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The Role of Trailerhitch in Mouse Germ Cell Development

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ABSTRACT

During oogenesis in the Drosophila ovary, a 16-cell cyst develops in which one cell grows to become the oocyte. The surrounding nurse cells transport mRNAs, proteins, and organelles to the oocyte during development and must be localized properly within the oocyte. A complex of proteins is responsible for mRNA localization, including the recently discovered protein Trailerhitch. Homologs of Trailerhitch exist in other species including C. elegans, yeast, mice, and humans, but the function is unknown. We are interested in studying the function of Trailerhitch in mouse germ cell development. Mice with a non-functional Trailerhitch gene were synthesized using a gene trapping method in which a β-geo vector was inserted randomly into the intron between exons two and three of the Trailerhitch gene. The coding sequence of the gene was thus interrupted and inactivated. The mutation was then transmitted through the germline of mice in order to analyze the effects. Using Western blotting, β-galactosidase staining, and Whole Mount Antibody staining, we have determined that Trailerhitch is expressed in both ovaries and testes of mice at all ages. In neonatal ovaries, Trailerhitch is expressed in the cytoplasm of oocytes and is highly concentrated in the Golgi. In neonatal testes, Trailerhitch is widely expressed in the cytoplasm of spermatagonia. However, Trailerhitch is expressed only in a concentrated region of the cytoplasm of spermatocytes in adult testes, which is thought to be the chromatoid body. During development, Trailerhitch is expressed widely throughout the embryo, which was determined by performing β-galactosidase staining during ages 7.5 dpc – 13.5 dpc. Trailerhitch expression in other organ systems of the mouse signifies that Trailerhitch may have a more general cellular function. Using PCR primer walking, we have identified the exact base pair location of the β-geo insertion. This discovery has allowed us to distinguish between heterozygous and homozygous mutants. According to genotyping, RT-PCR, and Western blot data, it appears that homozygous mutants are embryonic lethal prior to the age of 7.5 dpc (days post coitum). This finding must be confirmed and the exact age of lethality must be determined.

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ADVICE TO FUTURE HONORS STUDENTS

Performing research in Dr. Pepling's laboratory has become one of the most valuable aspects of my undergraduate career. Looking back on my experience, it is hard to imagine there was a time I considered not embarking on this endeavor. I recall meeting with Dr. Wolf for our advisor meeting and explaining to him that I was not interested in research because of a negative experience in high school. In addition, I was quite intimidated by the thought of approaching busy professors to ask for their time and guidance by allowing me a place in their lab. The thought of committing myself to a project for two years was also unnerving. Despite all my concerns, Dr. Wolf was quite firm in his belief that participating in research and preparing a thesis in the Honors Program here at Syracuse University would be an experience I would not regret. Thankfully, he could not have been more correct.

I was fortunate to have Dr. Wolf's guidance, and even more fortunate that Dr. Pepling decided to take on another undergraduate student. The process of finding a lab to work in can often be difficult, so it is important to contact professors as early as possible. I was also made aware of opportunities to perform research at Upstate if all of the labs at Syracuse University were full. As a sophomore, it was difficult to read through and attempt to understand descriptions of professors' research, and to decide which sounded most interesting. I would encourage students to speak to their professors about their research and to do some investigating in order to find a lab and a project they will thoroughly enjoy. Dr. Pepling's research caught my attention as I was searching for a lab. I was

fortunate enough to end up in a lab that was my first choice and also to have a research advisor that was extremely approachable and an excellent teacher. Dr. Pepling is incredibly dedicated to her lab. She makes time to teach her students the most minor tasks such as learning to use a pipette, and also the most intricate tasks such as dissecting 3.5 dpc embryos. Having a research advisor that provided such a relaxed and intriguing learning environment compelled me to dive head first into my research and to take full advantage of the opportunity that was made available to me.

I would encourage every student to find a lab that interests him or her and to become involved in research as early as possible. Besides the techniques and skills one can learn in a lab, one will develop relationships with other students and professors that I have found to be very valuable. Also, the attributes that I developed after almost two years in the lab are traits I will benefit from and carry with me for the rest of my life. Finally, preparing an honors thesis based on the progress accomplished in a lab is a very rewarding experience. It represents the culmination of much hard work and dedication, and is truly the capstone to a successful undergraduate career.

ACKNOWLEDGEMENTS

I would like to thank Dr. Pepling for allowing me to work in her lab the past two years and for providing so much support along the way. She created such a fun and relaxed lab environment, and insisted on taking the time to teach me just about everything I've learned in the lab. This resulted in a unique learning experience that has allowed me to gain skills and attributes I greatly value. She has become an extraordinary mentor, one whom I respect a great deal. I hope to eventually have the same dedication and passion for my own career. I would also like to thank the graduate students in the lab, Ying Chen and Leo Medico, for so willingly sharing their laboratory knowledge.

Dr. Wolf and Dr. Belote have also been tremendous mentors. I would like to thank them for the advice they have provided me, whether it was about my future career or about a particular research method. I also thank them for the past two years in the honors seminar.

I thank Dr. Erdman for showing his interest in my research project and for sharing his ideas about it with me. He has also been a meticulous second reader, and I greatly appreciate his comments and advice.

I would also like to acknowledge the Ruth Meyer Scholarship Fund for providing financial support for my research.

INTRODUCTION

Oogenesis is the process by which an oocyte, or egg cell, develops in the ovarian follicle of the ovary. Primordial germ cells are the precursor cells of oocytes. In *Mus musculus* (mouse), primordial germ cells migrate to the developing ovary at approximately 10.5 days post coitum (dpc) (Monk and McLaren 1981). Once these cells migrate to the genital ridge, or area of gonad development, they become organized into clusters of cells called germ line cysts. The cells of cysts divide synchronously until 13.5 dpc, but remain connected by intercellular bridges due to incomplete cytokinesis of each cell cycle (Pepling and Spradling, 1998) (Figure 1). Upon completion of this mitotic stage, the cells of cysts enter meiosis and become arrested during prophase I.

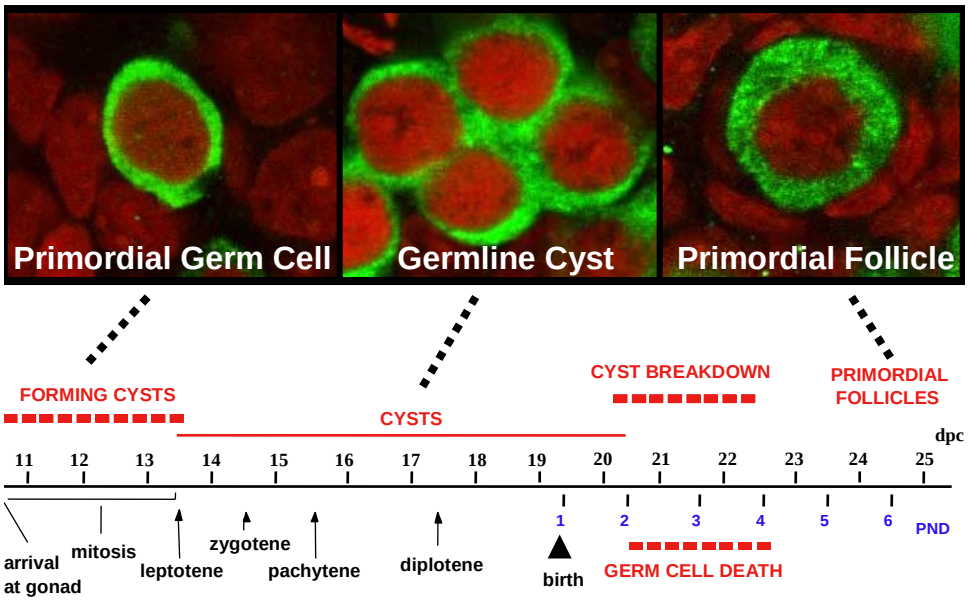


Figure 1. Timeline of Mouse Germ Cell Development (adapted from Pepling and Spradling, 2001). Propidium iodide (red) labels nuclei of all cells. Green staining is vasa, which is an oocyte-specific marker, and labels oocyte cytoplasm.

A similar cyst formation process occurs in *Drosophila melanogaster*. During oogenesis in *Drosophila*, a stem cell gives rise to a cystoblast, the founder of a 16-cell syncytial cyst. The cystoblast undergoes four successive divisions to produce the 16-cell cyst with interconnecting ring canals. Only one of these cells will develop into an oocyte while the rest form nurse cells (Figure 2).

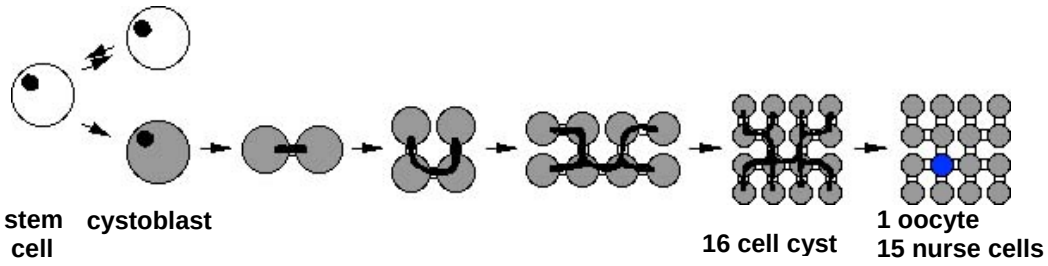


Figure 2. Oogenesis in the *Drosophila* ovary involves the formation of 16-cell cysts in which one cell develops into the oocyte while the others act as nurse cells. Nurse cells are responsible for providing the oocyte with mRNAs, proteins, and organelles (adapted from de Cuevas et al., 1996).

The nurse cells transport mRNAs, organelles, and proteins to the developing oocyte at the posterior end of the egg chamber (Figure 3). The oocyte grows larger during this transportation process, and eventually the nurse cells die. The formation of cysts in mice occurs somewhat differently, but with related underlying mechanisms. For instance, both processes involve incomplete cytokinesis resulting in cells connected by intercellular bridges, forming cysts in which many of the cells eventually die. These germline cysts remain fairly constant until birth.

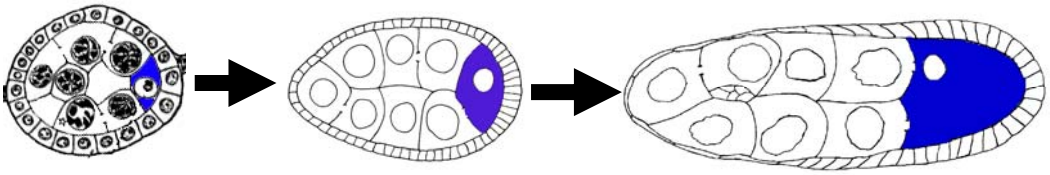


Figure 3. The *Drosophila* oocyte develops at the posterior end of the egg chamber (blue). Proteins, mRNAs, and organelles are transported to the developing oocyte, and proper localization is necessary for normal development (adapted from Koch et al., 1967).

Approximately two days after birth in the mouse, cysts begin to break down. Simultaneously, a process resulting in germ cell death occurs. These processes take place for a period of two days, or until about post-natal day (PND) four (Pepling and Spradling, 2001). Cyst breakdown occurs as cells of cysts die one by one, resulting in smaller and smaller cysts. Finally, only a few individual oocytes remain. Nearly two-thirds of the initial germ cells die due to apoptosis, a process also known as programmed cell death. Only one-third of the initial germ cell population will become surrounded by somatic cells to form primordial follicles, or oocytes (Pepling and Spradling, 2001). Several mammalian species undergo a similar process, losing one half to two thirds of their germ cell population before primordial follicles form (Baker, 1972). The mechanisms regulating cyst breakdown, germ cell death, and primordial follicle assembly are largely unknown.

In *Drosophila*, fertility is dependent on the successful transportation of mRNAs, organelles, and proteins to the developing oocyte within germline cysts. Equally important for normal oocyte development is the localization of these factors to proper areas within the oocyte. Within *Drosophila* oocytes, a ribonucleic protein (RNP) complex has been identified to be involved with the

localization of mRNAs in oocytes (Wilhelm et al., 2000). The proteins involved in this complex were identified using immunoprecipitation and mass spectrometry techniques, and include Ypsilon Schachtel (Yps, Y-box), Exuperantia, Poly A Binding Protein, Me31B (Dead box helicase), Orb (Cytoplasmic Polyadenylation Element Binding Protein), eIF4E, cup (eIF binding), Hsp70, Fragile-x mental retardation (FMR), Trailerhitch, and Winnebago (Wilhelm et al., 2000).

Previous studies of *Drosophila* involving Yps and Exu have identified these proteins to be key in the localization of oskar (osk) and bicoid (bcd) mRNA in the developing oocyte (Berleth et al., 1988) (St Johnston et al., 1989) (Wilhelm et al., 2000) (Figure 4). Osk protein is essential during development as it is responsible for recruiting additional components required for formation of the abdomen and germ cells (Ephrussi et al., 1991; Smith et al., 1992; Kobayashi et al., 1995; Breitwieser et al., 1996). It is localized to the posterior of the *Drosophila* oocyte by the Yps-Exu complex. Bcd protein is also important during development as it initiates a series of transcription programs establishing the anterior pattern of the embryo (St Johnston and Nusslein-Volhard, 1992). It is localized to the anterior of the *Drosophila* oocyte by the Exu complex. These studies have revealed that Exu mutants disrupt localization of both anterior and posterior mRNAs, as Exu is involved with both mechanisms (Berleth et al., 1988; St Johnston et al., 1989). Such studies suggest that the other proteins aggregated with Exu and Yps may serve similarly essential functions in *Drosophila* oocyte development and fertility.

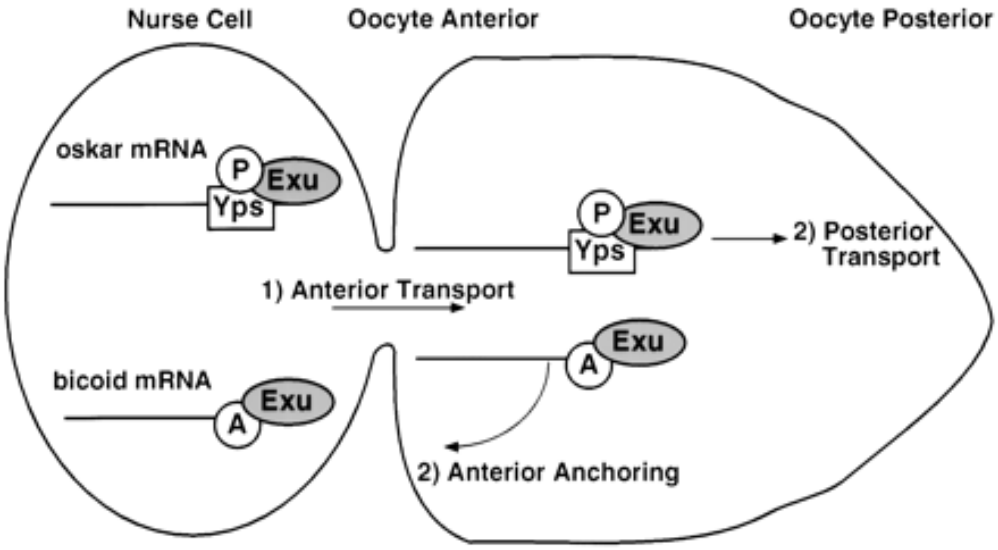


Figure 4. The Yps and Exu complex localize oskar mRNA to the posterior of the *Drosophila* oocyte. The Exu complex localizes bicoid mRNA to the anterior of the *Drosophila* oocyte. Exu mutants disrupt localization of both anterior and posterior mRNAs (Wilhelm et al., 2000).

Trailerhitch, a member of the *Drosophila* oocyte transport complex (Wilhelm et al., 2005; Wilhelm et al., 2000), is highly conserved in eukaryotes, including *C. elegans*, yeast, mice, and humans (Bong et al., 2005). Two conserved domains in the *trailer hitch* (*tral*) gene, the Sm domain and FDF domain, are present throughout these species. The Sm domain is found in proteins involved in RNA metabolism, such as splicing (Birney et al., 1993). The FDF domain is found in a family of proteins involved in regulation of mRNA decay (Anantharaman and Aravind, 2004). *Drosophila* and mouse Trailerhitch proteins are 74% similar within their amino-terminal Sm domain and are 59% identical overall (Ko et al., 2000) (Pepling et al., in preparation) (Figure 5). The protein product of the mouse *tral* gene is thought to directly interact with other

key proteins during oocyte development that have been found to localize specific mRNAs within the oocyte. It is also likely to be involved in RNA localization in other cell types, and for more general cellular functions. For instance, it has been found that human Trailerhitch localizes to P bodies (Yang et al., 2006), which are involved in RNA degradation (Cougot et al., 2004; Sheth and Parker, 2003; Wilczynska et al., 2005). Also, siRNA knockdown of the human *tral* gene was performed in cell culture, and it was found that P Body formation was disrupted (Yang et al., 2006). These studies signify that *tral* is involved in more general cellular functions.

Figure 5. Part A shows both the *Drosophila Tral* gene and the mouse *Tral* gene, highlighting the conserved Sm and FDF domains. Part B shows the amino acid similarities in a part of the *Drosophila* and mouse Sm domain (Pepling et al., in preparation).

An antibody generated against the *Drosophila* Sm domain recognized mouse Trailerhitch (Wilhelm et al., 2005). Extracts prepared from mouse ovaries and testes revealed a western blot band with a molecular weight of approximately

70 kd (Figure 6). Thus, the *Drosophila* antibody specifically recognizes the mouse Trailerhitch protein, which is present in mouse gonads.

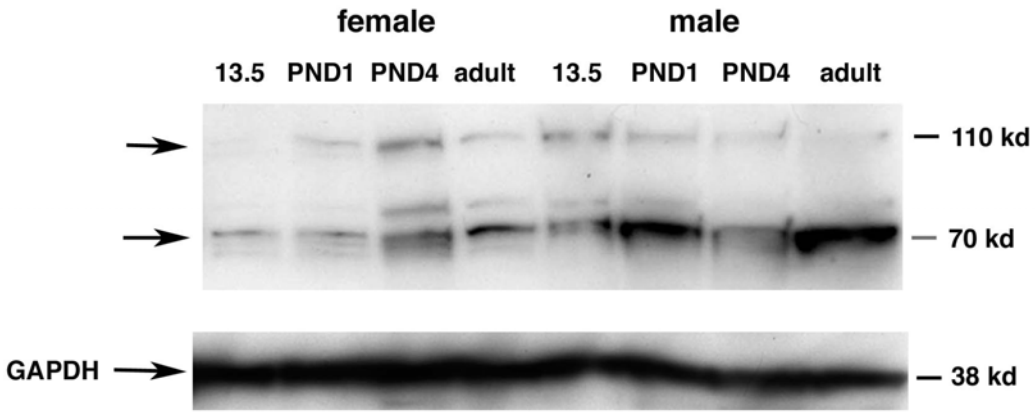


Figure 6. A western blot was preformed to determine the expression of the Trailerhitch protein in male and female germ cells. The protein is represented by 70 kd bands, the predicted molecular mass of Trailerhitch. Tissue extracts from 13.5 dpc, PND1, PND4, and adult mice were used (Pepling et al., in preparation).

The *Drosophila* Trailerhitch antibody has also been previously used in indirect immunofluorescence with neonatal mouse ovaries, and labeled the developing germ cells (Pepling, unpublished) (Figure 7). At post-natal day 3 (PND 3), Trailerhitch was shown to be localized in the cytoplasm of developing oocytes, especially in a particular concentrated region in the cytoplasm. This concentrated region of the Trailerhitch protein was proven to correspond with the Golgi apparatus at PND1 (figure 8) (Pepling et al., in preparation). However, the expression pattern of Trailerhitch in adult and developing testes has not been previously studied.

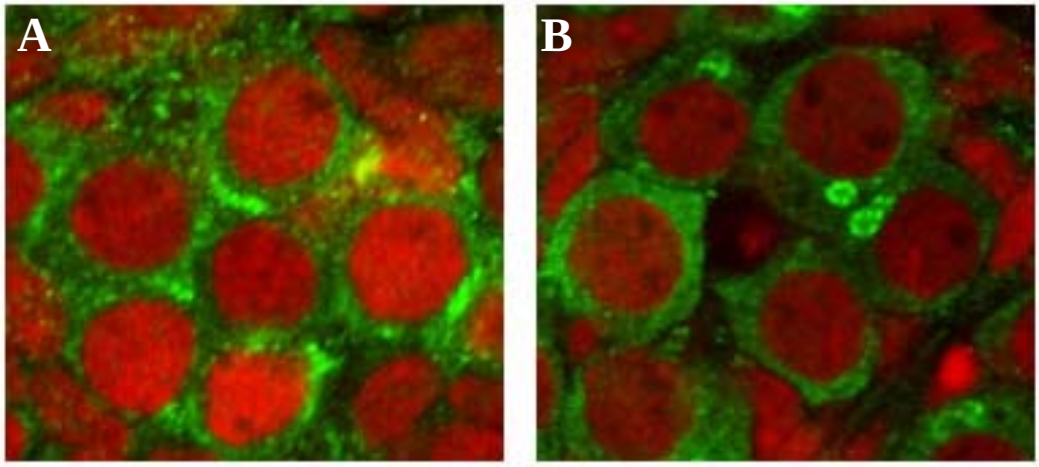


Figure 7. Oocytes were labeled with the Drosophila Trailerhitch antibody (green) and propidium iodide (red). Cytoplasm of oocytes as well as a more concentrated region within the oocyte expresses the Trailerhitch protein. A=18.5 dpc. B=PND 3 (Pepling et al., in preparation).

Figure 8. The Golgi is labeled with GM130 antibody in the two oocytes shown (seen as a green fluorescent stain), while the Trailerhitch protein is labeled with the *Drosophila* Trailerhitch antibody (seen as a red stain). The overlay of the two reveals that Trailerhitch is concentrated within the Golgi, marked by white arrows (Pepling et al., in preparation).

The function of *tral* has been studied in many different species, however, null alleles have not yet been found. For instance, P elements inserted in *tral* of *Drosophila* resulted in female sterility due to improper dorsal ventral patterning of the embryo (Wilhelm et al., 2005). In addition, RNAi of the *C. elegans* homologue of *tral* (CAR-1) results in increased germ cell death in hermaphrodites and causes cytokinesis defects and embryonic lethality (Boag et al., 2005). We

were interested in continuing the study of *tral* in mouse. A mouse *tral* gene is located on chromosome 7. Our studies with the *Drosophila* Trailerhitch antibody and neonatal mouse testes revealed the expression of Trailerhitch in developing germ cells. Our studies also revealed that the Trailerhitch protein is present in other organ systems of the mouse. We used a gene trapping method to knockout *tral*. We have studied the phenotype of this mutation and hypothesize that homozygous mutant mice are embryonic lethal. Our findings support the view that Trailerhitch is involved in universal molecular mechanisms of RNA metabolism present in most cells, as well as specific mechanisms of mRNA localization during development of germ cells.

MATERIALS AND METHODS

Mouse Strains

The *Mus musculus* Trailerhitch knockout strain was generated in C57BL/6 strain background. The strain was generated using a gene trapping method, which involves introducing insertional mutations in the genome of mouse embryonic stem cells. In this procedure, gene trap vectors are used to introduce an insertional mutation. Vectors are plasmids used to construct recombinant DNA molecules for introduction into living cells. The vectors contain a β-geo marker, which is a fusion of β-galactosidase and neomycin phosphotransferase II. The vectors insert randomly into introns and interrupt the coding sequence. Resulting mRNA transcripts contain upstream exon sequences joined to the now-linear vector containing the β-geo marker (Figure 9). Downstream exon sequences are not transcribed, and thus the coding sequence for the protein product is not transcribed correctly. The gene is nonfunctional or "trapped" after this procedure, and is considered to be "knocked out." Embryonic stem cells with a "trapped" Trailerhitch allele were obtained from BayGenomics, Inc. (San Francisco, CA).

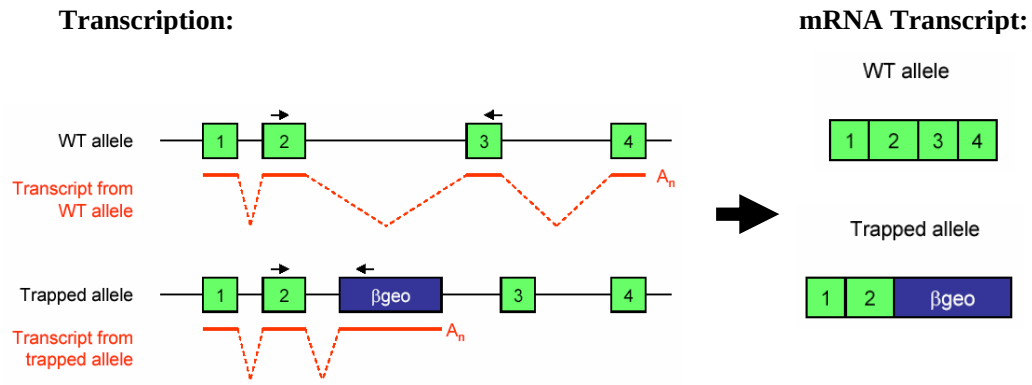


Figure 9. Exons from a Wildtype (WT) allele and from a Trapped allele are represented by green numbered boxes. The dashed red lines represent the sequences that are spliced out after transcription occurs. The thick red lines represent the sequences that are included in the final mRNA Transcript, which is shown on the right. The Trapped allele lacks downstream exons, as the β-geo vector interrupts transcription (Wellcome Trust Sanger Institute).

Mutations in selected genes may then be transmitted through the germline of mice to analyze any phenotypic effects (Figure 10). Heterozygous embryonic stem cells are selected according to their neomycin resistance. The cells are injected into a blastocyst, which is a formation of cells at the stage of development of 3.5 dpc. This blastocyst is injected into the uterus of a mouse. This mouse can produce chimeric mice after this process, which are then mated to wildtype mice to produce wildtype and heterozygous mutant mice. Finally, heterozygous mutants are crossed to produce wildtype, heterozygous mutants, and homozygous mutants. This gene trapping method is rapid and cost-effective, and compares favorably with gene-targeting approaches. It allows a large-scale functional analysis, as genome-wide mutagenesis can be undertaken with the vectors. It also allows the ability to analyze genome function in an intact organism. This method has been widely practiced, as almost two-thirds of all genes in mice have been "trapped." Blastocyst injections for this project were

performed at the University of Rochester, which is also where chimeric mice were generated.

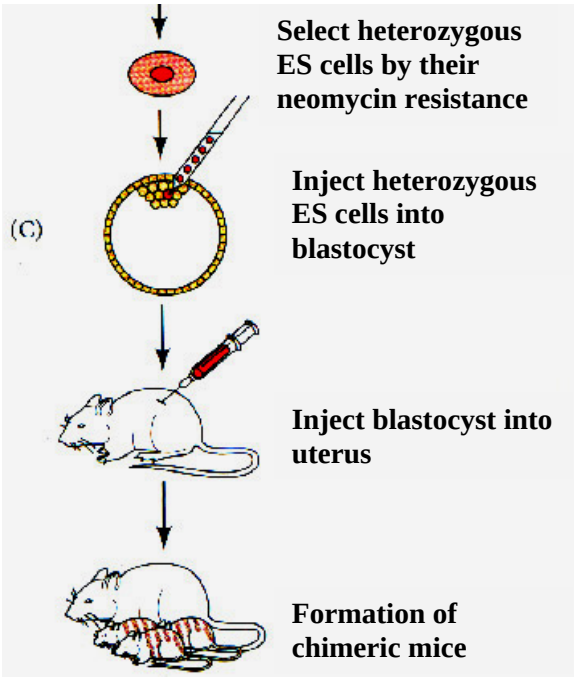


Figure 10. The procedure for forming chimeric mice from heterozygous ES cells is depicted.

Purification of DNA from Mouse Tails

In order to keep track of the Trailerhitch knockout mice, each mouse was given a tag and a tail snip was collected. Using a DNAeasy kit from QIAGEN, DNA was isolated from mouse tails. Each mouse tail was treated with 180 µl of Buffer AL and 20 µl of the enzyme proteinase K. These mixtures were placed in a 55°C water bath overnight, and then vortexed. A mixture of 200 µl Buffer ATL and 200 µl 100 percent ethanol was made and added to each sample, which were then vortexed. These samples were transferred to spin columns previously placed in collection tubes. 500 µl AW1 buffer plus ethanol was added to each column, which were centrifuged for one minute at 8,000 rpm. The flow-through was

discarded and the collection tubes were replaced with fresh tubes. 500 µl AW2 buffer plus ethanol was added to each column, which were centrifuged for three minutes at 14,000 rpm. The flow-through was again discarded and the columns were placed into fresh Eppendorf tubes. 100 µl of AE buffer was added to each column, and they were centrifuged one minute at 8,000 rpm to elute the DNA. The flow-through was collected and stored at -20°C.

Polymerase Chain Reaction (PCR)

PCR is a method used to amplify sequences of DNA using sequence-specific forward and reverse primers. Each PCR reaction consisted of 4 µl of 1.25 mM dNTP mixture (1.25 mM each of dATP, dTTP, dCTP, and dGTP), 2.5 µl of Buffer A, 1 µl of each primer, 0.5 µl of the enzyme Taq polymerase, 5 µl of template DNA, and a volume of distilled water that gave a total volume of 25 µl. The PCR reaction mixture was then placed in a thermocycler to undergo the PCR reaction.

The PCR reaction initially used to genotype the DNA isolated from Trailerhitch mouse tails included the use of four primers named 0013, 0014, 0015, and 0016. Primers 0013 and 0014 anneal to sequences of an unrelated gene, which is used as a control to determine if PCR has performed correctly. Primers 0015 and 0016 anneal to sequences within the neomycin resistance gene, which is present in the gene trap insertion of the *tral* intron, thus amplifying mutant DNA. The sequences for the primers follow:

Primer 0013: 5’ – CTT GGG TGG AGA GGC TAT TC – 3’

Primer 0014: 5' – AGG TGA GAT GAC AGG AGA TC – 3'

Primer 0015: 5' – CAA ATG TTG CTT GTC TGG TG – 3'

Primer 0016: 5' – GTC AGT CGA GTG CAC AGT TT – 3'

This PCR reaction was programmed into the thermocycler to follow these temperatures:

1) 5 minutes at 94°C

2) 1 minute at 94°C

3) 1 minute at 57°C

4) 1 minute at 72°C (Repeat steps 2-4 30 times)

5) 5 minutes at 72°C (Hold at 4°C)

The PCR reaction for genotyping Trailerhitch mice was modified to distinguish between heterozygous and homozygous mutants. Primers IR4, TH9A, TH10, and ER1 were used in these reactions. The sequences for these primers follow:

Primer IR4: 5' – ACA TCC ATG CTG AGG ATG AGG GAG – 3'

Primer TH9A: 5' – GGC AGA ATG CAC AGC ACA GTT CCT – 3'

Primer TH10: 5' – TGT TCT GCC TGC GTG TAT GCC TGC – 3'

Primer ER1: 5' – CCG AAC CAA CTG CAT TGC TCT GGG – 3'

This PCR reaction was programmed into the thermocycler to follow these temperatures:

1) 5 minutes at 94°C

2) 30 seconds at 95°C

3) 45 seconds at 60°C

4) 1 minute at 72°C (Repeat steps 2-4 30 times)

5) 5 minutes at 72°C (Hold at 4°C)

Gel Electrophoresis

The PCR product was verified using gel electrophoresis. Two percent agarose gels were prepared by dissolving 1 g of agarose in 50 ml 1 X TBE buffer using the microwave for two minutes. 2.5 µl of a 10 mg/mL working solution of ethidium bromide was added to the hot solution, which was then poured into a gel tray with a positioned comb for well formation. When the gel solidified, 1 X TBE buffer was poured over the gel and the comb was removed. A mixture of 2 µl 6x loading dye and 10 µl of each PCR reaction product was loaded into the wells of the gel. Also, a mixture of 2 µl 6x loading dye, 5 µl 1 X TBE buffer, and 5 µl 100 bp marker was loaded on the gel as a reference for band size. Gel electrophoresis took place for approximately one hour at 90-100 volts. Each agarose gel was analyzed for bands using a Kodak Image Station with UV light.

Primer Walking

To determine the exact location of the gene trap vector along the intron of the *tral* gene in mutant mice, primer walking was performed. Primers each 500 bp apart were made along the intron between exons two and three of the Trailerhitch gene in both the forward and reverse direction, as this was the expected location of the gene trap vector (Figure 11). Primers were also made in the forward and reverse direction on the gene trap vector (primers IR4 and IF1) as

well as on exons two and three (primers EF1 and ER1). The sequences for the primers follow:

Primer TH1F: 5' – TAC TGC TCT TAC CCA AGA CCC AGG – 3'

Primer TH2F: 5' – AAA AAT AGT GGA GAT CGC CGG GCG – 3'

Primer TH3F: 5' – CCA GTG TTG ACC TCT GGC CTT GAC – 3'

Primer TH4F: 5' – ACC CAG GGC CTC TGC ATG AGC AAT – 3'

Primer TH5F: 5' – GGG TCA CAA CTG CTG TCA GTC TTC – 3'

Primer TH6F: 5' – GAA GGA CCC TAA GTC CGT TTT GGC – 3'

Primer TH7F: 5' – GGG GGG GGG GAA ACC TGT GAA TTA – 3'

Primer TH8F: 5' – CCA GTA CCT GGA AAG CTG AGG CAG – 3'

Primer TH9F: 5' – GGT TCT GGG GAG CAG CAA AGT GTC – 3'

Primer TH10F: 5' – TGT TCT GCC TGC GTG TAT GCC TGC – 3'

Primer TH1R: 5' – CCT GGG TCT TGG GTA AGA GCA GTA – 3'

Primer TH2R: 5' – CGC CCG GCG ATC TCC ACT ATT TTT – 3'

Primer TH3R: 5' – GTC AAG GCC AGA GGT CAA CAC TGG – 3'

Primer TH4R: 5' – ATT GCT CAT GCA GAG GCC CTG GGT – 3'

Primer TH5R: 5' – GAA GAC TGA CAG CAG TTG TGA CCC – 3'

Primer TH6R: 5' – GCC AAA ACG GAC TTA GGG TCC TTC – 3'

Primer TH7R: 5' – TAA TTC ACA GGT TTC CCC CCC CCC – 3'

Primer TH8R: 5' – CTG GCT CAG CTT TCC AGG TAC TGG – 3'

Primer TH9R: 5' – GAC ACT TTG CTG CTC CCC AGA ACC – 3'

Primer TH10R: 5' – GCA GGC ATA CAC GCA GGC AGA ACA – 3'

Primer IR4: 5' – ACA TCC ATG CTG AGG ATG AGG GAG – 3'

Primer IF1: 5' – AGG AGA AAA TAC CGC ATC AGG CGC – 3'

Primer ER1: 5' – CCG AAC CAA CTG CAT TGC TCT GGG – 3'

Primer EF1: 5' – TGG GCA GCA GTT TGG TGC TGT TGG – 3'

A PCR reaction was performed with IR4 and each primer along the intron in the forward direction (and primer EF1) as well as with IF1 and each primer along the intron in the reverse direction (and primer ER1) in order to identify the gene trap vector. Any resulting bands were sequenced to determine the exact location of the gene trap vector. The PCR reaction used for primer walking was programmed into the thermocycler to follow these temperatures:

1) 5 minutes at 94°C

2) 30 seconds at 95°C

3) 45 seconds at 60°C

4) 1 minute at 72°C (Repeat steps 2-4 30 times)

5) 5 minutes at 72°C (Hold at 4°C)

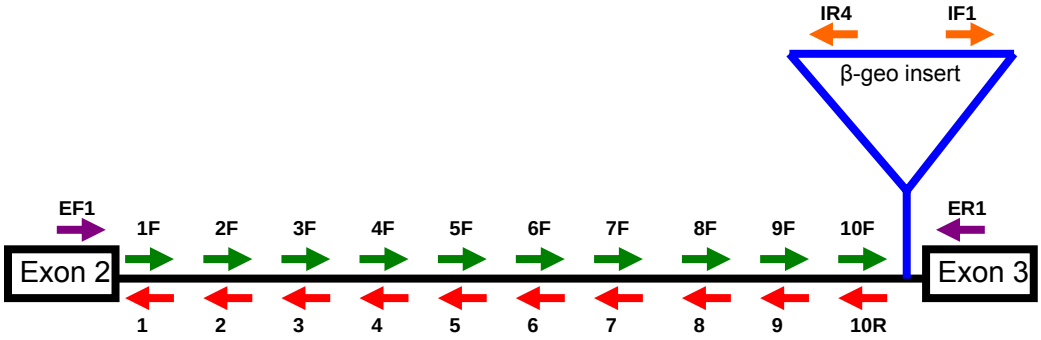


Figure 11. This represents the *tral* intron between exons two and three. Primers are represented by arrows and corresponding labels. These primers were used to perform primer walking in order to determine the exact location of the gene trap vector along the intron. Black signifies the *tral* gene and blue signifies the gene trap vector.

Dissection

Adult and neonatal (PND 4) ovaries and testes were dissected in 1 X phosphate buffered saline (PBS) using a microscope and were collected for further evaluation. Additionally, embryos of varying developmental ages (6.5 dpc through 13.5 dpc) were dissected in 1 X PBS using a microscope and were collected for evaluation.

X-gal Staining

X-gal staining was performed on tissue samples from ovaries, testes, and embryos of different ages to detect the presence of the β-geo vector insertion. After tissue was dissected in 1 X PBS, it was washed at room temperature in 1 mL 0.1 M phosphate buffer. The tissue sample was then fixed in 1 mL Fix buffer for 15 to 30 minutes at room temperature while mixing. Fix buffer consists of 0.14 mL 50% gluteraldehyde, 2.5 mL 100 mM EGTA (pH 8.0), 0.1 mL 1 M $MgCl_2$, and 47 mL 0.1 M phosphate buffer. The tissue sample was then washed 3 times for 15 minutes at room temperature in 1 mL Wash buffer while mixing. Wash buffer consists of 0.4 mL 1 M $MgCl_2$, 2 mL 1% deoxycholate, 2 mL 2% NP40, and 195.6 mL 0.1 M phosphate buffer. 1 mL of Staining buffer was then added, which consists of 2 mL 25 mg/mL X-gal in dimethylformamide, 0.106 g potassium ferrocyanide (Sigma P9387), 0.082 g potassium ferricyanide (Sigma P8131), and 48 mL Wash buffer. This solution was stored in a foil-covered tube. The tissue sample was incubated at 37°C for at least 1 hour. 1 mL of PBS was added to the tissue sample, and it was left at room temperature for storage. Tissue

samples were observed at this point or mounted on slides and viewed under a microscope. To mount on slides, the tissue was first soaked in 1 mL of a solution made of 50 percent glycerol and 50 percent 1 X PBS. The tissue was then mounted on slides with this solution. Tissue samples that stained blue signify the presence of β-galactosidase, and thus the β-geo vector. Therefore, blue tissue was isolated from either a homozygous or heterozygous mutant mouse. Tissue that did not stain was isolated from a wildtype mouse.

Whole Mount Antibody Staining of Neonatal and Adult Mouse Testes

Indirect immunofluorescence was used to stain neonatal mouse testes using the *Drosophila* Trailerhitch antibody at PND 4. Testes were dissected in 1 X PBS and were fixed in 600 µl of 5.3% formaldehyde for 1 hour at room temperature on a nutator. The testes were washed 2 times in 1 mL PT quickly, and then 1 time in 1 mL PT for at least 30 minutes at room temperature. They were then incubated in PT + 5% BSA for 30 minutes to 1 hour at room temperature on a nutator. The testes were next incubated with 500 µl of the primary antibody (*Drosophila* Trailerhitch antibody generated in rabbit) diluted in PT + 5% BSA overnight at 4°C on a nutator. *Drosophila* Trailerhitch Antibody was used at 1:500. Next, the testes were washed in 1 mL PT + 1% BSA for 30 minutes at room temperature on a nutator. They were then incubated in 10 µl of 10 mg/mL of RNase A in 1 mL PT + 1% BSA for 30 minutes. From this point on, the Eppendorf tubes containing the testes were wrapped in foil to prevent light exposure. The testes were incubated in 10 µl of 0.5mg/mL propidium iodide in 1

mL PT + 1% BSA for 20 minutes at room temperature. They were washed for 30 minutes in PT + 1% BSA. Incubation with 500 µl of preabsorbed fluorescent secondary antibody (α Rabbit-Alexa 488) diluted 1:200 in PT + 5% BSA for 2 hours at room temperature on a nutator was then performed. The testes were then washed 3 times in 1 mL PT + 1% BSA for 30 minutes at room temperature on a nutator. Finally, the testes were washed 1 time with 1 X PBS for 5 minutes. 100 µl of Vectashield was used to mount the testes on slides, which were stored at -20°C.

Confocal Microscopy

Indirect immunofluorescence of PND 4 testes was observed through a Zeiss Pascal confocal microscope. A nuclear marker (propidium iodide) and the *Drosophila* Trailerhitch antibody were used to stain the testes, and confocal analysis was performed.

Western Blotting

Western blotting was used to detect the Trailerhitch protein in samples of tissue from adult testes. Testes were dissected in 1 X PBS and stored on ice. Gonad extracts were made by homogenizing each testis on ice in 800 µl of 1 X Sample Buffer and complete mini protease inhibitors from Roche. Samples were then boiled for 5 minutes, cooled on ice, and centrifuged for 3 minutes at 6000 rpm. 2 µl of 2-mercaptoethanol was added to each sample per every 20 µl of

solution. The samples were boiled for 3 minutes, centrifuged for 1 minute at 6000 rpm, and placed on ice.

Gel electrophoresis was run on a 4-20% polyacrylamide gel, and 10 µl of Precision Plus Standard Protein marker was loaded into one well as a band size reference. After cleaning out the wells of the gel with a syringe, 20 µl of each extract was loaded into the wells. Empty wells were loaded with 20 µl of 2 X Sample Buffer. 2 X Sample Buffer consists of 4 mL 10% SDS, 2 mL Glycerol, 1 mL 0.1% Bromophenol Blue, 2.5 mL 0.5 M Tris (pH 6.8), and 0.5 mL 2-mercaptoethanol. Gel electrophoresis was performed at 100 volts for 1 hour using 1 X SDS Running Buffer. Running Buffer consists of 29 g of Tris base, 144 g of glycine, 10 g of SDS, and 1 liter of distilled water.

The gel was transferred to a Polyvilnidene fluoride (PVDF) membrane first by soaking the 6 x 8 cm PVDF membrane in methanol for 1 minute. The membrane was washed in distilled water for 5 minutes on a shaker, and then soaked in Transfer Buffer for 5 minutes. The Transfer Buffer consisted of 3.0 g of Tris base, 14.4 g of glycine, 200 mL of methanol, and distilled water up to 1 liter. 2 pieces of 6 x 8 cm filter paper and 2 fiber pads were soaked in transfer buffer for 5 minutes. The gel and PVDF membrane were assembled in a transfer cassette along with the filter paper and filter pads. The transfer cassette was placed into a transfer casket, which was placed into a transfer unit filled with Transfer Buffer. The transfer was performed at 4°C at 100 volts for 1 hour. Following the transfer, the gel was stained with Coomassie stain for 30 minutes and then treated with several changes of destain solution, which was left

overnight. The destain solution consisted of 450 mL of distilled water, 450 mL of methanol, and 90 mL of acetic acid. The gel was eventually dried on a frame using gel wrap. The membrane was blocked in Blocking Buffer on a shaker for 30 minutes at room temperature. Blocking Buffer consisted of 100 mL of 10 X PBS, 50 g of 5% nonfat dry milk, 0.5 mL of 0.05% Tween 20, and distilled water up to 1 liter. After changing the buffer, it was left overnight at 4°C.

To detect the Trailerhitch protein, the membrane was incubated in the 1° Trailerhitch Antibody diluted in Blocking Buffer (1:1,000; 5 ml total) for 1 hour on the nutator. The membrane was then washed in Blocking Buffer 3 times for 10 minutes on the shaker. Incubation with 2° Antibody (α-Rabbit HRP) diluted in Blocking Buffer (1:10,000; 5 ml total) was performed for 1 hour on the nutator. The membrane was washed in Blocking Buffer 2 times for 10 minutes, and then once in Wash Buffer for 10 minutes on the shaker. The Wash Buffer consisted of 0.05% Tween in 1 X PBS. The membrane was rinsed in 1 X PBS and incubated in 2.5 mL each of detection buffer and enhancer (1:1) for 5 minutes on the shaker. After rinsing in distilled water, the membrane was placed in a clear plastic cover and mounted to cardboard for exposure to film. It was exposed for 1 minute and then 5 minutes to visualize the bands.

RT-PCR

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) was performed to detect and quantify mRNA from the Trailerhitch gene in adult testes. It also allowed us to prove the location of the gene trap vector between

introns 2 and 3 of the Trailerhitch gene. Tissue samples were homogenized in 0.75 mL of Trizol LS Reagent per 50-100 mg of tissue. 20 µl of 1 mg/mL glycogen was added, and the samples were incubated for 5 minutes at 30°C to permit complete dissociation of nucleoprotein complexes. 0.2 mL of chloroform was added per 0.75 mL of Trizol, and tubes were shook vigorously by hand for 15 seconds. After incubating at 30°C for 2 to 15 minutes, the samples were centrifuged at 10,000 rpm for 15 minutes at 4°C. The mixture separated into 3 layers, and the upper aqueous layer was transferred to a clean tube. The RNA was precipitated from this aqueous phase by adding 0.5 mL of isopropyl alcohol per 0.75 mL of Trizol used initially. Samples were incubated at 30°C for 10 minutes and centrifuged at 10,000 rpm for 10 minutes at 4°C. A gel-like pellet forms on the bottom of the tube, which is the RNA precipitate. The supernatant was removed and the RNA pellet was washed with 1 mL of 75% ethanol per 0.75 mL of Trizol. The sample was mixed by vortexing and centrifuged at 8,000 rpm for 5 minutes at 4°C. The RNA pellet was air-dried for 5 to 10 minutes, but was not allowed to completely dry for increased solubility. The RNA was dissolved in 25 µl of PCR-grade water, and the solution was incubated for 10 minutes at 55°C. To determine if the isolation of RNA was successful, 1 µl of the sample was run on a 1% agarose gel using gel electrophoresis and a Kb ladder as a band reference. The mRNA transcripts for wildtype DNA and mutant DNA are shown in Figure 12.

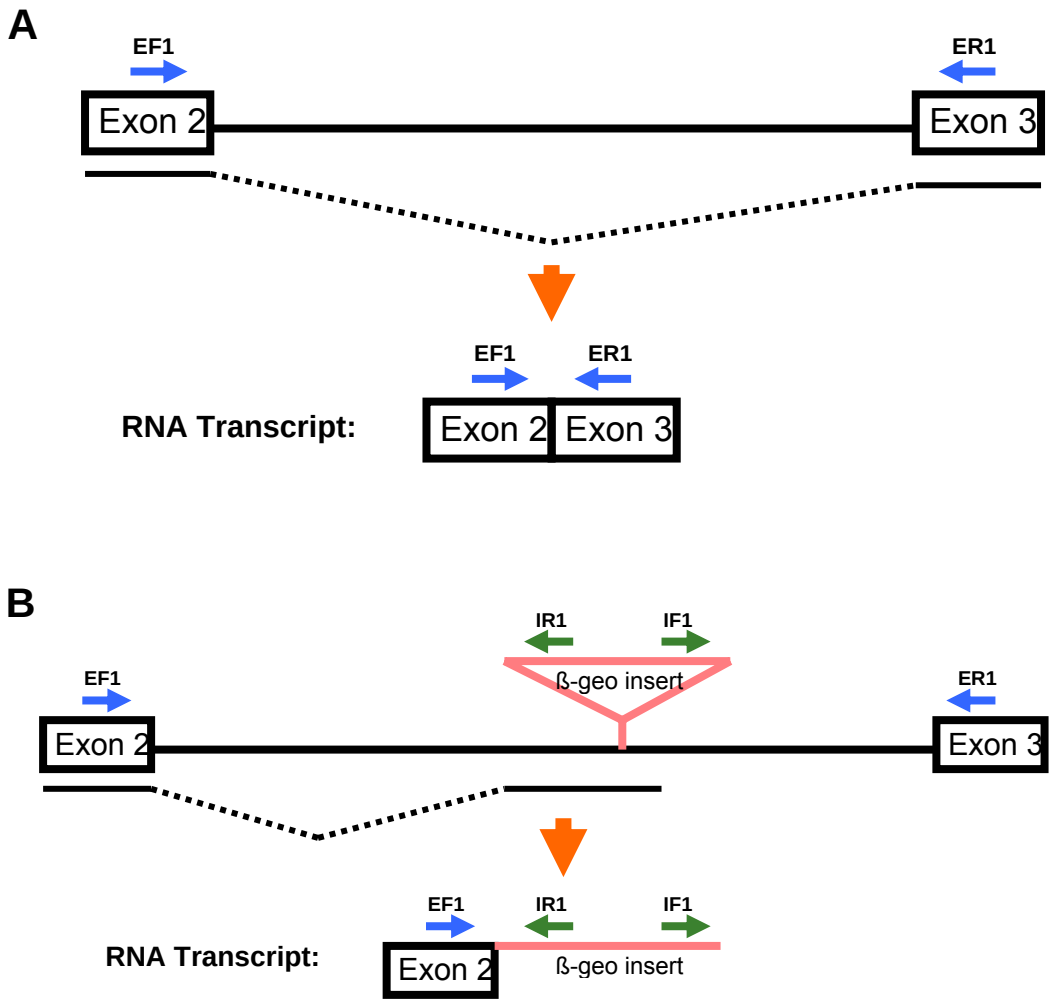


Figure 12. Part A depicts the mRNA transcript of a wildtype Trailerhitch gene between exons 2 and 3. Primers EF1 and ER1 should identify wildtype DNA. Part B depicts the mRNA transcript of a mutant Trailerhitch gene with a gene trap vector. Primers EF1 and IR1 should identify mutant DNA. Black indicates the Trailerhitch gene sequence and pink indicates the gene trap vector. Small arrows identify primers.

To determine the approximate concentration of the RNA sample, 1 µl of the sample was mixed with 100 µl TE for an optical density test. To perform the reverse transcriptase reaction, 5 µg of RNA in a 10 µl volume was heated at 65°C for 5 to 10 minutes, and then quenched on ice. The following components were inserted into an Eppendorf tube for a total volume of 40 µl: 10.0 µl heat denatured RNA, 3.0 µl 10 X PCR buffer B, 2.5 µl 10 mM dNTPs, 6.0 µl 25 mM $MgCl_2$, 2.0

µl 3' RACE, 0.5 µl SuperScript II reverse transcriptase, 0.5 µl RNase inhibitor, and 15.5 µl water. The samples were incubated at 25°C for 10 minutes and then at 42°C for 1 hour. Denaturation of the cDNA took place at 95°C for 5 minutes, and the tubes were placed on ice.

To amplify the cDNA, two PCR reactions were set up per initial reaction with the following components for a total volume of 25 µl: 6.0 µl cDNA, 2.5 µl 10 X PCR buffer A, 0.2 µl Taq polymerase, 5.62 µl distilled water, 3.34 µl of both the 0.15 µg/ µl forward and reverse primers, and 4.0 µl 1.25 mM dNTPs. The first PCR reaction included primers EF1 and IR1to amplify mutant cDNA. The second PCR reaction included primers EF1 and ER1to amplify wildtype cDNA. The primer sequences follow:

Primer EF1: 5' – CGT CGT CAT CTT CAT TCC AGT CGG – 3'

Primer IR1: 5' – CGC CAG GGT TTT CCC AGT CAC GAC – 3'

Primer ER1: 5' – CCG AAC CAA CTG CAT TGC TCT GGG – 3'

The PCR reaction was performed with 30 cycles of denaturation: 30 seconds at 95°C; annealing: 45 seconds at 60°C; and extension: 60 seconds at 72°C. The PCR products were run on a 2% agarose gel using gel electrophoresis for analysis (Figure 13).

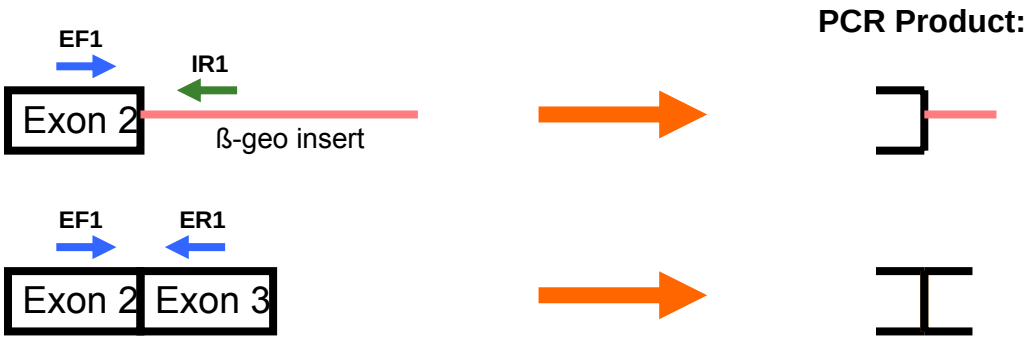


Figure 13. Both PCR reactions depicted here were performed on each sample to distinguish between wildtype, heterozygous mutant, and homozygous mutant DNA. Primers EF1 and IR1 amplify mutant cDNA and primers EF1 and ER1 amplify wildtype cDNA. The PCR products are depicted with black indicating Trailerhitch gene sequence and pink indicating gene trap vector sequence.

Inverse PCR

Inverse PCR was performed to amplify a specific, unknown region of DNA in the intron between exons 2 and 3 of the Trailerhitch gene by cleaving the sequence at a known cut-site and at an unknown cut-site within the flanking DNA region. The purpose of this method was to determine the exact location of the gene trap vector within the intron of the Trailerhitch gene. This was done by first digesting 2-5 µg of DNA template with an appropriate restriction enzyme for 2.5 hours at 37°C. 3 separate reactions were performed such that 3 different restriction enzymes were tested, which were HinPI, Sau3A, and MspI (Figure 14). This digestion mixture had a total volume of 20 µl, including 6 µl of distilled water, 10 µl of DNA, 2 µl of 10 X Buffer B, and 2 µl of the restriction enzyme. 5 µl of this product was run on a 1% agarose gel using gel electrophoresis to determine if the DNA was digested. Ligations were set up next to ligate the DNA fragments for 12-16 hours at 16°C (Figure 15). The ligations had a total volume of 20 µl, which included 11-15 µl of distilled water, 1-5 µl of cleaved DNA, 2 µl

of 10 X ligation buffer with ATP, and 2 µl of T4 DNA ligase (1 unit/ µl). A variety of ligations were performed for the 3 different reactions with the volume of DNA varying between 1-5 µl in order to determine the optimal ligation conditions. PCR reactions were then set up (Figure 15) to amplify the unknown sequence of DNA of interest using previously synthesized primers, whose sequences follow:

Primer IR4: 5' – ACA TCC ATG CTG AGG ATG AGG GAG – 3'

Primer IF3: 5' – CCC GGA TGT CCT CTG GTG CTC AAA – 3'

The PCR reaction had a total volume of 25 µl and consisted of 16 µl distilled water, 2 µl ligated DNA, 2 µl .2 mM dNTPs, 2.5 µl 10 X Buffer A, 1 µl 2.5 µM forward primer, 1 µl 2.5 µM reverse primer, and 0.5 µl Taq Polymerase. 5 µl of the PCR product was run on a 2% agarose gel using gel electrophoresis with 1 Kb ladder and 100 bp ladder for band size references.

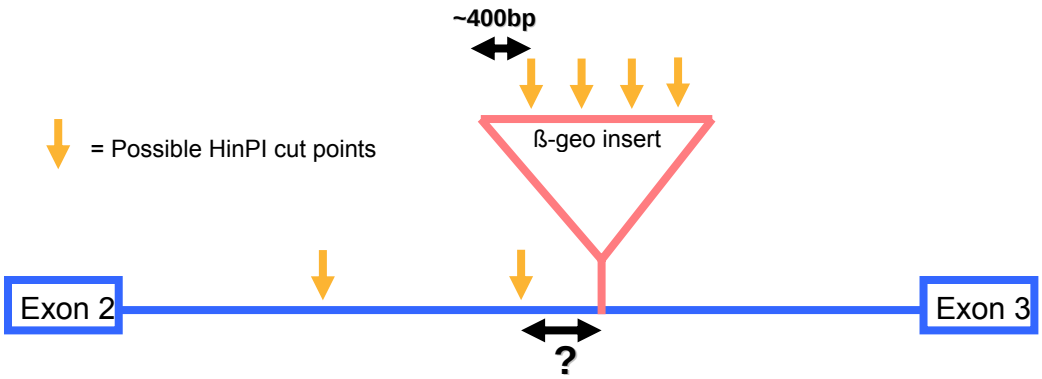


Figure 14. This diagram demonstrates the number of cut sites of the restriction enzyme HinPI (yellow arrows) on the gene trap vector (in pick) and intron of the Trailerhitch gene (in blue). The fragment of interest was an unknown size due to the unknown location of the gene trap vector along the intron.

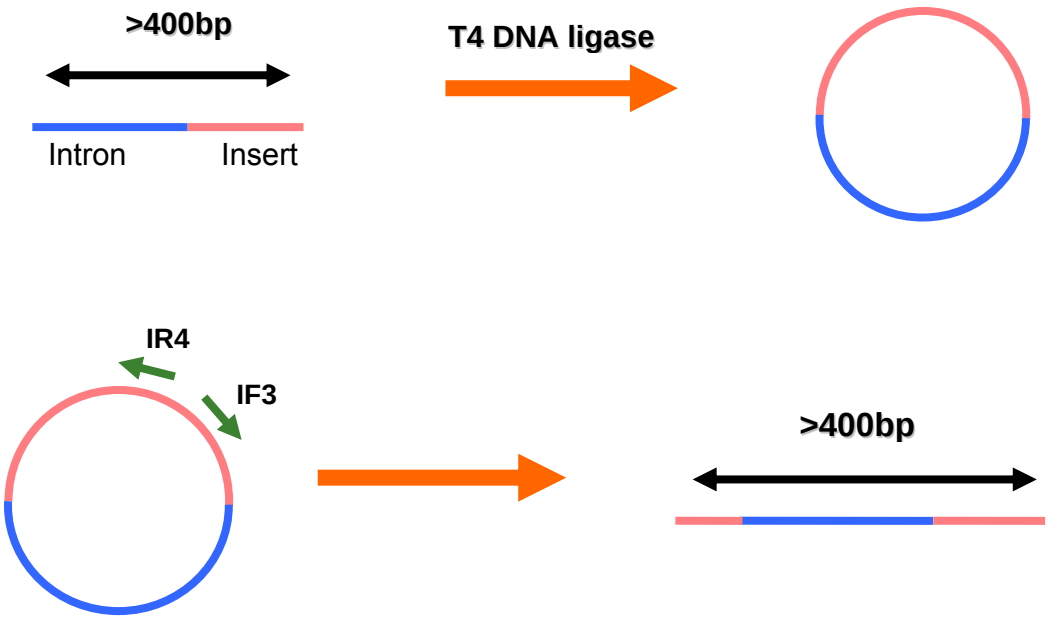


Figure 15. Digestion with a restriction enzyme produced a fragment that was at least 400 bp. The fragment was ligated with T4 DNA ligase. The ligated product was amplified with primers IR4 and IF3 (green arrows), thus amplifying the unknown sequence of interest. Blue indicates intron sequence and pink indicates insert sequence.

Spectrophotometry

A spectrophotometer is used to measure the amount of light of a specified wavelength which passes through a medium. The amount of light absorbed by a medium is proportional to the concentration of the absorbing material. The concentration of double stranded or single stranded DNA and RNA can be measured, as well as of protein. A spectrophotometer was used to determine the concentration of RNA isolated in RT-PCR. It was also used to determine the concentration of DNA samples for procedures like Inverse PCR. Absorbance was measured at a wavelength of 260 nm. An optical density of 1 measured for RNA may be converted to a concentration of 40 µg/mL, while an optical density of 1 measured for DNA may be converted to a concentration of 50 µg/mL.

DNA Purification for Sequencing

While performing such experiments as inverse PCR, RT-PCR, and primer walking, many DNA sequences were obtained. In order to sequence a segment of DNA obtained from PCR amplification, a large volume of the PCR product was run on an agarose gel with gel electrophoresis. The resulting band was excised from the gel using a scalpel under UV light. This piece of agarose gel contained the desired DNA fragment. The QIAquick protocol and kit for gel extraction was used to extract and purify the DNA. Three volumes of Buffer QG were added to 1 volume of the excised gel, and this was incubated at 50°C for 10 minutes. Vortexing was sometimes necessary to help the gel slice to dissolve. At this point, the color of the mixture was yellow, indicating an optimal pH of approximately 7.5 for DNA binding. One gel volume of isopropanol was added to the sample and mixed. This solution was applied to a QIAquick spin column placed in a 2 mL collection tube. After centrifugation for 1 minute at 13,000 rpm, the flow-through was discarded and the column was placed back into the collection tube. 0.5 mL of Buffer QG was again added to the QIAquick column, which was centrifuged for 1 minute at 13,000 rpm. The flow-through was discarded and the column was placed back into the collection tube. 0.75 mL of Buffer PE was added to the column to wash, and the column was centrifuged for 1 minute at 13,000 rpm. After the flow-through was discarded, the column was centrifuged for an additional 1 minute at the same speed. The column was placed into a clean 1.5 mL microcentrifuge tube and 50 µl of Buffer EB (10 mM Tris-Cl,

pH 8.5) was added to the column to elute the DNA. After centrifugation for 1 minute at 13,000 rpm, the DNA was eluted and prepared for sequencing.

RESULTS

The gene trap insertion interrupts *tral* between exons 2 and 3

Embryonic stem cells with a gene trap insertion in the *tral* gene were obtained and Trailerhitch mutant mice were generated at the University of Rochester. The gene trap insertion was reported to be between exons two and three of the *tral* gene. To confirm the location of the gene trap insertion, RT-PCR was performed. Testes and ovaries were collected from heterozygous mutant mice and RNA was isolated, which was reverse transcribed to cDNA. A PCR reaction was performed using primers that would amplify the 3' region of exon 2 and the 5' region of the gene trap insertion, if present. Another PCR reaction was performed using primers that would amplify the 3' region of exon 2 and the 5' region of exon 3 if the wildtype *tral* gene was present. The PCR products were run on an agarose gel using gel electrophoresis, and bands were viewed under UV light. To confirm the results, nested PCR was performed with primers located within the initial primers. Many different samples of DNA were tested, and a 169 base pair (bp) band resulted from each PCR reaction testing for the gene trap insertion (Figure 16). Also, a 106 bp band resulted from each PCR reaction testing for the wildtype *tral* gene. The 169 bp band was excised, the DNA eluted, and then sequenced. The sequence of this band matches the sequence of the 3' region of exon 2 and the 5' region of the gene trap insertion. This proves that the insertion was inserted between exons 2 and 3 of *tral*, and thus that the Trailerhitch mutant mice were made correctly. This, however, does not determine the insertion's exact location within the intron of the gene. These results from RT-

PCR also revealed that only heterozygous mutant mice seem to be present at an adult age. Every DNA sample tested resulted in a "wildtype" band, signifying that at least one copy of the *tral* gene is wildtype in these mice. Thus, this experiment suggests that homozygous mutants do not survive to adulthood.

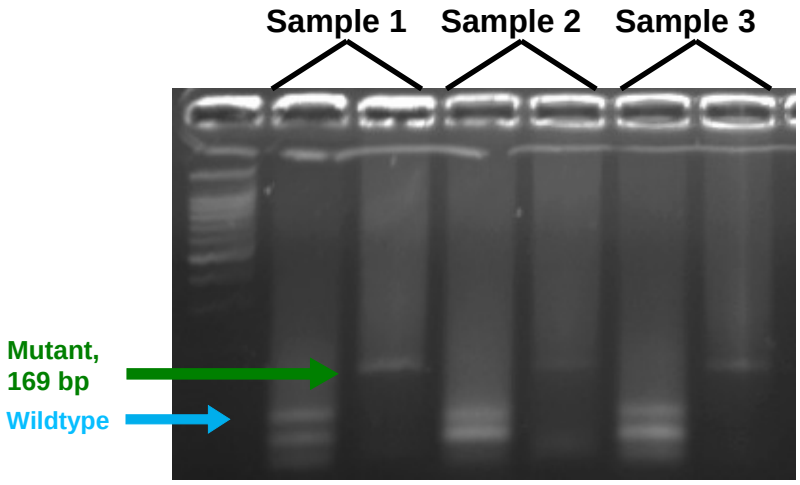


Figure 16. Three different DNA samples (#1, #2, #3) were tested with two different sets of primers. The first set amplified DNA and resulted in the band labeled "wildtype" only if wildtype DNA was present. The second set amplified DNA and resulted in the 169 bp band only if mutant DNA was present. The 169 bp was sequenced and confirmed the location of the gene trap insertion. This gel determines that these three DNA samples are from heterozygous mutant mice.

However, without knowledge of the gene trap insertion's exact location within *tral*, it is difficult to genotype homozygous and heterozygous mutant mice, and thus it is difficult to study the phenotype of this particular Trailerhitch mutant. To determine the exact location of the insertion, Inverse PCR was used to amplify the 5' region of the insertion and the flanking unknown 3' region of the intron. After the DNA was cut by the 3 restriction enzymes tested (HinPI, MspI, and Sau3A), the samples were run on a gel. Smearing was expected on the gel to represent the many pieces of cut DNA; but instead, the resulting band resembled the control uncut sample, suggesting that the restriction enzymes did not work

well (Figure 17A). After ligation and PCR were performed, DNA fragments only resulted from the samples cut with HinPI (figure 17B). Three fragments resulted, including 1 ~300 bp band and 2 ~500 bp bands. Each of these bands were cut out of the gel, purified, and sequenced. None of the sequences matched any t*ral* intron or insert sequences and were thought to be randomly amplified fragments.

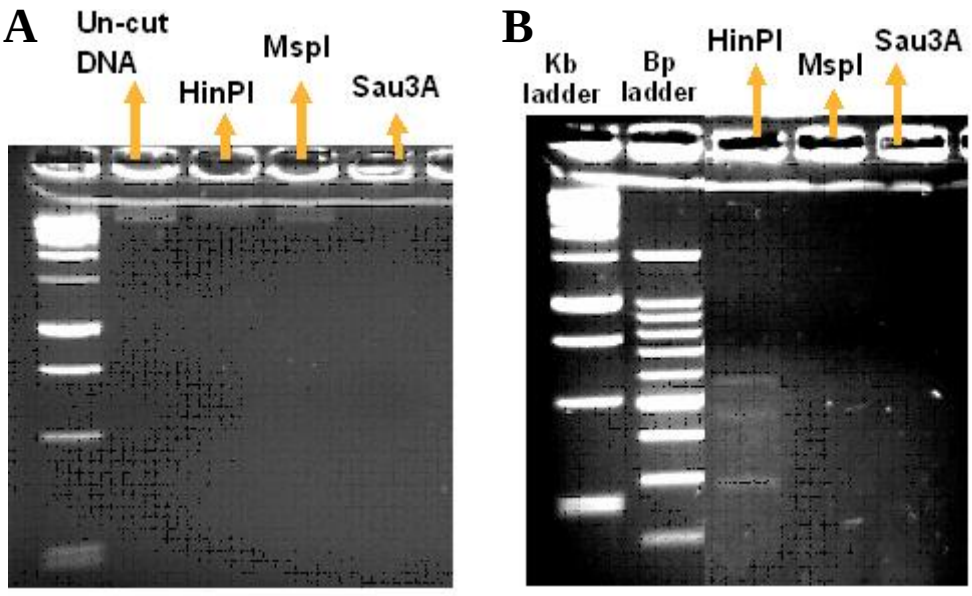


Figure 17. A) This gel shows the results from the digestion with three different restriction enzymes. None of the enzymes resulted in any smearing, suggesting that they did not work well. B) This gel shows the final results of inverse PCR for each enzyme tested. HinPI was the only enzyme to result in any amplified fragments.

As another approach to determine the exact location of the gene trap insertion, primer walking was performed. Primers were designed along the entire length of the *tral* intron between exons 2 and 3 in the 5' to 3' direction (primers 1 through 10). These were used in PCR reactions with a primer within the gene trap insertion in the 3' to 5' direction (primer IR4). PCR reactions were performed with samples of mutant Trailerhitch DNA to attempt to amplify a band that would

help us determine the approximate location of the gene trap insertion. A ~900 bp band was amplified using primers 9 and IR4. A ~200 bp band was amplified using primers 10 and IR4 (Figure 18). According to these results, the gene trap insertion is located very close to *tral* exon 3, on the 3' end of the intron between exons 2 and 3. An additional primer (9A) was designed between primers 9 and 10 in order to obtain a clear 500 bp band that could be sequenced. The resulting band from primers 9A and IR4 was excised, purified, and sequenced. This sequence matches a region of the *tral* intron between exons 2 and 3, very near exon 3, as suspected. Part of the sequence matching this region is shown in Figure 19. The gene trap insertion is located precisely 101 bp from the 5' end of exon 3 and 113 bp from the 3' end of primer 10 (Figure 20).

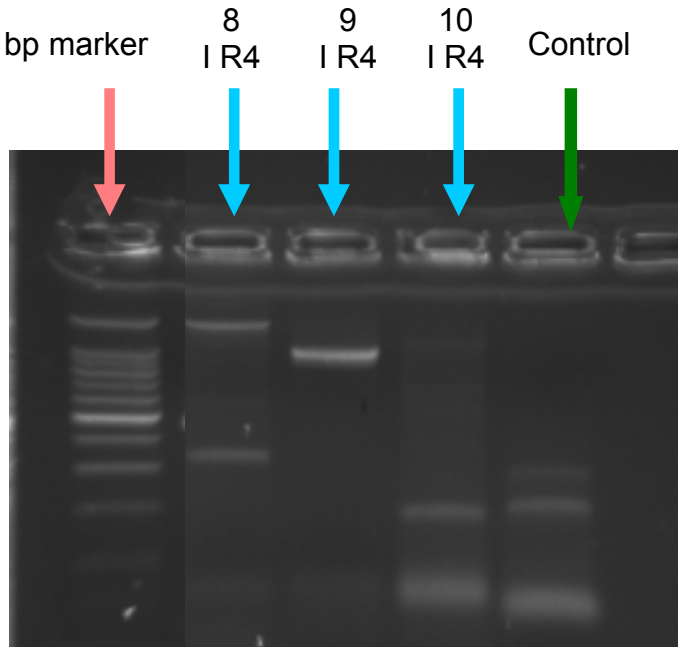


Figure 18. The PCR products run on this agarose gel allowed the determination of the relative location of the gene trap insertion. Primers 9 and IR4 amplify a ~900 bp band, suggesting that the gene trap insertion is ~900 bp away from the 3' end of primer 9. Primers 10 and IR4 confirm this as a ~200 bp band is amplified, suggesting that the gene trap insertion is ~200 bp downstream from the 3' end of primer 10.

TTTATTTATT TTGTATAGTG TTCTGCCTGC GTGTATGCCT GCAGGTCAGA
AGAGGGCTCT GATCCATCTC TCTAGCTCAG GATTTTTAAG TTTTATAAAG
TGCAGAGTGA CCAAGACAAT CAGTAAAATG CCTCACTGGC CAGGTGTAAG
CCAGGTCGTG GGTGCCGAGC CCTGCTCCCT CATCCTCAGC ATGGATTTGT

Figure 19. This sequence was obtained using primers 9A and IR4. Bases not underlined are part of a region of DNA in the *tral* intron, whereas bases underlined with a solid line are part of a region of DNA in the gene trap insertion. The dotted line indicates the sequence for primer TH10 while the curvy line indicates the sequence for primer IR4. This sequence allowed for precise mapping of the gene trap insertion in the *tral* intron.

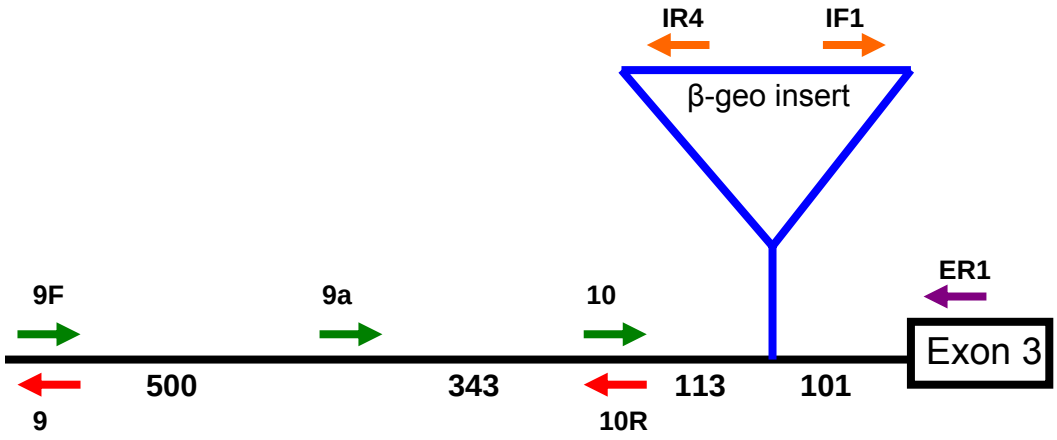


Figure 20. This diagram of a segment of the Trailerhitch intron demonstrates the exact position of the gene trap insertion. The β-geo insert is 101 bp from the 5' end of Exon 3 and 113 bp from the 3' end of primer 10F. Primers and their direction are indicated by arrows. Black indicates *tral* and blue indicates the β-geo insert.

Trailerhitch is expressed in both developing ovaries and testes

Studies in Drosophila and C. elegans suggest that Trailerhitch has an important, conserved role in germ cell development. Interest was thus provoked in the role of Trailerhitch in the development of mouse oocytes. In previous studies, Trailerhitch was shown to be localized in the cytoplasm of developing oocytes at PND 3, especially in a particular concentrated region in the cytoplasm. This concentrated region of the Trailerhitch protein was proven to correspond

with the Golgi apparatus at PND 1 (Pepling et al., in preparation). However, the expression pattern of Trailerhitch in developing testes was not previously studied.

A previous study using Western blots identified the Trailerhitch protein in adult mouse testes. To confirm this finding, testes were collected from 8 different adult males. A Western blot was performed to detect any Trailerhitch protein present in the testes. The Western blot identified a band at 70 kd corresponding to the Trailerhitch protein in all tissue samples (Figure 21); supporting the idea that Trailerhitch is present in male gonads. These results also suggest that homozygous mutants do not survive to adulthood as all collected tissues had at least one wildtype copy of the *tral* gene.

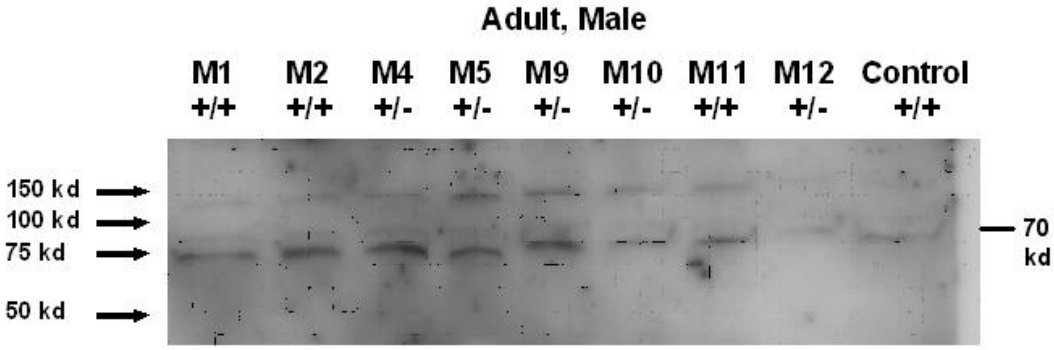


Figure 21. This western blot identifies the wildtype Trailerhitch protein at 70 kd. Eight different samples of testes tissue were isolated for this purpose (M1, M2, M4, M5, M9, M10, M11, M12). The genotype of each sample was determined, signified with +/+ (wildtype) and +/- (heterozygous mutant). No homozygous mutant tissue samples were found.

To determine the expression pattern of Trailerhitch in mouse testes, testes from mice of PND 4 and from adult mice were collected. Indirect immunofluorescence was performed and the expression of Trailerhitch was determined at these ages (Figure 22). Trailerhitch appears to be expressed throughout the developing seminiferous tubules, especially in the cytoplasm of

developing spermatagonia. However, adult Trailerhitch expression occurs only in small, circular structures resembling previously identified structures called chromatoid bodies located in the cytoplasm of mature sperm cells (Pepling et al., in preparation).

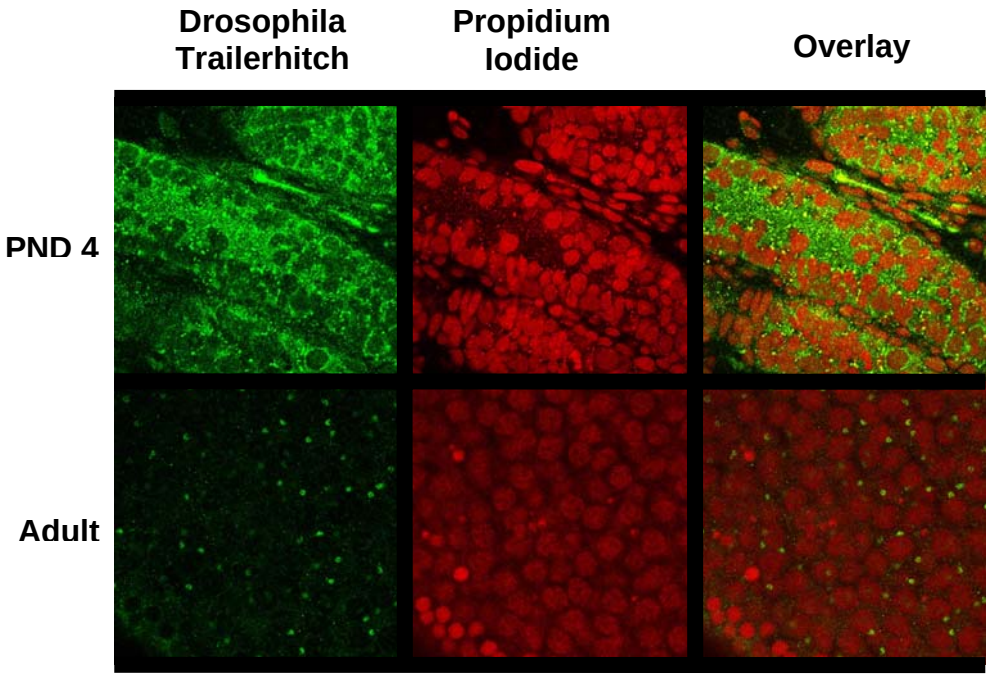


Figure 22. These are confocal images of post-natal day 4 and adult testes. Indirect immunofluorescence was performed on isolated tissues. Green fluorescence labels the Drosophila Trailerhitch Antibody while propidium iodide (red) labels the nuclei of cells.

Trailerhitch functions in many organ systems during development

Expression of Trailerhitch throughout ovaries and testes was demonstrated using a stain that identifies β-galactosidase in fixed tissues. The gene trap insertion causes formation of a fusion protein containing *tral* exons 1 and 2 as well as the β-geo insertion. Expression of β-galactosidase presumably reflects the expression pattern of endogenous protein in various cell types. PND 4 ovaries and testes were isolated and stained with X-gal (Figure 23). Gonads that did not

stain were identified as wildtype tissues while gonads that stained blue were identified as mutant tissues, and could be used to study Trailerhitch protein expression. Thus, this method confirms the expression of Trailerhitch in PND 4 gonads.

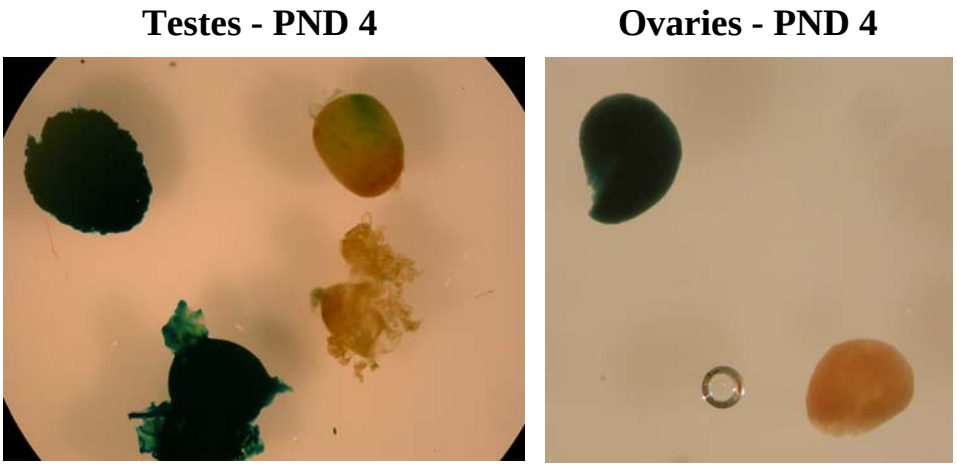


Figure 23. Testes and ovaries were stained with X-gal stain at post-natal day 4. Tissues resulting in blue color were identified as mutant, and those that did not stain were identified as wildtype. Staining allowed the study of Trailerhitch protein expression throughout the gonads.

To understand Trailerhitch's role in mice, it is important to examine not only its role in the reproductive system, but also its role in other organ systems. Its overall expression in developing and adult mice can signify a particular function for the Trailerhitch gene. Embryos of ages 7.5 dpc through 13.5 dpc were dissected and stained with X-gal to determine the expression pattern of the Trailerhitch protein during development (Figures 24 and 25). The staining results suggest that Trailerhitch might serve a role throughout the entire embryo during development between ages 7.5 and 13.5 dpc.

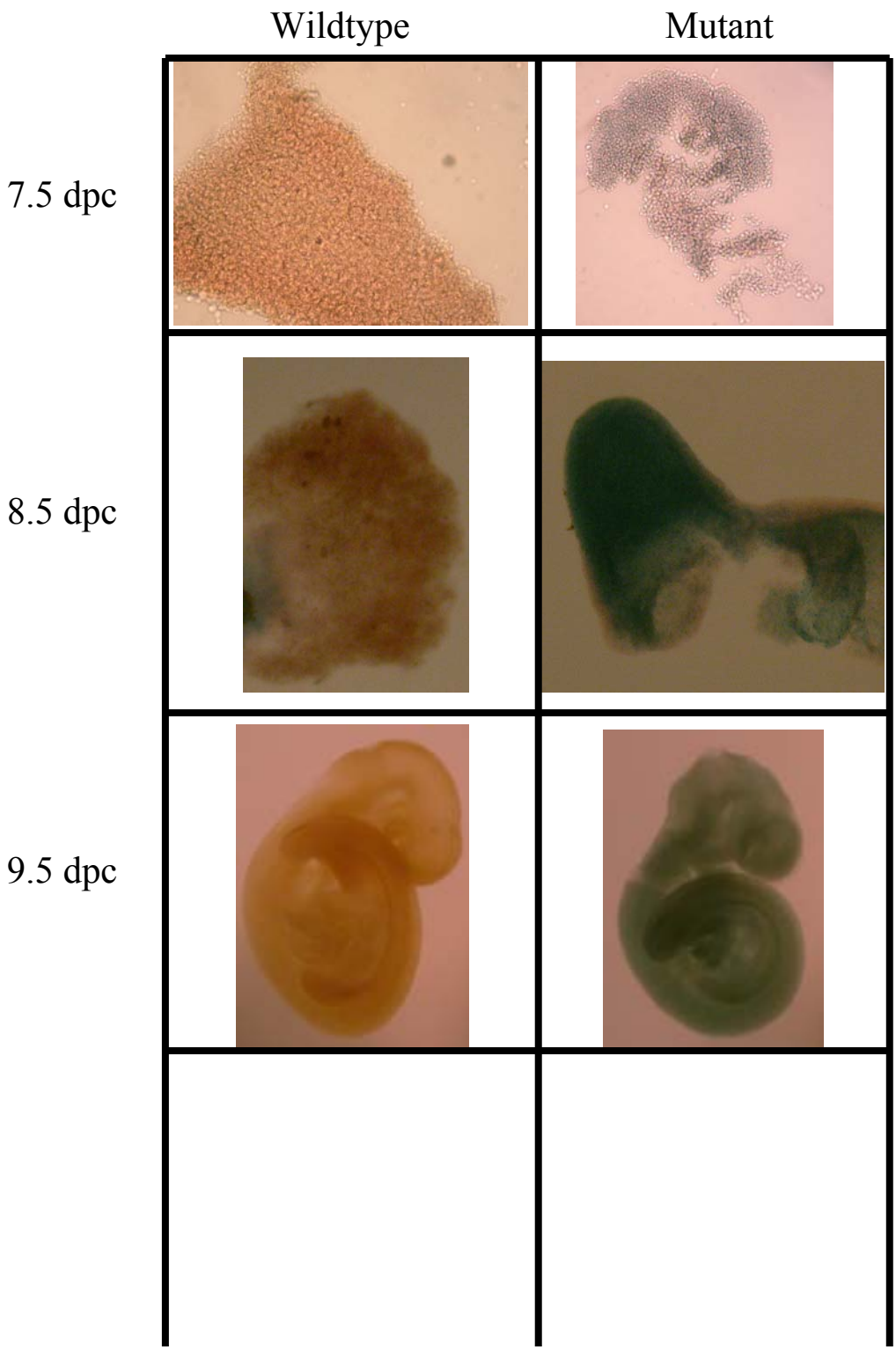
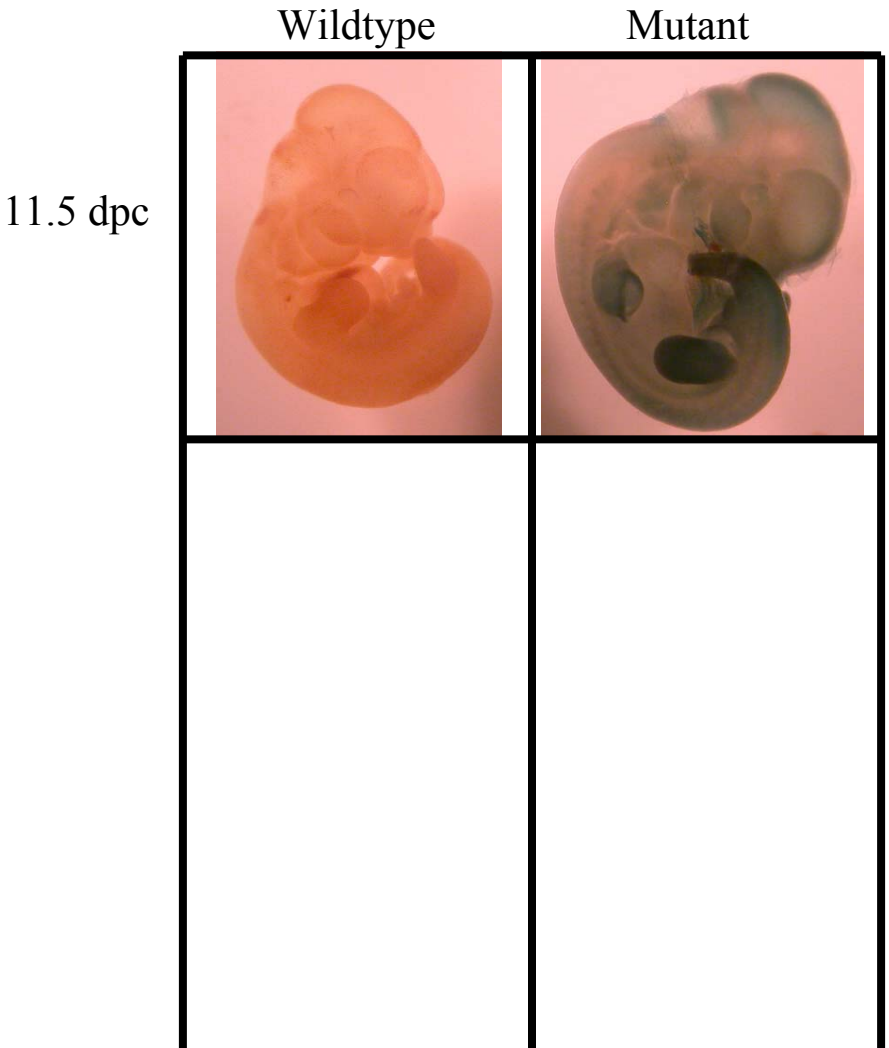

Wildtype
Mutant
7.5 dpc
8.5 dpc
9.5 dpc

Figure 24. Embryos of ages 7.5 dpc – 10.5 dpc were isolated and stained with β-gal stain to identify mutant (blue) and wildtype (un-stained) embryos. Stained mutant embryos allowed the study of Trailerhitch protein expression throughout the embryo. Trailerhitch appears to be expressed throughout the developing embryo.



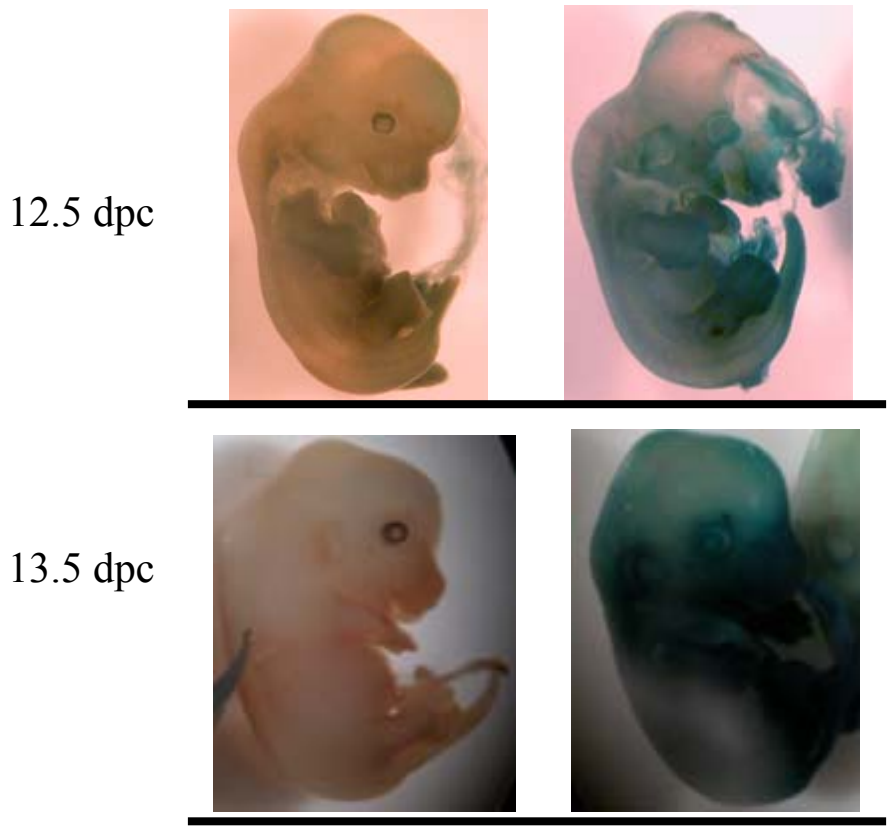


Figure 25. Embryos of ages 11.5 dpc – 13.5 dpc were isolated and stained with β-gal stain to identify mutant (blue) and wildtype (un-stained) embryos. Stained mutant embryos allowed the study of Trailerhitch protein expression throughout the embryo. Trailerhitch appears to be expressed throughout the developing embryo.

Homozygous Trailerhitch mutants may be embryonic lethal

Reproductive histories and genotyping data of Trailerhitch mutant mice were recorded thoroughly in order to determine if the knocked out *tral* gene had any effect on reproduction. After developing a proper method to genotype the mice that would distinguish between homozygous and heterozygous mutants, all collected DNA samples were genotyped. This included DNA from 24 litters of adult mice and 16 litters of embryonic mice. Of the 16 litters dissected, ages 7.5 dpc – 12.5 dpc were examined. The phenotype of the embryos was analyzed and development appeared to be occurring normally at these ages. Tissue was isolated for genotyping purposes, and with both adult and embryonic DNA, it was found that only heterozygous mutants existed. According to our genotyping, it appears that homozygous mutants are embryonic lethal prior to age 7.5 dpc.

To confirm the idea that homozygous mutants die at some point before birth, a chart was developed to analyze the genotyping data collected over a period of 20 months (Figure 26). This data seems to support the idea that homozygous mutants are embryonic lethal as the ratio of wildtype mice to mutant mice is approximately 1:2.4. We would expect a ratio of 1:2 if the homozygous mutants are embryonic lethal and a ratio of 1:3 if the homozygous mutants survived. However, upon analyzing the data with a Chi-Square test, it was revealed that this data is not statistically significant. Further data collection is necessary. However, in addition, the average number of mice per litter is significantly less for matings between heterozygous mutants. This supports the

idea that homozygous mutants are embryonic lethal, which results in smaller litter sizes.

Parent genotypes	**+/+ X +/-**	**+/- X +/-**
Total progeny +/+ genotype	**51**	**50**
Total progeny +/- genotype	**56**	**124**
Genotype ratio (+/+ : +/- and -/-)	**~ 1 : 1.1**	**~ 1 : 2.4**
Expected ratio (+/+ : +/- and -/-)	**1 : 1**	**1 : 3**
Total # of litters	**12**	**28**
Average # of mice per litter	**8.9**	**6.2**

Figure 26. This chart represents the genotyping data collected for Trailerhitch mice over the course of 20 months. Data from adult mice (24 litters) and sacrificed mice (16 litters) were included. Among the sacrificed litters, ages 7.5-12.5 dpc were studied, and none contained homozygous mutants. This data suggests that homozygous mutants are embryonic lethal prior to 7.5 dpc.

DISCUSSION

The overall goal of this project was to learn about the role of Trailerhitch in the mouse, and specifically in mouse germ cell development. Its role in other species such as *C. elegans* and *Drosophila* has been studied to some degree (Boag et al., 2005; Wilhelm et al., 2005; Wilhelm et al., 2000), but little was previously known about Trailerhitch's role in the mouse. Thus, our experiments were geared toward three main objectives. First, the location of the gene trap vector between exons 2 and 3 of the *trailer hitch* gene (*tral*) in the Trailerhitch knockout mice had to be confirmed. The exact base pair location of the gene trap vector also needed to be determined for further study of the mutant mice. Secondly, we aimed to study the expression patterns of Trailerhitch during development. This involved analyzing Trailerhitch expression in germ cells and in the entire embryo. Thirdly, we aimed to analyze the phenotype of the Trailerhitch mutants in order to determine the role of Trailerhitch in the mouse.

The gene trap vector was confirmed to be in its predicted position between exons two and three of the *tral* gene using RT-PCR. The 169 bp band that was sequenced from this experiment allowed us to be positive that the mice we were studying were mutants lacking a functional *tral* gene. However, this information was not specific enough for our study of Trailerhitch. The exact location of the gene trap vector in the *tral* gene needed to be determined for genotyping purposes. Inverse PCR was attempted for this reason, but failed to yield definitive results. Instead, a method called primer walking was attempted in

which primers were designed along the entire length of the *tral* intron between exons two and three. These primers were used in PCR reactions with primers in the gene trap vector in order to identify the approximate location of the gene trap vector. Specifically, primers 9A and IR4 amplified a ~500 bp band, which was sequenced. This sequence allowed us to determine the exact location of the gene trap vector, and thus to design primers that would allow us to accurately genotype mice. This is especially significant in distinguishing between heterozygous and homozygous mutant mice. Tools to distinguish between these mice have recently been made and are currently being employed for this purpose.

Genotyping data was gathered over the course of 20 months in order to determine a phenotype of the *tral* mutant mice. The genotype ratios were expected to differ between crosses of a wildtype mouse with a mouse carrying a mutant allele of *tral* and crosses of two mice carrying mutant alleles of *tral*, so the data for these crosses were examined separately. Genotypic ratios make a prediction about the phenotype of the *tral* homozygous mutants. The actual ratio of wildtype : heterozygous and homozygous mutants for wildtype/mutant crosses is ~1:1.1, which is very close to the expected 1:1 ratio. The actual ratio of wildtype : heterozygous and homozygous mutants for heterozygous mutant/mutant crosses is ~1:2.4, which is not very close to the expected 1:3 ratio. One explanation for the differing ratio is that homozygous mutants are not surviving to adulthood. By factoring in this hypothesis, the expected phenotypic ratio for heterozygous mutant/mutant crosses is 1:2. Although a Chi-Square test of our data indicated that it is presently not statistically significant to claim that

homozygous mutants are embryonic lethal, this still remains a possibility that a larger sample size may support. This hypothesis is also supported by the smaller average litter size of the heterozygous mutant/mutant crosses.

The expression of Trailerhitch in developing oocytes had previously been studied (Pepling et al., in preparation), but its expression in testes had not. The presence of the Trailerhitch protein in adult testes was confirmed by performing a Western blot using tissue samples isolated from adult males. The result indicated that each adult mouse studied had at least one normal copy of *tral*. This experiment further supports our hypothesis that homozygous *tral* mutants are not surviving to adulthood. The ß-geo allele should make a truncated version of the protein, and this epitope should also be recognized by the antibody. However, this larger protein, with exons 1 and 2 fused to ß-geo, was not observed.

In addition, the hypothesis that homozygous *tral* mutants are embryonic lethal is supported by our experiments with RT-PCR. After isolation of testes and ovaries from many different adult mice and performing RT-PCR, the cDNA samples were used in PCR reactions to amplify certain sequences that would identify wildtype or mutant DNA. Each DNA sample that amplified a band identifying mutant DNA also amplified a band identifying wildtype DNA, signifying that only heterozygous mutants existed as adults. From this data, it appears that homozygous mutants die at some point before birth.

To further study the expression of Trailerhitch in testes, indirect immunofluorescence was performed on isolated tissue from PND 4 and adult mice. The results show localization of Trailerhitch at PND 4 in the cytoplasm of

spermatagonia in developing seminiferous tubules. In adult testes, however, Trailerhitch expression is confined to a small, circular structure in the cytoplasm of spermatocytes, which has previously been identified as the chromatoid body (Pepling et al., in preparation). The chromatoid body has been speculated to be a specialized germ cell P body (Pepling et al., in preparation), and is similar to the Balbiani body. P bodies are involved in RNA metabolism, and specifically in RNA degradation (Cougot et al., 2004; Sheth and Parker, 2003; Wilczynska et al., 2005). The Balbiani body or mitochondrial cloud is made of a large, distinctive organelle aggregate. The aggregate consists of Golgi bodies surrounded by mitochondria and associated endoplasmic reticulum. It is found in developing oocytes of many species, including mice (Kloc et al., 2004). Trailerhitch has been found to be associated with the *Drosophila* Balbiani body, as well as with the mouse Balbiani body and chromatoid body (Pepling et al., in preparation). Human Trailerhitch has also been found to localize to P bodies (Yang et al., 2006). Thus, the association of Trailerhitch with P bodies signifies that it may be involved in RNA metabolism or a related cellular function.

The expression of Trailerhitch was further studied by examining its expression pattern in whole testes, ovaries, and developing embryos. This was done by staining these fixed tissues with X-gal, which is a substrate of β-galactosidase. Our examination of expression in whole PND 4 ovaries and testes did not reveal specific expression patterns in highly defined cell types, however, our analyses indicate that Trailerhitch is expressed broadly throughout the gonads. Similarly, results from staining embryos between ages 7.5 and 13.5 dpc allowed

us to conclude that Trailerhitch is widely expressed in the developing embryo. Trailerhitch appears more concentrated in the brain and spinal cord region of these embryos, as well as in the developing limbs. It is unknown whether this is a significant finding or if this is a result of uneven staining. However, the broad expression of Trailerhitch in the developing embryo allows us to conclude that in addition to its role in the development of germ cells, Trailerhitch probably serves a more general cellular function during development.

The results of X-gal staining embryos allowed us to determine Trailerhitch's general expression pattern, but did not allow us to determine exactly in which organ systems Trailerhitch is expressed. To do this, isolation and preparation of tissue from each organ system for a Western blot is necessary. Fortunately, a Mouse Tissue Protein Blot is available, which is a blot of denatured and electrophoresed protein lysates prepared from previously isolated tissues. In the near future, this blot will be used to analyze Trailerhitch protein expression in individual organ systems in order to learn more about the role(s) of Trailerhitch in the entire body.

The experiments we performed were geared towards understanding the role of Trailerhitch in mice, and the results allow us to make a number of conclusions. First, Trailerhitch is expressed in the developing germ cells of male and female mice. Its expression pattern is quite different during development and in adulthood. We can also conclude that our *tral* knockout mice have a gene trap vector interrupting the *tral* gene between exons 2 and 3, 101 bp upstream of exon 3. Thus, *tral* is in fact knocked out, and we can use the precise gene trap vector

information to help genotype and study the phenotype of heterozygous and homozygous knockout mice. Thirdly, the Trailerhitch protein is widely expressed throughout the embryo during development. X-gal staining of the β-galactosidase produced by the β-geo disruption allele has allowed us to study its broad expression in the embryo, and further study with the Mouse Tissue Protein Blot will give us a better understanding of Trailerhitch's expression in individual organ systems. Finally, according to our Western blot, RT-PCR, and genotyping data, we can conclude that homozygous mutants do not appear to survive to birth, suggesting that Trailerhitch plays an essential role in embryogenesis. To study the age of embryonic lethality, we have genotyped embryos from ages 7.5-12.5 dpc. During these ages, we have been unable to identify by PCR genotyping any homozygous mutants. Our genotyping method is currently being improved, and embryos from these ages will be re-genotyped to confirm this finding. Further study of embryonic lethality should involve embryos of ages 0.5-6.5 dpc, as this presently appears to be the most likely time period for embryonic death to occur due to lack of Trailerhitch function.

The results of our experiments allow us to draw many broad conclusions about Trailerhitch in mouse and to also speculate about its specific role in development. As it co-localizes with other proteins in *Drosophila* known to be crucial for mRNA localization and thus the polarity of the oocyte during development, it is plausible that mouse Trailerhitch serves a similar function. The fact that it is conserved across many species also suggests its importance in a conserved cellular process. Its association with the chromatoid body in

spermatocytes of adult mice and also with other specialized germ cell P bodies of other species suggests a role in RNA metabolism or a related cellular function. Also, because the homozygous mutant mice we are studying appear to die before birth, Trailerhitch likely plays a critical role in embryogenesis, such as in RNA metabolism. This role likely begins prior to age 7.5 dpc. Further research must be performed to better understand Trailerhitch's specific role in the developing embryo and in the adult mouse.

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