide a possible mechanism to augment this pathway, and there is considerable evidence that such a feedback loop occurs during ecdysone synthesis (Sakurai and Williams, 1989). Very little is known about the components of or direct targets of this positive feedback loop, but one study has suggested that Fucosyltransferase-A (FucTA) may be involved (Medvedova et al., 2003). Interestingly, FucTA is up-regulated by Phosphofructokinase (Pfk), which has also been proposed to be involved in ecdysone signaling (Li and White, 2003). A global yeast two-hybrid screen revealed that Trus interacts with both Pfk and FucTA (Giot et al., 2003). It is therefore possible that Trus might somehow act through these proteins to regulate the positive feedback loop.
Figure 4.1 Model of the role of Trus in control of ecdysone synthesis

When a stage of development is complete unknown clues signal the synthesis of PTTH which travels to the prothoracic cells of the ring gland and causes PTTH causes an increase in extracellular calcium and cAMP (Birkenbeil, 1998; Birkenbeil, 2000; Birkenbeil and Dedos, 2002; Gilbert et al., 2000; Smith et al., 1984). This increase in cAMP activates S6 Kinase, which phosphorylates and activates Ribosomal Protein Subunit 6 (Rps6) (Gilbert et al., 2000; Smith et al., 2003), a subunit of the 40s ribosome subunit. Our Tap-tagging results suggest that Trus is bound to Sop (another 40s subunit) and Ef1-α and cytology and western blots show Trus highly expressed in the prothoracic cells of the ring gland. I therefore, hypothesize that Trus is playing a role in regulating translation of mRNAs that a required for the synthesis of ecdysone although I do not know where in the cascade it acts.

After ecdysone is synthesized it is exported into the hemolymph where it is modified to the active for of ecdysone (20HE). 20HE then goes on to bind and activate Ecdysone Receptor (EcR) and Ultraspriical (USP). This trimer then upregulates the ecdysone immediate genes, which then upregulate the ecdysone secondary genes, which results in ecdysis.
Although the \textit{trus}\textsuperscript{\textdagger} mutation appears to be null or severely hypomorphic, homozygous mutant animals cannot be completely devoid of ecdysone. If this was the case, \textit{trus}\textsuperscript{\textdagger} homozygotes would die during embryogenesis instead of at the third instar larval stage. The most likely explanation for this apparent paradox is that maternally supplied Trus would allow development until those stores are exhausted. It is also conceivable that Trus has a partially overlapping role with another protein, or that ecdysone synthesis is not absolutely dependent on Trus regulation because of the existence of an alternative pathway. For example, similar to some other molting insects (Gatti et al., 1974; Titschack, 1926), \textit{trus}\textsuperscript{\textdagger} mutants may be able to override checkpoints and employ alternative pathways to molt to the next stage if enough time has passed between molts. \textit{trus}\textsuperscript{\textdagger} mutants do not exhibit the substantially smaller size seen in all known cases of this override, but at the wandering third instar stage the mass of \textit{trus}\textsuperscript{\textdagger} mutants is only 74\% of normal.

\textbf{Trus, Ecdysone and PTTH titers}

My results provide considerable circumstantial evidence that Trus regulates ecdysone synthesis, but I believe that actual measurements of ecdysone titers in the \textit{trus}\textsuperscript{\textdagger} mutants would give direct support for such a role and further insight into the type of defect. Since development occurs at a delayed and highly variable rate in the mutants, it is more difficult to determine when peaks of ecdysone would be expressed than in wild type animals. It would therefore be necessary to measure ecdysone titers at frequent intervals over the life of the larvae.

Future studies may also wish to look at PTTH titers in \textit{trus} mutants. As PTTH is synthesized in the brain it is unlikely that Trus directly regulates PTTH
synthesis, but it is possible that larvae might increase PTTH titers in an attempt to synthesize ecdysone to compensate for deficiencies in the pathway.

**trus** as a possible tool in studying the ecdysone pathway

If Trus does in fact regulate the synthesis of ecdysone after PTTH signaling, this gene may become an important tool for future dissection of the ecdysone pathway. Currently, the only mutants known to cause a decrease in the ecdysone titer are in the gene *ecdysoneless*. However, the *ecd* allele is difficult to work with since it is a temperature sensitive allele and does not completely knockout ecdysone synthesis under restrictive conditions. Despite repeated attempts, no null allele of *ecd* has yet been identified (Gaziova et al., 2004).

**Ring gland-specific rescue**

Interesting insights into the function of ecdysone pathway proteins such as Broad (Zhou et al., 2004) have been achieved by over-expression of the protein specifically in the ring glands. I have attempted to obtain flies in which Trus is over-expressed in the ring gland by first cloning the full-length *trus* cDNA clone RE69372 into the pTWG-GW vector (Clontech, Mountain View, CA), which placed *trus* under the control of a Gal4 promoter and should produce a Trus protein containing a C-terminal GFP tag. The vector was injected into embryos by Genetic Services Inc, and screened based on eye color for integration.

Unfortunately, presumptive over-expression of Trus using the Gal4 P0163 and PO206 drivers (which both should be ring-gland specific) did not cause a visible phenotype. I nonetheless thought it might be possible to exploit
this system to see whether specific expression of the transgene in the ring gland might be sufficient to rescue the *trus'* phenotype. I attempted to perform this rescue multiple times using the cross scheme shown in Figure 4.2, but I never obtained the desired PO163/+; +/Tb flies intermediate in this cross scheme, and so I was never able to test this rescue. While there is no obvious explanation of my inability to obtain these flies, it is possible that PO163 is not at its proposed location on the second chromosome or that there is some mutation in its background on the third chromosome that makes it incompatible with the TM6B balancer. However, given the high expression level of Trus in the ring gland, I still believe this would be an informative result to obtain in future research.

**Insights into the function of Trus through interactors**

The Tap-tagging experiments described in Chapter 2 were conducted with Kc cells that were originally isolated from embryos. This cell type may not be biologically relevant to Trus' involvement in ecdysone synthesis (although a background level of Trus expression is seen in Western blots of extracts made from these cells). Ideally, one should perform Tap-tagging or similar affinity chromatography studies in cell culture types that are more responsive to ecdysone or in ring glands or whole larvae; it is possible that additional interactors might be found in such studies that provide new clues concerning Trus' control of ecdysone synthesis.
Figure 4.2 Cross scheme for ring gland specific rescue

To determine if ring gland specific expression can rescue the \textit{trus}\(^{1}\) mutation, I designed the cross scheme shown in the figure, using a Trus-GFP fusion under the control of a UAS promoter (Xi) and GAL4 driver expressed exclusively in the ring gland (PO163). After the final cross, I would in theory observe what happens to homozygous \textit{trus}\(^{1}\) larvae expressing Trus in the ring gland (GFP expressing non-Tubby animals). Unfortunately I have unexpectedly never been able to obtain the PO163/+; +/Tb flies required by the cross scheme.
Studying Trus homologs in mammals

The study of ecdysone signaling in *Drosophila* has important implications for hormone signaling in higher organisms, even though ecdysone is only found in molting insects and crustaceans. This is because several ecdysone pathway proteins are conserved and utilized in other hormone pathways in other organisms (Gilbert et al., 2000; Hsu and Schulz, 2000; Laudet et al., 1992). Often these proteins even have the same or similar interacting partners, as is the case with PTTH, S6 kinase, and Rps6 (Gilbert et al., 2000). Also, since ribosome structure is conserved in eukaryotes, it is likely that Trus-related proteins could play a fundamentally similar role in another hormone pathway.

We had hoped to elucidate some information about the function of Trus in mammals by using RNA *in situ* analysis to define the domains in which the Trus homolog is expressed in the mouse embryo. However, our data is insufficient to propose a function for PDCD2L (mouse Trus). The localized expression pattern within the developing nervous system does, however, suggest that PCDC2L is playing a cell-type specific role. Further *in situ* hybridizations conducted at later stages of development might provide a better idea of function. It would seem reasonable to continue to focus attention on the brain, as many neuroendocrine hormone signals are transmitted through brain tissues. The mouse knockout consortium has PDCD2L on its list of future knockouts to be made (Austin et al., 2004); the phenotype of such a knockout might eventually be extremely informative. However, if the gene is essential for early embryogenesis, the animals would die too early for detailed
observations. This possibility of course could be overcome by making conditional or tissue specific knockouts, but such studies would require more knowledge of the pattern of PDCD2L expression.

Research on Trus in both *Drosophila* and mammals certainly has the potential to provide a greater understanding of conserved mechanisms controlling hormone signaling.

**II. HYBRID INCOMPATIBILITY**

We have shown that, in contrast with previously published research, the incompatibility that causes lethality in hybrid males between *Drosophila melanogaster* and its sibling species is not due to an arrest in mitosis. We found that the brains of hybrid males are fragile and highly sensitive to environmental stress. However, the cellular or physiological basis of the lethality associated with this incompatibility remains unclear.

**Mitotic defects in hybrid females and rescued hybrid males**

Interestingly, hybrid males rescued from lethality by a deletion of *Drosophila* Hmr do have a mitotic defect. The brains still have a lower mitotic index than either single species and about a quarter of the cells in mitosis display spindly chromosome morphology (see Chapter 3, Figure 3.6 for images). Hybrid females that are *Hmr*⁺ also display a mitotic defect, but this phenotype has a higher than normal mitotic index, and many of the chromosomes appear puffy and under-condensed. The deletion of *Hmr* from these hybrid females results in a “wild type” mitotic index as well as a normal appearance of mitotic chromosomes. However, since the deletion of *Hmr* cannot rescue the males fully, *Hmr* cannot be the sole gene in *D.*
*melanogaster* responsible for the incompatibility and cell cycle problems, but it does play an important role in them.

**Chromosome condensation defects as a potential explanation for hybrid incompatibility**

As previously discussed in Chapter 3, two recent studies suggest that incompatible hybrids may have defects in chromatin structure. Pal-Bhadra *et al.* (2006) found that dosage compensation proteins fail to localize to the hybrid male X-chromosome. However, this is unlikely to be the sole explanation of hybrid lethality because this lethality is also fully penetrant in females that are homozygous for the *D. melanogaster* X chromosome. These results do, however, suggest that the hybrid male chromosome may have an aberrant chromatin structure (Akhtar, 2003). Brideau *et al.* (2006) recently found that the *Lethal hybrid rescue (Lhr)* gene encodes a protein that interacts and colocalizes with Heterochromatin Protein 1 (HP1). Greil *et al.* (Greil et al., 2007) also independently found that *Lhr* (alternatively referred to as HP3) colocalizes with HP1 and depends on HP1 for its correct localization on heterochromatin in cell culture cells. The requirement for HP1 in the maintenance of heterochromatin states suggests the possibility that hybrid lethality may result from an altered structure of heterochromatin. It is plausible that interactions between Hmr, Lhr, and HP1 affect the ability of DNA to properly condense in male hybrids. Condensation defects that are either more limited in severity or specific to certain tissues might also explain the sterility of hybrid females (Foe, 1989; Gatti and Goldberg, 1991; Schupbach and Wieschaus, 1991). Improper chromosome condensation during interphase could alter gene expression needed for cellular physiology or for cell cycle
progression during interphase, or otherwise activate checkpoint mechanisms that could prevent cells from entering mitosis.

**Exploring hybrid incompatibility through interactors of Hmr and Lhr**

Some mitotic defects have been shown to cause female sterility since replication is such an essential part of oocyte development (Foe, 1989; Gatti and Goldberg, 1991; Schupbach and Wieschaus, 1991). The apparent mitotic defect in hybrid females may, therefore, partially explain the sterility seen in these animals. To further understand the mechanism of female sterility, however, I feel that an understanding of the role of Hmr, Lhr, and their interacting proteins in the single species is essential. While studies have looked at the function of HP1, little is known about Hmr or Lhr in the organism.

**Determination of timing of the cell cycle arrest**

I am currently using antibodies to Cyclin A and Cyclin B to stain hybrid and single species brains in hopes of determining when in the cell cycle the cells are arresting. Cyclin A is expressed from late G1 until early M, whereas Cyclin B is expressed in G2 until the metaphase/anaphase transition. Both Cyclin A and Cyclin B in association with Cdk1 are involved in triggering mitosis (Edgar and Lehner, 1996). The results of immunostaining for the two Cyclins could be particularly informative if many cells in the hybrid brains stain for Cyclin A but not Cyclin B. Such a result would suggest that the cells are arresting due to action of a DNA damage checkpoint. Staining for Grapes (a homolog of the checkpoint protein Chk1) might reveal if the G2/M checkpoint is being turned on in many cells in hybrid brains.
When do the proliferation defects begin?

It is important to remember that while my work has focused on the cells in the brain of the hybrids, the hybrid males are also missing imaginal discs and likely other cell types. Determining when these defects begin could provide additional information concerning the nature of the physiological defects in hybrids. This could be achieved by staining early embryos (and if cell types are present later animals) with antibodies to proteins expressed in the precursors of the imaginal discs and optic lobes. Such studies could determine if these cell types are created but never able to properly propagate, or instead if they are never formed.

Greater implications of hybrid incompatibility in *Drosophila*

This mechanism of *Drosophila* hybrid lethality and sterility has been of interest since Sturtevant first described these phenomena (Sturtevant, 1920; Sturtevant, 1929). It is likely that a different set of genes and/or mechanisms underlies the separation of every set of diverging species. For example, Hmr and Lhr evolve so rapidly that homologs have not been identified even in species as closely related to *Drosophila* as the mosquito. However, even if Hmr and Lhr functional orthologs do not exist outside the genus *Drosophila*, it is still possible that altered chromatin structure might be the basis of hybrid incompatibility in many evolutionary clades. These suppositions must await verification by more direct tests for aberrations in chromatin structure in a variety of hybrids.
LITERATURE CITED


APPENDIX 1

THE DROSOPHILA LKB1 KINASE IS REQUIRED FOR SPINDLE FORMATION AND ASSYMMETRIC NEUROBLAST DIVISION

ABSTRACT

We have isolated lethal mutations in the dlkb1 gene, the Drosophila homologue of C. elegans par-4 and human LKB1 mutated in Peutz-Jeghers syndrome. We show these mutations disrupt spindle formation, resulting in frequent polyploid cells in larval brains. In addition, dlkb1 mutations affect asymmetric division of larval neuroblasts (NBs); they suppress unequal cytokinesis, abrogate proper localization of Bazooka, Par-6, DaPKC and Miranda, but affect neither Pins/Gαi localization nor spindle rotation. Most aspects of the dlkb1 phenotype are exacerbated in dlkb1 pins double mutants, which exhibit more severe defects than those observed in either single mutant. This suggests that Dlkb1 and Pins act in partially redundant pathways to control the asymmetry of NB divisions. Our results also indicate that Dlkb1 and Pins function in parallel pathways controlling the stability of spindle microtubules. The finding that Dlkb1 mediates both the geometry of stem cell division and chromosome segregation provides novel insight into the mechanisms underlying tumor formation in Peutz-Jeghers patients.

5 This appendix is modified from the following paper; Silvia Bonaccorsi1 Violaine Mottier1, Maria G. Giansanti1, Bonnie J. Bolkan2, Byron Williams2, Michael L. Goldberg3 and Maurizio Gatti. 2007. The Drosophila Lkb1 kinase is required for spindle formation and asymmetric neuroblast division. Development. 134(11):2183-93. Printed with permission.

Initial characterization of the dlkb1 mutants was done by Dr. Byron Williams. I completed the mapping of the dlkb1 mutants and identified them as CG9374. After identification I cloned and purified Dlkb1 for antibody production and tested and purified the antibody. I also performed the western blot that is shown as Figure A8.1A displaying the lack of dLKB1 in the mutants. All other work in this section was completed in the laboratory of Dr. Gatti.
INTRODUCTION

*Drosophila* neuroblasts (NBs) are one of the best model systems for the study of the control of cell polarity and asymmetric cell division. During *Drosophila* embryogenesis NBs delaminate basally from the neuroectodermal epithelium and divide asymmetrically along the apical/basal axis to produce another NB and a smaller ganglion mother cell (GMC). The newly generated apical NB divides repeatedly in an asymmetric fashion, while the basal GMC divides symmetrically only once to generate equal-sized daughter cells that differentiate into neurons or glia (reviewed by Betschinger and Knoblich, 2004; Wodarz, 2005).

The asymmetric division of *Drosophila* NBs is regulated by several proteins that concentrate at the cell cortex. The basal cortex is enriched in the cell fate determinants Prospero (Pros) and Numb, as well as their respective adaptor proteins Miranda (Mira) and Partner of Numb (Pon). These proteins are preferentially segregated into the GMC following NB cytokinesis. Localization of Pros/Mira and Numb/Pon at the basal cortex is mediated by a large multiprotein complex that concentrates at the apical cortex. This complex includes two functionally distinct subcomplexes. One of them contains Bazooka (Baz; Par-3 in *C. elegans*), DaPKC (*Drosophila* atypical protein kinase C) and Par-6; this assembly is hereafter called the Baz/Par-6 subcomplex. The other subcomplex includes the Gαi subunit of the heterotrimeric G protein complex and Partner of inscuteable (Pins), and is hereafter named the Pins/ Gαi subcomplex. The Baz/Par-6 and Pins/Gαi subcomplexes are integrated in a larger apical complex by the Insuc
(InsC) protein that binds both Pins and Baz (reviewed by Betschinger and Knoblich, 2004; Wodarz, 2005).

Recent genetic analyses have shown that the Baz/Par-6 subcomplex is mainly involved in the control of proper basal localization of Pros/Mira and Numb/Pon. The Pins/Gαi subcomplex is instead required for spindle orientation during NB divisions. Both complexes, however, cooperate in controlling cleavage furrow positioning during asymmetric NB divisions. Mutations that disrupt either the Baz/Par-6 or the Pins/Gαi pathway have little or no effect on asymmetric cytokinesis. However, mutations that disrupt both pathways completely abrogate spindle displacement during telophase, leading to symmetric cytokinesis (Cai et al., 2003; Izumi et al., 2004; Shaefer et al. 2000; Yu et al. 2000; 2003).

In this study, we have addressed the role of the Drosophila LKB1 kinase in NB division. This kinase is mutated in the Peutz-Jeghers syndrome, an autosomal dominantly inherited disorder characterized by the formation of intestinal polyps and a high incidence of various cancer types. Somatic mutations in the LKB1 gene have also been detected in sporadic adenocarcinomas (reviewed by Alessi et al., 2006; Baas et al., 2004b). There is evidence that LKB1 plays a conserved role in the control of cell polarity. Recent work has unambiguously shown that activation of LKB1 leads to rapid and complete polarization of human intestinal epithelial cells (Baas et al., 2004a). Similarly, Par-4, the C. elegans homologue of LKB1, is required for correct polarity and asymmetric division of one-cell embryos (Watts et al., 2000). Furthermore, Drosophila LKB1 (Dlkb1) mediates determination of anterior/posterior polarity of both egg chambers and embryos, as well as proper polarity of follicle cells (Martin and St. Johnston, 2003). Here, we
demonstrate that Dlkb1 controls many asymmetries that characterize the mitotic division of larval NBs. dlkb1 mutations also disrupt mitotic spindle assembly, leading to the formation of frequent polyploid cells. Thus, in addition to cell polarity and the geometry of cell division, Dlkb1 directly or indirectly regulates the stability of spindle microtubules (MTs).

**MATERIALS AND METHODS**

**Fly strains and genetic manipulations**

The $dlkb1^{315}$ mutant allele was isolated from a collection of 1600 third chromosome late lethals induced by ethylmethanesulfonate (EMS) in C. Zuker’s laboratory (Koundakjian et al., 2004). The $dlkb1^{7}$ allele is associated with the chromosome carrying $Df(3R)su(Hw)7$. This and all the deficiencies used for mapping were obtained from the Bloomington Stock Center. The $pins^{P62}$ null allele and the $asf^{e}$ mutation have been described previously (Bonaccorsi et al., 1998; Yu et al., 2000); $dlkb1^{315}$ $pins^{P62}$ and $dlkb1^{315}$ $asf^{e}$ double mutants were generated by recombination. All mutations were maintained over the $TM6B$ balancer, and mutant larvae were identified based on their non-$Tubby$ phenotype. Genetic markers and special chromosomes are described in FlyBase (http://www.flybase.org/). Genemline transformation was performed as previously described (Vernì et al., 2004).

**Antibodies and immunoblotting**

The anti-Dlkb1 Antibody was generated in guinea pig using a Maltose Binding Protein (MBP)/Dlkb1 fusion protein. Expression of the fused protein in *E. coli*, and the production and purification of antibodies against this fusion was according to Vernì et al. (2004). Immunoblotting was performed as
described (Vernì et al., 2004); the anti-Dlkb1, anti-Pins and anti-Giotto (Giansanti et al., 2006) antibodies were diluted 1:2000, 1:1000 and 1:5000, respectively.

Cytology

Brains from third instar larvae were dissected and fixed according to Bonaccorsi et al. (2000). After several rinses in phosphate-buffered saline (PBS), brain preparations were incubated overnight at 4 °C with a monoclonal anti-a tubulin antibody (Sigma-Aldrich), diluted 1:1000 in PBT (PBS with 0.1% TritonX-100), and any of the following rabbit antibodies, also diluted in PBT: anti-centrosomin (1:300; gift of T. Kaufman), anti-Deadpan (1:400; gift of Y. Jan), anti-Miranda (1:500; gift of Y. Jan), anti Bazooka (1:50; gift of F. Matsuzaki); anti-Gαi (1:200; gift of J. Knoblich); anti-Par-6 (1:1000; gift of J. Knoblich); anti-DaPKC (1:x100; Santa Cruz Biotechnology) and anti-Mud (1:200; gift of F. Matsuzaki). After two rinses in PBS, primary antibodies were detected by a 1 hour incubation at room temperature with FITC-conjugated anti-mouse IgG + IgM (1:20; Jackson Laboratories) and Alexa 555-conjugated anti-rabbit IgG (1:300; Molecular Probes), diluted in PBS.

For double Centrosomin/Pins immunostaining, brains were incubated overnight at 4 °C with the rabbit anti-Centrosomin antibody (1:300) and a rat anti-Pins antibody (1:100; gift of W. Chia) diluted in PBT. Detection was performed by 1 hour incubation at room temperature with Alexa 555-conjugated anti-rabbit IgG (Molecular Probes) and FITC-conjugated anti-rat IgG (Jackson Laboratories) diluted 1:300 and 1:20 in PBS, respectively.

For Dlkb1 immunostaining, brain preparations were incubated overnight at 4 °C with the anti-Dlkb1 antibody (1:100 in PBT) and, after rinsing in PBS,
incubated 1 hour at room temperature with Alexa 555-conjugated anti-guinea pig IgG diluted 1:500 in PBS.

In all cases, immunostained preparations were mounted in Vectashield medium H-1200 (Vector Laboratories) containing the DNA dye DAPI (4,6 diamidino-2-phenylindole). Preparations were examined with a Zeiss Axioplan microscope, equipped with an HBO100W mercury lamp and a cooled charged-coupled device (CCD camera; Photometrics CoolSnap HQ). Grayscale images were collected separately, converted to Photoshop (Adobe Systems), pseudocolored and merged.

Spindle measurements were taken on enlarged digital images and scaled down to their size in mm. In preparations stained for Cnn, measurements were taken from centrosome-to-centrosome. In the absence of Cnn staining, measurements were taken from pole-to-pole in anastral spindles; in the presence of asters, measurements were taken from the center of the astral MT array.

RESULTS

Isolation and characterization of mutations in the dlkb1 gene

In the course of a screen aimed at the isolation of mitotic mutants (see Materials and Methods), we identified a lethal mutation that causes frequent polyploid cells in larval brains (see below). Animals homozygous for this mutation die at the larval/pupal transition, as do most mitotic mutants; most probably, they exploit maternally supplied products to survive until late larval stages (Gatti and Baker, 1989). Deficiency mapping showed that this mutation is uncovered by both Df(3R)urd and Df(3R)26c, which define a map interval that contains only 16 annotated genes. During these mapping studies, we also
identified another mutant allele of this same mitotic gene. This allele is associated with the chromosome that carries \(Df(3R) su(Hw)7\), but is independent of the deficiency. We next sequenced the candidate genes and found that both mutant stocks carry lesions in the \(dlkb1\) gene (also known as CG9374). This gene encodes a 567 amino acid serine/threonine kinase homologous to the Par-4 kinase of \(C.\ elegans\) and to the human LKB1 kinase mutated in the Peutz-Jeghers syndrome (Martin and St. Johnston, 2003). The \(dlkb1^{315}\) mutant allele isolated in our screen carries a frameshift mutation resulting in a truncated Dlkb1 protein of 234 amino acids; the \(dlkb1^{7}\) allele, associated with \(Df(3R) su(Hw)7\), has a stop codon that truncates Dlkb1 to a 346 amino acid polypeptide (Figure A1.1). A genomic fragment including sequences that extend roughly 1 kb on either side of \(dlkb1\) (Figure 1A) rescued both the lethality and the mitotic phenotypes of \(dlkb1^{315}/dlkb1^{315}\) and \(dlkb1^{315}/dlkb^{7}\) mutants.

**Mutations in the \(dlkb1\) gene affect spindle formation**

\(Drosophila\) brains contain mostly two types of dividing cells: NBs and GMCs (Goodman and Doe, 1993). Wild type larval NBs are characterized by many asymmetries that develop during the course of mitosis. Prometaphases and metaphases of larval NBs exhibit centrosomes and asters of similar sizes at the two cell poles. However, as NBs progress through anaphase and telophase, the MTs of the basal aster shorten dramatically while those of the apical asters elongate slightly (Figure A1.1B). Concomitantly, the basal centrosome becomes smaller than the apical one (Bonaccorsi et al., 2000; see Figure A1.4A below).
Figure A1.1. Mutations in the *dlkb1* gene disrupt spindle organization of both NBs and GMCs.

(A) A molecular map of the *dlkb1* gene and its genomic region. P[dlkb1+] designates the genomic fragment that rescues both the lethality and the cytological defects associated with the *dlkb1* mutation. Black boxes correspond to protein-coding exons of the genes, and the arrows indicate the direction of transcription. The positions of the stop codons causing the *dlkb1*<sup>315</sup> and *dlkb1*<sup>7</sup> mutations are indicated by vertical lines.

(B) Mitotic spindle morphology of NBs and GMCs from wild type (wt) and *dlkb1* brains. Cells were stained for tubulin (Tub, green) and DNA (blue).

(C) Spindle morphology of wild type (wt), *dlkb1*, *asl* and *asl dlkb1* metaphases stained for tubulin (green), DNA (blue) and centrosomin (red). Note that the spindle density in *asl dlkb1* double mutants is substantially lower than in *asl* single mutants. Scale bar for all panels, 5mm.
These changes in aster and centrosome morphology are accompanied by a progressive displacement of the central spindle towards the basal pole, resulting in unequal cytokinesis (Giansanti et al., 2001). GMCs display equally-sized centrosomes and very small asters throughout mitosis, and divide symmetrically (Figure A1.1B; Bonaccorsi et al., 2000).

To determine the mitotic defect leading to polyploid cell formation in $dlkb1$ mutants, we examined larval brain preparations from $dlkb1^{315}/dlkb1^{315}$, $dlkb1^{315}/Df(3R)urd$ and $dlkb1^{315}/dlkb1^{7}$ larvae stained for both tubulin and DNA. These mutant combinations showed identical mitotic aberrations. Most strikingly, mutant spindles showed an overall MT density that is substantially lower than that seen in wild type spindles (Figure A1.1B). In approximately 80% of mutant spindles, asters were absent or severely reduced; in control brains, the frequency of spindles without asters, or with very small asters, was 49% (Figure A1.1B; Table1). In addition, most mutant prometaphase and metaphase figures were characterized by low densities of both kinetochore and interpolar MTs, and ana-telophases displayed central spindles thinner than their wild type counterparts (Figures 1B and 3D below). Mutant brains also showed an increase in the relative frequency of metaphase figures with respect to wild type, suggesting that $dlkb1$ mutations lengthen metaphase duration (Table A1.1).

Finally, mutant brains displayed approximately 20% polyploid cells (not shown); in wild type brains, the frequency of polyploid cells is virtually zero (Table A1.1). The phenotype of $dlkb1^{315}$ homozygotes was qualitatively and quantitatively similar to that observed in $dlkb1^{315}/Df(3R)urd$ hemizygotes, indicating that $dlkb1^{315}$ is a null mutation (Table A1.1).
Table A1.1. Mitotic parameters in larval brains from *dlkb1* mutants.

<table>
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<th>Genotype (a)</th>
<th># of cells (b)</th>
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<th>anaphases %</th>
<th>telophases %</th>
<th>sym. telo (c) %</th>
<th>no asters (d) %</th>
<th>polyploid cells %</th>
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<td>15.6</td>
<td>35</td>
<td>49</td>
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<tr>
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(a) Oregon R, wild type stock used as control; 315/315, *dlkb1*<sup>315</sup>/*dlkb1*<sup>315</sup>; 315/Df, *dlkb1*<sup>315</sup>/Df(3R)urd; 315/7, *dlkb1*<sup>315</sup>/*dlkb1*<sup>7</sup>. (b) The numbers of cells scored refer only to diploid mitotic figures; polyploid cells were recorded but not used for calculation of the frequencies of different types of mitotic figures. (c) sym telo, relative frequencies of symmetric telophases; (d) no asters, diploid cells without asters or with very small asters.
The spindle phenotypes observed in *dlkb1* mutants could be due either to a defect in MT elongation and/or stability, or to a defect in centrosome function. To distinguish between these possibilities, we sought to eliminate centrosome function in *dlkb1* mutants. We have previously shown that brain cells of *asterless* (*asl*) mutants fail to assemble functional centrosomes and nucleate astral MTs. Nonetheless, *asl* NBs and GMCs manage to form robust anastral spindles that are able to mediate chromosome segregation (Bonaccorsi et al., 2000; Giansanti et al., 2001). We thus constructed *dlkb1 asl* double mutants and compared their phenotype to those exhibited by *dlkb1* and *asl* single mutants. The anastral spindles from *asl* single mutants displayed a MT density comparable to wild type (Figure A1.1C). In contrast, in *dlkb1 asl* double mutants, the density of spindle MTs was substantially reduced with respect to wild type and similar to that observed in *dlkb1* single mutants (Figure A1.1C). These results strongly suggest that the low density of spindle MTs observed in *dlkb1* mutants does not depend on centrosome dysfunction. Thus, the spindle phenotype of *dlkb1* mutants is likely to be attributable to either a decreased rate of MT growth or an increased MT instability.

***dlkb1* mutations disrupt the asymmetry of NB division leading to a reduction in NB size**

Observation of mitotic divisions stained for tubulin and DNA revealed that the spindles of *dlkb1* mutant cells are generally smaller than in wild type (Figure A1.1). In addition, the frequency of asymmetric telophases in mutant brains (35-37%) was significantly lower than in wild type brains (65%) (Table A1.1). These phenotypes could reflect a partial loss of morphological asymmetry during NB division, resulting in smaller than normal daughter NBs.
We thus examined in greater detail the pattern of cell division in \textit{dlkb1}^{315}\textbackslash Df(3R)\textit{urd} brains, and compared this pattern to those observed in wild type, \textit{partner of inscuteable (pins)} and \textit{asl} brains. Comparison between \textit{dlkb1} and \textit{pins} mutants was prompted by two previous findings. First, mutations in \textit{pins} partially suppress the asymmetry of NB divisions, leading to a progressive reduction in NB size (Cai et al., 2003; Parmentier et al., 2000). Second, the \textit{Drosophila} and the human LKB1 kinases interact with the orthologous proteins Pins and AGS3, respectively (Blumer et al., 2003). In addition, more detailed comparisons between \textit{dlkb1} and \textit{asl} mutants would allow us to assess more precisely the role of astral MTs in asymmetric NB divisions.

To unambiguously distinguish between NB and GMC spindles, we immunostained preparations from control and mutant brains for both tubulin and the NB marker Deadpan (Dpn), (Bier et al., 1992; Figure A1.3A below). The analysis of Dpn-positive cells showed that the \textit{dlkb1} and \textit{pins} NBs are indeed defective in aster formation. However, the two mutants displayed different patterns of spindle defects. In brains homozygous for the \textit{pins}\textsuperscript{P62} null mutation (Yu et al., 2000), most NB prometaphases and metaphases showed normal asters but most ana-telophases were characterized by an abnormally small apical aster (Figure A1.2A-D). Despite this defect in astral MTs, the density of the spindle MTs in \textit{pins} NBs was comparable to that observed in their wild type counterparts (compare Figure A1.2A-D with Figure A1.1B). In contrast, the spindles of \textit{dlkb1} NBs not only showed a reduction in MT density but also displayed small asters in both metaphase and ana-telophase figures (Figures A1.1B and A1.2E).
Figure A1.2. Mutations in pins affect aster formation without altering the density of spindle MTs.

Cells were stained for tubulin (green), DNA (blue) and Deadpan (not shown, but see Figure A1.3A below) to identify NBs. (A) Prometaphase, (B) metaphase, (C) anaphase and (D) telophase from pins$^{P62}$ mutant brains. Scale bar, 5mm. (E) Frequencies of NBs displaying normal asters in wild type, dlkb1 and pins brains.
Figure A1.3. Mutations in *dlkb1* disrupt unequal cytokinesis and reduce the average size of NB population within mutant brains.

(A) Wild type and *dlkb1* metaphases stained for tubulin (Tub) DNA and Deadpan (Dpn). Note that the NBs are more intensely stained by the anti-Dpn antibody than GMCs. Scale bar, 5mm.

(B and C) Size distribution of metaphase (B) and ana-telophase (C) spindles in wild type, *dlkb1*315/Df(3R)urd, *pins*P62/pinsP62 and *asl*/*asl* brains. Dpn-positive (NB) and negative (GMC) spindles are depicted in red and green, respectively. Size (mm) classes in (B): A, 4.7-6.9; B, 7.0-9.2; C, 9.3-11.5; D, 11.6-13.8; E, 13.9-16.1; F, 16.2-18.4; G, 18.5-20.7; H, 20.8-23.0; I, 23.1-31.7. Size (mm) classes in (C): J, 6.7-11.2; K, 11.3-15.8; L, 15.9-20.4; M, 20.5-25.0; N, 25.1-29.6; O, over 29.7. The numbers on top of each column correspond to the number of cells observed in each size class.

(D) Criterion used for measuring the asymmetry index of NB divisions. The difference between the length of the long (a) and the short (b) spindle axis (a-b) was divided it by the total length of the two axes (a+b). Scale bar, 5mm.

(E) Asymmetry indexes in wild type, *dlkb1*315/Df(3R)urd, *pins*P62/pinsP62 and *asl*/*asl* NBs of different sizes. The bars correspond to the SEM. Size (mm) classes: P, 11.3-15.8; Q, 15.9-20.4; R, 20.5-25.0; S, 25.1-29.6; T, over 29.7.
In *pins* and *asl* mutants, the spindles of Dpn-negative GMCs displayed a normal morphology and were indistinguishable from their wild type counterparts (data not shown). However, in *dlkb1* mutants, GMC spindles were characterized by low MT density just as those of the NBs (Figure A1.1B). Thus, the wild type function of *dlkb1* is required for proper spindle formation in both NBs and GMCs.

We next measured the spindle length of metaphase and ana-telophase figures in both NBs (Dpn-positive) and GMCs (Dpn-negative). In *dlkb1* and *pins* mutant brains, the average sizes of NB spindles are substantially smaller than those measured in either *asl* or wild type brains. This is mainly due to the absence of large NBs, as both *dlkb1* and *pins* mutants lack NB metaphases and telophases longer than 19 and 26 mm, respectively; these large NBs represent approximately 20% of the NBs found in wild type or *asl* mutant brains. In contrast, the average sizes of the GMC spindles observed in *dlkb1*, *asl* and *pins* mutants were very similar and comparable to those of wild type controls (Figure A1.3B, C). An explanation for these results is that in both *dlkb1* and *pins* mutants NBs divide more symmetrically than either in *asl* or wild type. To test this possibility, we directly evaluated the degree of asymmetry of NB telophases showing strong Dpn staining. The asymmetry index was determined using the formula \(a-b/a+b\), where \(a\) is the long axis of the spindle and \(b\) its short axis (Figure A1.3D). This analysis (Figure A1.3E) clearly shows that the NBs from *dlkb1* and *pins* mutants divide more symmetrically than those of either *asl* or wild type. Collectively, these results indicate that mutations in either *dlkb1* or *pins* partially suppress the asymmetry of NB division, leading to a reduction in the NB size at each cell division cycle.
To ask whether mutations in \textit{dlkb1} and \textit{pins} affect centrosome size, brain preparations were stained for centrosomin (Cnn), an integral component of \textit{Drosophila} centrosomes (Megraw et al., 2001). Observations were restricted only to those cells that, according to our analysis of spindle size distribution (Figure A1.3B, C), were likely to be NBs (wild type, \textit{dlkb1} and \textit{pins} metaphases longer than 12 mm, and ana-telophases longer than 16 mm). This analysis (Figure A1.4A, B) revealed that 88\% \((n = 76)\) of wild type NBs displays centrosomes of different sizes at their poles. In contrast, only in 34\% \((n = 180)\) of \textit{dlkb1} NBs and 39\% \((n = 100)\) of \textit{pins} NBs is the centrosome at the apical pole larger than that at the basal pole. These results indicate that \textit{dlkb1} and \textit{pins} control asymmetry in centrosome size during NB division.

\textbf{\textit{dlkb1} mutations affect Mira and Baz/Par-6/DaPKC but not Pins/G\textsubscript{\textit{a}}i localization in dividing NBs}

We examined whether \textit{dlkb1} and \textit{pins} mutations affect the distribution of Miranda in dividing NBs. Larval brain preparations were simultaneously stained for both tubulin and Mira and analyzed for Mira localization (Figure A1.5A). We again restricted our analysis to large mitotic figures that are likely to be NBs by the criteria just employed. Both \textit{dlkb1} and \textit{pins} mutant NBs revealed abnormal Mira distributions, but the patterns of Mira mislocalization were different (Figure A1.5A, B). In wild type, 93\% of NB metaphases and ana-telophases displayed a clear Mira crescent at the basal pole, while the remaining 7\% showed diffuse Mira staining. In contrast, in \textit{dlkb1} and \textit{pins} mutants the frequencies of NBs with a basal Mira crescent were 47\% and 26\%, respectively. Most (97\%) of \textit{dlkb1} mutant NBs lacking a Mira crescent displayed a diffuse cytoplasmic localization of Mira.
Figure A1.4. Mutations in the *dlkb1* gene affect centrosome size during NB division.

(A) Metaphases and telophases from wild type and *dlkb1*<sup>315/Df(3R)urd</sup> mutant brains stained for tubulin (green), DNA (blue) and centrosomin (red). Note the differently-sized and the equally-sized centrosomes at the poles of wild type and *dlkb1* mutant cells, respectively. Scale bar, 5mm.

(B) Frequency of NBs displaying differently-sized centrosomes in wild type (wt), *dlkb1*<sup>315/Df(3R)urd</sup> and, *pins<sup>P62</sup>/pins<sup>P62</sup> brains.
Figure A1.5. Mutations in the *dlkb1* gene affect Mira localization at the NB basal pole

(A) Mira localization in wild type, *dlkb1*315/*dlkb1*315 and *pins*P62/*pins*P62 NBs. Cells were stained for tubulin (Tub), DNA and Mira. Scale bar, 5mm.

(B) Distribution of Mira in dividing NBs from wild type (wt), *dlkb1*315/*dlkb1*315 and *pins*P62/*pins*P62 brains; regular, regular Mira crescent at the basal pole; cortical, Mira associated with the entire cell cortex; diffuse, Mira dispersed in the cytoplasm.
A

wild type | pins | dlkb1

Mira

Mira Tub DNA

telophase

B

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However, while the majority (67%) of *pins* mutant NBs without a Mira crescent had this same pattern, a substantial minority (33%) of these cells showed a diffuse cortical distribution of Mira (Figure A1.5B).

We next determined whether *dlkb1* mutations affect the localization of Pins and Gαi at the apical cortex of dividing NBs (NBs were again identified by their size). A regular Pins signal was observed in 95% (n = 60) of the wild type NBs and in 84% (n = 145) of the *dlkb1* NBs (Figure A1.6A, B). Gαi formed a crescent at the apical pole of 96% of wild type NBs (n = 61) and 74% of *dlkb1* mutant NBs (n = 75) (Figure A1.6A, B). Consistent with previous results (Cai et al., 2003; Shaefer et al., 2001), we observed a Gαi apical crescent only in 4% (n = 50) of *pins* mutant NBs (data not shown). Thus, while *dlkb1* mutations affect Mira localization at the basal cortex, they have little or no effect on Pins and Gαi localization at the apical cortex.

We also analyzed Baz, DaPKC and Par-6 localization in both wild type and *dlkb1* mutant NBs. In wild type larval brains, the Baz signal was rather weak and only 48% (n = 63) of the dividing NBs displayed a clear Baz crescent at the apical pole. However, in *dlkb1* mutant brains, only 5 of the 106 NBs scored showed a discernible Baz crescent (Figure A1.6A, B). The DaPKC and Par-6 apical crescents were observed in 96% (n = 24) and 71% (n = 35) of wild type NBs, respectively, but most *dlkb1* NBs did not show apical accumulations of these proteins; DaPKC and Par6 crescents were detected only in 7% (n = 30) and 12% (n = 33) of *dlkb1* mutant NBs. These results suggest that the wild type function of *dlkb1* is required for the localization of the Baz, DaPKC and Par-6 at the NB apical pole.
Figure A1.6. Mutations in the \textit{dlkb1} gene disrupt Baz/Par-6/DaPKC but not Pins/G\alpha_i localization at the NB apical pole.

(A) Pins, G\alpha_i, Baz, Par-6 and DaPKC localization in wild type and \textit{dlkb1}^{315}/\textit{dlkb1}^{315} mutants. NBs in the first row were simultaneously stained for Pins, centrosomin (Cnn) and DNA (blue). Cells shown in the subsequent rows were stained for tubulin (Tub), DNA (blue) and either G\alpha_i, Baz, Par-6 or DaPKC. Scale bar, 5mm.

(B) Frequencies of NBs with Pins, G\alpha_i, Baz, Par-6 and DaPKC crescents in wild type and \textit{dlkb1}^{315}/\textit{dlkb1}^{315} mutant brains.
A

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B

[Graph showing comparison between wild type and dlk1 for different proteins]
Recent work has suggested that DaPKC delocalization from the apical cortex can result in NB overproliferation (Lee et al., 2006b). Consistent with this idea, the brains from third instar larvae of dlkb1 null mutants exhibit a dramatic hyperplasia of both the hemispheres and the ventral ganglion; this phenotype has been attributed to a reduction in developmental apoptosis during embryogenesis (Lee et al., 2006a). We observed a clear brain overgrowth in all our dlkb1 mutant alleles, confirming that Dlkb1 regulates Drosophila brain size (data not shown). It is likely that the brain hyperplasia elicited by dlkb1 mutations results from both defective apoptosis and DaPKC-related NB overproliferation.

**Dlkb1 is not required for NB spindle rotation**

We examined 128 metaphases of dlkb1 mutant NBs; 53 of them displayed a Mira crescent but only in one case was this crescent incorrectly oriented with respect to the spindle axis (Figure A1.5B). In contrast, this crescent was misoriented with respect to the spindle axis in 9 of the 29 pins NB metaphases with a Mira crescent (Figure A1.5B). These results confirm that Pins is required for proper spindle rotation during NB division and indicate that the Dlkb1 kinase is not involved in this process.

Recent work has shown that that spindle rotation is regulated by Mud (Mushroom body defect), a protein related to vertebrate NuMa that interacts with both Pins and the spindle microtubules. In embryonic NBs Mud forms an apical crescent and accumulates at the spindle poles; in larval NBs the cortical localization of Mud is weak or undetectable but the protein remains enriched at the spindle poles (Bowman et al., 2006; Izumi et al., 2006; Siller et al., 2006).
Figure A1.7. Mud localization in wild type and dlkb1 mutant NBs. Cells were stained for Mud, tubulin (Tub) and DNA (blue).

(A and B) wild type prophase (A) and metaphase (B). (C and D) dlkb1315/dlkb1315 prophase (C) and metaphase (D). Scale bar, 5mm.
Immunostaining for Mud revealed that the protein is enriched at the centrosomes and the astral MTs in 93% (n = 45) of prophase and early prometaphase NBs (Figure A1.7A). With progression through mitosis, Mud localization becomes more diffuse and 62% (n = 45) of NB metaphase figures do not exhibit clear Mud accumulations at the spindle poles (Figure A1.7A); however, Mud relocalizes at the pericentrosomal regions of most anaphases and telophases (83%, n = 30; not shown). In dlkb1 mutant NBs, Mud accumulates at the centrosomes/asters in 91% (n = 35) of prophase and early prometaphase NBs (Figure A1.7C), and remains associated with the spindle poles in 78% (n = 37) of the metaphases (Figure A1.7D) and 85% (n = 20) of the ana-telophases (not shown). Thus, mutations in dlkb1 do not affect Mud localization during metaphase and ana-telophase but appear to increase Mud concentration at the spindle poles during metaphase.

**Subcellular localization of Dlkb1**

To determine the subcellular localization of Dlkb1, we raised a guinea pig antibody against the entirety of Dlkb1. Western blot analysis showed that this antibody recognizes a band of the expected size (~ 63 kDa) in larval, embryonic and S2 cell extracts. This band is absent in both dlkb1^{315}/Df(3R)urd and dlkb1^{315}/dlkb1^{7} larvae (Figure A1.8A), demonstrating that it corresponds with Dlkb1. Since the truncated forms of Dlkb1 encoded by the dlkb1^{315} and dlkb1^{7} mutant alleles were not observed in mutant animals, either the mutant transcripts or the truncated proteins are unstable. These findings provide a strong support for the genetic data (Table A1.1), indicating that the dlkb1^{315} mutant allele is functionally null.
Figure A1.8. Expression and intracellular localization of the Dlkb1 kinase.

(A) Western blot showing that our anti-Dlkb1 antibody recognizes a band of approximately 63 kDa. This band is absent in extracts from either \textit{dlkb1}^{315}/\textit{Df}(3R)\textit{urd} or \textit{dlkb1}^{315}/\textit{dlkb1}^{7} mutant larvae. \(\alpha\)-tubulin was used as a loading control (LC).

(B) Immunostaining of wild type dividing NBs for Dlkb1. Note that Dlkb1 is diffuse in the cytoplasm. Scale bar, 5mm.
Immunolocalization experiments revealed that Dlkb1 is dispersed in both the nucleus and the cytoplasm of interphase larval brain cells, and in the cytoplasm of both NBs and GMCs undergoing mitotic division. Immunostaining of \textit{dlkb1}^{315}/Df(3R)\textit{urd} mutant cells did not reveal any clear cytoplasmic signal, confirming the specificity of the antibody (Figure A1.8B and data not shown). The diffuse localization of Dlkb1 in brain cells is not consistent with its cortical localization in Drosophila oocytes (Martin and St. Johnston, 2003). However, the Dlkb1 localization pattern in brain cells does not depend on the quality of our antibody, as the same antibody revealed a cortical accumulation of Dlkb1 in oocytes (data not shown).

\textbf{Dlkb1 and Pins function in different pathway controlling NB division}

The findings that Dlkb1 and Pins co-precipitate (Blumer et al., 2003), and that \textit{dlkb1} and \textit{pins} mutations cause similar (but not identical) phenotypes, prompted us to perform an epistasis analysis. We thus compared the phenotype of the \textit{dlkb1}^{315} \textit{pins}^{P62} double mutant with those of the single mutants by examining brain preparations stained for tubulin, Dpn and DNA. In \textit{dlkb1} \textit{pins} mutant brains, the spindles of both NBs and GMCs are much more defective than those observed in either of the single mutants (Compare Figure A1.9 with Figures 1 and 2). In addition to cells with severely defective spindles (Figure A1.9A, C, F, G, I), we also observed many (50%, \textit{n} = 300) mitotic figures in which the spindle morphology was barely recognizable (Figure A1.9B, D, E, H); the frequency of the latter type of cells was only 4% (\textit{n} = 201) in the \textit{dlkb1} single mutant.
Figure A1.9. *dlkb1* and *pins* function in different pathways controlling the stability of spindle MTs.

Mitotic figures from brains of *dlkb1*<sup>315</sup> *pins*<sup>P62</sup> double mutants were stained for tubulin (Tub, green), DNA (blue) and centrosomin (red). (A – F) NBs, (G – I) GMCs. (A, B) metaphases; (C – E) anaphases; (F) telophase (G) metaphases; (H) anaphase; (I) telophase. The arrow in (E) points to a lagging X chromosome with unseparated sister chromatids. Note the extremely defective spindle structures of the NBs shown in (B, D, E and H). Scale bar, 5mm.

(J) Size distribution of metaphase spindles in wild type and *dlkb1* *pins* brains. Dpn-positive (NB) and negative (GMC) spindles are depicted in red and green, respectively. Size (mm) classes: A, 4.7-6.9; B, 7.0-9.2; C, 9.3-11.5; D, 11.6-13.8; E, 13.9-16.1; F, 16.2-18.4; G, 18.5-20.7.

(K) Expression of Pins and Dlkb1 in brains from third instar larvae of *dlkb1*, *pins* and *dlkb1* *pins* mutants. Note that the Dlkb1 protein is undetectable in larval brain extracts of both *dlkb1*<sup>315</sup> homozygotes and *dlkb1*<sup>315</sup> *pins*<sup>P62</sup> double mutants. Similarly, Pins cannot be detected in brain extracts of both *pins*<sup>P62</sup>/pins<sup>P62</sup> mutants and *dlkb1*<sup>315</sup> *pins*<sup>P62</sup> double mutants. The Giotto proteins (Giansanti et al., 2006) was used as a loading control (LC).
In *dlkb1 pins* metaphases, the MT density is extremely low, the spindle poles have a characteristic pointed appearance and the asters are completely absent (Figure A1.9A). The ana-telophases are also devoid of asters and display few and sparse central spindle MTs, which are never pinched in the middle (Figure A1.9C, F). These results indicate that *dlkb1* and *pins* function in parallel pathways to control spindle formation. The absence of central spindle pinching, which suggests an accompanying failure of cytokinesis, prevented a reliable assessment of the degree of asymmetry of NB divisions. However, NB spindles of the double mutant are smaller than in wild type (Figure A1.9J). In addition, the analysis of centrosome size in large metaphase figures (longer than 14 mm), most of which are likely to be NBs, revealed that 90% (n = 106) of them have equally sized centrosomes. In wild type, *dlkb1* and *pins* the frequencies of NB metaphases with centrosomes of equal size were 12%, 72% and 61%, respectively (Figure A1.4B). Finally, only 3% (n = 120) of the *dlkb1 pins* NB metaphases were characterized by a Mira crescent; in the remaining cells, Mira was either diffuse in the cytoplasm (85%) or associated with the entire cell cortex (12%). Thus, the Mira mislocalization phenotype observed in *dlkb1 pins* double mutants is stronger than that seen in the single mutants (see Figure A1.5B).

Although the *dlkb1*<sup>315</sup> and *pins*<sup>P62</sup> alleles are both functionally null, it cannot be excluded that the brains of the *dlkb1*<sup>315 pins</sup><sup>P62</sup> double mutants retain residual amounts of the maternally supplied Dlkb1 and Pins proteins. We thus performed a Western blotting analysis of extracts from third instar larval brains of *dlkb1*<sup>315</sup>, *pins*<sup>P62</sup> and *dlkb1*<sup>315 pins</sup><sup>P62</sup> mutants. As shown in Figure A1.9K, *dlkb1*<sup>315</sup> and *dlkb1*<sup>315 pins</sup><sup>P62</sup> brains do not exhibit detectable amounts of the Dlkb1 kinase, consistent with the results shown in Figure A1.8A. Similarly, the
Pins protein appears to be completely absent in pinsP62 and dlkb1315 pinsP62 brains. Thus, the phenotypes observed in the single and double mutants reflect a complete loss of the wild type function of either Dlkb1 or Pins or both.

Collectively, our results suggest that dlkb1 and pins act in different pathways to control the asymmetry of NB division. These genes also function in parallel pathways involved in MT stability and spindle formation. Whether the latter pathways are the same that control the asymmetry of NB mitosis remains to be determined.

**DISCUSSION**

**Dlkb1 controls the stability of spindle MTs**

Our results indicate that mutations in the dlkb1 gene disrupt spindle formation in both NBs and GMCs. In addition, the finding that the imaginal discs of dlkb1 mutant larvae are small and misshapen suggests a defect in imaginal cell mitosis. Previous studies have shown that late larval lethality and small imaginal discs are diagnostic of abnormalities in mitotic divisions (Gatti and Baker, 1989). Thus, the dlkb1 phenotype strongly suggests that the Dlkb1 kinase plays an important mitotic role not only in NBs, but also in other somatic cell types. Despite the low density of spindle MTs, most mutant metaphases enter anaphase (the frequency of anaphases in dlkb1 mutants and in wild type controls was 10-13% and 16%, respectively; see Table A1.1), suggesting that in a substantial fraction of mutant cells the spindle checkpoint is either not induced or only transiently activated. However, the defects in NB spindles are likely to lead to the formation of polyploid cells. These cells could arise through two different mechanisms. Cells blocked in metaphase due to either reduced MT density or activation of the spindle checkpoint could revert
to interphase and become polyploid after an additional round of DNA replication. Alternatively, cells that enter anaphase but assemble an abnormally thin central spindle may be unable to undergo cytokinesis and thus produce polyploid cells (Verni et al., 2004).

The precise function of Dlkb1 in spindle formation and/or maintenance is currently unclear. However, the finding that the spindles of dlkb1 asl double mutants display a lower MT density than asl single mutants argues for a defect in MT stability and not in centrosome function. Studies in mammalian cells have shown that LKB1 is a master kinase that phosphorylates at least 14 kinases all related to AMP-activated kinases (AMPK). Kinases of the AMPK family include regulators of cellular energy levels, as well as four Microtubule Affinity Regulating Kinases (MARK; reviewed by Alessi et al., 2006; Baas et al., 2004b). The MARK enzymes are the mammalian homologues of C. elegans and Drosophila Par-1. However, Drosophila Par-1, which controls MT stability in oocytes (Shulman et al., 2000), appears to act upstream of Dlkb1/Par4 (Martin and St. Johnston, 2003). It is therefore unlikely that the Dlkb1 substrate required for the stability of spindle MTs is Par-1. Further evidence that Dlkb1 does not act via Par-1 phosphorylation comes from RNAi experiments showing that Dlkb1 depletion, but not Par-1 depletion, causes defects in spindle morphology (Bettencourt-Dias et al., 2005). Thus, current data indicate that the Dlkb1 kinase regulates the activity of an unknown factor required for the stability of the spindle MTs; this factor could either be a direct substrate of Dlkb1 or a substrate for one of the kinases acting downstream Dlkb1.

Our cytological analyses have shown that the spindles of dlkb1 pins double mutants display a MT density that is substantially lower than that
observed in either single mutant. Here again, highly defective spindles were observed in both NBs and GMCs. In addition, doubly mutant larvae showed extremely reduced imaginal discs, suggesting an underlying mitotic defect. Given that the $dlkb1^{315}$ and $pins^{P62}$ alleles used in the analysis are both functionally null and that the corresponding proteins were undetectable in mutant brains (Yu et al., 2000; this study), these results indicate that Pins and Dlkb1 function in different pathways for the control of MT stability. The observation that the spindles of $pins$ mutants display a normal MT density further suggests that Pins plays a redundant role in the maintenance of MT stability. A role of Pins in spindle formation and/or stability has never been demonstrated in *Drosophila*. However, the mammalian homologue of Pins binds NuMa and regulates mitotic spindle organization and positioning (Du et al., 2001).

**Dlkb1 controls the asymmetry of NB division**

We have analyzed the phenotypic consequences of $dlkb1$ mutations in larval brain NBs. In contrast with embryonic NBs that display small, regularly-sized spindles (their metaphase spindles are approximately 5 mm long), brain NBs exhibit spindles of very different sizes (ranging from 5 to 32 mm for metaphase spindles). Nonetheless, dividing brain NBs exhibit the same asymmetries of their embryonic counterparts, including asymmetries in aster and centrosome size, localization of specialized protein complexes and positioning of the cleavage furrow (Bowman et al., 2006; Giansanti et al., 2001; Lee et al., 2006b; Parmentier et al., 2000; Rolls et al., 2003; Siller et al., 2006; this study). However, the degree of asymmetry of brain NB division is directly related to the cell size, so that large NBs divide more asymmetrically.
than the small ones (Figure A1.3E). This is likely to render large brain NBs particularly sensitive to mutations that affect cleavage furrow positioning. Consistent with this hypothesis, mutations in pins have mild effects on the asymmetry of embryonic NB divisions (Cai et al., 2003) but disrupt unequal cytokinesis in most larval brain NBs (Parmentier et al., 2000; Figure A1.3E). dlkb1 larval NBs also divide more symmetrically than their wild type counterparts, leading to larval brains devoid of large NBs. In addition, most dlkb1 NBs display centrosomes of equal size and very small asters at both poles. However, the symmetric cytokinesis of dlkb1 NBs cannot result from their short astral MTs, as asl NBs divide asymmetrically in the complete absence of asters (Figure A1.3B, C, E).

dlkb1 mutant NBs are also characterized by the abnormal distribution of several components of the apical and basal complexes. In dlkb1 mutant brains, most NBs display normal Pins and Gαi crescents at their apical pole but fail to accumulate Baz, DaPKC and Par-6 at the same pole. In addition, most dlkb1 mutant NBs fail to exhibit a normal Mira crescent at the basal pole cortex. A normal localization of Pins and Gαi has been already observed in most embryonic NBs defective in the Baz/Par6 pathway (Cai et al., 2003; Izumi et al., 2004; Schaefer et al., 2000; Yu et al., 2000; 2003). Moreover, studies on embryonic NBs have suggested that Baz, Par-6 and DaPKC function as a complex, are interdependent for their localization at the NB apical pole, and required for the formation of the Mira crescent at the basal pole (Petronczki and Knoblich, 2000; Wodarz et al., 2000). However, subsequent work on second instar larval NBs has shown that these proteins are not mutually dependent for the formation of the Baz/Par-6/DaPKC apical crescent; they accumulate at the apical cortex in a hierarchical fashion, with
Baz and Par-6 mediating proper DaPKC localization (Rolls et al., 2003). Mutations that disrupt the Pins/Gαi pathway prevent asymmetrical localization of either Pins or Gαi in embryonic NBs but do not substantially affect Mira accumulation at the basal pole (Cai et al., 2003). However, it should be noted that mutations in pins partially disrupt asymmetric Mira localization in larval brain NBs (Parmentier et al., 2000; this study), suggesting that larval NBs differ from embryonic NBs in some aspects of the control of Mira localization. Thus, taking into account the differences between embryonic and larval NBs, our results indicate that mutations in the dlkb1 gene and those that disrupt the Baz/Par6 pathway affect similar aspects of NB mitotic division.

Our analyses have shown that in dlkb1 pins double mutants the NBs divide more symmetrically than in the corresponding single mutants. This indicates that the dlkb1 and pins genes act in different pathways that mediate unequal cytokinesis. Previous studies have shown that the asymmetry of NB cytokinesis depends on the Baz/Par6 and the Pins/Gαi redundant pathways. When only one of these pathways is impaired, NBs still divide asymmetrically, but they divide symmetrically when both are disrupted (Cai et al., 2003). The simplest interpretation of our findings is that dlkb1 acts in the Baz/Par6 pathway. In addition, the observation that Dlkb1 is required for proper localization of Baz, Par-6 and DaPKC suggest that this kinase acts at the top of the hierarchical mechanism that mediates accumulation of the Baz/Par6 complex at the apical cortex. However, although we favor the hypothesis that Dlkb1 acts in the Baz/Par6 pathway, we cannot exclude the possibility that this kinase functions in both the Baz/Par6 and Pins/Gαi pathways, or in a third pathway different from the Baz/Par6 pathway.
In this context, it is important to note that our results exclude the possibility that \textit{dlkb1} acts via Pins phosphorylation. Previous studies have shown that mammalian LKB1 coprecipitates and phosphorylates AGS3, the mammalian orthologue of Pins. Dlkb1 and Pins coimmunoprecipitate as well, but it is currently unclear whether Pins is phosphorylated by Dlkb1 (Blumer et al., 2003). Regardless of whether Pins is a substrate of Dlkb1, the phenotypes elicited by \textit{dlkb1} mutations cannot be the consequence of an impairment of Pins function. \textit{dlkb1} and \textit{pins} mutant NBs do in fact differ in a number of phenotypic traits, including spindle organization and the pattern of Mira localization, and do not belong to the same epistasis group.

**Dlkb1 is not required for NB spindle rotation**

In vivo imaging has shown that the spindles of embryonic NBs rotate during metaphase to become aligned with the center of the Pins apical crescent (Kaltschmidt et al., 2000). In contrast, the spindles of larval NBs align with the Pins crescent at prophase (Siller et al., 2006). Failure of proper rotation of larval NB spindles results in spindles that are misoriented with respect to the apical (Pins) and basal (Mira) crescents (Giansanti et al., 2001; Siller et al., 2006). There is also evidence that proper positioning of larval NB spindles depends on astral MTs, since in approximately 50% of \textit{asl} NB metaphases the Mira crescent is misoriented with respect to the spindle axis (Giansanti et al., 2001; this study).

Our results indicate that spindle rotation occurs normally in \textit{dlkb1} mutant NBs. In addition, we have shown that prophase/prometaphase larval NBs of \textit{dlkb1} mutants normally accumulate the Mud protein, which mediates proper spindle alignment in both embryonic and larval NBs (Bowman et al.,
2006; Izumi et al., 2006; Siller et al., 2006). Together, these results indicate that the Dlkb1 kinase is not required for spindle rotation and that the short astral MTs of \textit{dlkb1} mutant NBs can mediate proper spindle positioning. These results are consistent with the idea that the Pins/Gαi, but not the Baz/Par6 pathway is involved in spindle rotation (Izumi et al., 2004; Siegrist and Doe, 2005) and provide further support for the hypothesis that Dlkb1 functions in the latter pathway.

Recent work has shown that in the absence of the Baz/Par6 pathway, astral MTs can mediate the localization of Pins/Gαi at the basal cortex (Siegrist and Doe, 2005). Assuming that Dlkb1 acts in the Baz/Par6 pathway, the finding that this kinase is not required for the formation of Pins/Gαi crescents indicates that the short astral MTs of \textit{dlkb1} NBs retain the ability to mediate Pins/Gαi cortical localization.

**Conclusions and perspectives**

Our results indicate that Dlkb1 and Pins function in partially redundant pathways controlling the stability of spindle MTs. These proteins are also required for the asymmetry of NB divisions and, here again, they appear to function in different pathways. Pins acts in a common pathway with Gαi, while Dlkb1 is likely to function in the Baz/Par6 pathway. Intriguingly, recent work has shown that simultaneous loss of \textit{pins} and \textit{baz} functions results in the formation of abnormally small embryonic NB spindles that lack astral MTs at both poles (Fuse et al., 2003). Thus, the embryonic NBs of \textit{baz pins} double mutants have a spindle phenotype reminiscent of that observed in \textit{dlkb1} larval NBs. These findings raise the question of whether the Pins/Gαi and Baz/Par6 pathways redundantly control spindle organization as they do for the
asymmetry of NB divisions. The extant data do not provide a clear answer for this question. The analysis of the roles of the two pathways in spindle formation and their precise relationships with the Dlkb1 kinase will be an interesting problem to be addressed in future studies.

Previous studies in Drosophila and mammalian cells have led to the suggestion that loss of epithelial cell polarity is ultimately responsible for the Peutz-Jeghers cancer syndrome (Martin and St. Johnston, 2003; Baas et al., 2004b). Here, we have shown that Dlkb1 plays an essential mitotic role and is required for the asymmetry of NB division. These results lead us to propose that tumor development in Peutz-Jeghers patients depends on the impairment of multiple processes, including cell polarity, the asymmetry of stem cell division and the fidelity of chromosome segregation during mitosis.

ACKNOWLEDGEMENTS

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LITERATURE CITED


INTRODUCTION

Cell division is an essential, highly regulated process that allows every living organism to grow, develop, and produce progeny. The cell cycle is simply described as containing of G1 (gap 1 phase), S (DNA Synthesis), G2 (gap 2), and M (mitosis). G1, S, and G2 together constitute interphase, the period that separates successive mitotic divisions, and during which cells grow and replicate their DNA. Mitotic cell division is the intricate process by which the replicated DNA and cellular components of one parental cell are equally divided to become two daughter cells.

Although mitosis is a continuous process, it is often split into more specific stages for the sake of discussion. Cells exit interphase and enter prophase, when the chromosomes begin to condense mostly because of the phosphorylation of certain histones. Prometaphase is characterized by further condensation of the chromosomes, nuclear envelope breakdown, and the initial stages of the attachment of microtubules to the kinetochores of the chromatids. Metaphase is considered to have begun when the sister chromatids are bidirectionally attached to microtubules so that the chromosomes align along the metaphase plate. A checkpoint exists which holds a cell in metaphase until all chromatids are aligned properly and kinetochore microtubules achieve a proper amount of tension across the
chromosomes. Separation of the sister chromatids occurs during anaphase, allowing the individual chromatids to move along the kinetochore microtubules to opposite poles of the cell. When the chromatids are fully separated, the cells enter telophase and the nuclear envelopes are reformed while the chromosomes decondense. The two daughter cells are physically separated during cytokinesis, which occurs (in animal cells) by the formation of a cleavage furrow between the asters of the spindle apparatus. With mitosis complete, the cells then re-enter interphase. The length of time spent in interphase depends on the cell type and developmental stage of the organism, but it can be as short as a couple of minutes, as occurs during early Drosophila embryogenesis (Foe and Alberts, 1983) or as long as forever, since a newly formed cell may remain quiescent for the rest of the life of the organism.

**Drosophila and mitosis**

*Drosophila* is an ideal model organism for studying mitosis, in large part because maternal contributions can allow certain mutants to live to a stage in development at which mitosis can be examined by high-resolution cytological techniques. In *Drosophila* larvae only three cell types are undergoing mitosis: the imaginal discs, abdominal histoblasts and the neuroblasts (Gatti and Goldberg, 1991). By comparing the development of these tissues and mitosis of these dividing cells in mutant and wild type flies, the role of individual proteins in mitosis can be elucidated.

Many proteins important for cell division in *Drosophila* are provided in sufficient concentrations within the egg for homozygote mutant zygotes to develop to the third instar larval stage, when the maternally supplied stores of
the protein are depleted (Baker et al., 1982). These late larval lethal mutants can show many types of aberrant behavior during cell division, resulting in an increase or decrease in the mitotic index in mitotically active tissues, small or absent imaginal discs, and cells that are aneuploid or polyploid, or that show defects in chromosome condensation (Gatti and Goldberg, 1991).

In this Appendix, I will describe my initial characterization of six different mutant strains that were provided to us by Dr. Fiammetta Verní in the laboratory of Professor Maurizio Gatti at the University of Rome. Dr. Verní screened the Zucker collection of EMS-induced mutants (Koundakjian et al., 2004) for strains showing late larval lethality that also displayed potential chromosome condensation defects (see Chapter 2 for more details). My characterization involved genetic mapping of the mutation in question as well as a preliminary cytological analysis of the mutant phenotype. The toys are us (trus) mutation described in Chapter 3 is a seventh mutant stock uncovered from this same screen.

**MATERIALS AND METHODS**

**Wild Type Flies**

All wild type (WT) flies discussed in this Appendix are from the *Drosophila melanogaster* laboratory stock OregonR (OR). The mitotic index is defined as the number of mitotic cells visualized per field of view at 1000 X. In a WT third instar larval brain, the average mitotic index is $0.62 \pm 0.06$. Images of WT brain squashes showing normal mitotic figures are provided (Figure A2.1 A-D) for comparison to the mutants that will be discussed in this chapter.
**Figure A2.1 Phenotype of wild type brains**

(A-B) Orcein-stained brain squashes showing the range of normally condensed mitotic chromosomes in metaphase. (C) DAPI stained mitotic chromosomes are condensed and identifiable as individual chromosomes. (D) A wild type anaphase showing DNA stained with Hoerst (blue) and spindles stained with anti-Tubulin (green).
Cytology of brains for analysis of mitosis

Larval brains were dissected, fixed, and squashed as described in Williams et al. (1992), except that incubation in 3.7% formaldehyde was decreased to 20 min. Incubations with antibodies were done overnight at 4°C in phosphate saline buffer (PBS) containing 0.1% Triton X (PBT). Primary antibodies were rabbit anti-Phosphohistone H3 (Upstate, Charlottesville, VA) used at a 1:500 dilution and mouse anti-α-Tubulin (Upstate, Charlottesville, VA) at a 1:100 dilution. Brains were incubated in primary antibody overnight at 4°C. These primary antibodies were detected by the following secondary antibodies: TRITC (tetrarhodamineisothiocyanate)-conjugated anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA) and Cy2 (cyanine 2) conjugated anti-mouse IgG (Jackson Immunoresearch), both at a 1:500 dilution for 3 hrs at room temperature. DNA was detected by staining with 0.05 µg/ml Hoechst 33742 (Sigma, St. Louis, MO) in PBS for 5 minutes. Imaging was performed on an Olympus BX-50 microscope equipped with a Qimaging Retiga Exi CCD camera (Burnaby, BC, Canada) and MetaMorph 6.1 software (Universal Imaging, Downington, PA).

For aceto-orcein staining, larval brains were dissected in 0.7% NaCl and transferred immediately to a drop of aceto-orcein stain (2% orcein in 45% acetic acid) on a cover slip and squashed onto a glass slide (Gatti and Goldberg, 1991). In some experiments, brains were incubated in 0.5 x 10^{-5} M colchicine in 0.7% NaCl for 0.5, 1, or 2 hours after dissection prior to transfer to aceto-orcein. Mitotic indices were determined as previously described (Williams et al., 1992); briefly, the mitotic index of a sample was defined as the number of PH3-staining mitotic cells per standard field of view at 1000X.
RNAi

RNAi was done in Kc cells as described by Clemens et al. (Clemens et al.). Briefly, with a few minor changes, dsRNA was produced using the MEGAscript T3 & T7 High Yield Transcription Kits (Ambion, Austin, TX) according to the manufacturer's instructions. Primers used to amplify the cdc6 cDNA LD25083 clone were the same used by Kiger and colleagues (2003). This primer pair contained both T7 and T3 promoter sequence and gene specific sequences. Kc cells were propagated in HyQ-CCM3 media (HyClone, Logan, UT) and were diluted to a concentration of 1 x 10^6 cells/mL before plating 1 mL per well in 6 well plates. dsRNA was added to the cells to a concentration of 30 nM and immediately swirled for mixing. A final 2 mL of media were added, and the cells were allowed to grow for 3 days to allow for the turnover of protein products.

Cytology for analysis of Kc cells and RNAi

Cells were spun down in Eppendorf tubes and the excess supernatant aspirated away between each step before transfer to slides. One mL of cells in media was fixed in 3.7% formaldehyde in PBS for 15 min. Cells were then dehydrated by treating with 45% acetic acid for 30 sec, followed by 60% acetic acid for 3 min. All but 45 µL of the acetic acid was removed, and 20 µL of the resuspended solution was pipeted onto a clean coverslip. A slide was inverted onto the coverslip, and the sandwich was then squashed between filter paper to spread cells and chromosomes. Slides were placed in liquid nitrogen after squashing and then refixed in methanol for 2 min before being staining with Hoechst 33742 as described above.
RESULTS AND DISCUSSION

The B38 mutant phenotype is caused by mutations in the *Drosophila* homologue of *cell division cycle 6* (*dcdc6*)

Initial mapping by our collaborators in the Gatti laboratory localized the mutation in the B38 stock to a 217.5 kb region between 66D8 and 66E2. Using recently created Exelexis and Drosdel deletions, which have well defined breakpoints, I was able to delimit this area to a region containing 21 genes (Figure A2.2 A). Lethal mutations in three of these genes complemented the B38 mutation, decreasing the number of possible genes to 18. Of these genes, CG5971 (*Drosophila melanogaster* homologue of *cell division cycle 6* (*cdc6*)) was chosen as a candidate gene and sequenced; this analysis revealed two missense mutations, E33K and Q222R in the B38-bearing chromosome. The E33K mutation is located in the cdc binding domain and is next to a possible site of phosphorylation, Serine 32 (Figure A2.3A,B). The change of a carboxyl group to an amine at amino acid position 33 conceivably could alter both charge and steric shape so as to disrupt phosphorylation of S32 and thus the function of cdc6.

In yeast, Cdc6 is known to bind the Origin Recognition Complex (ORC) and is thus regarded as one of the factors involved in replication initiation (Diffley, 1994; Lisziewicz et al., 1988). When bound by Clb2/Cdc28 at the cdc binding domain, Cdc6 becomes unable to assemble the pre-replication complex (pre-RC) and thus prevents over-replication (Mimura et al., 2004). Cdc6 also functions in the G2-mitosis checkpoint (Figure A2.3C) in combination with other proteins monitoring DNA damage (Lau et al., 2006; Lydall and Weinert, 1997). Cdc6 inhibits Cdc2 though the Wee1 pathway and must be degraded for entry into mitosis (Bueno and Russell, 1992).
Figure A2.2 B38 map and mitotic phenotype

(A) Modified from Flybase 2007. A gene map of the cytological region 66D8 to 66D15 of chromosome 3, the region containing the \textit{cdc6} gene (CG5971). Deficiencies and genes in green represent deletions that complemented the B38 mutation, while ones in red represent deletions that failed to complement. The blue vertical lines delineate the region to where the mutation was delimited. Cdc6 is highlighted in pink. (B-D) Orcein staining of B38 brains showing precocious sister chromatid separation and lagging chromosomes in anaphase (arrow).
Figure A2.3 Homologs of *cdc6* and the nature of the B38 mutation

(A) Homologs of the *cdc6* gene. Regions in purple show the highly conserved *cdc6* binding domain. Red lines mark known sites of phosphorylation and the red triangle represents a putative site of phosphorylation in *Drosophila*. Positions of the two amino acid substitutions associated with the B38 allele are shown. (B) The glutamic acid to lysine substitution caused by the B38 mutation could be sufficient to disrupt the S32 putative phosphorylation site. (C) *cdc6* acts both in the G2-M checkpoint maintenance and clamp loading of the DNA replication machinery at the beginning of S phase.
In *Drosophila* no research has been published on *cdc6*; B38 thus appears to be the first known mutant allele of this gene. Our observations show that this allele is third instar lethal and results in brains with a high mitotic index. Even though the mitotic index is almost double that of WT at $1.17_{-0.24}^{+1.0}$, the size of the brains is essentially normal, suggesting that the cells are not over-proliferating but are instead becoming arrested in mitosis. When individual mitotic cells are observed, it is not a surprise that they are unable to undergo proper mitotic divisions. Many neuroblasts in mutant brains are highly aneuploid and have under-condensed DNA (Figure A2.2 B-D). Even in cells that have proper chromosomal numbers (Figure A2.2B), the chromosomes appear under-condensed. Usually cells get arrested in metaphase, but I presume that in this case, the cells are arrested in a G2/prophase-like state. Also, when dissected brains are treated with colchicine (a mitotic drug which arrests cells in metaphase), it becomes apparent that sister chromatids have separated precociously. Not surprisingly (given the apparent G2/prophase arrest), less than half of the normal frequency of anaphases was observed, and 86% of these anaphases showed clear evidence of misbehavior such as lagging chromosomes (Figure A2.2D).

To look further at the function of Cdc6 in *Drosophila*, we utilized RNAi to eliminate the protein in Kc cells. Three days of this treatment resulted in a high level of cell death.
Figure A2.4  *cdc6* RNAi causes under-condensation in Kc cells.

(A) Wild type cells stained with DAPI for DNA show normal DNA condensation in mitosis. (B) Kc cells treated with *cdc6* RNAi fail to condense properly and are likely also polyploid.
Although we do not have an antibody to verify the knockdown of dCdc6, the extreme phenotype suggests a severe decrease in protein levels, since no other control RNAi done at the same time caused cellular lethality. Cells that remained alive showed a high level of aneuploidy and under-condensed chromosomes (Figure A2.4A-B), consistent with the phenotype observed in brain neuroblasts. This finding strengthens the argument that mutation of the dcdc6 gene indeed causes the mitotic effects observed in the B38 mutant stock.

The MA41 mitotic phenotype is likely caused by a mutation in DNA polymerase-delta

My results indicate that, similar to the B36 mutation, MA41 is caused by a mutation in a gene whose product is essential for DNA replication. The MA41 mutant was characterized as a mitotic mutant due to its low mitotic index of 0.37 +/- 0.11 [slightly over half of the wild type value (0.67 +/- 0.06)], a high frequency of aneuploid cells, under-condensed chromosomes, and a high frequency of chromosomes with broken arms. By deficiency mapping, I narrowed the region containing the causative mutation to 71F1-72D10, a 540 kd region containing 19 genes (Figure A2.5A). Of these genes, DNA Polymerase delta (DNApol-δ) was a logical candidate for involvement with the phenotype. Sequencing confirmed that the MA41-bearing chromosome contains a point mutation within this gene that causes a C496Y amino acid replacement.

Drosophila DNApol-δ shares high homology with, and probable orthology to, its mammalian counterpart (Aoyagi et al., 1994). Using purified DNApol-δ in biochemical assays, Aoyagi and colleagues (1994) proposed that in
*Drosophila* DNApol-δ is essential for leading strand elongation during replication; they also concluded that it functions also as a proofreading exonuclease. Since the MA41 mutants are not embryonic lethal, it is unlikely that this allele of DNApol-δ is completely non-functional. Also, the broken chromosome morphology as well as aneuploidy in mutants (Figure A2.5B-F) suggests that the lesion more likely disrupts the proofreading activity of DNApol-δ. As DNA breaks and mutations build up in the cells, loss of chromosome arms would become visible and would also affect the ability of chromosomes to properly segregate to their respective daughter cells (Figure A2.5A,C). Checkpoints for DNA damage during replication would also explain the low level of cells entering mitosis. Presumably the operative checkpoint in this case would be that regulating the transition between the S and G2 phases of the cell cycle.

Also of note, I found that the previously identified mutation *l(3)72Ac*<sup>110</sup>, which maps to the same region, failed to complement MA41, suggesting that it constitutes a second allele of DNApol-δ. No sequencing of the gene in this mutant strain has yet been done, but if an additional aberration in the gene is found, this would constitute strong evidence for the identity of the mutant phenotypes with DNApol-δ.

Future characterizations of the putative DNApol-δ mutations as well as *cdc6(B38)* are being conducted by Dr. Tim Christensen (East Carolina University) in connection with their effect on the replication initiation factor Mini chromosome maintenance 10 (Mcm10).
Figure A2.5 MA41 map and mitotic phenotype

(A) Modified from Flybase 2007. A gene map of the cytological region 72A5 to 72D1 of chromosome 3, the region containing the DNApol-δ gene. Deficiencies and genes in green complemented the MA41 mutation, while deletions in red failed to complement MA41. The blue vertical lines delineate the region to where the mutation was delimited. DNApol-δ is highlighted in pink. (B,C) Orcein stained MA41 brains show lagging chromosomes in anaphase (arrows) and over condensed and polyploid DNA in metaphase. (D,E) DNA staining shows a similar phenotype to orcein staining, but Tubulin staining appears normal, suggesting that improper spindle formation is not the cause for the mitotic arrest seen in the MA41 mutant strain.
Table A

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Images B, C, D, E
The M374 mutation causes high levels of polyploidy

The M374 mutation maps somewhere between 68A8 and 68C6 (Figure A2.6A). Unfortunately, this 470 kb region is sparse in deficiencies, known lethal genes, and even known transcriptional units. We first tested candidate genes in the no optic lobe family, since they have known alleles exhibiting mitotic defects that prevent proliferation of the optic lobes (Koizumi, 1995); however, none of these genes contained mutations in their coding sequences in the M374 strain. Of course, it is still possible that one of these genes is responsible for the phenotype and that the mutation is in a regulatory region; however, this possibility is difficult to determine from sequence information alone. In the future, fine scale P-element mapping might be used to narrow down the region responsible, perhaps to a single gene.

Despite our failure to locate the gene responsible for the M374 phenotype, the mutant deserves mention due to the extreme polyploidy seen in brain cells (Figure A2.6B-D). Cells with a hundred or more chromosomes are common, and the majority of cells display at least some degree of polyploidy and chromosomes that appear over-condensed. The extreme polyploidy suggests that these cells are capable of exiting mitosis without completing cell division and yet are competent to complete another round of the cell cycle; it is likely that cytokinesis is affected. Over-condensation is common in mutants that arrest in metaphase, since the chromosomes continue to condense throughout this stage. In the future, visualizing the spindle with antibodies against Tubulin will allow us to get a better idea of how mitosis is progressing, particularly during telophase and cytokinesis. Similarly, the use of GFP marked histones and live imaging (Yu et al., 2004) would provide more clues to mitotic progression in this mutant.
Figure A2.6 M374 map and mitotic phenotype

(A) Modified from Flybase 2007. A gene map of the cytological region 68A7 to 68C7 of chromosome 3, the region containing the as-yet unidentified M374 mutation. Deficiencies and genes in green complemented the M374 mutant, while deletions in red failed to compliment. The blue vertical lines delineate the region to where the mutation was delimited. (B-D) Orcein staining of M374 brains showing the high levels of polyploidy in most of the mitotic cells.
MA9 mutants have small fragile brains with very few cells in mitosis

Originally, the MA9 mutation appeared to map to the region between 87F12-F15 and 88A1 on chromosome 3. However, using newly developed deficiencies with more precise endpoints, I was able to remap the gene to the region 88B1-88C1 (Figure A2.7B). This region is 110 kb long and contains 12 genes (Figure A2.7A). No candidate genes were sequenced in this region, and the identity of MA9 remains unknown.

The MA9 homozygous mutants have small brains that are very fragile when dissected. In squashed preparations, orcein staining revealed a mitotic index of only 0.19, less than 1/3 of the wild-type value. The cells that were in M-phase appeared morphologically normal, although I observed some apparently improper chromosome condensation. Interestingly, similar to the trus mutants, these larvae spend an extended time as larvae, although this delay has never been quantified. It is possible that these mutants also have a developmental hormone signaling deficiency, thus explaining their low mitotic index and lack of imaginal discs. If the developmental delay is indeed suggestive of a problem in the ecdysone pathway, it will be interesting to determine whether the phenotype can be rescued by feeding with ecdysone.
Figure A2.7 MA9 map and mitotic phenotype

(A) Modified from Flybase 2007. A gene map of the cytological region 88B1 to 88C1 of chromosome 3, the region containing the currently unidentified MA9 mutation. Deficiencies and genes in green complemented the MA9 mutation, while deletions in red failed to complement. The blue vertical lines delineate the region to where the mutation was delimited. (B) Map of a larger area of chromosome 3 showing deletions that were used for complementation analysis to narrow MA9 to the region shown in panel A. (C-D) Orcein staining of MA9 brains showing the over-condensation phenotype.
Mit1174 and M370x are likely alleles of Polo kinase

Dr. Verní previously mapped both Mit1174 and M370x to 77B2-6, a region of chromosome 3 containing 9 transcriptional units including the well-known mitotic gene polo. Both of these mutations complemented lethal alleles of polo in terms of viability, so it was originally believed that polo was not the gene responsible for the mutant phenotype. However, I repeated this complementation analysis and found that the transheterozygotes for either of the mutations and strong polo alleles were female sterile. Since alleles of polo can have separable functions (Riparbelli et al., 2000), it is likely that these two mutations represent new alleles of polo. However, the chromosomes carrying these mutations were never sequenced to verify this supposition.

CONCLUSION

The seven mutants I have described (including trus) illustrate the diversity of genes that, when mutated, can result in late larval lethality and the inability to complete the cell cycle properly.
LITERATURE CITED


