Exploring the molecular mechanisms of *Drosophila* dTRIM32 implicated in pathogenesis of Limb-Girdle Muscular Dystrophy 2H

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Abstract

The E3 ubiquitin ligase TRIM32 is a member of tripartite motif (TRIM) family of proteins involved in various processes including differentiation, cell growth, muscle regeneration and cancer. TRIM32 is conserved between vertebrates (humans, mouse) and invertebrates (*Drosophila*). The N-terminus of this protein is characterized by a RING domain, B-box domain, and Coiled-Coil region, while the C-terminus contains six NHL repeats. In humans, mutations that cluster in the NHL domains of TRIM32 result in the muscle disorders Limb-Girdle Muscular Dystrophy type 2H (LGMD2H) and Sarcotubular Myopathy (STM). Mutations in the B-box region cause Bardet-Biedl Syndrome (BBS), a clinically separate disorder that affects multiple parts of the body. A comprehensive genetic analysis in vertebrate models is complicated by the ubiquitous expression of TRIM32 and neurogenic defects in *TRIM32-/-* mutant mice that are independent of the muscle pathology associated with LGMD2H. The model organism *Drosophila melanogaster* possesses a TRIM32 [dTRIM32/Thin (Tn)/Abba] homolog highly expressed in muscle tissue. We previously showed that dTRIM32 is localized to Z-disk of the sarcomere and is required for myofibril stability. Muscles form correctly in *Drosophila tn* mutants, but exhibit a degenerative muscle phenotype once contraction ensues. Mutant or RNAi knockdown larvae are also defective in locomotion, which mimics clinical features associated with loss of TRIM32 in LGMD2H patients. It is predicted that mutations in the NHL domain either affect protein structure or are involved in protein-protein interactions. However, the molecular mechanism by which these mutations affect the interaction properties of dTRIM32 is not understood. Biochemical pulldown assays using the bait fusion protein GST-dTRIM32-NHL identified numerous dTRIM32 binding proteins in larval muscle tissue. Many key glycolytic enzymes were present in the dTRIM32 pulldowns and not in control experiments. Glycolytic

genes are expressed in the developing *Drosophila* musculature and are required for myoblast fusion. Strikingly, many glycolytic proteins are also found at the Z-disk, consistent with dTRIM32 localization. Our biochemical and genetic studies provide evidence that there is direct interaction between dTRIM32 and glycolytic proteins (Aldolase and PGLYM). dTRIM32 also regulates glycolytic enzyme levels and protein localization at their sites of action. These data together suggest a role for dTRIM32 in coordinating glycolytic enzyme function, possibly for localized ATP production or to maintain muscle mass via glycolytic intermediates.

Table of Contents

List of Figures

List of Tables

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Dedication

To

My Parents

&

My Grandparents

Chapter 1 - Introduction

Muscular Dystrophies are a heterogeneous group of inherited diseases characterized by progressive weakness and degeneration of the voluntary muscles. However, quite often other muscles are also affected, including the heart and respiratory muscles. Onset of disease varies from childhood, adolescence or even later after the age of 30 years. Common symptoms include muscle weakness, lack of coordination and progressive crippling. Mutations in genes involved in dystrophies play important role in muscle maintenance, function and homoeostasis. Key features of dystrophic muscles include central nucleation, fiber splitting, Z-line streaming and myofibrillar disorganization (Figure 1.1). MDs comprise over 30 different inherited disorders which can be X-linked recessive, autosomal dominant or autosomal recessive. At present, no treatment is available to stop or reverse any form of muscular dystrophy (MD). Certain medication and therapies can improve the quality of life of the patients. Some of these are drug therapy, physical therapy and corrective surgery.

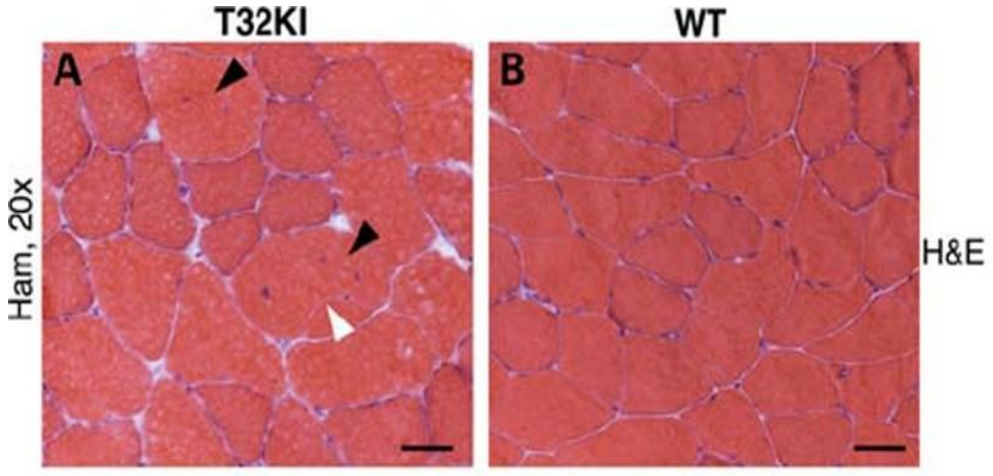

Figure 1.1 Myopathic muscle histology
Myopathic features in TRIM32KI mice similar to that of patients with LGMD2H dystrophy. Wild type (B), shows nucleus arranged nicely at the periphery of myofibers. However, in TRIM32KI mice muscle (A), black arrowhead represents central nucleation and white arrowhead indicates fiber splitting. Reprinted with permission from (KUDRYASHOVA et al. 2011).

1.1 Classification of Muscular Dystrophies

MDs are classified into nine specific types depending on the clinical and pathological conditions. Table 1.1 shows the different types of MDs and their common features.

Table 1.1 Types of MDs and their common features

S.No	Muscular Dystrophies	Clinical Features
1	Becker Muscular Dystrophy	Severe form of DMD, caused by partially functional dystrophin.
2	Congenital Muscular Dystrophy	Molecularly heterogenous, symptoms include several muscle weaknesses, joint deformities.
3	Duchenne Muscular Dystrophy	X-linked inherited, caused by absence of dystrophin, results in progressive weakness of skeletal and cardiac muscles.
4	Distal Muscular Dystrophy	Onset 20 to 60 years, symptoms include wasting and weakness of muscles of hands, arms and lower legs.
5	Emery-Dreifuss Muscular Dystrophy	X-linked inherited disorder, normally occurs in children, muscle weakness and wasting in distal limbs.
6	Facioscapulohumeral Muscular Dystrophy	Autosomal recessive inheritance affects muscles of face, shoulders, upper arms.
7	Limb-Girdle Muscular Dystrophy	Inheritance-Autosomal recessive and dominant. Primarily affects the pelvic and shoulder muscles. Age of onset: childhood to adulthood.
8	Myotonic Muscular Dystrophy	Autosomal dominant MD. Affects different body parts other than skeletal muscles like heart, endocrine organs.
9	Oculopharyngeal Muscular Dystrophy	Affects muscles of eyelids, throat, patients have problem in swallowing and breathing. Onset is late between 40 to 70 years.

Since, muscular dystrophies are genetically inherited, they can be dominant or recessive and cause progressive degeneration and weakness of the voluntary muscles. Figure 1.2 represents the different muscles affected in MDs.

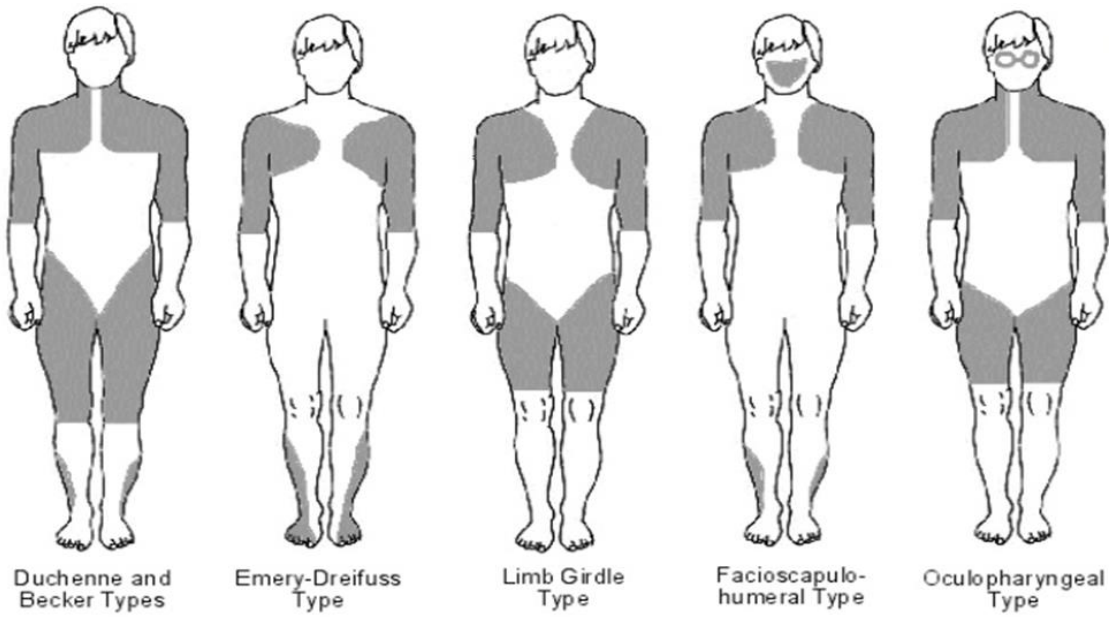

Figure 1.2 Main areas of muscle weakness in different types of Muscular Dystrophies
Figure represents the different areas in body affected in MDs (highlighted grey). In Limb-Gridle Muscular Dystrophy, primarily pelvic and shoulder muscles are affected (https://patient.info/health/muscular-dystrophies-an-overview).

1.2 Limb-Girdle Muscular Dystrophy

Limb-Girdle Muscular Dystrophy (LGMD) refers to a heterogenous group of inherited muscular disorders characterized by progressive weakness and degeneration of the skeletal muscle primarily affecting pelvic and shoulder musculature. Some LGMD subtypes also have additional symptoms like abnormal cardiac and respiratory function. The onset of disease varies. It can begin in childhood, adolescence or later in adult life. Since LGMD is an inherited autosomal disorder, it is sub-categorized as autosomal dominant LGMD type 1 (1A-H) or autosomal recessive LGMD type 2 (2A-Y).

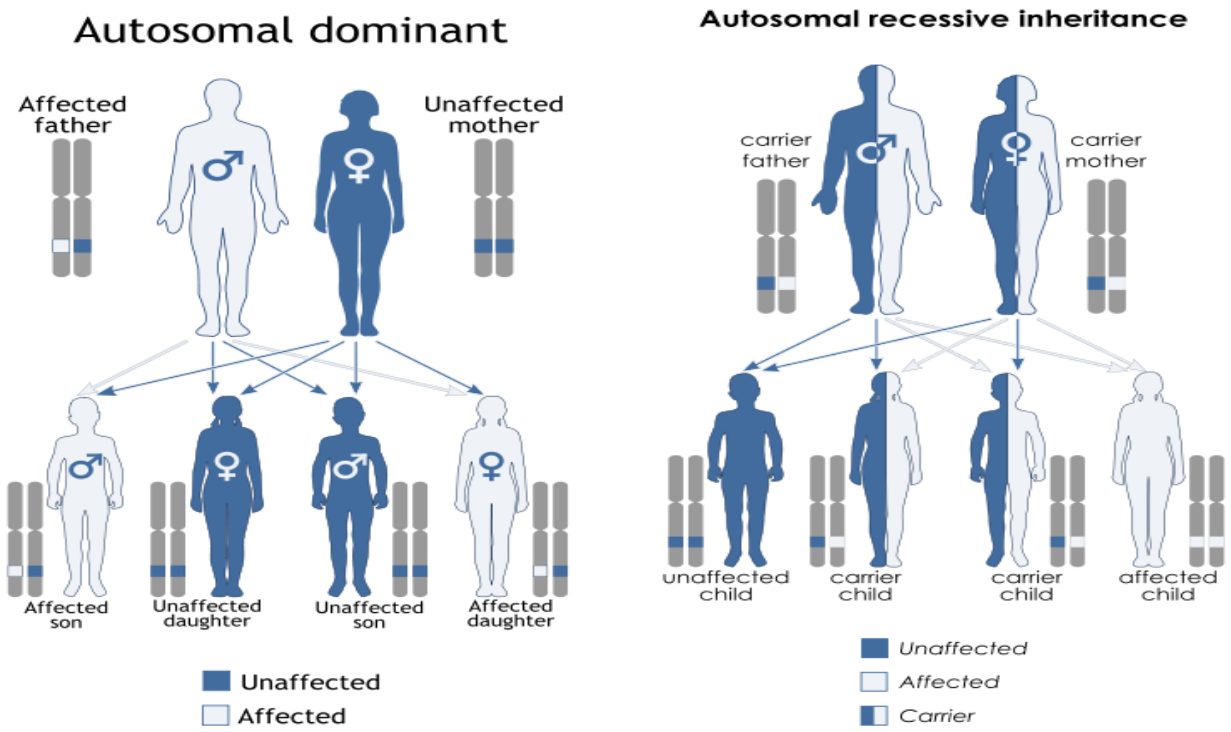

Figure 1.3 LGMD categorized as type 1 and type 2 depending on the inheritance pattern
LGMD type 1 (1A-H) are categorized as autosomal dominant and LGMD type 2 (2A-Y) are autosomal recessive (*https://en.wikipedia.org/wiki/Dominance_(genetics)).*

The autosomal dominant form of LGMD type 1 starts typically in adulthood. Type 1 can result from mutations in gene such as myotilin, which is a Z-disk associated protein (HAUSER *et al.* 2000). Mutation in gene encoding Lamin A/C results in LGMD1B, also known as laminopathies, that cause proximal leg weakness and cardiomyopathies (NIGRO and SAVARESE 2014; TURK *et al.* 2013)

The autosomal recessive LGMD type 2 (2A-2H) occurs when both copies of a gene are mutated. All the forms of LGMD type 2 show different clinical features. LGMD2A is caused by mutation in gene encoding Calpain protein (CAPN3), which is a muscle specific protein (JIA *et al.* 2001). LGMD (2C-F) are caused by the loss of function mutations in the sarcoglycan complex of skeletal muscle. LGMD2H is caused by mutations in TRIM32 (FROSK *et al.* 2002). LGMD (2I-P) together are called dystroglycanopathies and are caused by mutations in six

different genes that differ in severity and disease onset. Each type of LGMD (2I-P) shows different clinical conditions like brain and eye abnormalities, cardiomyopathies and respiratory disorder (NIGRO and SAVARESE 2014)

1.3 Limb-Girdle Muscular Dystrophy Type 2H

LGMD2H is a recessive autosomal muscular disorder which was first studied in the Hutterite population in central Canada and the Dakotas of the USA. The onset of the disease is slow and usually begins during the 2^{nd} - 3^{rd} decade of life (SHOKEIR and KOBRINSKY 1976; WEILER *et al.* 1998). The condition results in progressive weakness of the pelvic and shoulder muscles with differences in the fiber size, central nucleation, Z-line streaming and high levels of creatinine kinase. Comparison of sequences from affected patients and haplotype analysis from the Human Genome Project lead to the identification of a candidate region for pathogenic mutations that identified TRIM32 as the gene affected in LGMD type 2H. (FROSK *et al.* 2002)

1.3.1 TRIM32 Protein

Human TRIM32, a 72kDa protein, is a member of tripartite motif family. It is characterized by the presence of an N-terminal three domain module that includes a RING domain, one or two B-box domains, a Coiled-Coil region, while its C-terminal portion contains 6 NHL repeats (REYMOND *et al.* 2001) (Figure 1.4). TRIM32 is ubiquitously expressed and is localized to the Z-disk of the skeletal muscle (NICKLAS *et al.* 2012). TRIM32 is an E3 ubiquitin ligase because of the presence of the N-terminal RING domain. The B-box and Coiled-Coil region typically mediate the formation of high order complexes. The C-terminal NHL repeats are known to have protein-protein interaction properties (MERONI and DIEZ-ROUX 2005). The NHL repeats were identified in *C. elegans,* NCL-1 (B-box type zinc finger protein), LIN-41 (Abnormal cell lineage protein 41) and human protein HT2A (Zinc finger protein). The NCL-1

protein is involved in regulating the size of the nucleolus. LIN-41 promotes cell division of seam cells and HT2A protein is a transcription factor that mediates the transcription of HIV protein (SLACK and RUVKUN 1998)

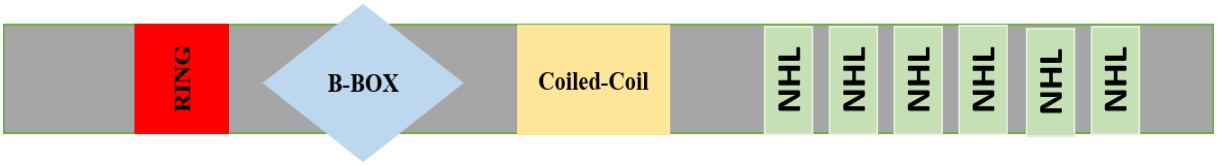

Figure 1.4 Schematic representation of TRIM32 protein
Red: ring domain, Blue: B-Box, Yellow: Coiled-coil, Green: NHL repeats.

Consistent with its ubiquitous expression, TRIM32 protein is involved in various biological processes, including cell growth, differentiation, immunity and development (LAZZARI and MERONI 2016). The E3 function of TRIM32 is known to ubiquitinate muscle related proteins such as tropomyosin and actin (KUDRYASHOVA *et al.* 2011). TRIM32 is also known to regulate levels of p53 in tumor cells and cells undergoing stress. (Figure 1.5). (LIU *et al.* 2013)

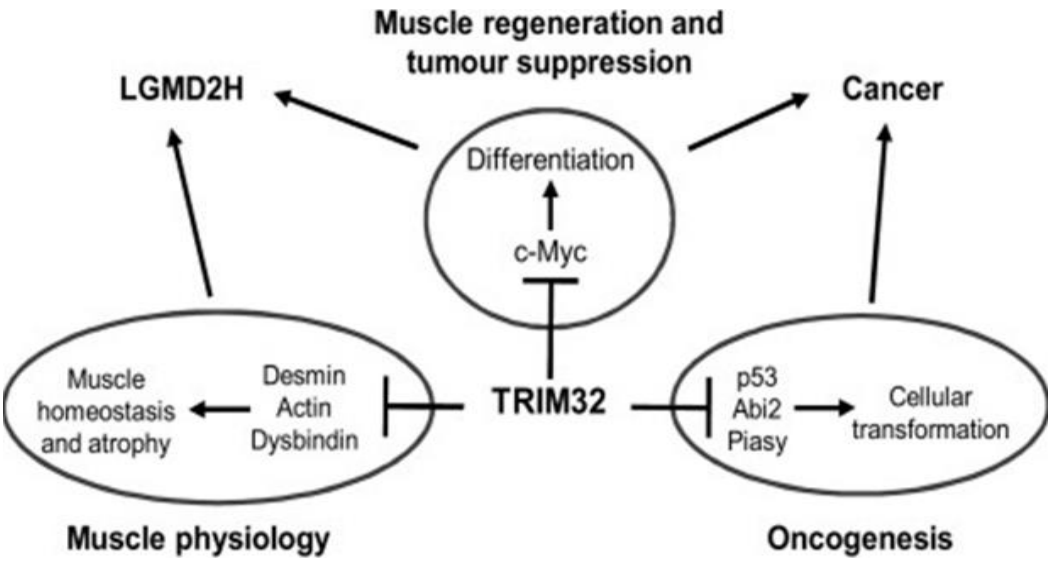

Figure 1.5 Role of TRIM32 in muscle physiology, regeneration and cancer
Myc is involved in the cell proliferation, satellite cell differentiation and regeneration of damaged muscle. TRIM32 dependent degradation of myc could lead to progressive muscle loss and degradation of the tumor suppressor substrates by TRIM32 could lead to cancer. TRIM32

dependent ubiquitination of muscle protein maintains muscle homeostasis and plays a critical role in pathogenesis of LGMD2H. Reprinted with permission from (LAZZARI and MERONI 2016).

1.3.2 Mutations in TRIM32 Results in Two Diverse Diseases

The six C-terminal NHL repeats in TRIM32 are predicted to be involved in protein-protein interaction. A homozygous mutation in third NHL repeat (D487N) causes LGMD2H in a Manitoba Hutterite population (FROSK *et al.* 2002). A new homozygous mutation (P130S) in the B-box region of TRIM32 protein was revealed by SNP microarray genotyping analysis in Israeli family that resulted in Bardet Biedl Syndrome type 11 (BBS11) (CHIANG *et al.* 2006; COSSÉE *et al.* 2009). Three novel mutations (p. R394H, p.T520TfsX13 and p.D588del) were identified in the non-Hutterite population in the first, fourth and fifth NHL repeats of TRIM32 gene (Figure 1.6).

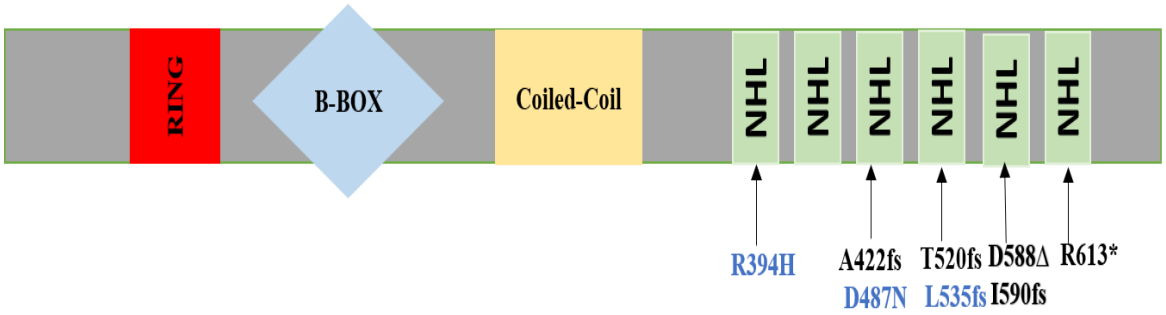

Figure 1.6 Mutations in TRIM32-NHL domain
Arrows indicate the mutations in the first, third, fourth and fifth NHL repeat of TRIM32 protein that caused LGMD2H in Hutterite and Non-Hutterite population. Red: ring domain, Blue: B-Box, Yellow: Coiled-coil, Green: NHL repeats.

TRIM32 protein has the ability to self-associate, however the protein loses this ability when the D487N mutation is present in the NHL domain. The other mutations also show weaker interactions. This suggests that the mutations in the NHL repeats could abolish the self-interaction property of TRIM32 protein and normal function. (SACCONE *et al.* 2008)

1.4 Models Used to Study LGMD2H

The model organisms used to study LGMD2H are explained in the following sections.

1.4.1 TRIM32 Knockout (KO) and Knockin (KI) Mouse Model

Human and murine TRIM32 are highly conserved, they share 95% identity at the protein level (KUDRYASHOVA *et al.* 2009). To study the pathogenesis of LGMD2H, genetically modified mice lacking the *TRIM32* gene were generated. *TRIM32KO* mice muscle histology analysis revealed myopathic phenotypes such as fiber splitting and mislocalized nuclei (Figure 1.7). Further, transmission electron microscopy (TEM) analysis revealed Z-line streaming, loss of sarcomeric patterning followed by myofibrillar degeneration and a dilated sarcotubular system (Figure 1.8) (KUDRYASHOVA *et al.* 2009). The myopathy in *TRIM32KO* mice replicates the phenotypes of LGMD2H and STM human patients. However, *TRIM32KO* also show neurogenic defects with decreased neurofilaments in the brain and reduced axon diameter. No reports of patients suffering with LGMD2H have shown signs of neurological problems.

Further studies were conducted to understand the role of TRIM32 protein in muscle, based on information provided by the predicted structure of conserved NHL domain. TRIM32 KI mouse carrying D487N mutation was generated. The analysis of the muscles revealed mild dystrophic changes in muscle with centrally located nuclei, fiber splitting. The *TRIM32KI* mice also mimic the LGMD2H phenotype with mild myopathic and neurologic components (KUDRYASHOVA *et al.* 2011). A comprehensive genetic analysis in vertebrate models is complicated by the ubiquitous expression of TRIM32 and neurogenic defects in *TRIM32-/-* mutant mice that are independent of the muscle pathology associated with LGMD2H.

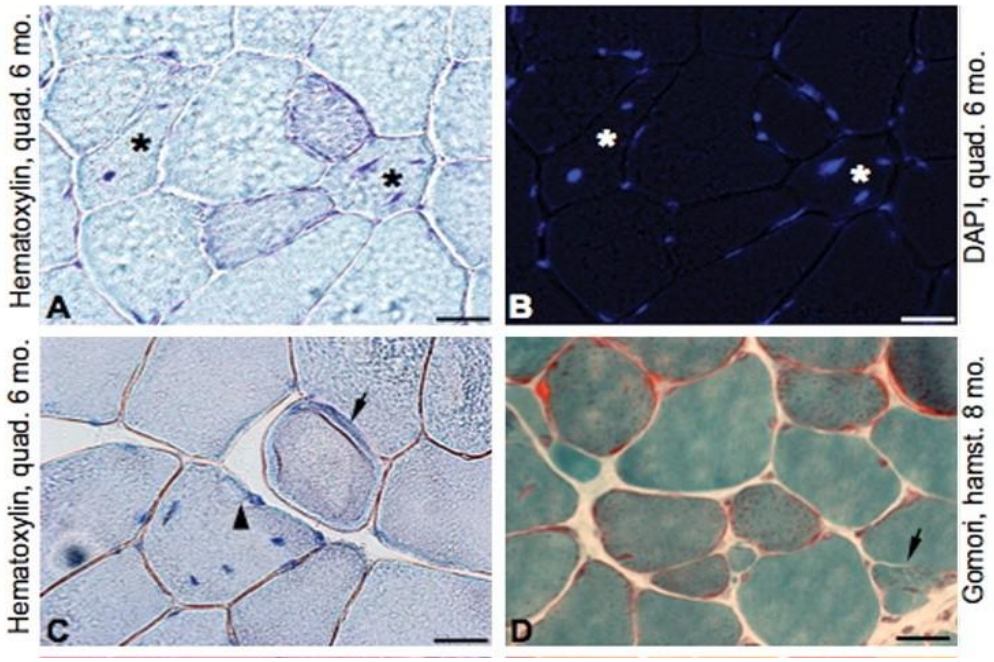

Figure 1.7 Histology of *TRIM32KO* muscles

(A and B) Hematoxylin, DAPI stained internalized nuclei marked by asterisk. (C and D) Hematoxylin, Gomori stained muscles shows fiber splitting indicated using arrow head. Adapted from Reprinted with permission from (KUDRYASHOVA et al. 2011)

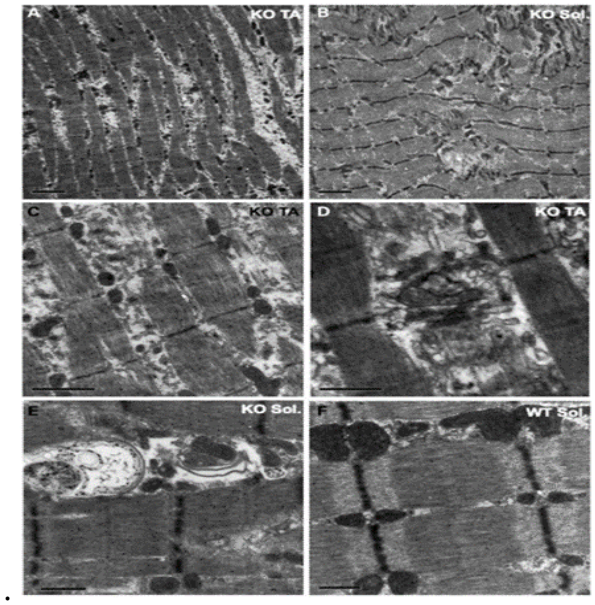

Figure 1.8 Transmission electron microscopy of *TRIM32KO* skeletal muscles

(A and B) KO TA, Sol muscles shows increased myofibrillar space and Z-line streaming. (C, D and E) KO TA, Sol muscles shows dilated sarcotubular system and autophagic vacuoles. (F) WT muscle depicting normal sarcomeric organization in skeletal muscle. Reprinted with permission from (KUDRYASHOVA et al. 2011)

1.4.2 *Drosophila Melanogaster dTRIM32-/-*

The model organism *Drosophila melanogaster* possesses a TRIM32 (dTRIM32) homolog which is highly expressed in muscle. The *CG15105* locus encodes for the dTRIM32/Abba/Tn protein. dTRIM32 protein is 132kDa and shares structural similarity to human TRIM32 proteins. Both contain the N-terminal Ring domain, B-box and Coiled-Coil region followed by C-terminal NHL repeats. Thin protein is homologous to human TRIM32 and shares overall 34% homology. (Figure 1.9)

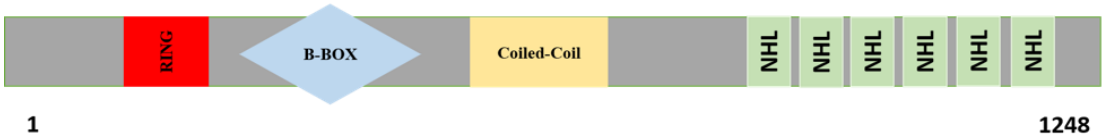

Figure 1.9 Schematic of dTRIM32 protein with functional domains
Protein size 1248aa, Red: RING finger domain, Blue: B-box, Yellow: CC region, Green: NHL repeats.

tn was identified as the uncharacterized muscle degenerative mutant in *Drosophila* (BALL *et al.* 1985). The mutation was mapped to a gene called *CG15105* and is also called *abba* (another B-box affiliated family). *tn* mutants exhibit thin, elongated pupae and L3 larvae shows thinner, looser muscles with irregular sarcomeric patterning (Figure 1.10). Muscles are formed properly during embryogenesis in *tn* mutants and progressively degenerates as the embryo transitions to the larval stage (LABEAU-DIMENNA *et al.* 2012). Thin is expressed in both larval and adult muscles. Immunolocalization studies revealed that Thin protein is localized to Z-disk and M-line of the sarcomere as it colocalizes with various sarcomeric proteins (MLP84B, kettin and zormin) (DOMSCH *et al.* 2013) (Figures 1.11 and 1.12). The costameres in *Drosophila* provide structural stability and support to the muscles during contraction and relaxation (LABEAU-DIMENNA *et al.* 2012). The costameres are compromised in *tn* mutants exhibiting loose myofibrils, thus, suggesting the role of Thin protein in myofibrillar stability.

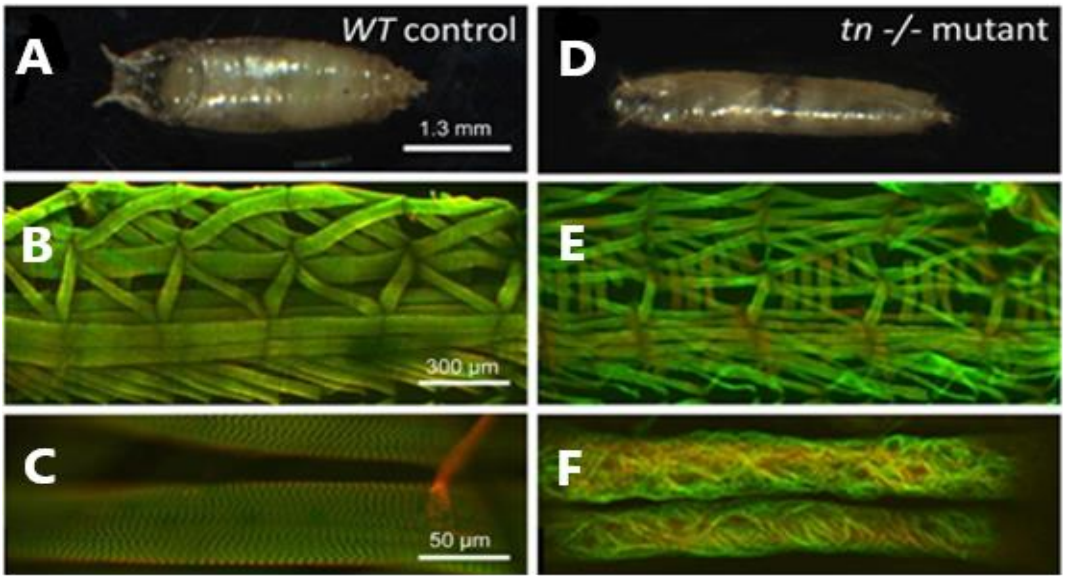

Figure 1.10 A decrease in dTRIM32 results in muscle degeneration
(A) Pupal case and normal sarcomere patterning in WT L3 larval muscles (C and E). (B) Defective muscle contraction in tn-/- mutants leads to an elongated pupal case and thinner, dystrophic muscles (D and F) Reprinted with permission from (LABEAU-DIMENNA et al. 2012).

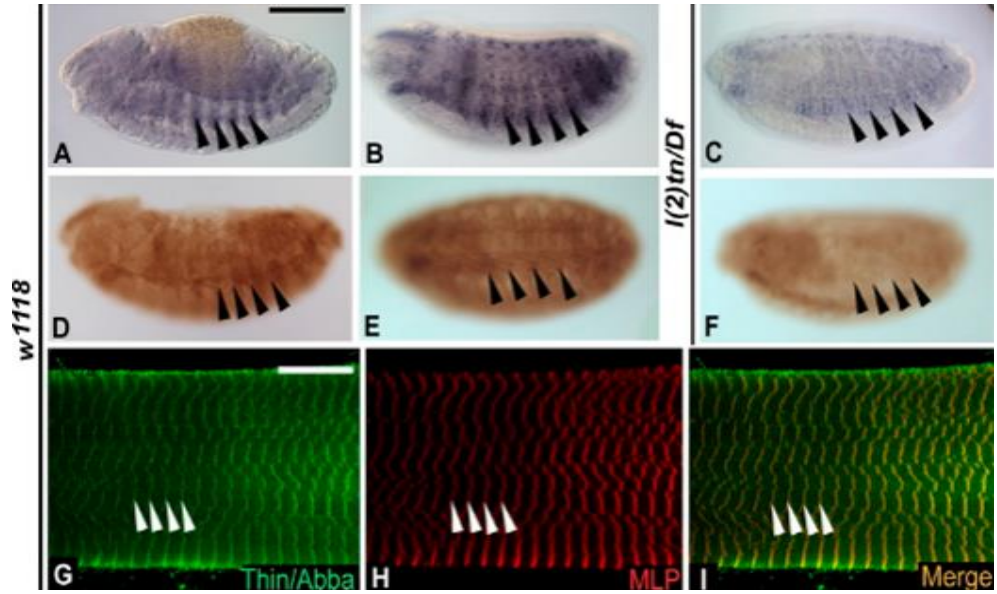

Figure 1.11 *thin* is expressed in developing and mature muscles in *Drosophila*
(A, B, D and E) Shows the expression of Thin *protein in WT embryo. (G) WT preparation shows that Thin is present in L3 larval muscle (green) in a repeated pattern within the sarcomeres. (H) Co-staining the muscle with MLP84 which is a Z-disk protein, suggests Thin is also found at the Z-disk of the sarcomere. Reprinted with permission from (LABEAU-DIMENNA et al. 2012).*

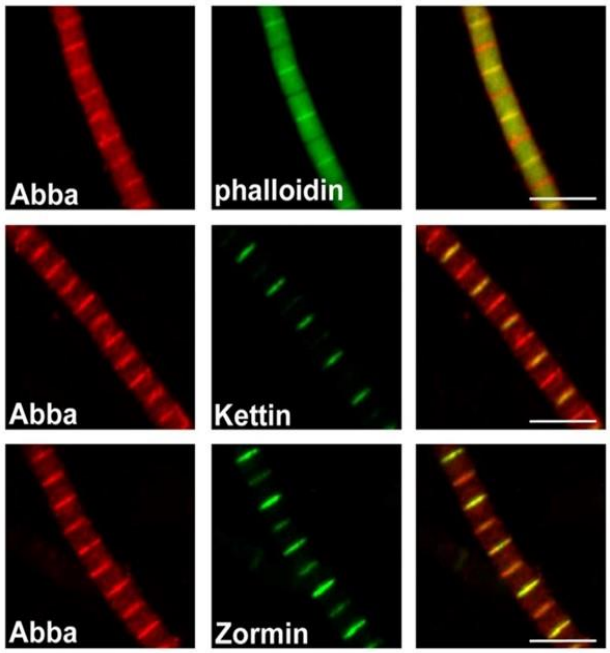

Figure 1.12 Abba is detected at Z-disk and M-lines in adult myofibrils
*Myofibrils dissected from adult thoracic muscles and double-labelled for Abba and F-actin (phalloidin), Kettin (at Z-disk) or Zormin (at Z-disk and M-lines). Abba is prominent at both Z-disk and M-Lines. Reprinted with permission from (*DOMSCH *et al. 2013).*

1.4.2.1 Structural and Functional Importance of the NHL Domains in TRIM32 Protein

The software predicted model of 6 NHL repeats is a six-bladed β-propeller based upon the *Drosophila* Brain tumor protein (BRAT) three-dimensional structure. BRAT is a member of conserved NHL family proteins and act as a translational repressor. Each blade consists of four highly twisted antiparallel β-sheets. To analyze the structural change due to NHL mutations, the TRIM32-NHL WT structure was compared to the mutated TRIM32-NHL domains. The 1st and 3rd NHL repeat with p.R394H or p.D487N mutation results in a change in the shape of the NHL domain. However, p.D588del significantly alters the structure (SACCONE *et al.* 2008). The predicted changes in the structure suggest that these mutations could abolish the self-association

and protein-protein interaction property of TRIM32 protein (EDWARDS *et al.* 2003) (Figure 1.13).

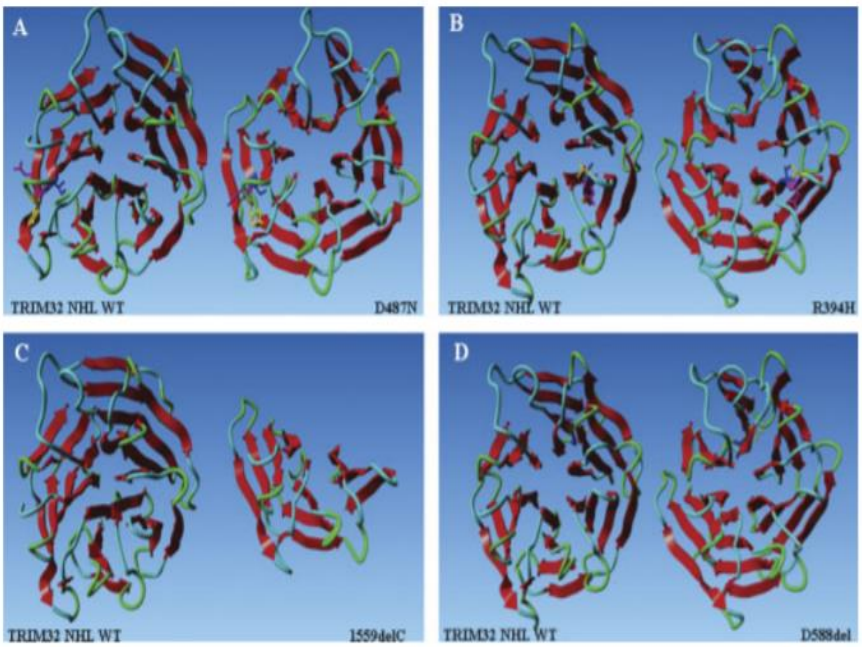

Figure 1.13 Predicted WT TRIM32-NHL structure compared to TRIM32 p.D487N (A); p.R394H (B); c.1559delC (C) and p.D588del alleles (D)
The mutations alter the structure significantly as compared to WT TRIM32-NHL. Reprinted with permission from (SACCONE et al. 2008)

In *Drosophila*, UAS/GAL4 system allows us to modulate the temporal and spatial expression of the gene of interest in tissue of choice (Figure 1.14). GAL4 is a transcription factor and binds upstream of UAS and drives the expression of the gene in specific tissue. Domsch, et al., used this UAS/GAL4 system to study the importance of NHL domain of dTRIM32 in muscle. UAS-based *abba/tn* constructs generated encoding full length protein and lacking NHL domain to carry out the rescue experiments. These experiments implicated the requirement of the NHL domain for proper functioning of the Thin protein. The construct lacking NHL domain was not able to provide the rescue of myofiber unbundling compared to the construct encoding the full-length protein (DOMSCH *et al.* 2013) (Figure 1.15).

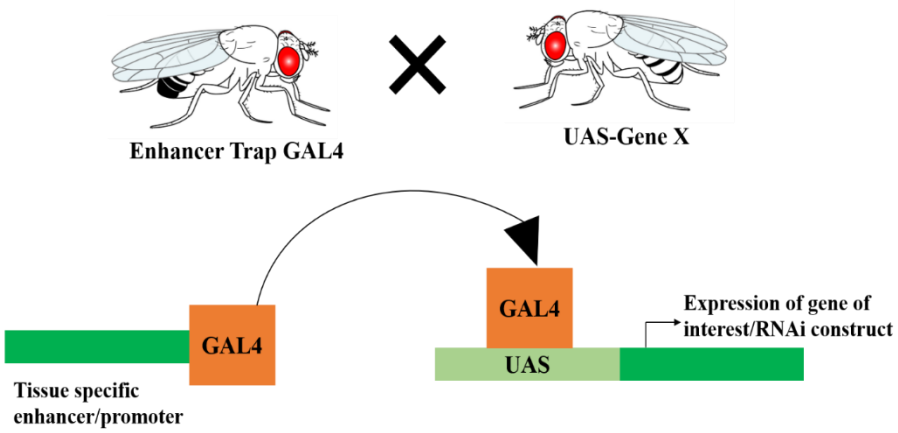

Figure 1.14 UAS/GAL4 system work flow

The transgene (gene of interest) is activated by crossing to the flies that express GAL4 (enhancer trap GAL4). GAL4 binds upstream to the promotor region and drives the expression of the transgene in the tissue of interest

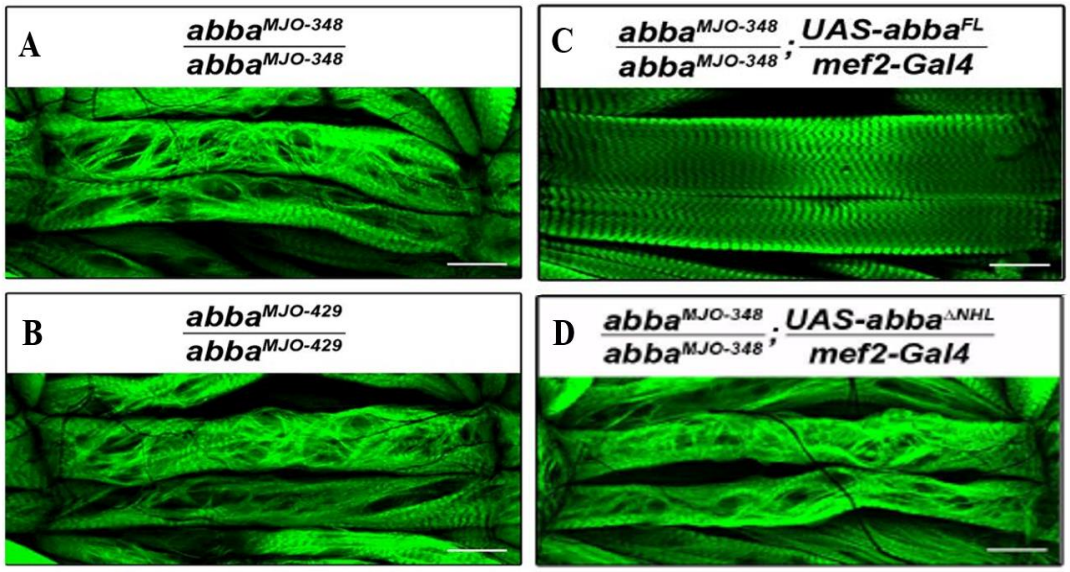

Figure 1.15 Abba/Thin requires NHL repeats for proper function

(A and B) Mutant lacking NHL repeats show striation defects. All third instar larvae stained with phalloidin to visualize muscles. (C) UAS-construct encoding full length provides full rescue of the mutant phenotype. However, UAS-Abba construct lacking NHL repeats unable to rescue the defects. Reprinted with permission from (DOMSCH et al. 2013)

1.5 Role of Glycolytic Proteins in *Drosophila* Muscle Development

The role of metabolic genes in muscle development and function has not been elucidated.

A better understanding is required to comprehend the role of some of the genes that are involved

in metabolic myopathies. Mutations in *Phosphofructokinase* (pfk) and *Phosphoglycerate mutase 2* (*pgam2*) affects the body's ability to break down sugar and results in myopathy marked by muscle cramps, exercise intolerance which supports the role of glycolysis in normal muscle function (BERARDO *et al.* 2010; RABEN and SHERMAN 1995; NAINI *et al.* 2009). Glycolysis provides a source of energy for proliferating and differentiating cells. The role of glycolytic enzymes in muscle development has been studied using *Drosophila* as a model organism. Gene expression based profiling in *Drosophila* reveals genes that encodes for protein involved in metabolic pathway including glycolytic genes. Embryonic studies implicated the activation of glycolytic genes during muscle development (Figure 1.16). Further knockdown of one of the glycolytic enzyme *PGLYM78* results in *thin* muscle phenotype (TIXIER *et al.* 2013) (Figure 1.17).

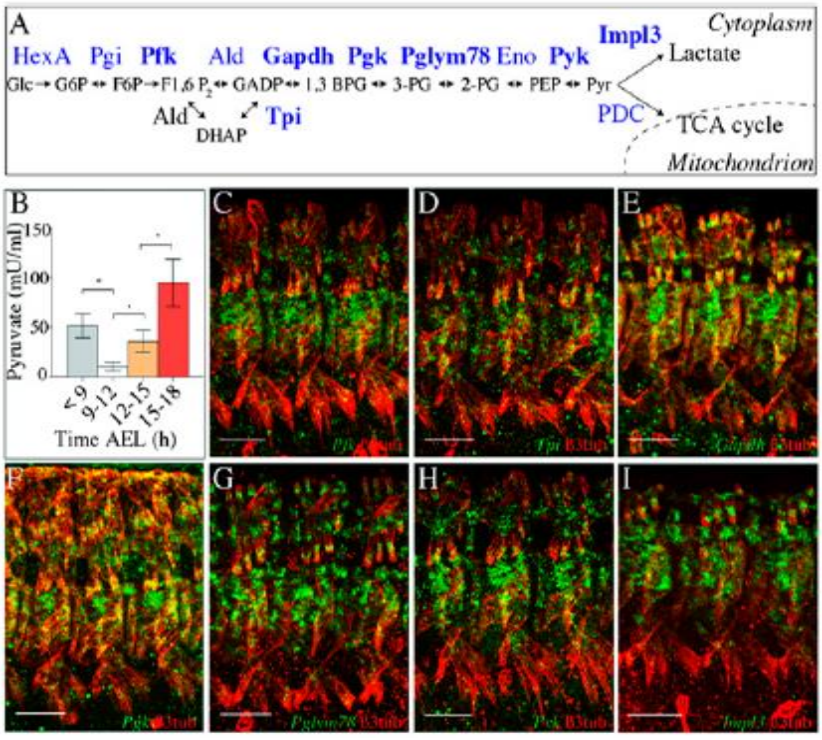

Figure 1.16 Expression of glycolytic genes in developing muscles
(A) Schematic of glycolytic pathway. (B) Pyk activity during embryogenesis. (C-I) Expression profile of glycolytic genes (green) in developing embryos (stage 15) (red) β-3 tubulin to reveal muscles. Reprinted with permission from (TIXIER et al. 2013).

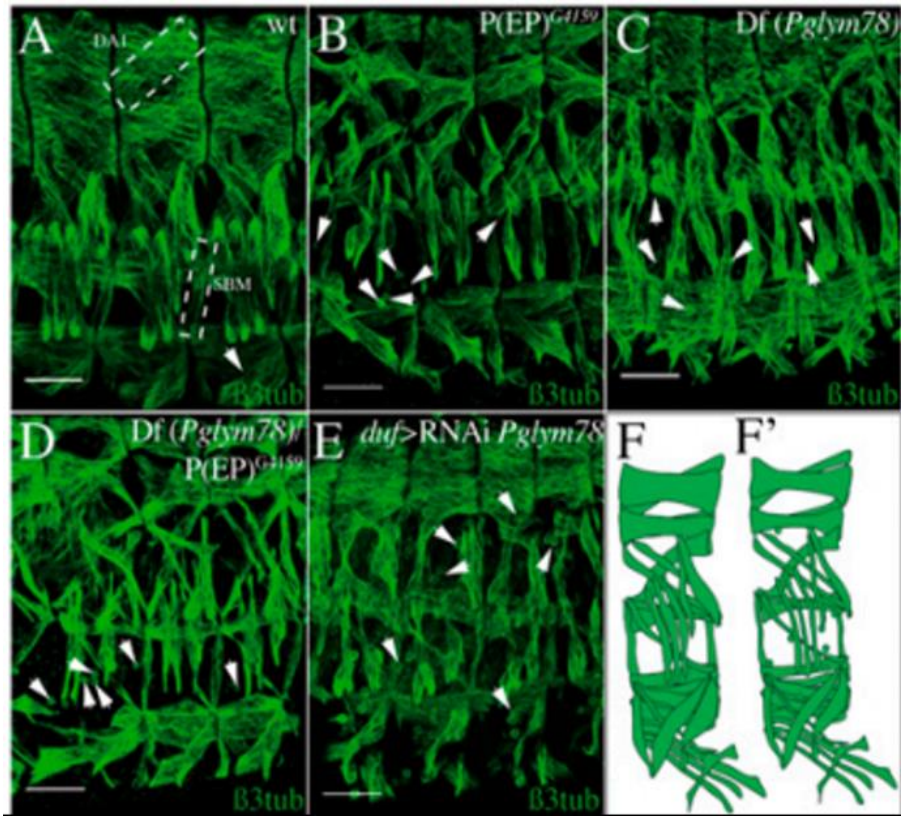

Figure 1.17 Loss of *PGLYM* results in thinner muscles and myoblast fusion defects
Muscle pattern in wild type (A), *in P(EP)G4159 (B), in Df(Pglym78) (C) and in trans heterozygous Df(Pglym78)/P(EP)G4159 (D) mutant embryos. (E) Muscle phenotype observed upon RNAi knockdown of Pglym78. Reprinted with permission from (*TIXIER *et al. 2013)*

The localization studies performed in *Drosophila* flight muscle indicates that the glycolytic enzymes (GAPDH, PGK, PGLYM and Aldolase) are co-localized to the Z-disk and M-line of the sarcomere and are required for normal flight function (SULLIVAN *et al.* 2003) (Figure 1.18). Mutants null for GPDH affected the localization of other enzymes. This suggests that these enzymes are interdependent. The flight ability is affected in the flies when these enzymes are not co-localized. During mid-embryogenesis there is upregulation of glycolysis to support the growth of the animal throughout the larval stage (TENNESSEN *et al.* 2014). From the embryonic to the end of the larval stages, there is about 200-fold increase in the size of the animal. There is constant need for synthesis of lipids, amino acids and nucleotides to support the

rapid growth of the animal. Aerobic glycolysis provides precursors for biomass accumulation for rapid growing cells (TENNESSEN *et al.* 2011). The other function of glycolysis in muscle is to provide ATP to myosin ATPase for contraction. However, the role of glycolysis in co-localization and muscle contraction is not clear.

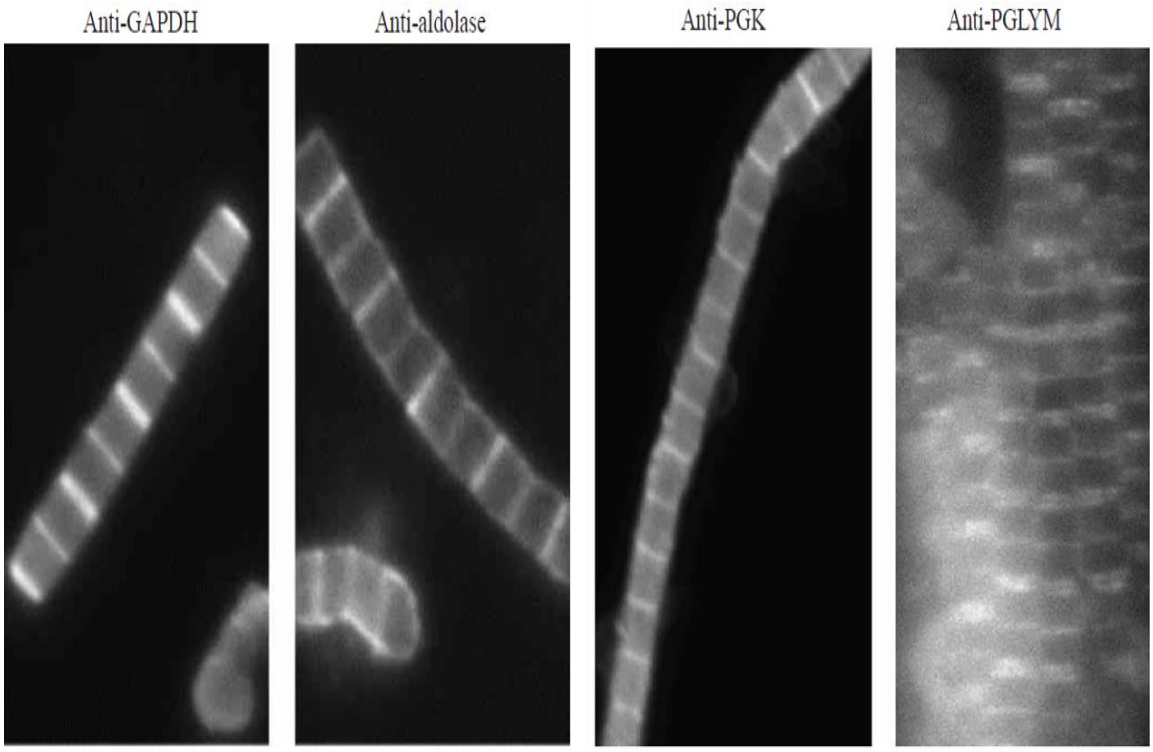

Figure 1.18 Localization of glycolytic enzymes in the indirect flight muscle of *Drosophila*
GAPDH, Aldolase, PGK and PGLYM antibody stained adult myofibrils. Reprinted with permission from (SULLIVAN et al. 2003).

Chapter 2 - Material and Methods

2.1 *Drosophila* Stocks

Drosophila fly stocks were cultured on standard cornmeal medium at 25°C. To test the genetic interactions using the UAS/GAL4 system, crosses were maintained at 28.5°C and 29°C. The following stocks were obtained from Bloomington Stock Center: y, w (BL-1495); *mef2*-gal4 (BL-27390); UAS-*PGK* RNAi (BL-28053); UAS-*PGLYM* RNAi (BL-41603) and UAS-*GAPDH* RNAi (BL-62212). The following stock was obtained from Vienna Drosophila Resource Center: UAS-*tn* RNAi (v19291). The *mef2* > UAS-*tn* RNAi stock was generated in lab using standard genetic crosses.

2.2 Protein Expression and Purification

2.2.1 Protein Cloning, Expression and Purification of C-Terminal dTRIM32-NHL Domain

Previous studies have shown that mutation in the first (R394H), third (D487N), fourth (T520TfsX13) and fifth (D588del) NHL repeats of TRIM32 results in LGMD 2H. The C-terminal NHL region of TRIM32 was amplified by PCR. The size of the amplified dTRIM32-NHL domain was 828bp as expected (Figure 2.1). The amplified C-terminal TRIM32-NHL domain was sub cloned into Sal1 (right primer) and Not1 (left primer) sites present in the protein expression vector PGEX-5X-2 that contain an N-terminal glutathione transferase tag (Appendix A). The plasmid containing the DNA fragment was transformed into *E. coli* DH5α cells and the correct clones were further selected and verified by sequencing.

Primers used :-

LEFT PRIMER - GGG ATC CCC GGA ATT CCC CTG CGC AAG CGC CAG CAG CTG TTC

RIGHT PRIMER - GTC CAC ATG GAC GCG TTC GCG GTC CGC CGG CGT AAG AAT A

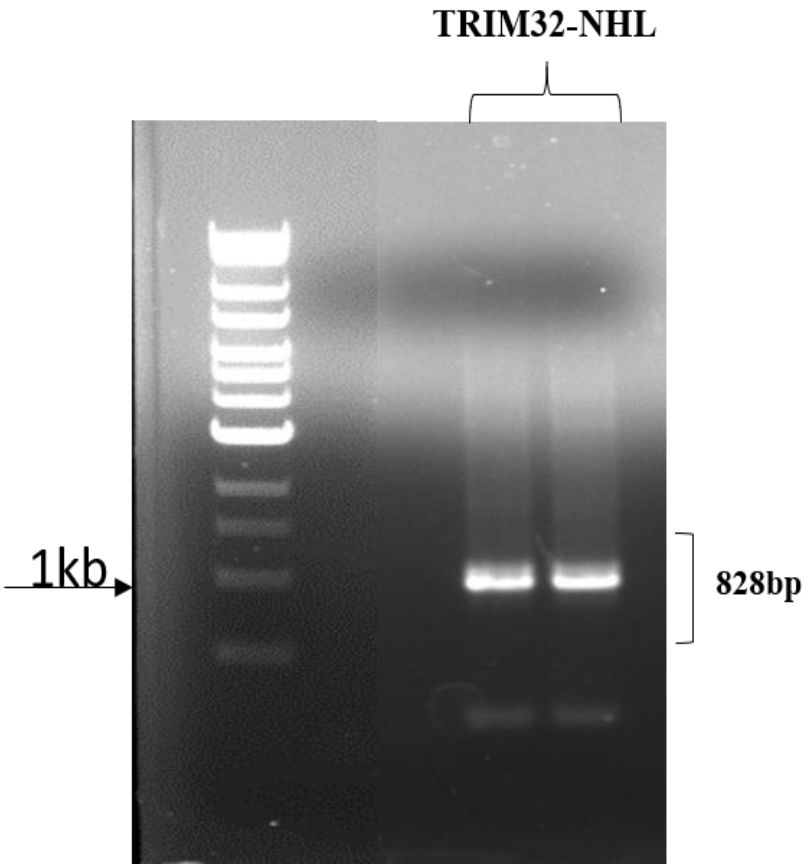

Figure 2.1 PCR amplified dTRIM32 NHL domain

2.2.2 Expression and Solubility Test of C-Terminal TRIM32-NHL Protein

The sequenced verified dTRIM32-NHL domain was cloned into the protein expression vector PGEX-5X-2 and was transformed in BL21 cells and selected on ampicillin resistant plates. A single colony was picked and inoculated into LB media. Once the OD600 of the culture reached between 0.6 - 1.0, a 1000µL aliquot was taken out to run on SDS page and the remaining culture was induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG). The cells were allowed to grow for 3 hours at 37°C and 16-18 hours at 18°C. After incubation at 37°C and 18°C, 1000µL aliquots were taken for SDS gel analysis. The before and after induction samples

were loaded on SDS gel for analysis. Expression of protein in the induced samples (37°C and 18°C) is shown in Figure 2.2. The expected size of the protein is 56 kDa.

2.2.3 Large Scale Protein Expression and Purification

After small scale protein expression, GST-dTRIM32-NHL was present in the soluble fraction. Thus, large scale expression of protein was carried out. After 18 hours of incubation at 18°C, cells were centrifuged and stored at -80°C for further processing. The cells were lysed using micro fluidizer cell lysis machine. The lysed cells were then spun at high speed to get a clear supernatant. The supernatant was loaded onto GST affinity column with glutathione beads. The eluted protein was further purified using gel filtration and Ion-Exchange Chromatography. Different elutions were analyzed using SDS page. The samples of interest were pooled together and concentrated to the final volume of 2mL using Amicon centrifuge columns. The final concentration of the protein calculated using nanodrop was 0.6mg/mL. The purified protein was stored at -80°C for future experiments.

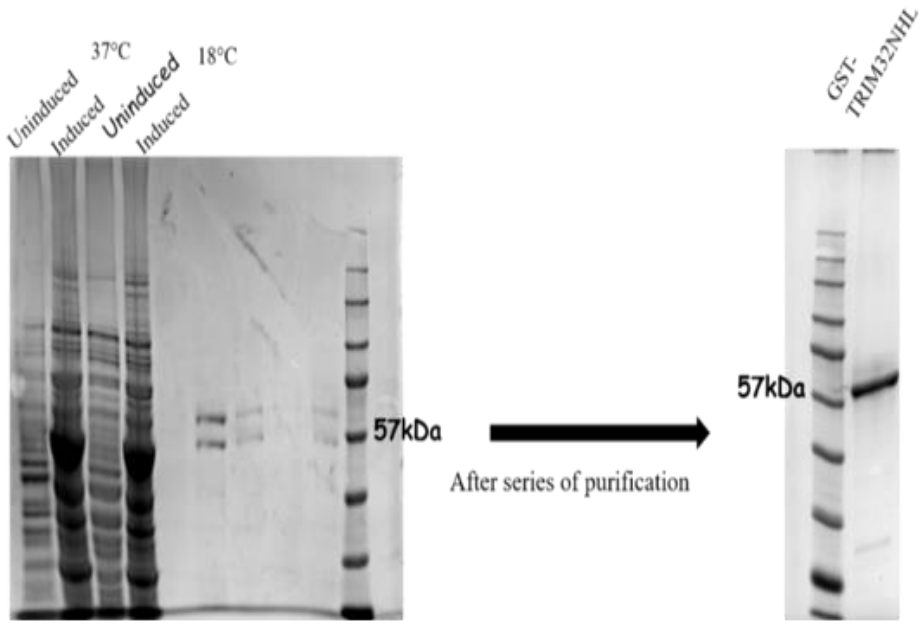

Figure 2.2 SDS PAGE image of purified GST-TRIM32-NHL protein

2.2.4 Protein Cloning, Expression and Purification of Thin Filament Protein (Tropomyosin) and Glycolytic Proteins

Tropomyosin and the glycolytic proteins Aldolase and PGLYM were amplified by PCR. The size of the amplified Tropomyosin, Aldolase and PGLYM (Figure 2.3). The PCR amplified products were sub cloned into protein expression vector pT7HMT using restriction sites mentioned below for each protein (GEISBRECHT *et al.*). For large scale expression and purification of Tropomyosin, Aldolase and PGLYM proteins, procedure similar to purification of dTRIM32-NHL protein was followed (Figure 2.3).

List of primer sequences:-

PGLYM - Sal5'-F Not3'-R

LEFT - CAGGGGTCGACAATGGGCGGCAAGTACAAGATC

RIGHT - AAGGAAAAAAGCGGCCGCTTACTTGGCCTTGCCCTGGGC

ALDOLASE: BamH5'-F Not3'-R

LEFT - ACAGGATCCATGACGACCTACTTCAACTACC

RIGHT - AAGGAAAAAAGCGGCCGCTCAATACCTGTGGTCATCCAC

TROPOMYOSIN: BamH5'-F Not3'-R

LEFT - ACAGGATCCATGGACGCCATCAAGAAGAAG

RIGHT - AAGGAAAAAAGCGGCCGCTTAGTAGCCAGCCAATTCGGC

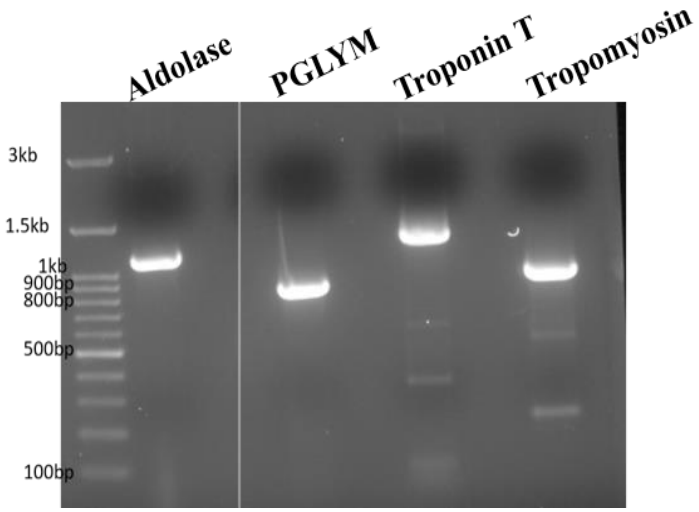

Figure 2.3 PCR amplified Aldolase, PGLYM, Troponin T and Tropomyosin

Table 2.1 Product size of amplified products

S.NO	PCR Product	Product Size
1	Aldolase	1095bp
2	PGLYM	815bp
3	TROPONIN T	1035bp
4	TROPOMYOSIN	900bp

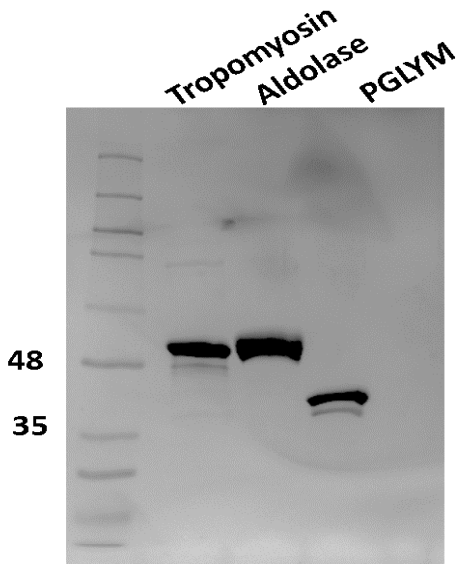

Figure 2.4 SDS page image of affinity purified thin filament (Tropomyosin) and glycolytic proteins (Aldolase and PGLYM)

2.3 Western Blots

L3 larvae were homogenized in 3X SDS sample buffer (188mM Tris-HCl pH 6.8, 3% SDS, 30% glycerol, 0.01% bromophenol-blue and 15% β-mercaptoethanol). The homogenized larvae were heat boiled at 95°C for 10 minutes and centrifuged at 15000 xg for 1 minute. To analyze overall protein levels, lysate was run on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to polyvinyl difluoride (PVDF) membranes (Pierce Biotechnology, Inc.). The membrane was then probed with following primary antibodies: rabbit -PGLYM, rabbit-Aldolase, rabbit-PGK, rabbit-GAPDH (1:1000, a kind gift from Sullivan DT) and mouse anti-ATPase 5α (1:10 000, Mitosciences). The membrane was then incubated with Horseradish Peroxidase (HRP) conjugated secondary antibodies (1:3000–1:5000, GE Healthcare). Protein detection was carried out using the ECL Plus western blotting detection system (Pierce).

2.4 Immunostaining and Confocal Imaging

L3 larvae were filleted and fixed using 4% formaldehyde for 20 minutes at room temperature. The fillets were washed 3 times with PBST and immunostained using following primary antibodies: rabbit-PGLYM, rabbit-PGK, rabbit-GAPDH, rabbit-Aldolase (1:1000, a kind gift from Sullivan DT) and phalloidin (1:400, Molecular probes). Secondary antibodies used were Alexa Flour 488 and Alexa Flour 568 (1:400, Molecular Probes). Immunostained larval preparations were imaged using Zies 700 confocal microscope. The images were analyzed using the Adobe Photoshop and Zen software.

2.5 Statistics

To quantitate western blots, densitometry analysis was performed using ImageJ. The data were normalized to ATPase 5α which served as a loading control. For western blot analysis, the

raw data was imported to Graph Pad Prism 6 and bar graphs were generated. The error bars represent mean ± S.E.M. To determine the statistical significance either t-tests, Mann-Whitney tests or one-way ANOVA followed by a Bonferroni post-hoc analysis was used. Differences were considered significant if the p value was <0.05.

2.6 Preparation of Larval Lysate for Pulldown Experiments

The wild type flies were cultivated in large fly cages to collect 3rd instar larvae. The container consisted of fly food with yeast extract. After four days, the 3rd instar larvae were separated from the food plate and washed with NaCl and 1% triton (wash buffer). After three consecutive washes, larvae were transferred to homogenizer. Larvae were homogenized in lysate buffer (50mM Tris HCL pH 7.5, 100mM NaCl, 10% glycerol and 1mM EDTA) and inhibitors (1mM Na3VO4, 5mM NPPS, 2mM PMSF, 2ug/ml Leupeptin, 10uM MG132, 1x Halt Pro inhibitor cocktail). The larval lysate was then stored at -80°C for further experimentation.

2.7 Coupling of GST-dTRIM32-NHL Protein to Cyanogen Bromide Activated Sepharose Beads for Pulldown Assay

The beads (0.06gm) were washed with 10mL of 1mM HCL followed by a single wash of 0.1M sodium phosphate buffer (pH6.5 - 7.5). The resin was spun down immediately after the washes. GST-dTRIM32-NHL protein was incubated with activated CNBr beads overnight shaking on a platform at 4°C. Next day, beads were washed with 0.4M monoethanolamine to block the reactive sites. To carry out the pulldown assay, GST-dTRIM32-NHL bound to CNBr beads (Experiment) and CNBr beads alone were incubated with 100mg of larval lysate for 4 hours at 4°C on a rocking platform. After incubation, beads were washed with wash buffer (50mM Tris HCL pH 7.5, 100mM NaCl, 10% glycerol, 1mM EDTA and 1% Triton).

2.8 Binding Assay to Assess Protein-Protein Interaction

2.8.1 Protein-Protein Interaction Between dTRIM32 and Tropomyosin

Full length Tropomyosin 42kDa and the C-terminal dTRIM32-NHL 37kDa protein were purified using affinity chromatography. To assess the interaction of the Tropomyosin with dTRIM32-NHL protein, His pulldown assays were performed by binding dTRIM32-NHL and His protein to Ni-NTA beads (Figure 3.4). First dTRIM32-NHL protein (experiment) or His-SCIN protein (control) were incubated with Ni-NTA magnetic beads for 1 hour and 30 minutes. The unbound protein was then washed off using wash buffer (PBS, 75mM imidazole). Next, Tropomyosin was incubated with dTRIM32-NHL and His protein bound to beads for 2 hours and 30 minutes at 4°C on shaking platform. Then washes were performed (3 x5 minutes each) to remove non-specific binding. The bound proteins were eluted by boiling at 100°C in 6X Laemmli buffer for 10 minutes. The binding was analyzed on SDS PAGE.

2.8.2 Protein-Protein Between dTRIM32 and Aldolase

To validate the interaction between Aldolase and dTRIM32-NHL protein, Ni-NTA pulldown assay was carried out by binding Aldolase and His protein to Ni-NTA beads (Figure 3.4). The experiment began by immobilizing Aldolase and His protein to Ni-NTA magnetic beads for 1 hour and 30 minutes. The unbound protein was then washed off using wash buffer (PBS buffer). dTRIM32-NHL was then incubated with Aldolase and His-SCIN protein bound to beads for 30 minutes at 4°C on shaking platform. Then, the nonspecific binding was removed by performing washes (6 x5 minutes each with 500mM NaCl, PBS, 1% Triton). The bound proteins were eluted by boiling at 100°C in 6X Laemmli buffer for 10 minutes. The binding was analyzed on SDS PAGE. It was observed that Aldolase binds only to dTRIM32-NHL protein.

2.8.3 Protein-Protein Interaction Between dTRIM32 and PGLYM

To assess the interaction of PGLYM with dTRIM32-NHL protein, His pulldown assays were performed by binding PGLYM and His protein to Ni-NTA beads (Figure 3.4). First, His tagged PGLYM protein (experiment) or His-SCIN protein (control) were incubated with Ni-NTA magnetic beads for 1 hour and 30 minutes. The unbound protein was then washed off using wash buffer (PBS buffer). Next, dTRIM32-NHL was incubated with PGLYM and His protein bound to beads for 1 hour and 30 minutes at 4°C on shaking platform. Then washes were performed (7 x5 minutes each with 500mM NaCl, PBS, 1% Triton) to remove non-specific binding. The bound proteins were eluted by boiling at 100°C in 6X Laemmli buffer for 10 minutes. The binding was analyzed on SDS PAGE. PGLYM binds only to dTRIM32-NHL protein.

Chapter 3 - Results and Discussions

3.1 Determination of Importance of dTRIM32-NHL Domain in Proper Functioning of dTRIM32 Protein

The C-terminal NHL domain of the TRIM32 protein is conserved among different species and is known to play role in protein-protein interaction and protein dimerization. Mutations in the NHL repeats results in LGMD2H. Therefore, to understand the molecular mechanism behind these diseases, the first part of this thesis focuses on identifying novel protein partners of dTRIM32-NHL protein using proteomics approach.

3.1.1 To Identify Biochemical Interactors of dTRIM32-NHL to Determine its Mechanism of Function Using Proteomics Approach

A large number of genes have been identified that are involved in various muscle wasting and neuromuscular disorders using proteomics approaches. It is of utmost importance to figure out the underlying cellular and molecular mechanisms of muscle atrophy and dystrophy.

3.1.2 Pulldown Assay Using CNBr Bound GST-dTRIM32-NHL Protein and Larval Lysate to Identify Interacting Proteins

Pulldown assays are an invaluable technique used to identify physical interactions between one or more proteins. It is the most commonly used method for initial screening of novel proteins and their unknown function. The pulldown assay was carried out by incubating GST-dTRIM32-NHL bound to CNBr beads (Experiment) and CNBr beads alone (Figure 3.1). The experiment was done in triplicate and the beads were snap frozen using liquid nitrogen. Apart from this, one set of experiment was run on SDS PAGE (Figure 3.2). The beads and SDS PAGE were sent off to Oklahoma State University for mass spectrometry analysis.

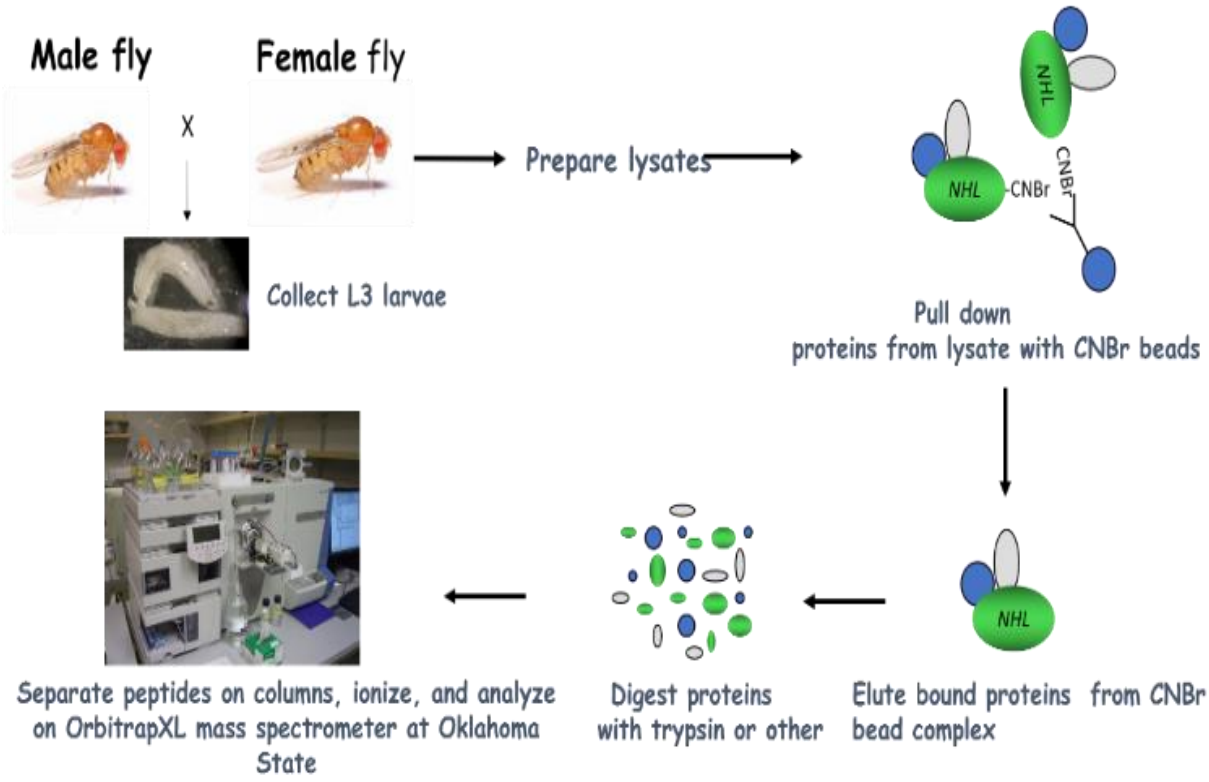

Figure 3.1 Schematic representation of the pulldown assay

Larval lysate incubated with beads only and GST-TRIM32-NHL protein coupled to CNBr beads. Eluted protein was sent to Oklahoma Mass Spec facility for further analysis.

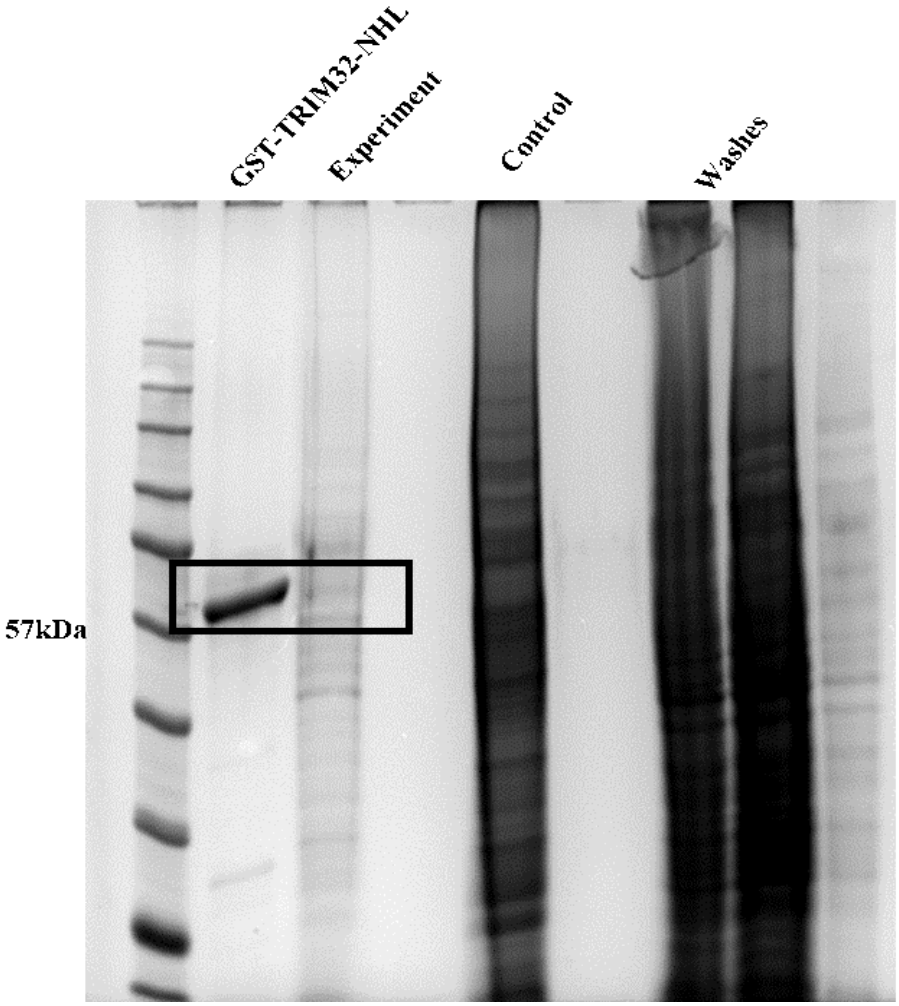

Figure 3.2 SDS-PAGE of CNBr pulldown assay
GST-TRIM32-NHL protein bound to CNBr beads incubated with the larval lysate (experiment). CNBr beads incubated with lysate (control). The experiment lane shows distinct bands as compared to control with recovery of the bait protein.

3.1.3 Analysis of Mass Spectrometry Data Using Scaffold Software

The pulldown assays were performed in triplicate. All three scaffold experiment files contain the raw peptide identification data were combined into one file which resulted in a total of 185 identified proteins. Next, the proteins with a cut-off peptide probability greater than 95% lowered the list to 157 proteins. Statistical significance was determined by ANOVA followed by a Bonferroni post-hoc analysis. Out of 157 proteins, 86 proteins with significant difference in the

p value ($p<0.05$) were finalized for further analysis. The samples that were higher in controls or equal between control and experiment (40 proteins) were removed followed by a required unique peptide match for each protein ≥ 3. The mass spec data analysis resulted in 37 proteins which were considered as significant hit. Table 3.1 shows 17 of the shortlisted proteins.

Table 3.1 Mass Spec data analyzed using Scaffold Software

	Experiment #1		**Experiment #2**		**Experiment #3**		ANOVA test
	Control beads	Trim32-NHL	Control beads	Trim32-NHL	Control beads	Trim32-NHL	(P-value)
Thin (tn), isoform C	**0.0%**	**7.3%**	**0.0%**	**14.9%**	**0.0%**	**9.2%**	**<0.0001**
Tropomyosin 1 (TM1), isoform E	1.8%	15.3%	10.4%	23.6%	9.6%	25.0%	<0.0001
Tropomyosin 2 (TM2), isoform E	14.0%	34.0%	35.0%	62.6%	29.6%	62.3%	<0.0001
Toponin I/Wings up A (wupA), isoform J	3.6%	14.6%	0.0%	28.0%	8.0%	27.6%	<0.0001
Toponin T/Upheld (up), isoform O	0.0%	22.3%	12.6%	27.6%	14.4%	34.6%	<0.0001
Troponin C (TpnC73F), isoform 3	0.0%	24.7%	23.0%	58.7%	7.6%	52.0%	<0.0001
Troponin C (TpnC47D), isoform 2	21.0%	21.0%	20.3%	48.3%	15.0%	62.6%	<0.0001
Zipper (zip), isoform F	0.0%	3.2%	0.0%	7.2%	0.0%	16.6%	<0.0001
Myosin regulatory light chain 2 (Mlc2), isoform E	0.0%	21.0%	31.6%	49.6%	39.6%	55.0%	<0.0001
Myosin-2 essential light chain (Mlc-c)	0.0%	0.0%	0.0%	34.3%	0.0%	65.7%	<0.0001
Phosphoglycerate mutase (Pglym78)	2.3%	9.8%	0.0%	9.8%	0.0%	29.3%	<0.0001
Fructose-bisphosphate aldolase (Ald)	50.0%	39.2%	2.5%	34.0%	2.3%	14.4%	<0.0001
Glycerol-3-phosphate dehydrogenase (Gpdh)	28.3%	24.7%	1.3%	11.2%	12.8%	12.6%	<0.0001
Pyruvate kinase (Pyk)	0.0%	0.0%	0.0%	0.0%	6.4%	23.5%	0.001
Tiggrin (Tig)	2.7%	1.0%	8.7%	12.3%	1.4%	21.6%	<0.0001
Pericardin (prc)	1.1%	0.0%	1.3%	14.9%	1.1%	7.3%	<0.0001
Papilin (Ppn)	0.0%	1.0%	0.0%	2.2%	0.0%	9.2%	<0.0001
Lamin-C (LamC)	13.3%	0.0%	0.0%	10.6%	3.4%	66.0%	<0.0001

3.1.4 Discussion

The *Drosophila* TRIM32 protein is homologous to human TRIM32 gene which causes Limb-Girdle Muscular Dystrophy type 2H. The mutations occur in NHL repeats of the protein which is highly conserved among different species. The NHL domain is known to be involved in protein-protein interaction. The mutations in the NHL repeats could affect the binding of the target proteins and their ubiquitination to the proteasome pathway. By using proteomics based approach, different classes of proteins that have role in muscle development and function were identified.

3.2 Analysis of the Proteins Identified Using the Proteomics Approach

3.2.1 Glucose Metabolism Genes and Thin Filament Proteins Identified from the Mass Spec Data Analysis

In addition to recovery of our dTRIM32 (NHL) bait protein, we detected peptides corresponding to thin filament proteins [i.e., Tropomyosin (TM) and Troponin T (TnT)] known to interact with mammalian TRIM32. Surprisingly, we also observed enrichment in glycolytic enzymes as candidate binding proteins for dTRIM32. TRIM32 in vertebrate literature is known to ubiquitinate thin filament proteins (Tropomyosin, Troponin and Actin) and promote their degradation during muscle atrophy. Just like Thin protein, glycolytic genes are also localized to Z-disk and M-line of the sarcomere. In *Drosophila,* the loss of glycolytic genes during embryo development resulted in *thin* muscle phenotype. Muscles have high energy demand to produce contractions and energy is derived from ATP produced in the muscle fibers. Glycolysis is one of the source through which the muscle generates ATP for contraction. The altered metabolism results in muscle distress, weakness and impairs repair and regeneration of the damaged muscle tissue. (Figure 3.3 and Table 3.2)

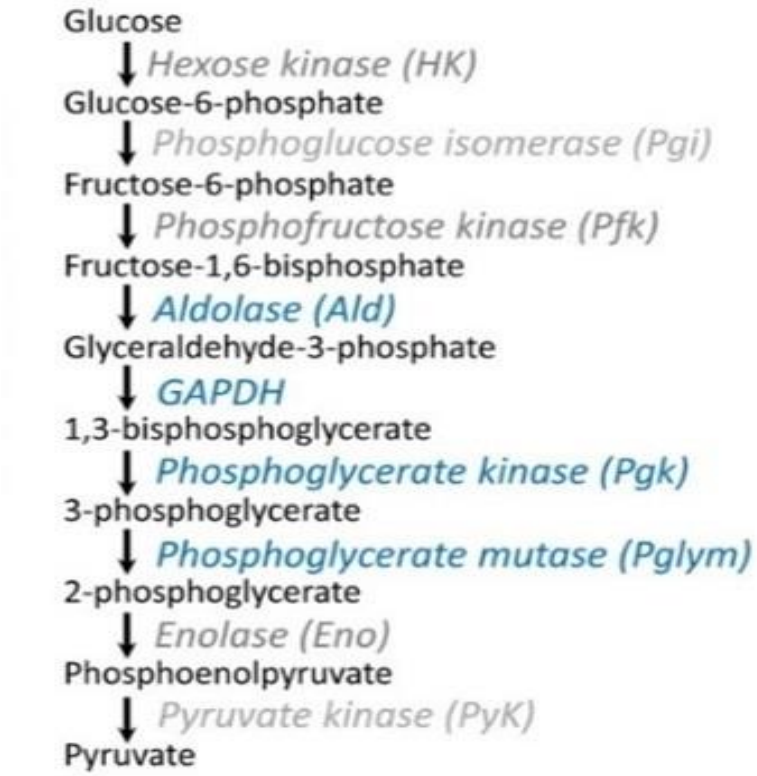

Figure 3.3 Glycolytic enzymes found in a biochemical complex with dTRIM32
The glycolysis pathway enzymes in blue were identified as candidate dTRIM32 interactors by proteomics approaches.

Table 3.2 Proteins isolated with the NHL repeats in dTRIM32

	TRIM32 binding candidates	Unique/Total peptides	Fold change[1]	T-Test ($p < 0.05$)
Bait	Drosophila Thin/TRIM32(NHL)	7/12	INF[2]	0.00098
Glycolytic enzymes	Fructose-biophosphate aldolase (Ald)	4/7	INF	0.0003
	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)[3]	4/7	1.3	0.05
	Phosphoglycerate kinsae (Pgk)	74/123	INF	0.0027
	Phosphoglycerate mutase 1 (Pglym78)	8/12	INF	0.00019
Muscle proteins	Tropomyosin 2 (TM2)	27/47	4.2x	< 0.0001
	Troponin (TnT)	10/14	2.9x	< 0.0001

[1]Fold change (ratio of raw spectra in TRIM32 pulldown/control pulldown)

[2]INF = Infinite (not detected in control pulldown)

[3]Proteins downregulated in TRIM32KO mouse

3.3 Biochemical Interactions of Glycolytic Genes and Thin Filament Protein with dTRIM32-NHL

Few protein targets of TRIM32 have been verified. Our mass spec data analysis shows enhancement of glycolytic and thin filament proteins specifically bind to the NHL domain. Mammalian literature provides evidence that TRIM32 ubiquitinates thin filament proteins and is involved in protein turnover. Therefore, our data suggests that these proteins could be possible interactors of dTRIM32.

3.3.1 Tropomyosin Binds Directly to dTRIM32-NHL Protein

Ni-NTA binding assay was performed to assess the interaction between dTRIM32 and Tropomyosin (Figure 3.4). The binding was analyzed on SDS PAGE. Tropomyosin only binds with dTRIM32-NHL protein. This data indicates direct interaction between dTRIM32-NHL and Tropomyosin, which is a thin filament protein. (Figure 3.5)

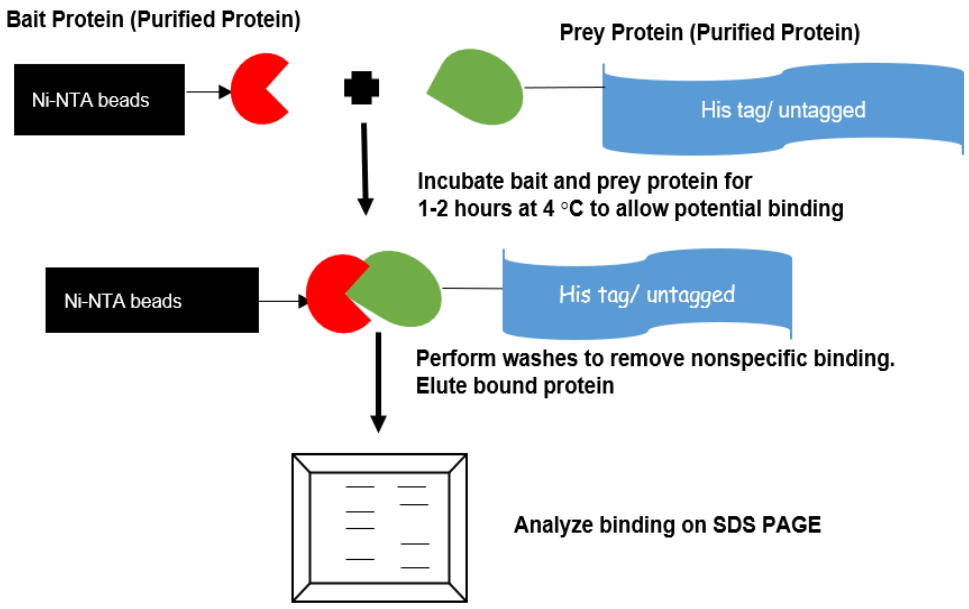

Figure 3.4 Schematic representation of the Binding Assay

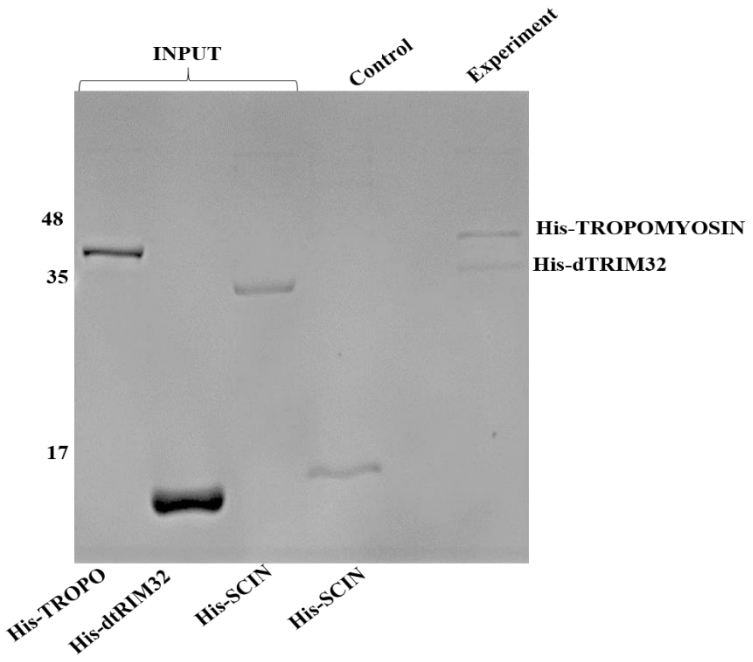

Figure 3.5 Pulldown of Tropomyosin by dTRIM32-NHL protein
Tropomyosin incubated with dTRIM32-NHL and His protein bound to Ni-NTA magnetic beads at 4°C, washed and analyzed by SDS PAGE.

3.4 Interaction of Glycolytic Proteins with dTRIM32-NHL

3.4.1 Aldolase Shows Direct Interaction with dTRIM32-NHL Protein

The His-tagged proteins Aldolase and SCIN attached to Ni-NTA beads were incubated with dTRIM32 to validate the interaction (Figure 3.4). The binding was analyzed on SDS PAGE. It was observed that Aldolase binds only to dTRIM32-NHL protein. This data indicates direct interaction between dTRIM32-NHL and Aldolase. (Figure 3.6)

3.4.2 Phosphoglycerate Mutase Binds Directly to dTRIM32-NHL Protein

The binding was carried out by immobilizing PGLYM (experiment) and SCIN (control) protein on to Ni-NTA beads and incubating with dTRIM32-NHL protein (Figure 3.4). The binding was analyzed on SDS PAGE. PGLYM binds only to dTRIM32-NHL protein. This data indicates direct interaction between dTRIM32-NHL and PGLYM. (Figure 3.7)

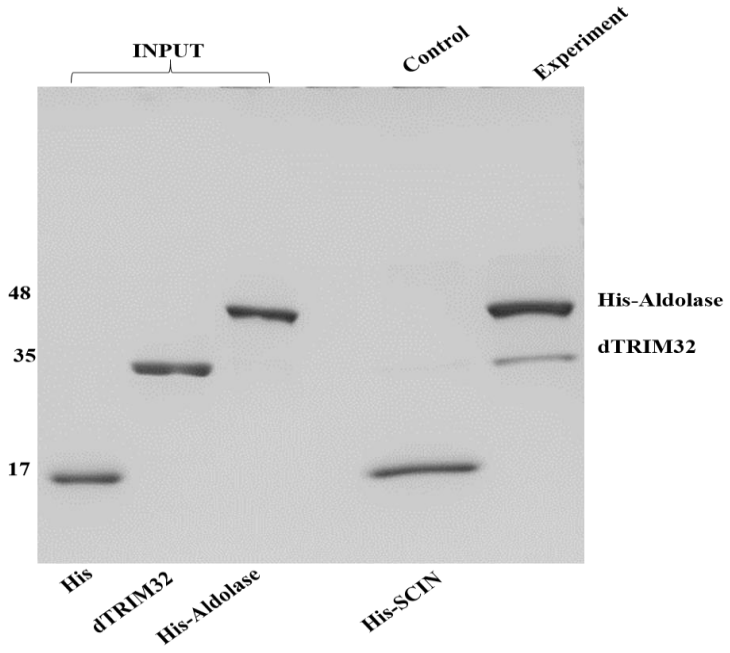

Figure 3.6 Pulldown of dTRIM32-NHL by Aldolase protein
dTRIM32-NHL incubate with Aldolase and His protein bound to Ni-NTA magnetic beads at 4°C, washed and analyzed by SDS PAGE.

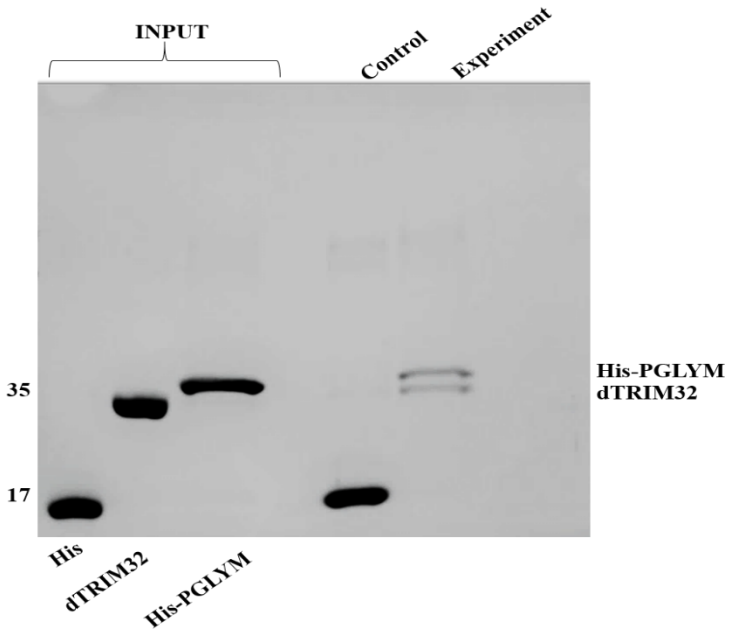

Figure 3.7 Pulldown of dTRIM32-NHL by PGLYM protein
dTRIM32-NHL incubated with PGLYM and His protein to Ni-NTA magnetic beads at 4°C, washed and analyzed by SDS PAGE.

3.5 Loss of *thin* Affects the Localization of Glycolytic Proteins in Third-Instar Larval Musculature

Similar to dTRIM32, glycolytic proteins are colocalized to M-line and Z-disk of both *Drosophila* Indirect Flight muscle and larval muscle. Our biochemical analysis provides evidence that there is direct interaction between dTRIM32-NHL protein and glycolytic proteins (Aldolase and PGLYM). To test the possibility of loss of *thin* affecting the localization/expression of these glycolytic proteins, 3rd instar larvae of *tn-/-* mutants and *WT* were dissected, double stained with phalloidin and glycolytic antibodies (PGLYM, GAPDH, Aldolase and PGK). The results were analyzed using fluorescence confocal microscopy and shown in Figures 3.8, 3.9, 3.10 and 3.11. These glycolytic proteins were localized along the repeated pattern of sarcomere in *WT* larvae. However, throughout the degenerated myofibrils in *tn-/-* mutants glycolytic proteins were mislocalized.

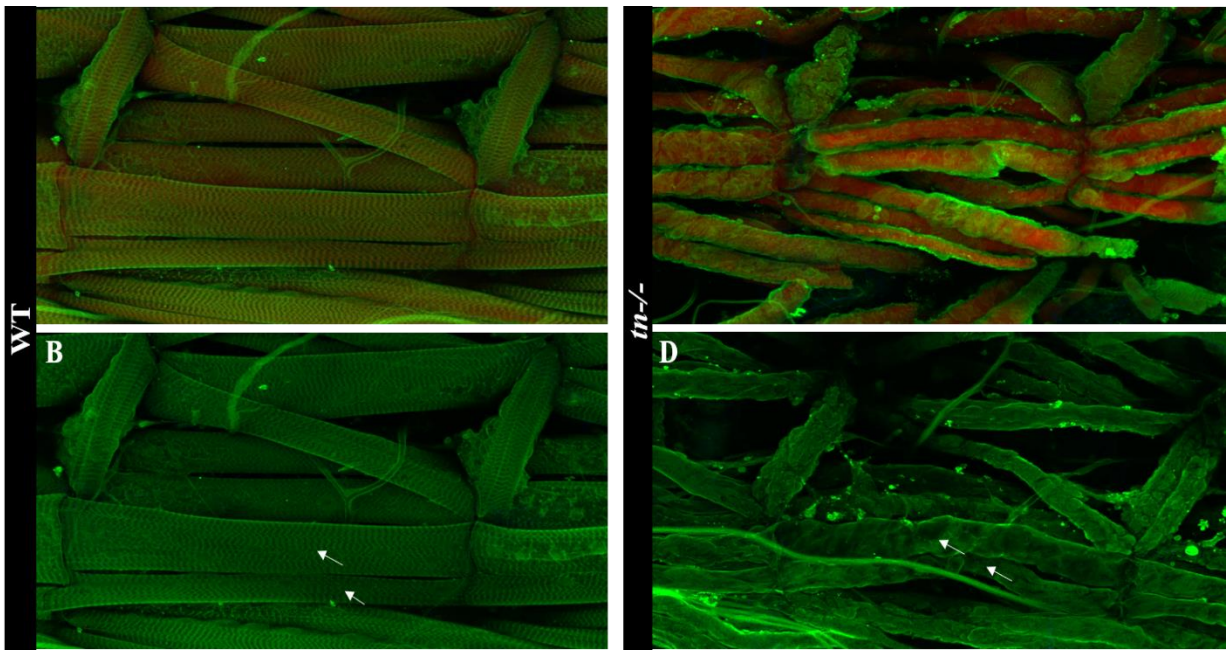

Figure 3.8 Loss of dTRIM32 affects the localization of GAPDH protein
(A and B) GAPDH is localized to sarcomeres in WT muscle; green (GAPDH), red (phalloidin). (C and D) GAPDH is absent in tn-/- mutants; green (GAPDH), red (phalloidin).

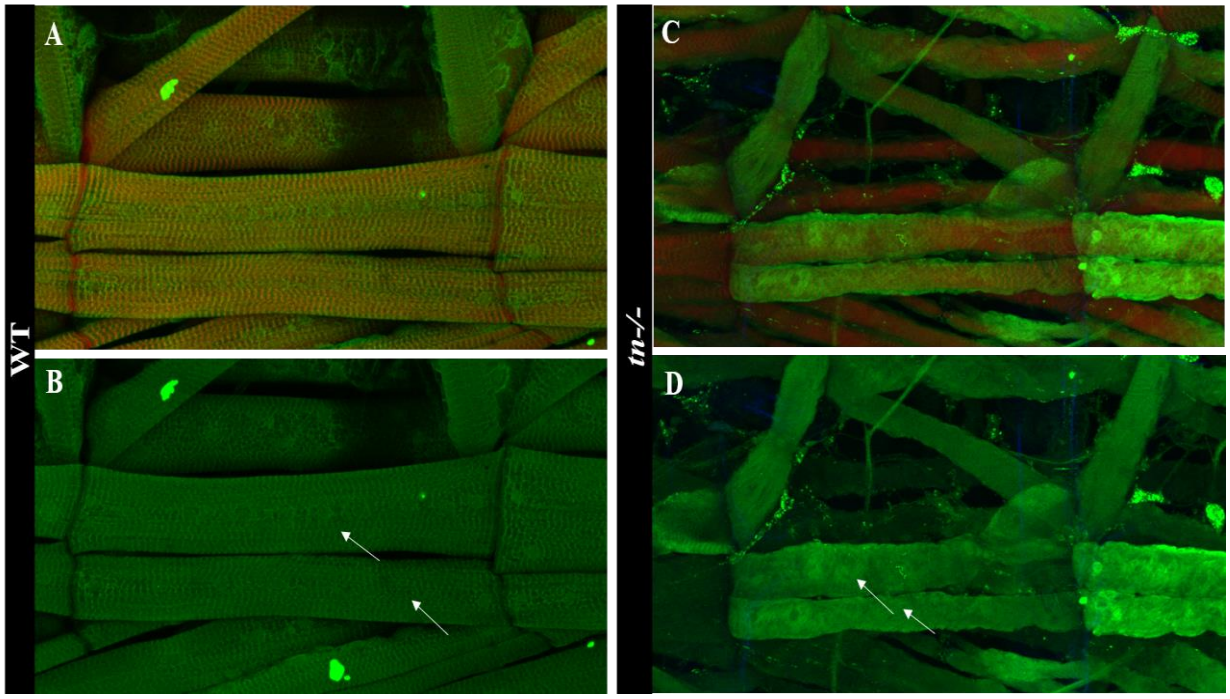

Figure 3.9 Loss of dTRIM32 affects the localization of PGLYM protein
(A and B) PGLYM is localized to sarcomeres in WT muscle; green (PGLYM), red (phalloidin). (C and D) PGLYM is absent in tn-/- mutants; green (PGLYM), red (phalloidin).

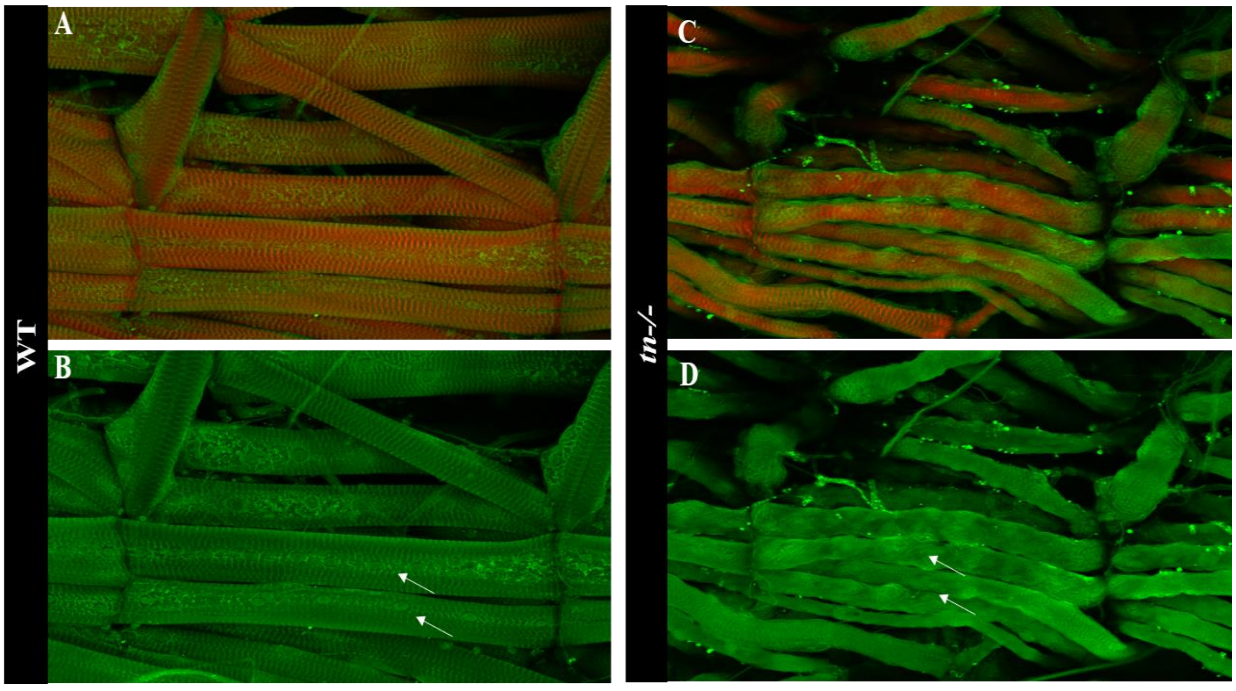

Figure 3.10 Loss of dTRIM32 affects the localization of PGK protein
(A and B) PGK is localized to sarcomeres in WT muscle; green (PGK), red (phalloidin). (C and D) PGK is absent in tn-/- mutants; green (PGK), red (phalloidin).

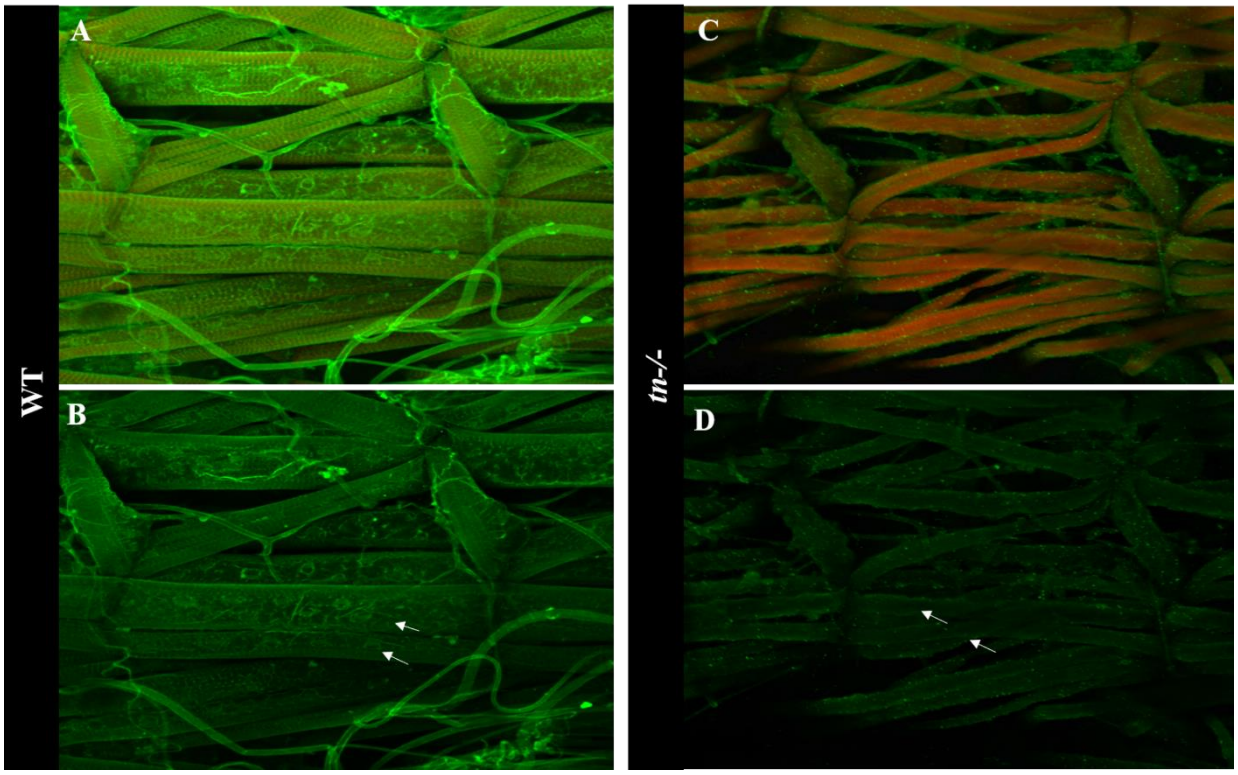

Figure 3.11 Loss of dTRIM32 affects the localization of Aldolase protein
(A and B) Aldolase is localized to sarcomeres in WT muscle; green (Aldolase), red (phalloidin). (C and D) Aldolase is absent in tn-/- mutants; green (Aldolase), red (phalloidin).

3.6 Glycolytic Proteins are Downregulated in *thin* Mutants

Since, glycolytic proteins (Aldolase, PGK, PGLYM and GAPDH) are mislocalized in *tn* mutants, it was important to look at the steady state protein levels. Five L3 larvae of *WT*, *thin* mutant and *mef2> tn* RNAi were lysed and boiled in 3X SDS lammeli buffer. Whole larval lysates (*WT*, *tn* mutant and *mef2> tn* RNAi) were immunoblotted using antibodies against each glycolytic protein and ATP5α served as loading control. The glycolytic proteins (Aldolase, PGK and PGLYM) were downregulated in *tn* mutants except for GAPDH. (Figure 3.12 and 3.13)

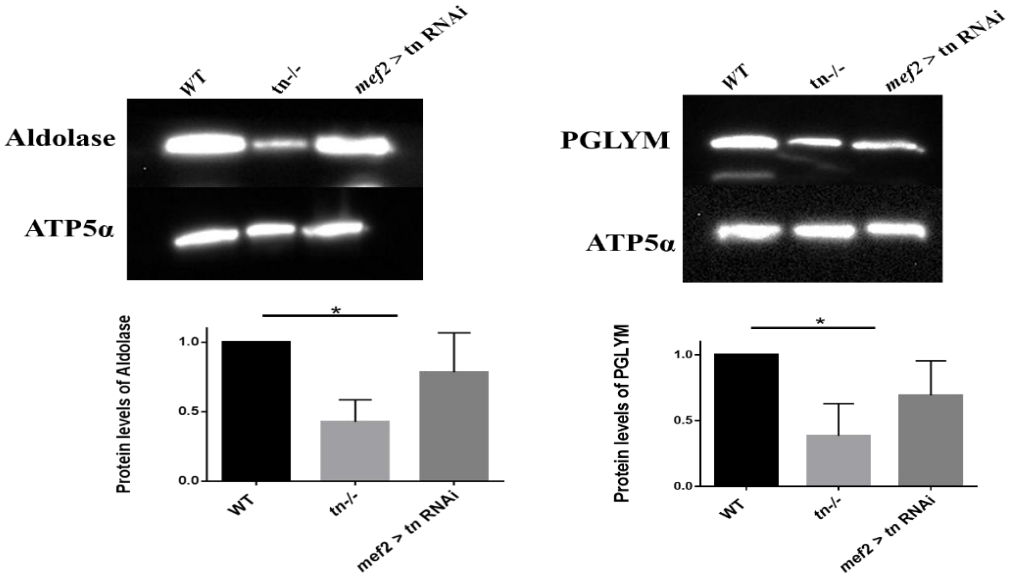

Figure 3.12 Western blot analysis of the glycolytic protein levels in *tn-/-* mutants

Immunoblot staining for (A) Aldolase and (C) PGLYM shows significant reduction in protein levels in thin mutants as compared to WT. Quantification of the protein levels (B) and (D) $p<0.05$.

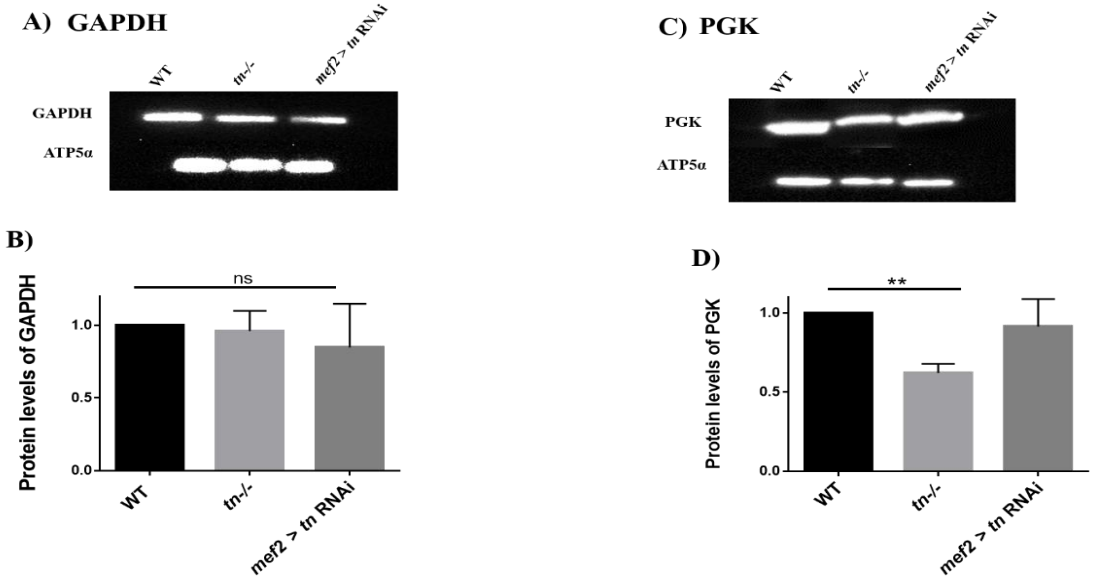

Figure 3.13 Western blot analysis of the glycolytic protein levels in *tn-/-* mutants

Immunoblot staining for (E) GAPDH shows no reduction in protein levels in thin mutant as compared to WT, (G) PGLYM shows significant reduction in protein levels in thin mutant as compared to WT. Quantification of the protein levels for (F) GAPDH and (G) PGK. For PGK $p<0.05$, GAPDH $p>0.05$.

3.7 Genetic Interaction of Glycolytic Proteins with *dTRIM32*

3.7.1 Glycolytic Proteins Genetically Interacts with dTRIM32 Protein During Muscle Formation

Glycolytic proteins were identified as candidate binding partners of dTRIM32-NHL in mass spec data analysis. Further *in vitro* studies provide evidence that Aldolase and PGLYM binds directly to dTRIM32. Also, loss of *tn* affects the localization and expression levels of these glycolytic proteins in muscle tissue. Therefore, the i*n vitro* and localization data suggests that these proteins may functionally cooperate with dTRIM32. Next, we tested for genetic interactions between *tn* and glycolytic genes using an RNAi approach.

3.7.1.1 Testing Genetic Interaction Between *tn* and *PGLYM*

The knockdown of *tn* in muscle at lower temperature results in mild unbundling of myofibers. We tested if the knockdown of glycolytic genes in this *tn* RNAi background would further enhance or suppress the unbundling phenotype. First, knockdown of *PGLYM* RNAi line was assessed with the muscle *mef2*-Gal4 driver. L3 larvae fillets were stained with phalloidin and analyzed using fluorescence confocal microscopy. Larval muscles show a normal striation pattern with no defects (Figure 3.14C). Previous studies done by our lab have already shown that knockdown of *tn* using RNAi phenocopies the larval muscle phenotype present in *tn*-/- mutants . (Figure 3.14A). The double RNAi experiment that decreases *tn* and *PGLYM* enhanced the unbundling of the myofibers (Figure 3.14B) by approximately 40% as compared to the single *tn* RNAi experiment. This experiment indicates that *tn* and *PGLYM* genetically interact during larval muscle development. (Figure 3.14).

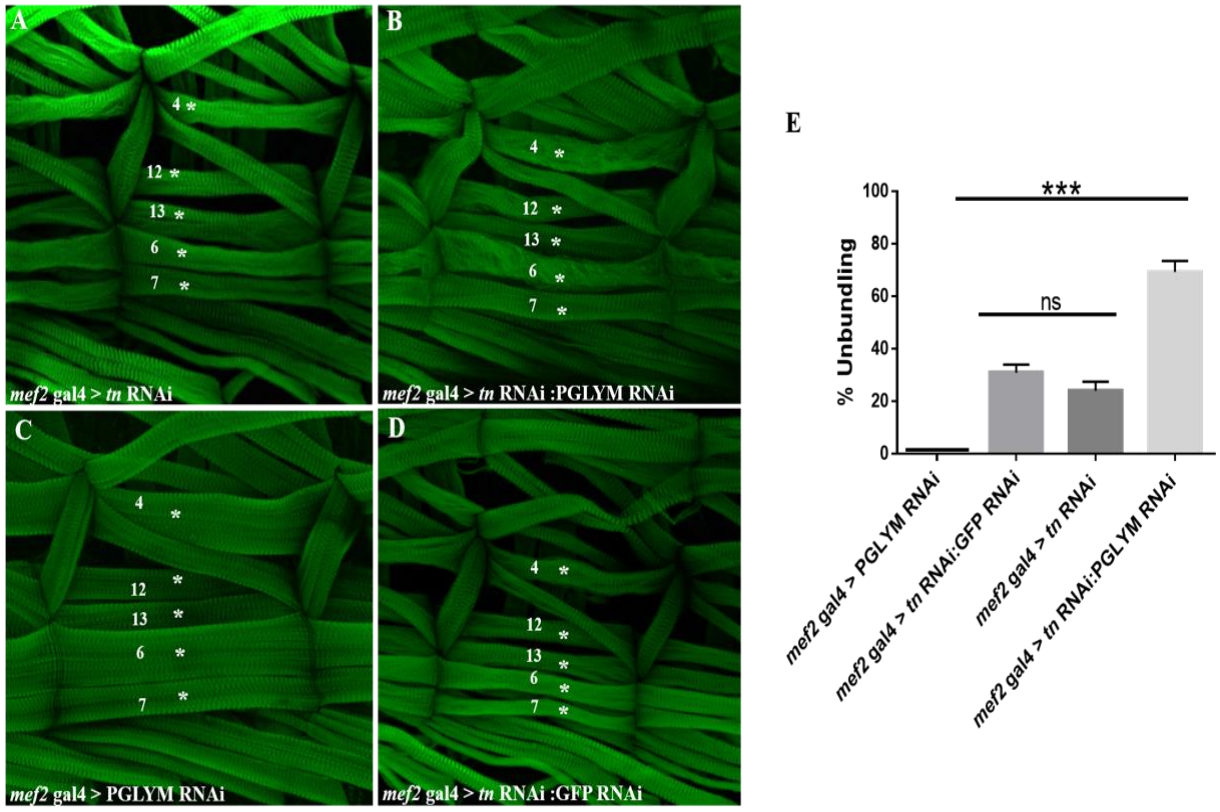

Figure 3.14 *PGLYM* interacts with *thin* during muscle formation
(A) Muscle specific expression of tn RNAi at 28.5°C results in an intermediate dystrophic muscle phenotype. (B) Knockdown of PGLYM RNAi in background of tn RNAi enhances the unbundling of the muscles. (C and D) PGLYM RNAi alone and GFP RNAi in mutant background of tn RNAi does not result in dystrophic muscle. (E) Quantitation of enhancement of degenerative muscles $p<0.05$.

3.7.1.2 Testing Genetic Interaction Between *tn* and *GAPDH*

The *mef2*-Gal4 muscle driver was used to analyze the muscle phenotype using *GAPDH* RNAi. Larval muscles show normal striation pattern with no defects (Figure 3.15C). Knockdown of *tn* using RNAi shows a mild phenotype at the intermediate temperature of 28.5°C (Figure 3.15A). The double RNAi experiment with *tn* and *GAPDH* enhanced the unbundling of the myofibers (Figure 3.15B) by approximately 50% as compared to the single *tn* RNAi experiment. This experiment indicates that there is genetic interaction between *tn* and *GAPDH* during larval muscle development. (Figure3.15)

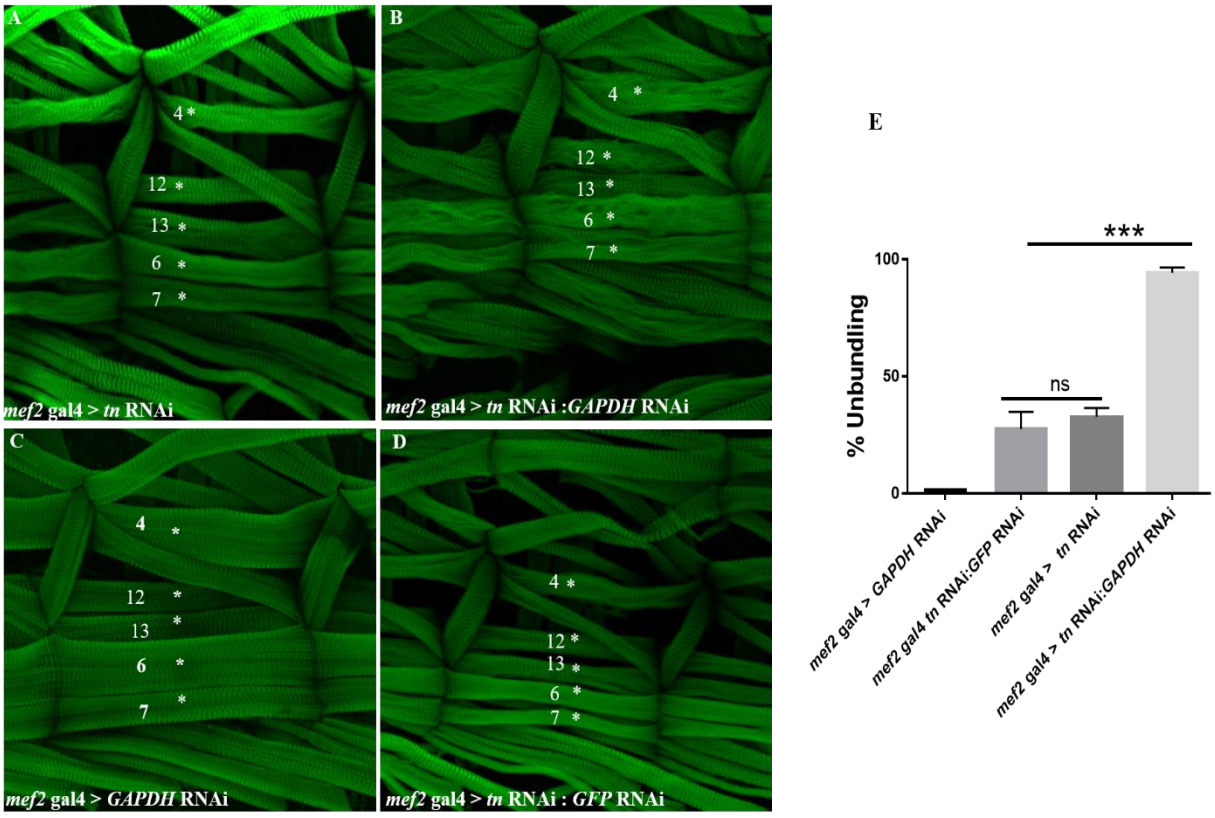

Figure 3.15 *GAPDH* interacts with *thin* during muscle formation
(A) Muscle specific expression of tn RNAi at 28.5°C results in an intermediate dystrophic muscle phenotype. (B) Knockdown of GAPDH RNAi in background of tn RNAi enhances the unbundling of the muscles. (C and D) GAPDH RNAi alone and GFP RNAi in mutant background of tn RNAi does not result in dystrophic muscle. (E) Quantitation of enhancement of degenerative muscles $p<0.05$.

3.7.1.3 Testing Genetic Interaction Between *tn* and *PGK*

Apart from PGLYM, Aldolase and GAPDH PGK was also enriched in the experiment (GSTdTRIM32-NHL) in the pull-down assay. Since, knockdown of *PGLYM* and *GAPDH* in the background of *tn-/-* mutant enhanced the unbundling phenotype. We further decided to test the genetic interaction between *tn* and *PGK*. To assess the effects of knockdown of *PGK* in muscle, RNAi line was expressed using muscle specific driver *mef2*-gal4. L3 larval fillets were stained with phalloidin. Larval muscles show normal striation pattern with no defects (Figure 3.16C).

The results were analyzed using fluorescence microscope. The knockdown of *PGK* in the background of *tn* RNAi enhanced the unbundling phenotype. (Figure 3.16)

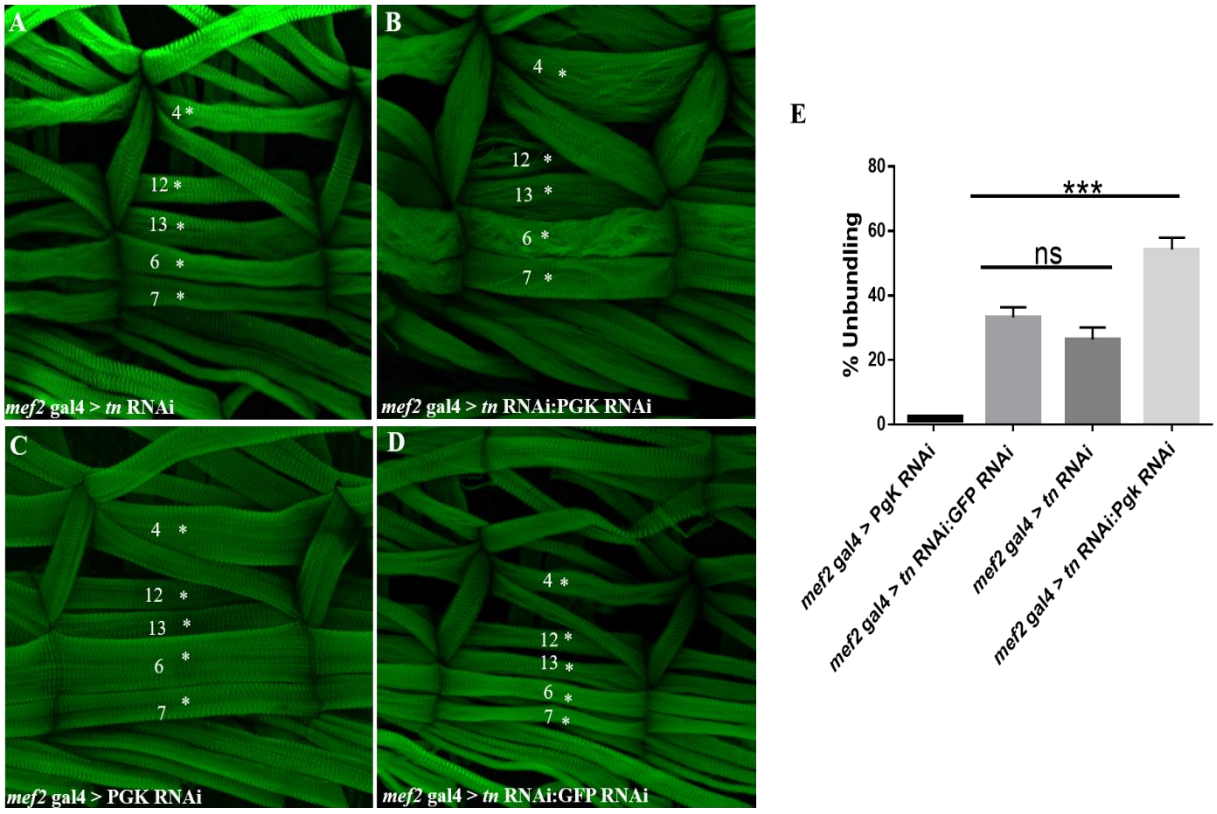

Figure 3.16 *PGK* interacts with *thin* during muscle formation

(A) Muscle specific expression of tn RNAi at 28.5°C results in an intermediate dystrophic muscle phenotype. (B) Knockdown of PGK RNAi in background of tn RNAi enhances the unbundling of the muscles. (C and D) PGK RNAi alone and GFP RNAi in mutant background of tn RNAi does not result in dystrophic muscle. (E) Quantitation of enhancement of degenerative muscles $p<0.05$.

Chapter 4 - Mechanism of Action

Ubiquitination is a post-translational modification which results in addition of ubiquitin proteins to a substrate protein. Different modes of ubiquitination can lead to different substrate fates. Both polyubiquitination and monoubiquitination is known to regulate various physiological and pathological processes. For example, poly-ubiquitination can direct substrates to proteasome degradation pathway. However, monoubiquitination is known to play role in DNA damage repair and the subcellular localization of proteins (SADOWSKI and SARCEVIC 2010). TRIM32 an E3 ubiquitin ligase protein can bind and ubiquitinate thin filament proteins. During atrophy, TRIM32 degrades the components of Z band which results in loosening and ultimately degradation of thin filaments (COHEN *et al.* 2012).

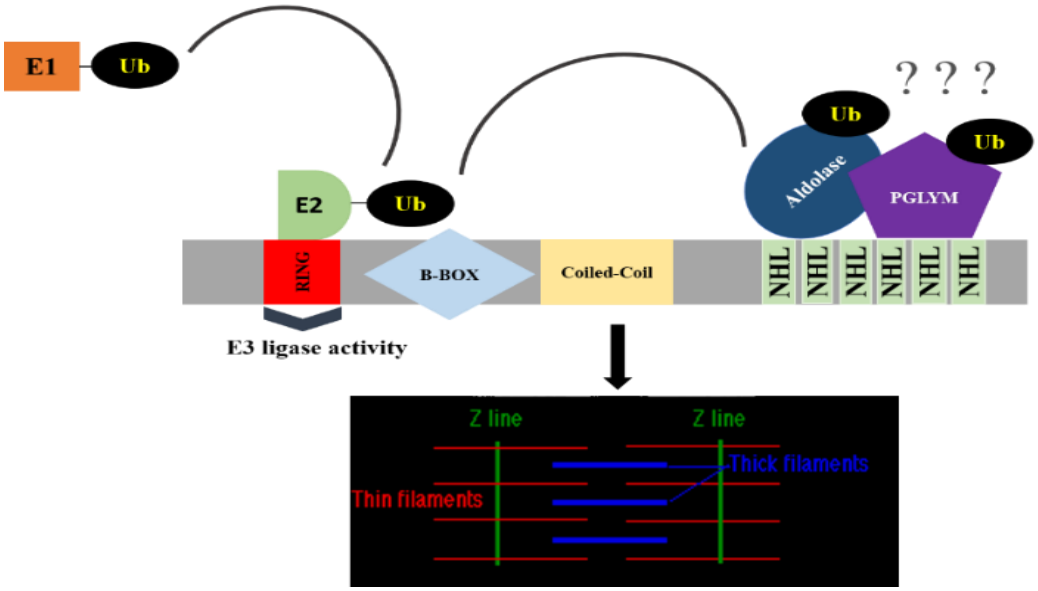

Figure 4.1 Model for mechanism of action of dTRIM32 on glycolytic enzymes
Binding of glycolytic enzymes (Aldolase & PGLYM) to the NHL repeats of dTRIM32 could facilitate the mono-ubiquitination of these proteins and determine their sarcomeric localization within muscle.

In our study, loss of *tn* results in altered glycolytic protein levels. TRIM32 N-terminal ring domain is known to polyubiquitinate actin, tropomyosin and troponin and is known to

regulate the protein turnover (KUDRYASHOVA *et al.* 2005). If dTRIM32 is regulating glycolytic protein levels through polyubiquitination, we should have observed elevated glycolytic proteins levels (Aldolase, PGLYM and PGK). However, upon loss of *tn*, we saw a significant reduction in the protein levels and a lack of localization to the sarcomere. We anticipate that these glycolytic proteins first bind to the NHL repeats of dTRIM32 protein, which results in the monoubiquitination of glycolytic proteins through ring domain and directs their localization within the sarcomere of the muscles. (Figure 4.1)

Future Direction

Our studies provide evidence that the NHL domain(s) of dTRIM32 physically and genetically interact with glycolytic proteins. Our future studies will identify whether the human point mutations at the NHL repeats, which are conserved in *Drosophila*, would alter the binding of thin filament protein (Tropomyosin) and glycolytic proteins (Aldolase, PGLYM) to dTRIM32. To do this, we will generate a C-terminal dTRIM32-NHL construct with point mutations in NHL repeats and repeat the binding assay to see if these mutations alter the binding of these proteins to dTRIM32 (Figure 4.2). Our *in vivo* data suggests that loss of *tn* affects the sarcomeric localization of these proteins within myofibrils. We are further interested to determine whether human point mutations causative for LGMD2H alter glycolytic enzyme localization. We would also like to test if ubiquitination mechanistically accounts for loss of glycolytic enzymes within muscle tissue.

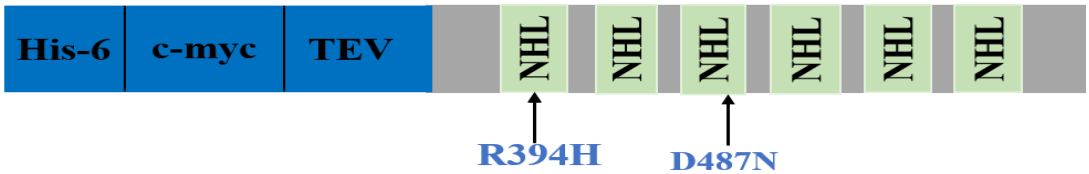

Figure 4.2 Schematic representation of construct of dTRIM32-NHL with human point mutations

Chapter 5 - Summary and Conclusions

Mutations in NHL domain of TRIM32 protein results in Limb-Girdle Muscular Dystrophy type 2H. It has already been reported in the mammalian literature that TRIM32 is localized to the Z-disk of the sarcomere, directly interacts with myosin and ubiquitinates thin filament proteins (ref). However, its role in pathogenesis of LGMD2H has not been understood. Previous studies in our lab revealed that muscle defective genes exhibit abnormal pupal morphology due to their inability to initiate normal muscle contraction during pupariation. As proof of principle, one such gene, *Drosophila thin* (*tn*), encodes for the human ortholog of tripartite motif 32 (TRIM32), a gene mutated in patients with Limb-Girdle Muscular Dystrophy type 2H. *tn* mutants are pupal lethal and examination of muscles in *tn* mutants (*tn-/-)* revealed unbundled and loosely arranged myofibers. The functional domain analysis of dTRIM32/Thin protein using Tn protein lacking NHL domain did not recuse the unbundling phenotype. This experiment suggests that NHL domain is important for proper functioning of the dTRIM32 protein. The mutations in NHL domain are thought to destabilize the protein and affect the binding efficiency of TRIM32 to other proteins. Therefore, the main goal of this project was to identify binding partners of dTRIM32 protein that could play a significant role in the pathogenesis of LGMD2H.

5.1 Proteomics Approach to Identify Novel Proteins Binding dTRIM32-NHL

We utilized a proteomics approach to identify proteins that physically interact with the NHL domain of *Drosophila* TRIM32 (dTRIM32). A GST-dTRIM32-NHL fusion protein was incubated with larval lysates and associated proteins were analyzed using mass spectrometry. In addition to recovery of our dTRIM32 (NHL) bait protein, we detected peptides corresponding to thin filament proteins [Tropomyosin (TM) and Troponin T (TnT)] known to interact with

mammalian TRIM32. We also observed a significant enhancement in binding of glycolytic enzymes as candidate binding partners to dTRIM32.

5.2 Pulldown Assays to Investigate Protein-Protein Interactions

To validate the mass spectrometry data, a biochemical approach was used. Ni-NTA pull down assays were carried out. First, we tested the binding of Tropomyosin by incubating it with immobilized dTRIM32 and His proteins on Ni-NTA magnetic beads. Second, to test the binding of glycolytic proteins (Aldolase and PGLYM) to dTRIM32, both Aldolase and PGLYM along with His-SCIN protein were immobilized on Ni-NTA beads. dTRIM32 was incubated with the glycolytic proteins. The binding assays validated that Tropomyosin, Aldolase and PGLYM are *bona fide* dTRIM32 binding proteins.

5.3 Localization and Expression Studies of Glycolytic Proteins in *tn* Mutants

Our *in vitro* data provides evidence that both Aldolase and PGLYM are binding partners of dTRIM32. It is also known from literature that both Tn and glycolytic proteins localize to Z-disk and M-line of the sarcomere. Further studies were done to analyze if, dTRIM32 regulates the localization of these glycolytic enzymes to their site of action and/or affects the expression level of the glycolytic proteins. Immunostaining of the *tn* mutant larval muscles with glycolytic specific antibodies (GAPDH, Aldolase, PGLYM and PGK) revealed localization defects. Analysis of protein expression levels was performed using whole larval lysates. Western blot analysis suggests loss of *tn* results in reduced glycolytic protein levels in Aldolase, PGLYM and PGK. However, there was no effect on GAPDH protein level.

5.4 Testing Genetic Interactions Between *tn* and Glycolytic Proteins

The *in vitro* and localization data suggests that the glycolytic enzymes might be acting through dTRIM32. We used the UAS/GAL4 system to assess the *in vivo* requirement for dTRIM32 and glycolytic enzymes in muscle degeneration. RNAi knockdown of dTRIM32 results in thinner muscles with a mild dystrophic phenotype at 28.5°C. This defect was further enhanced by knocking down *PGK*, *GAPDH* or *PGLYM* in the background of *tn* RNAi. Our studies provide evidence that dTRIM32 and glycolytic enzymes together are required for maintaining myofibrillar stability and normal muscle function.

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Appendix A - Vector Maps

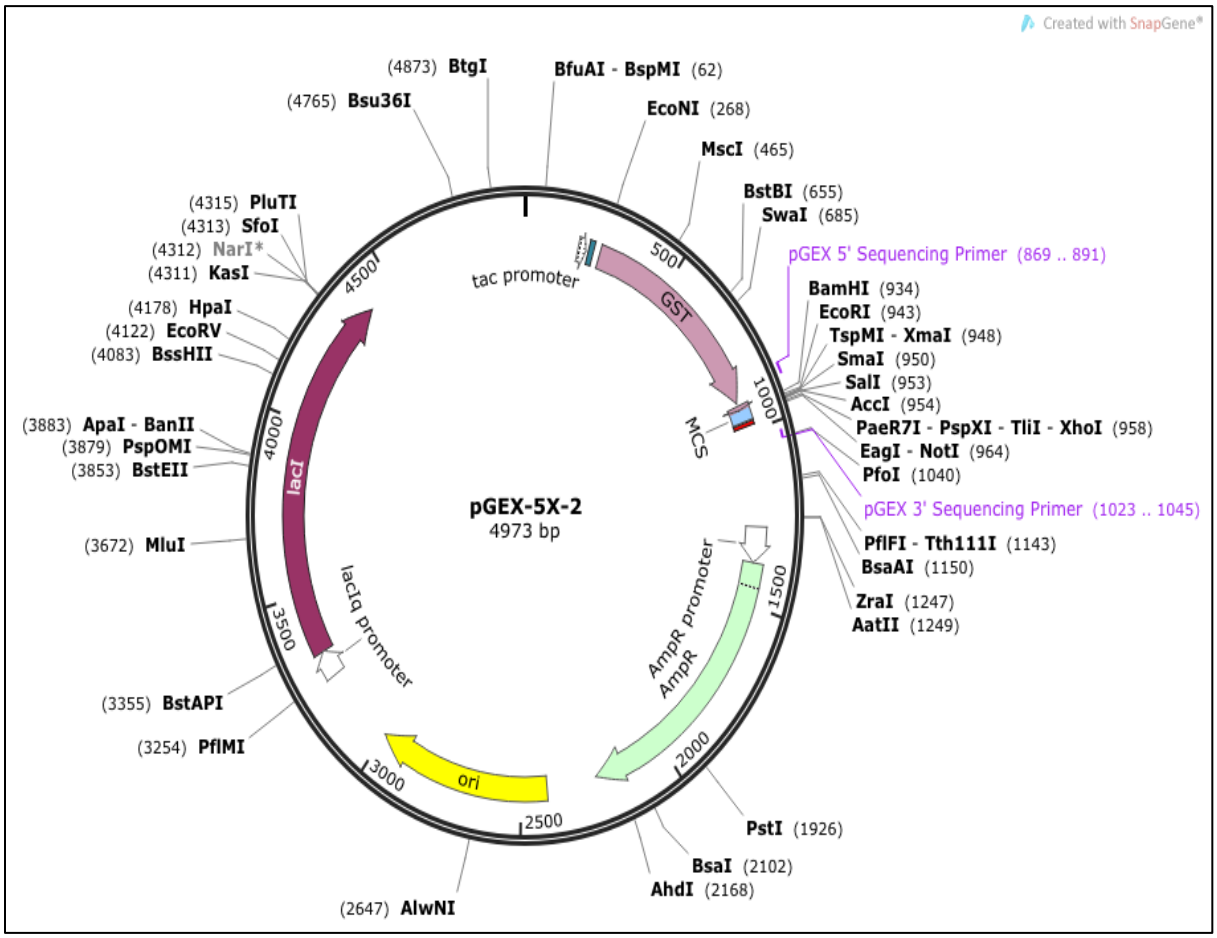

Figure A.1 pGEX-5X-2

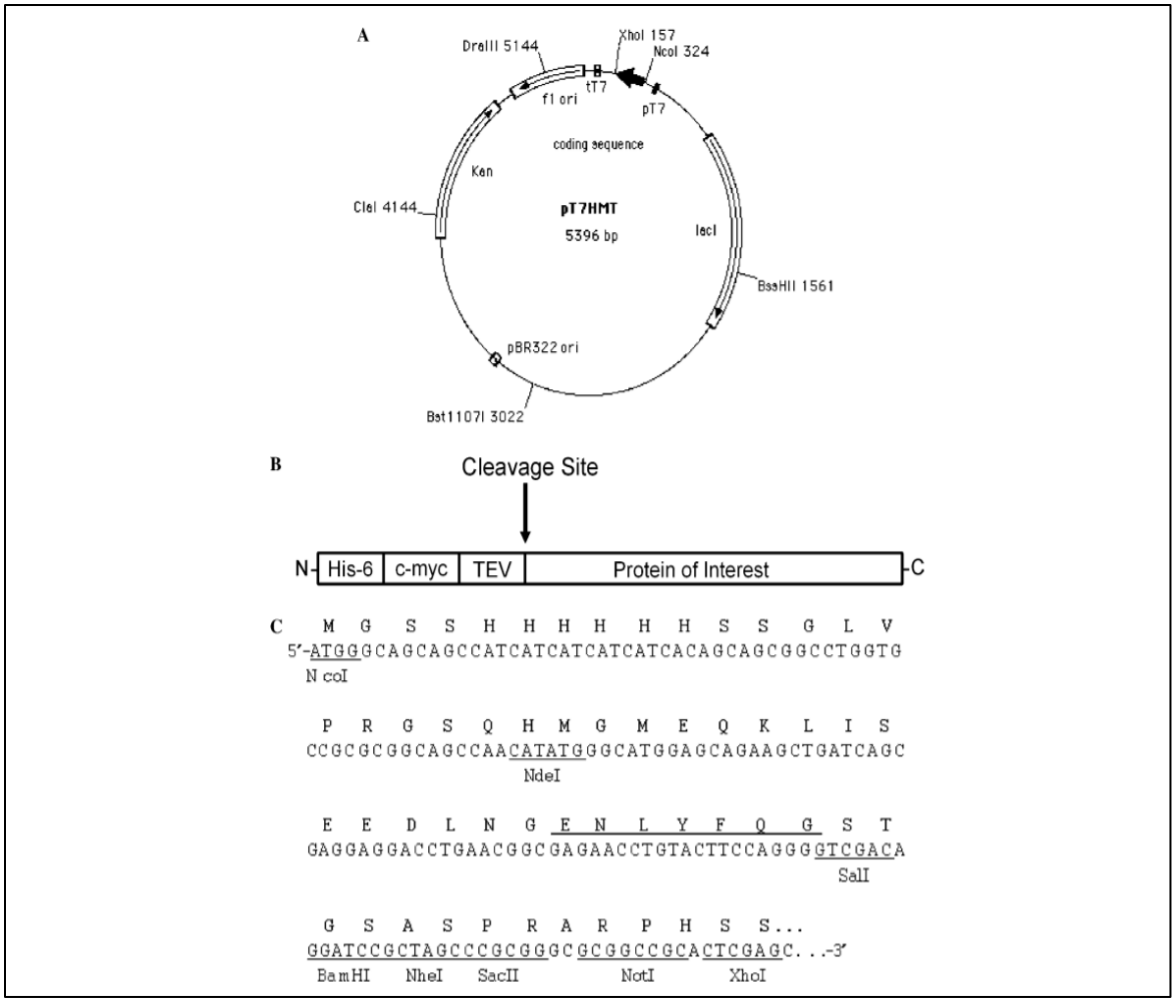

Figure A.2 pT7HMT