Analysis of pkc-2 gene of Caenorhabaditis elegans and screen for serotonin resistant mutant in pkc-2(ok328) background

Yu Qian

N° d'ordre 136-2009

Année 2009

THESE DE L'UNIVERSITE DE LYON

Délivrée par

L'UNIVERSITE CLAUDE BERNARD LYON 1 et EAST CHINA NORMAL UNIVERSITY

ECOLE DOCTORALE ET SCHOOL OF LIFE SCIENCE

DIPLOME DE DOCTORAT EN CO-TUTELLE

(arrêté du 7 août 2006)

soutenue publiquement le 28, Septembre, 2009

Par

Mme. Yu QIAN

Analysis of the *pkc-2* gene of *Caenorhabditis elegans* And Screen for serotonin resistant mutants in *pkc-2(ok328)* background

Directeurs de thèse: Dr. Laurent SEGALAT, Prof. Fukang GU

JURY: M. Zhenqin ZHU
M. Laurent SEGALAT
Mme Kathrin GIESELER
M. Xuguang XI
M. Fukang GU
M.Liansheng HOU

N° of Order 136-2009

Year 2009

THESIS OF L'UNIVERSITE DE LYON

Awarded by

GRADUATE SCHOOL, L'UNIVERSITE CLAUDE BERNARD LYON 1

And

SCHOOL OF LIFE SCIENCE, EAST CHINA NORMAL UNIVERSITY

CO-TRAINED DEGREE OF DOCTOR OF PHILOSOPHY

(Law of 7 August 2006)

Defense in public on Septembre 28, 2009

Presented by

Mme. Yu QIAN

Analysis of the *pkc-2* gene of *Caenorhabditis elegans*

And

Screen for serotonin resistant mutants in *pkc-2(ok328)* background

Supervisors: Dr. Laurent SEGALAT, Prof. Fukang GU

JURY COMMITTEE: M. Zhenqin ZHU
M. Laurent SEGALAT
Mme Kathrin GIESELER
M. Xuguang XI
M. Fukang GU
M.Liansheng HOU

ABSTRACT

Duchenne Muscular Dystrophy (DMD) is an X-linked progressive muscle disease which is caused by mutations in the dystrophin gene. Until now, there is no effective therapy for DMD. As the largest gene in human beings, it produces a 427-kDa cytoskeleton protein: Dystrophin. Dystrophin links actin and dystrophin associated protein complex (DAPC) in muscles. Currently, there are 3 hypotheses to explain the mechanisms of DMD. They suggest that the absence of dystrophin could lead to periodic muscle cell membrane ruptures, or affect the distribution and function of ion channels, or perturb signal transduction pathways.

In *Caenorhabditis elegans*, there is only one homologue of mammalian dystrophin gene named *dys-1*, and the nematode protein DYS-1 presents 37% similar to the human one. The double mutant *dys-1; hlh-1* exhibits a severe progressive muscle degeneration. The protein composition of the sarcomere has been studied and it has revealed a high degree of similarity with mammalian sarcomere. These allow *C. elegans* be a relevant animal model to study DMD.

To understand why the lack of dystrophin induces muscle degeneration in mammals and worms, and to find new drugs that might help in reducing muscle degeneration, L. Ségalat and his coworkers performed several screens for drugs and genes suppressing muscle degeneration. An interesting gene *pkc-2* came out and was considered as a possible regulator in the process of muscle degeneration in *C. elegans*. The protein that is encoded by this gene in *C. elegans* is an orthologous of the human gene Protein Kinase C Alpha (PKC), which belongs to the family of serine/threonine specific protein kinases. To study the function of *pkc-2*, we generated different recombinant constructs, analyzed the expression pattern of *pkc-2* with immunocytochemistry, and performed yeast two-hybrid to search for PKC-2 binding partners. In addition, a neurotransmitter serotonin (5-HT) was found by drug screening to be an active blocker of striated muscle degeneration. As *C. elegans* lacking PKC-2 displays a severe blister phenotype in exogenous 5-HT, studying the correlation between PKC-2 and 5-HT therefore seems to be an opportunity to explore the reasons of muscle degeneration. A genetic screen with EMS (ethane methyl sulfonate) to search serotonin resistant mutant in strain *pkc-2 (ok328)* would help us study further about the role of *pkc-2*.

In this thesis, different clones *myo3::pkc-2* and *pkc-2::gfp* were made to inject into wild-type animals. The results revealed that *pkc-2* expressed intensely in neurons and pharynx, but was not found in body-wall muscles. Mutants *dys-1;hlh-1* fed with *pkc-2* RNAi did not reduce muscle degeneration statistically comparing to triple mutant *pkc-2;dys-1;hlh-1*. This indicated that PKC-2 may be dominantly acting in neurons. A yeast two-hybrid screen identified the gene Y59A8A.3, which is a homologue to mammalian filamin A interacting protein 1 isoform 3, as a binding partner of PKC-2. Filamin A is a cytoskeleton protein, anchoring various trans-membrane proteins to the actin cytoskeleton and may also function as an important signaling scaffold. The result suggested that PKC-2 may therefore modulate filamin A activity through the filamin interacting protein 1. Genetic screen by EMS presented 8 candidates named *cx253*, *cx254*, *cx259*, *cx263*, *cx267*, *cx268*, *cx270*, cx276, which were mapped on chromosomes by SNP mapping using a polymorphic *C. elegans* strain, but time was too short to identify these genes formally. The experiment also offered possibilities of searching links between PKC-2 and serotonin pathways.

In summary, this work studied the gene *pkc-2* in order to reveal the function of PKC-2 and its involvement in muscle degeneration. The present results answered some questions about *pkc-2*, and needed further researches to elucidate the *in vivo* role of PKC-2 protein and its interaction with other proteins in the mechanism of muscle dystrophy in *C. elegans.*

KEY WORDS: dystrophin, Duchenne Muscular Dystrophy, *Caenorhabditis elegans*, Protein Kinase C, *pkc-2*, ethane methyl suffocate, single nucleotide polymorphisms mapping

RESUME

La myopathie de Duchenne est une maladie génétique qui se caractérise principalement par une dégénérescence progressive des muscles squelettiques dont la cause est l'absence de dystrophine fonctionnelle dans les muscles. A ce jour, il n'existe toujours pas de traitement efficace contre ces maladies. Comme le plus grand gène connu chez l'Homme, la dystrophine code pour une protéine de 427kDa. La protéine connecte l'actine avec le DAPC (Dystrophin Associated Protein Complex) dans les muscles striés. Pour l'instant, il y a 3 hypothèses concernant le mécanisme du DMD. L'absence de la dystrophine peut supprimer le lien physique entre les protéines structurales de la membrane basale (laminines) et les protéines structurales du cytosquelette (filaments intermédiaires et actine), ou la distribution et la fonction des canaux ioniques, ou des voies de signalisation nécessaires à la survie du muscle.

Caenorhabditis elegans ne possède qu'un homologue du gène de la dystrophine humaine, le gène *dys-1*. La protéine DYS-1 présente 37% d'homologie avec la dystrophine humaine. Le double mutant *dys-1(cx18) ; hlh-1(cc561)* présente une forte dégénérescence musculaire. Comme le sarcomère de *C. elegans* ressemble au sarcomère de mammifère, *C. elegans* est modèle pertinent d'étude la maladie.

En vue de comprendre la raison du DMD chez les mammifères et chez les vers, le groupe L. SEGALAT a effectué des cribles pour identifier les molécules et les gènes qui peuvent supprimer la dégénérescence musculaire. On a trouvé un gène *pkc-2* qui est capable de supprimer la dégénérescence musculaire chez *C. elegans*. La protéine PKC-2 est l'orthologue de la Protein Kinase C Alpha (PKC) humaine et appartient à la famille du serine/threonine protéine kinase. Afin d'étudier la fonction du gène *pkc-2*, on a analysé l'expression du gène avec les construits différents *in vivo* et a utilisé la technique de double-hybride dans la levure. De plus, le crible par EMS (éthane méthyle sulfonâtes) a identifié une molécule sérotonine (5-HT) qui est un neuromédiateur, et supprime partiellement la dégénérescence musculaire des doubles mutants *dys-1; hlh-1*. La sérotonine a aussi un effet fort sur le mutant *pkc-2(ok328)*, puisqu'elle provoque un phénotype *blister*. Ça nous permet de rechercher le lien entre la signalisation sérotoninergique et *pkc-2*. Le crible génétique peut contribuer à la connaissance du rôle *pkc-2*.

Des constructions *myo3::pkc-2* et *pkc-2::gfp* ont été réalisées et injectées dans des vers sauvage. L'expression a montré que le gène *pkc-2* est exprimé fortement dans les neurones et pharynx, mais n'a pas été vu dans les muscles striés longitudinaux. Quand on inactive l'expression du *pkc-2* par RNAi chez les double mutants *dys-1 ;hlh-1*, on n'observe peu de diminution de cellules absentes ou abîmées chez les descendants par rapport au triple mutant *pkc-2 ;dys-1 ;hlh-1*. Les résultats suggèrent que *pkc-2* agit principalement dans les neurones. Les expériences de double-hybride dans la levure ont identifié un gène nouveau, Y59A8A.3, qui est l'homologie du filamin A interacting protéine 1 isoform 3 mammifère. Filamin A est un des protéines de cytosquelette associée à plusieurs protéines transmembranaires. Elle sert aussi de plate-forme de voie de signalisation intracellulaire. L'identification de Y59A8A.3 propose la possibilité que *pkc-2* modifie la filamin A par l'intermédiaire de la filamin A interacting protéine 1. Le crible génétique par EMS pour rechercher des suppresseurs de l'effet blister de la sérotonine sur les mutants *pkc-2(ok328)* a donné 8 candidats sur 5000 F1s : *cx253, cx254, cx259, cx263, cx267, cx268, cx270, cx276*. Les mutations ont été localisées sur les chromosomes par SNP mapping avec une souche de *C. elegans* très polymorphe, mais le temps a manqué pour leur identification exacte. L'expérience valide notre approche à étudier le lien entre la signalisation sérotoninergique et *pkc-2*.

En résumé, le but de la thèse était de rechercher la fonction du gène *pkc-2* dans les mécanismes moléculaires conduisant à la nécrose musculaire en absence de dystrophine. Les résultats présentés dans la thèse apportent des réponses aux questions fondamentales sur *pkc-2* et aussi demandent des expériences supplémentaires afin de élucider plus avant les mécanismes de la dégénérescence musculaire dystrophine-dépendante.

MOTS-CLES: dystrophine, myopathie de Duchenne, *Cœnorhabditis elegans*, Protéine Kinase C, *pkc-2*, éthane méthyle sulfonâtes, single nucléotide polymorphismes mapping

Table of Contents

Introduction

Chapter1 Muscle: The Development and the Structural Characteristic

1.1 Introduction

There are 3 types of muscles in vertebrate animals: heart muscle, also called cardiac muscle, makes up the wall of the heart; smooth muscle is found in the walls of all the hollow organs of the body (except the heart); skeletal muscle, also called striated muscle, is the muscle attached to the skeleton.

1.2 The development of skeletal muscle in vertebrate: the principal rules

It is widely accepted that all the skeletal muscle in vertebrate are derived from progenitors present in the somites (Christ and Ordahl, 1995). Somites are transient mesodermal units, and each newly formed somite rapidly differentiates into a ventral sclerotome and a dorsal dermomyotome from which myogenic precursors originates.

After the onset of somito-genesis, some myogenic precursors cells differentiate into mononucleated muscle cells, myocytes, in the myotome. The myotome cells are the myoblast-committed muscle cell precursors. Skeletal myogenesis begins with myotome fomation followed by fusion of myoblasts to form primary fibers. Some of the myoblasts do not fuse into primary fibers but continue to proliferate and differentiate into secondary (fetal) fibers. At the same time, a basal lamina begins to form around each fiber and it is only after its formation that satellite cells can be morphologically identified as mononucleated cells laying between the basal lamina and the fiber plasma membrane. During peri- and post-natal development, satellite cells divide at a slow rate and part of the progeny fuse with the adjacent fiber to contribute new nuclei and to increase size of muscle fibers whose nuclei cannot divide.

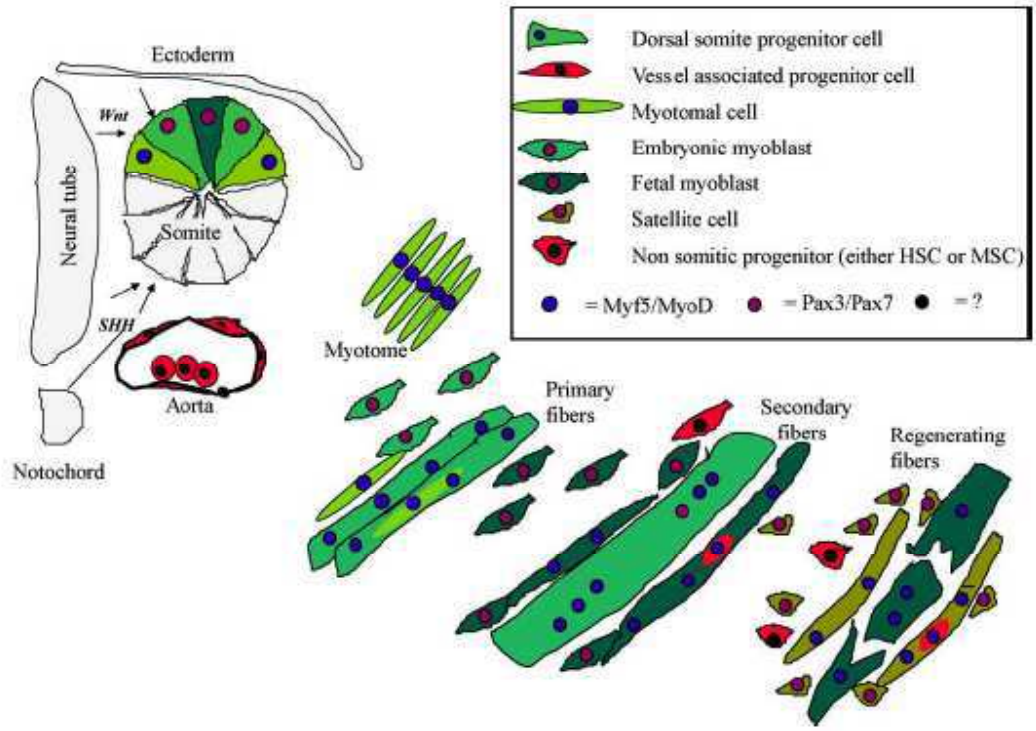


Figure 1-1: Development of Muscle. Skeletal myogenesis begins with myotome formation that is follozed by fusion of myoblasts to form primary fibers. Some of the myoblasts do not fuse into primary fibers but continue to proliferate and differentiate, giving rise to secondary (fetal) fibers. At the same time a basal lamina begins to form around each fiber and it is only after its formation that satellite cells laying between the basal lamina and the fiber plasma membrane (Cossu and Biressi, 2005).

1.3 Signaling pathways during myogenesis

During vertebrate development, signaling pathways regulate the balance between proliferation, specification, and differentiation of the myogenic lineage.

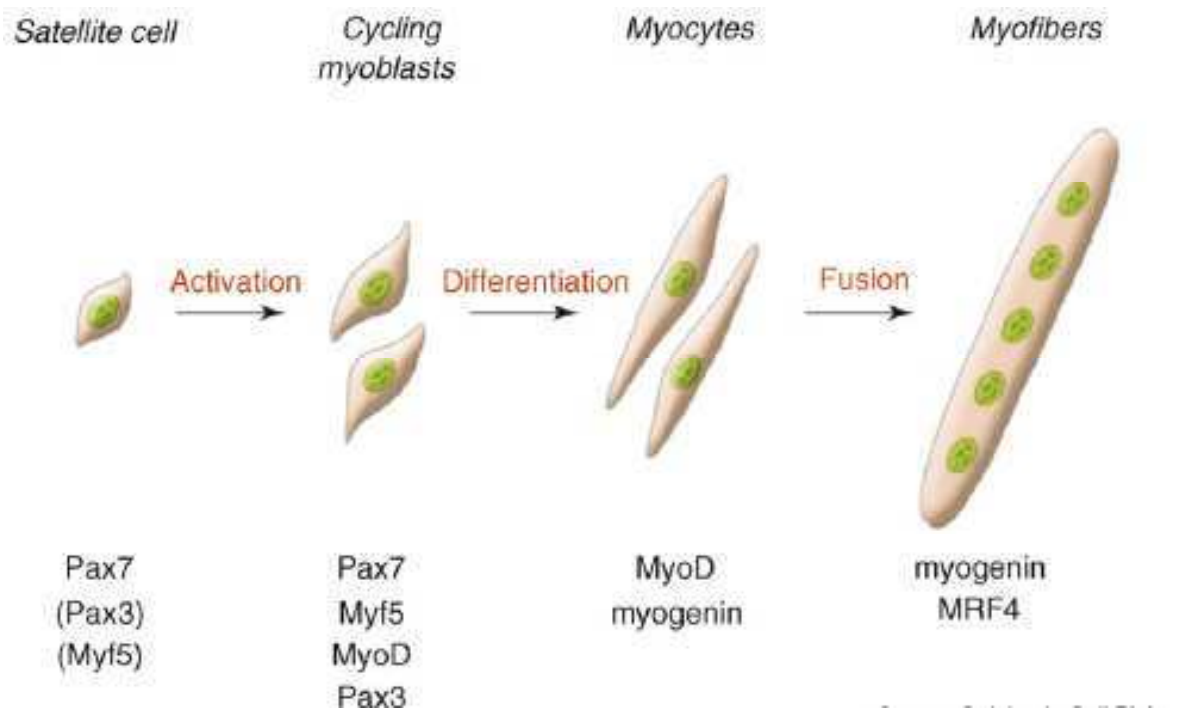


Figure 1-2: Schematic representations of adult myogenesis (Le Grand and Rudnicki, 2007). Skeletal muscle cell becomes activated. Their proliferating progeny; the skeletal myoblast, express the paired-box transcriptions factor Pax7 and Pax3, as well as the myogenic regulatory factors, Myf5 and MyoD. Once committed to differentiation; myoblast stop cycling and stop expressing of Pax7, Pax3 and Myf5. Differentiating myocytes will then align and fuse to form multinucleated myofibers.

Myogenic precursors are specifically committed depending on the function of myogenic transcription factors such as Pax3 and Pax7 (Goulding *et al.*, 1994; Cossu *et al.*, 1996a; Cossu *et al.*, 1996b; Borycki *et al.*, 1999). Once committed, somite-derived cells migrate to multiple sites of embryonic myogenesis and begin to express the myogenic basic helix-loop-helix transcription factors Myf-5 and MyoD (Birchmeier and Brohmann, 2000). Once committed to differentiation, myoblasts stop cycling and lose expression of pax-3, pax-7 and Myf-5. Differentiating myogenitive myocytes will then align and fuse to multinucleated myofibers. MRF-4 is further involved in hypertrophy of the new fibers.

1.4 Skeletal muscle fiber structure

A myofiber is a multinucleated single muscle cell. The cell is densely packed with contractile proteins, energy stores where many are involved in different signaling mechanisms. Within each myofiber is a network of myofibrils. They contain the proteins that do the actual force production. Each myofibril is constructed by arrays of parallel filaments: the thick filaments composed of myosin extend from M line produce the dark A band. The thin filaments composed of actin extend in each direction and form the Z line produce the light I band. The entire array of thick and thin filaments between 2 Z lines is called sarcomere, which is a unit of muscle contraction (Figure 1-3).

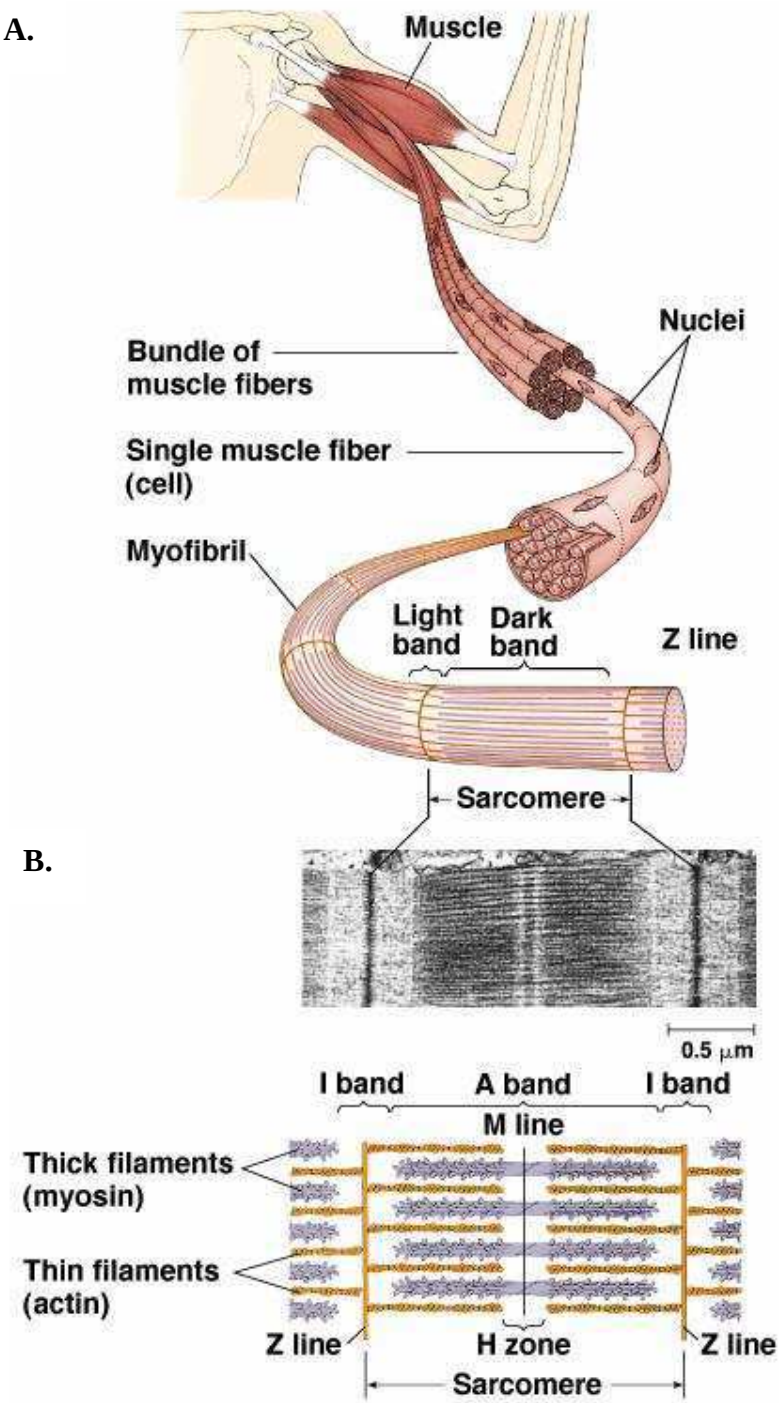


Figure 1-3: Skeletal Muscle Structure.

(http://fajerpc.magnet.fsu.edu/)

(A). A muscle fiber is multi-nucleated single muscle cell, which consist of myofibrils.

(B). Sarcomere of myofibril. It presents the arrangement of actin and myosin filaments in a single sarcomere.

Activation of the muscle fiber causes the myosin heads to bind to actin. An allosteric change occurs which draws the thin filament a short distance past the thick filament. Then the linkages break (for which ATP is needed) and reform farther along the thin filament to repeat the process.

Together with myosin, actin, tropomyosin and troponin construct over three-quarters of the protein in muscle fibers. Some two dozen additional proteins complete the rest. These serve functions as attaching and organizing the filaments in the sarcomere and connecting the sarcomere to the plasma membrane and the extra-cellular matrix.

1.5 The Muscular Dystrophies (MD)

Muscular dystrophy refers to a group of genetic, hereditary muscle diseases that cause progressive muscle weakness. Muscular dystrophies are characterized by progressive skeletal muscle weakness, defects in muscle proteins, and the death of muscle cells and tissues. (Emery, 2002). Some diseases including congenital, limb girdle, distal, myotonic, oculopharyngeal, Duchenne, Becker, etc, are always classified as muscular dystrophy.

Many forms of congenital muscular dystrophies are caused by defects in proteins that are thought to have some relationship to the dystrophin-glycoprotein complex and to the connections between muscle cells and their surrounding cellular structure. Some congenital muscular dystrophy show severe brain malformations, such as lissencephaly and hydrocephalus (Schachter *et al.,* 2004).

Limb-girdle muscular dystrophy is also called LGMD. Many forms of LGMD have been identified, showing different patterns of inheritance (autosomal recessive vs. autosomal dominant). Some of the recessive forms have been associated with defects in proteins that make up the dystrophin-glycoprotein complex (Guglieri *et al.*, 2008).

Miyoshi myopathy, one of the distal muscular dystrophies, causes initial weakness in the calf muscles, and is caused by defects in the same gene responsible for one form of LGMD (Limb Girdle Muscular Dystrophy) (Bashir *et al.*, 1994).

Myotonic dystrophy results from the expansion of a short repeat in the DNA sequence (CTG in one gene or CCTG in another gene). While the exact mechanism of action is not known, this molecular change may interfere with the production of important muscle proteins (Bachinski *et al.*, 2003).

Oculopharyngeal MD has been attributed to a short repeat expansion in the genome which regulates the translation of some genes into functional proteins (Abu-Baker and Rouleau, 2007).

Chapter2 Review of Duchenne Muscular Dystrophy

2.1 Introduction

Duchenne muscular dystrophy (DMD) is a severe X-linked recessive, progressive muscle-wasting disease, affecting ~1 in 3500 boys(Emery, 1993). The symptoms usually begin to appear around the age of 5, and patients are usually confined to a wheelchair before the age of 12 and die in their late teens or early twenties usually of respiratory failure in the most severe cases. A milder form of the disease, Becker Muscular Dystrophy (BMD), has a later onset and a much longer survival. Both disorders are caused by mutations in the DMD gene that encodes a 427-kDa cytoskeletal protein called dystrophin. The vast majority of DMD mutations result in the complete absence of dystrophin, while BMD is from mutations that do not eliminate qualitatively all dystrophin production.

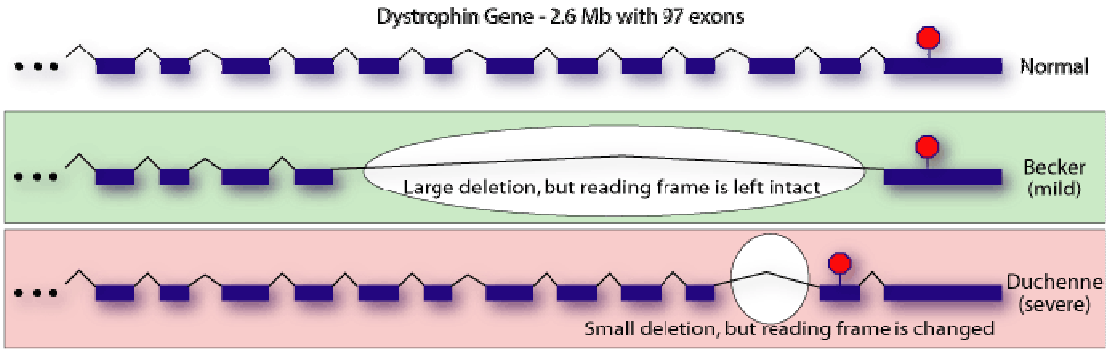


Figure 2-1: The different type of deletion of dystrophin gene. Only deletions which create a translational frame shift will lead to low levels of dystrophin expression in a DMD phenotype (Monaco *et al.*, 1988). Otherwise a smaller, but functional version of dystrophin could be produced, which would be consistent with a BMD phenotype.

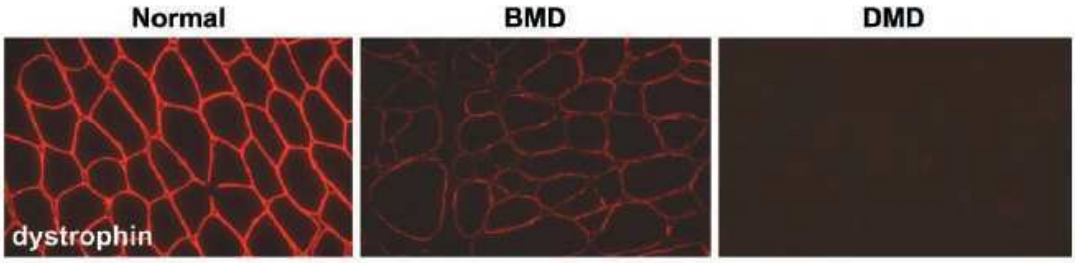


Figure 2-2: Dystrophin in normal and dystrophic muscle & Immunohistochemistry visualization of dystrophin in human muscle (Blake *et al.*, 2002). The images were taken from healthy controls and patients with Becker's and Duchenne's muscular dystrophy.

In addition to these diseases, mutations in the gene encoding many components of the dystrophin-associated protein complex (DAPC) can also cause the phenotype which is similar to that seen with DMD and BMD mutations. However, mutations in these genes produce only muscular dystrophy and cardiomyopathy, but not affect the central nervous system or smooth muscle involvement disorders that caused by DMD or BMD.

There is no effective therapy for DMD currently, although various strategies are being developed, including gene correction strategies, gene replacement strategies, etc.

2.2 Dystrophin: gene and protein

2.2.1 Gene character

The dystrophin gene was identified in 1986 on X chromosome, spanning 2.5 million base pairs and is composed of 80 exons. It is located on the short (p) arm of the X-chromosome at position 21.2(Boyd and Buckle, 1986).

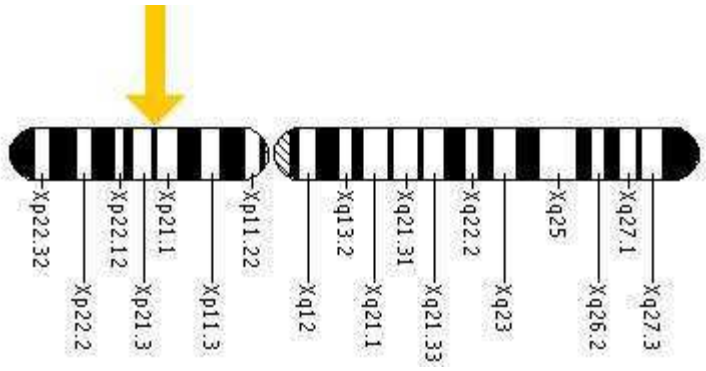


Figure 2-3: Cytogenesis Location: Xp21.2. (http://ghr.nlm.nih.gov/gene=dmd)
Molecular Location on the X chromosome: base pairs 31,047,265 to 33,267,646

There are 3 active promoters at the 5'-end of the gene and additional internal promoters (depicted by arrows) that drive expression of smaller amino-terminally truncated gene products. The three products are designated Dp427 (Brain), Dp427 (Muscle), and Dp427 (Purkinje cells) to reflect their tissue-specific expression pattern. The smaller isoforms are produced from distally located promoters expressed in the retina (R: Dp260), brain (B3:Dp140); Schwann cells (S: Dp116) or are general (G: Dp71) ubiquitously expressed.

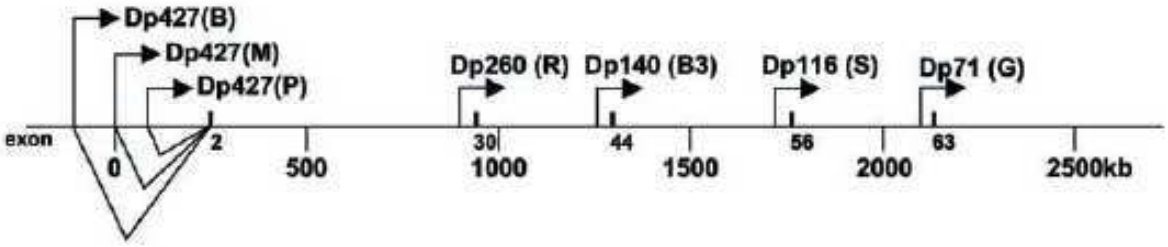


Figure 2-4: Schematic showing the expression pattern of dystrophin gene (Blake *et al.*, 2002).

Gene deletion testing is the basis of clinical diagnosis but detects only 60% of mutations (Dent *et al.*, 2005). The remaining 40% of cases involve missense/nonsense mutations or small insertions or deletions.

2.2.2 Dystrophin protein

Dystrohin protein is a 427-kDa cytoskeleton protein that is a member of the β-spectrin/α-actinin protein family (Koenig *et al.*, 1988). The identifiable domains in the cysteine-rich region and COOH terminus (CYS) of dystrophin are identified. Dystrophin is organized into four separate regions based on sequence homologies and protein-binding capabilities. They are actin binding-domain at NH2 terminus; the central rod domain; the cysteine-rich domain (CYS) and the COOH-terminal domain (CT). The identifiable domains in the CYS and CT region are WW domain, the EF hands, the ZZ domain, and the paired coiled-coil (CC). The amino-terminal actin-binding domain is responsible for anchoring dystrophin to cytoskeletal, filamentous γ-actin (Rybakova *et al.*, 2000). The central rod domain of dystrophin consists of 24 spectrin-like repeats. Like other spectrin repeats, three helix bundles align to form each repeat unit and provide structural stiffness. Flexibility of the rod region is thought to derive from breaks in the spectrin repeat pattern at four hinge regions. At the COOH-terminus, the CYS and CT region interact with the intracellular portion of the trans-membrane protein β-dystroglycan (DG), syntrophin (SYN), and the dystrobrevin. The organization of utrophin protein is very similar to dystrophin, whereas the DRP2 and the dystrobrevin proteins only have sequences similar to COOH-terminal of dystrophin.

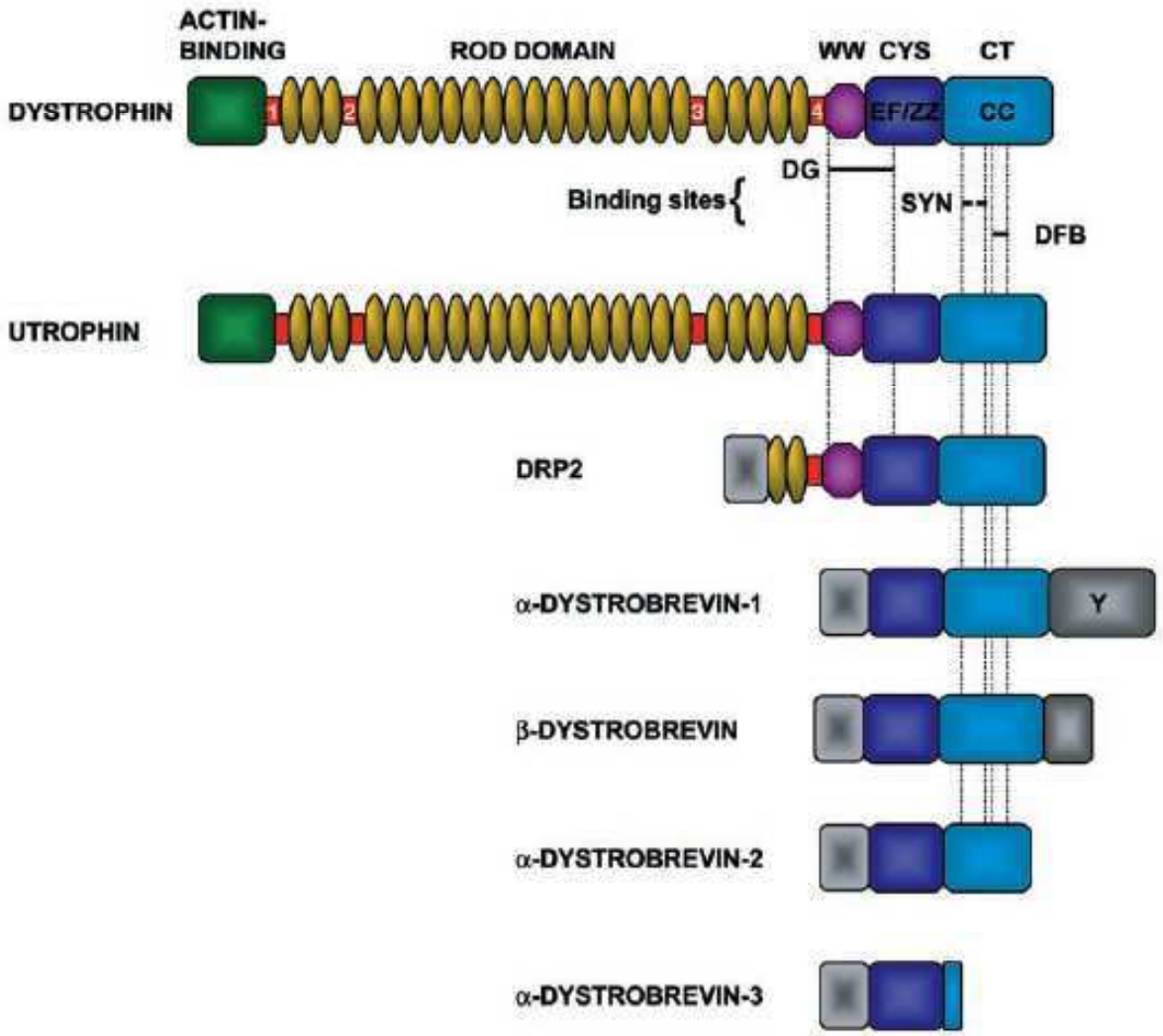


Figure 2-5: Structure of dystrophin and related proteins (Blake *et al.*, 2002). Utrophin is highly similar to dystrophin. DRP2 and the dystrobrevin proteins only have sequence similar to the COOH-terminal region of dystrophin as shown. The α-dystrobrevin-1 isoform has an additional site for tyrosine phosphorylation (Y) in C-terminal.

2.3 Dystrophin Associated Protein Complex (DAPC)-Protein and Location

The DAPC is composed of trans-membrane, cytoplasmic and extra cellular proteins. The DAPC links the extra-cellular matrix (ECM) to the actin network underlying the muscle membrane. A disruption of this link is thought to cause the myopathy observed in DMD

patients. With the emergence of data on numerous and diverse components of the protein complex and how they interact with each other, it has become increasingly clear that the DAPC holds both structural and signal transduction properties. Known components of DAPC include dystrophin, sarcoglycan, dystroglycan, dystrobrevin, syntrophin, and sarcospan.

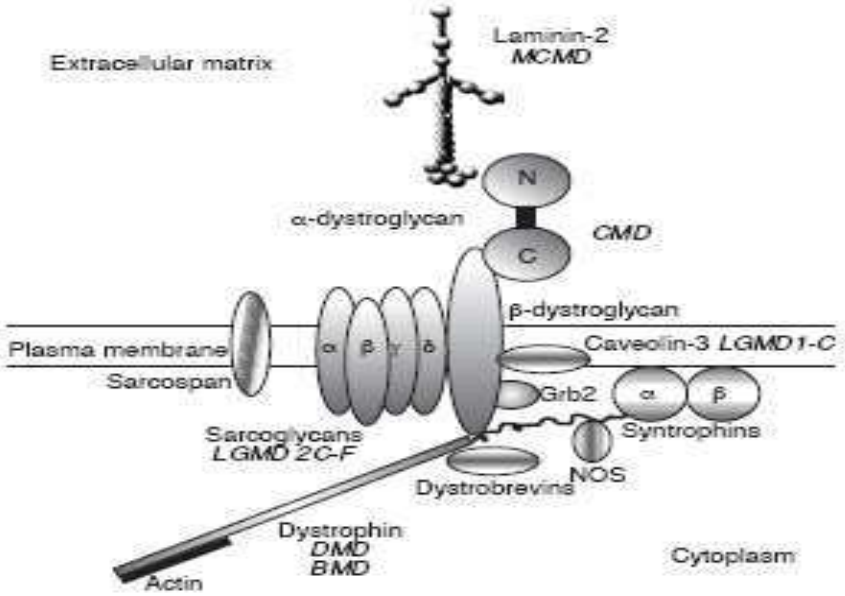


Figure 2-6: The interaction of DAPC (Sciandra *et al.*, 2003). The integral components of the DAPC include: dystrophin, dystrobrevin, sarcospan, dystroglycan, syntrophin; other proteins are associated with the DAPC: NOS, Grb2, Caveolin-3, etc.

2.3.1 Dystroglycan and the Dystroglycan Complex

Dystroglycan is a core protein of the DAPC because it connects the cytoskeletal components of DAPC to the extra cellular matrix (Henry and Campbell, 1999). The protein is produced from a single gene and is posttranslational cleaved to produce α and β subunits. α-dystroglycan forms an important connection to the extra cellular matrix through its interactions with the α2 chain of laminin 2. There are studies also reveal the interactions between α-dystroglycan and agrin, perlecan with varying affinities (Hohenester *et al.*, 1999). These interactions are Ca^{2+} dependent, and calcium is found bound to the edge of the LG5 interaction face of laminin α2-chain.

α-dystroglycan binds the N-terminus of β-dystroglycan. β-dystroglycan has a single trans-membrane domain and is inserted into the muscle plasma membrane with the COOH terminus binding to the cysteine-rich region of dystrophin (Suzuki *et al.*, 1992; Jung *et al.*, 1995; Rentschler *et al.*, 1999).

The binding partners of dystroglycan have been identified. They are: Grb2, caveolin-3, and rapsyn. Their roles in DAPC involved signal transduction will be discussed below.

2.3.2 Sarcoglycan complex and Sarcospan

In skeletal and cardiac muscles, the sarcoglycan complex is composed of six sarcoglycans: α, β, γ, δ, ε, ζ and a member of the tetraspan family of proteins called sarcospan (Lim and Campbell, 1998; Crosbie *et al.*, 2000). The presence of the sarcoglycan complex is required for the stability of sarcospan at the plasma-membrane. α-and ε-sarcoglycan belong to type I trans-membrane protein, which has a single trans-membrane stretch of hydrophobic residues with the portion of the polypeptide on the N-terminal side of the TM domain exposed on the exterior side of the membrane and the COOH-terminal portion exposed on the cytoplasmic side; while the rest of them belong to type II trans-membrane protein which are similar to the type I class in that they span the membrane only once, but they have their amino terminus on the cytoplasmic side of the cell and the carboxy terminus on the exterior. In vitro experiments, α, β, γ and δ-sarcoglycan relate to the correct assembly and trafficking of sarcoglycan complex to the membrane (Chan *et al.*, 1998; Holt and Campbell, 1998). Evidence showed over-expression of γ-sarcoglycan in mice is associated with an increase in the levels of α- and β-sarcoglycan, which causes muscular dystrophy (Zhu *et al.*, 2001).

Sarcospan-null mice maintain the proper assembly of entire DAPC and show normal muscle function and histology that is due to the highly diverse of tetraspanin family, so additional sarcospan-like proteins may accommodate the loss of sarcospan (Crawford *et al.*, 2000; Lebakken *et al.*, 2000).

It is now considered that sarcoglycan-sarcospan complex is important for anchoring or stabilizing the DAPC in sarcolemma. Both α-sarcoglycan-null mouse and γ-sarcoglycan-null

mouse display the depleted levels of dystroglycan, but the normal localization of dystrophin and laminin (Iwata *et al.*, 1993; Roberds *et al.*, 1993; Hack *et al.*, 1998; Straub *et al.*, 1998).

2.3.3 Syntrophins

The syntrophin family has five members: α, β1, β2, γ1, γ2. They have a similar domain structure consisting of a split PH (pleck-strin homology) domain and intact PH domain, a PDZ domain, and the syntrophin unique region at the COOH terminus (Adams *et al.*, 1993; Ahn *et al.*, 1994; Ahn *et al.*, 1996; Piluso *et al.*, 2000).

They are potentially involved in a number of cellular functions. The syntrophin interacts different molecular mostly with PDZ domain. It binds dystrophin and α-dystrobrevin in their COOH-terminal but binding nNOS between the peptide groove of the PDZ domain in α-syntrophin and β-hairpin finger of nNOS (Hillier *et al.*, 1999; Tochio *et al.*, 1999; Newey *et al.*, 2000). Besides, sytrophin also binds SAPK3, voltage-gated sodium channel, calmodulin, SAST/MAST205, and Erb4 according to yeast two-hybrid research (Newbell *et al.*, 1997; Gee *et al.*, 1998; Iwata *et al.*, 1998; Hasegawa *et al.*, 1999; Lumeng *et al.*, 1999; Garcia *et al.*, 2000).

2.3.4 Dystrobrevin

The dystrobrevin family is encoded by 2 different genes, located in human chromosome 2, 18 respectively; correspond to α- and β-dystrobrevin. α-dystrobrevin gene encodes at least 5 different protein isotypes (Blake *et al.*, 1996; Sadoulet-Puccio *et al.*, 1996). α-dystrobrevin-1 and -2 are found in neuromuscular junction and sarcolemma (Metzinger *et al.*, 1997; Nawrotzki *et al.*, 1998; Peters *et al.*, 1998). α-dystrobrevin-3 is a component of skeletal and cardiac muscle, location unknown. More evidences suggest that α-dystrobrevin is associated with the sarcoglycan complex and mediated by the N-terminal region, anchoring all three dystrobrevin isoforms to the DAPC (Yoshida *et al.*, 2000).

α-dystrobrevin binds directly to dystrophin with coil-coil domain. α-dystrobrevin deficient mice displayed mild muscular dystrophy without perturbing the assembly of the other components of DAPC at the sarcolemma. Transgenic *mdx* mice expressing dystrophin lacking dystrobrevin binding domains displayed normal muscle function (Crawford *et al.*, 2000). Taken together, dystrobrevin can bind to DAPC without dystrophin and to prevent the development of a myopathy.

2.4 Dystrophin Associated Protein Complex (DAPC)-Binding Partners and related Signaling Pathway

Many studies suggest that muscular dystrophies are related to the changed signal pathway that caused muscle cell death. The DAPC thus plays an essential role in those pathways. First, people found that DAPC or their homolog can express either muscle specific or non-muscle tissues, which suggesting a general function (Durbeej and Campbell, 1999; Kachinsky *et al.*, 1999; Ort *et al.*, 2000). Second, numerous signaling molecules are associated with DAPC as mentioned above and the list continues to expand. Interestingly, their mutations cause muscular dystrophies. People suppose that the muscular dystrophies arise from defects in enzymes or the disruption of the DAPC structure, but finally, the finding that apoptosis is an early feature of muscular dystrophies due to DAPC defects (Tidball *et al.*, 1995; Tews and Goebel, 1997) supports that DAPC may be a part of the signaling pathway that regulates cell survival and cell death.

Table1 2-1: DAPC and their binding partners involved in signaling pathways

DAPC Component	Binding Partner	Role of Signal Pathway in Muscle
Dystrophin	F-actin	Structural stability
	Aciculin	provide an additional cytoskeletal-matrix trans-membrane link
	calmodulin	regulatory of phosphorylation, probably involved in apoptosis
Dystrobrevin	Dysbindin	Anchoring dystrobrevin to DAPC
	Syncoilin Desmuslin	provide a link between DAPC and intermediate filament cytoskeletal network in muscle
α-dystroglycan β-dystroglycan	Agrin	infects the stabilization of the AchR cluster and the formation of the specialized extra¬junction sarcolemma
	Perlecan Laminin	provide a link between extra-cellular matrix and DAPC
	Grb2	represent a link between DAPC defects and apoptotic muscle cell death via Ras/MAPK signaling pathway
	Rapsyn	required for the clustering of AchR at the developing neuromuscular junction
	Caveolin-3	critical for the localization of Src
α¬syntrophin	nNOS	mediate cGMP production and regulates diverse physiological response, regulate cell death
	Calmodulin	participate in regulation of muscle cell survival with calcium-calmodulin-dependent protein kinase (Cam Kinase II)
β-syntrophin	SAPK3	ND
	Voltage-gated sodium channels	ND
	MAST205/SAST	ND
	ErbB4	ND

2.4.1 Pathway with Dystrophin

Dystrophin is the target of a variety of proline-direct and serine/theronine kinase (Luise *et al.*, 1993; Milner *et al.*, 1993; Walsh *et al.*, 1995). There are many potential phosphorylation sites in the C-terminal region of the protein. The phosphorylation of dystrophin changes its affinity for actin (Senter *et al.*, 1995). The physiological significance of the phosphorylation of dystrophin and whether this plays a role in signal transduction or the recruitment of signaling molecules to the membrane has yet to be determined. Besides its binding actin and DAPC proteins, dystrophin also binds 3 other molecules:

A. Aciculin

Aciculin is a 60-kDa cytoskeletal protein, highly homologous to the glycolytic enzyme phosphoglucomutase type 1. Aciculin expression in skeletal muscle is developmentally regulated, and this protein is particularly enriched at cell-matrix adherens junctions of muscle cells (Belkin and Burridge, 1994). Dystrophin was shown to be a major aciculin-associated protein in skeletal muscle. Association of aciculin with dystrophin (utrophin) in various cell types might provide an additional cytoskeletal-matrix trans-membrane link at sites where actin filaments terminate at the plasma membrane.

B. Calmodulin

Calmodulin binding sites have been found both in dystrophin and syntrophins (Anderson *et al.*, 1996). Calmodulin binds to dystrophin sequence 1-385 in a Ca^{2+}-dependent manner and competitively inhibits F-actin binding (Jarrett and Foster, 1995).

The interaction regulates not only dystrophin-actin interaction, but also the components of DAPC. The binding of calmodulin to DAPC proteins is particularly relevant to signaling functions because of the association of a calcium-calmodulin-dependent protein kinase (Cam Kinase II) with the DAPC. CaM Kinase II phosphorylate a site within the C-terminals of dystrophin which inhibits their syntrophin binding in vitro, suggesting a regulatory role for phosphorylation (Madhavan and Jarrett, 1999). Many protein kinases, including CaM Kinase

II, participate in cellular processes that regulate apoptosis (Franklin and McCubrey, 2000). The DAPC may serve as a scaffold for calmodulin and CaM Kinase II involved in the regulation of muscle cell survival.

C. α-actinin-2

α-actinin-2 was found by screening the human skeletal muscle yeast two-hybrid cDNA library with the carboxyl-terminal region (the last 200 amino acids) of dystrophin. This is the first identification of a direct interaction between α-actinin-2 and C-terminal of dystrophin (Hance *et al.*, 1999).

2.4.2 Pathway with Dystrobrevin

There are several tyrosine kinase consensus sites in the C-terminal region of certain dystrobrevin isoforms. This suggests that the protein-protein interactions may be modulated by tyrosine phosphorylation (Balasubramanian *et al.*, 1998). Specially, dystrobrevin phosphorylation has the prosperity to increase its affinity for other dystrophin family proteins via coiled-coil motifs.

The precise role of the dystrobrevin in relation to DAPC at the sarcolemma is unclear, but they are proposed to play a role in intra-cellular signal transduction in this context by studying its binding partners: dysbindin (Benson *et al.*, 2001), syncoilin (Newey *et al.*, 2001) , desmuslin (Mizuno *et al.*, 2001).

Dysbindin is an evolutionary conserved 40-kDa coiled-coil-containing protein that binds to alpha- and beta-dystrobrevin in muscle and brain. Dysbindin co-localizes with alpha-dystrobrevin at the sarcolemma and is up-regulated in dystrophin-deficient muscle. It may provide an alternative route for anchoring dystrobrevin and the DPC to the muscle membrane. Considering syncoilin and desmuslin are both predicted to be intermediate filament protein,α-dystrobrevin may provide a link between DAPC and intermediate filament cytoskeletal network in muscle.

2.4.3 Pathway with Dystroglycans

The dystroglycan complex plays an important role in muscle by acting as a receptor for the extra-cellular matrix protein. Dystroglycan involves in many cellular processes, such as epithelial development and viral adherence/infection and neuromuscular junction formation (Chamberlain, 1999; Henry and Campbell, 1999). The binding partners of dystroglycan have been identified and listed below:

A. Agrin

The organization of the extra-cellular matrix appears to be a feature of dystroglycan function, based on the studies on agrin, which infects the stabilization of the AchR cluster and the formation of the specialized extra−junction sarcolemma (Jacobson *et al.*, 2001).

B. Grb2

Grb2 is a 25-kDa protein composed of a Src homology 2 (SH2) domains flanked by two Src homology 3 (SH3) domains and participates in signal transduction pathways involved in receptor tyrosine kinases and Ras signaling (Lowenstein *et al.*, 1992; Chardin *et al.*, 1995). Grb2 binds to praline-rich sequences in the COOH-terminus of β-dystroglycan. Through this interaction, β-dystroglycan can participate in the transduction of extra−cellular-mediated signals to the muscle cytoskeleton (Yang *et al.*, 1995). As disruption of DAPC leads to muscle cell apoptosis, where muscle cell attachment to the EMC is perturbed, Grb2 may represent a link between DAPC defects and apoptotic muscle cell death via Ras/MAPK signaling pathway (Tidball *et al.*, 1995; Tews and Goebel, 1997).

C. Caveolin-3

Caveolae are involved in many cellular processes. Its characteristic protein coat, caveolin, demonstrates the target to the plasma membrane is required for normal caveolar formation (Park *et al.*, 2002). In the three caveolin isoforms, caveolin-3 is a muscle-specific isoforms (Tang *et al.*, 1996). Caveolae are thought to be docking sites for signaling proteins, and

caveolin-3 is critical for the localization of Src (Smythe *et al.*, 2003), which is involved in apoptosis. The study showed the absence of caveolin-3 increases myofiber apoptosis by causing accumulation of Src.

D. Rapsyn

The 43 kDa AchR-associated protein rapsyn is required for the clustering of AchR at the developing neuromuscular junction (Cartaud *et al.*, 1998). The hypothesis is that rapsyn may be a molecular link connecting the AchR to the DAPC (Apel *et al.*, 1995). Activation of AchRs leads to calcium influx via the ligand-gated ion channel (i.e.,AchRs) or indirectly via sodium influx-stimulated calcium from intracellular stores （Allard et al.,1996; Cherednichenko et al., 2004）.

Interestingly, a recent study shows that the AChR-associated protein rapsyn interacted with calpain in an agrin-dependent manner, and this interaction inhibited the protease activity of calpain (Chen *et al.*, 2007). Calpains are a family of cytosolic, Ca^{2+}-activated proteases that may prove significant in cell death (necrosis and/or apoptosis) associated with increased intracellular Ca^{2+}. Cholinergic stimulation increases the activity of calpain in muscle cells.

2.4.4 Pathway with Syntrophins

Syntrophin is phosphorylated by a calcium-calmodulin-dependent protein kinase (CaM Kinase II) and by stress-activated protein kinase-3 (SAPK-3) (Hasegawa *et al.*, 1999; Madhavan and Jarrett, 1999).

A. nNOS

Activity of the enzyme nitric oxide synthase (nNOS) leads to the production of NO, which modulates many intracellular signaling pathways. nNOS is located beneath the sarcolemma of skeletal muscle fibers. A clinically relevant aspect of nNOS is its absence from the skeletal muscle sarcolemma of patients with Duchenne muscular dystrophy (DMD). A concept is presented which suggests that, as a consequence of the disruption of the dystrophin-

glyoprotein complex in DMD; nNOS fails to become attached to the sarcolemma and is subject to downregulation in the cytosol. In muscle, NO mediated cGMP production and regulates diverse physiological response, such as contractile function, glucose metabolism, and calcium mobilization (Grozdanovic and Baumgarten, 1999). A potential role of nNOS in the pathogenesis of DMD is its regulation of vascular smooth muscle tone (Thomas and Victor, 1998; Sander *et al.*, 2000). A hypothesis supposed that DAPC defects and nNOS deficiency lead to muscle cell death. NO itself has been implicated as a regulator of apoptotic and necrotic cell death, suggesting that alteration in nNOS might be helpful to disease pathogenesis and another potential link between DAPC and DMD/BMD (Brune *et al.*, 1999).

2.5 Utrophin

Utrophin is an autosomal paralogue of dystrophin. Over-expression of Utrophin as a transgene in the *mdx* mouse (the mouse model of DMD) has demonstrated that Utrophin can prevent the muscle pathology.

2.5.1 The Utrophin gene and protein

The utrophin gene has a large genomic region about ~1Mb with multiple small exons (Pearce *et al.*, 1993). The cDNA of utrophin derives from an autosomal gene (chromosome 6 in humans, 10 in mice) (Buckle *et al.*, 1990). It is 13kb long and encodes a protein with 3433 amino acids and a predicted molecular mass of 395kDa (Tinsley *et al.*, 1992). The structure of utrophin protein is similar to dystrophin protein (Figure 2-5).

2.5.2 Functional Domains and Binding Partners of Utrophin

The NH2 terminus of utrophin binds to actin, similar to dystrophin but has some differences: utrophin lacks the additional actin-binding activity associated with the dystrophin rod domain (Amann *et al.*, 1999); the NH2 terminus of utrophin contains a short extension not exist in dystrophin (Moores and Kendrick-Jones, 2000).

The COOH-terminal of utrophin binds members of DAPC (Tinsley *et al.*, 1992), such as β-dystroglycan, α-dystrobrevin-1, syntrophins, and sarcoglycans.

2.5.3 The Utrophin Function

Considering the similarity of structure and binding domains between utrophin and dystrophin, the hypothesis that utrophin is sufficiently like dystrophin to substiture for it and ameliorate the pathology of muscular dystrophies has been tested by generating several lines of *mdx* mice harboring utrophin trans-genes (Tinsley *et al.*, 1996; Tinsley *et al.*, 1998). These studies show that increased muscle fiber utrophin significantly reduces pathology, and abnormalities of calcium homeostasis and membrane permeability are also corrected.

2.6 Three hypotheses about the mechanism of DMD

2.6.1 The structural hypothesis

According to the location of DAPC in the trans-membrane of muscle cell, one hypothesis for explaining how the absence of dystrophin leads to the extensive muscle necrosis characteristic of late-stage DMD indicates that dystrophin plays an important structural role in ensuring that the membrane stresses associated with muscle contraction and tension development do not lead to membrane rupture and subsequent muscle necrosis (Engel and Franzini-Armstrong, 1994).

It suggests that periodic membrane ruptures in dystrophic muscle result in the loss of muscle enzymes and an exchange with extracellular fluids that would alter intracellular ionic concentrations and thereby eventually lead to cell death (Elbrink and Malhotra, 1985). Focal lesions in the plasma membrane overlying wedge-shaped defects in muscle fibers (“delta lesions”) are an early pathological change in Duchenne muscular dystrophy (DMD). Abnormalities in the plasma membrane have been suggested as a cause of these lesions and of the degeneration of muscle fibers in DMD. The presence of membrane “delta lesions”, which identified DMD as a membrane disease prior to the discovery of the cytoskeletal protein

dystrophin (Engel and Franzini-Armstrong, 1994), is partly support the hypothesis. Recently, evidence found in *C. elegans* shows that the disruption of the muscle cell adhesion is probably an early event during muscle degeneration caused by absent of dystrophin (Lecroisey *et al.*, 2008)

2.6.2 The Ion Channel hypothesis

Regarding to the subsarcolemmal localization of dystrophin, it is reasonable to enquire if the distribution and function of ion channels might be affected by the absence of dystrophin. This hypothesis considers the dystrophic process as a consequence of the loss of intracellular calcium homeostasis and the subsequent activation of autoproteolytic activities in dystrophin-deficient fibres (Duncan, 1978; Turner *et al.*, 1988). Ca^{2+} handling in skeletal muscle is tightly controlled by the membrane potential which is set by sarcolemmal ion channels activity.

Mariol MC *et al* observed that in *Caenorhabditis elegans*, a gain-of-function mutation in the *egl-19* calcium channel gene dramatically increases muscle degeneration in dystrophin mutants. Conversely, RNAi-mediated inhibition of *egl-19* function reduces muscle degeneration by half. Therefore, it demonstrates that calcium channel activity is a critical factor in the progression of dystrophin-dependent muscle degeneration in *C.elegans* (Mariol and Segalat, 2001). Up to now, however, no one has been able to determine whether the increased calcium influx through these channels is the cause of- or just an associated phenomenon with the degeneration process.

2.6.3 The Signal Transduction hypothesis

As discussed above, the DAPC provides a means of communicating from the extra-cellular matrix to the cytoskeleton and the members of this protein complex are related to many signal pathways.

Among these pathways, that relevant to muscle atrophy are focused on, such as Grb2 and calmodulin which are mentioned above; even dystrophin itself is capable of mediating signals

related to muscle atrophy and hypertrophy (Acharyya *et al.*, 2005). There is a growing body of literature demonstrating that alterations of normal signal transduction pathways play critical roles in the pathogenesis of various muscular dystrophies (Rando, 2001), but there is no evidence that a signal pathway predominates or that there is a final common pathway for cell death in the dystrophies.

Further studies will be necessary to examine the interactions among the various signal transduction pathways to determine the relative contribution of these pathways to the pathogenetic mechanisms of muscular dystrophies.

Chapter3 Overview of Protein Kinase C

3.1 Introduction

Protein kinases are kinase enzymes that modify other proteins by removing a phosphate group from ATP and covalently attaching it to a protein that have a free hydroxyl group (phosphorylation).

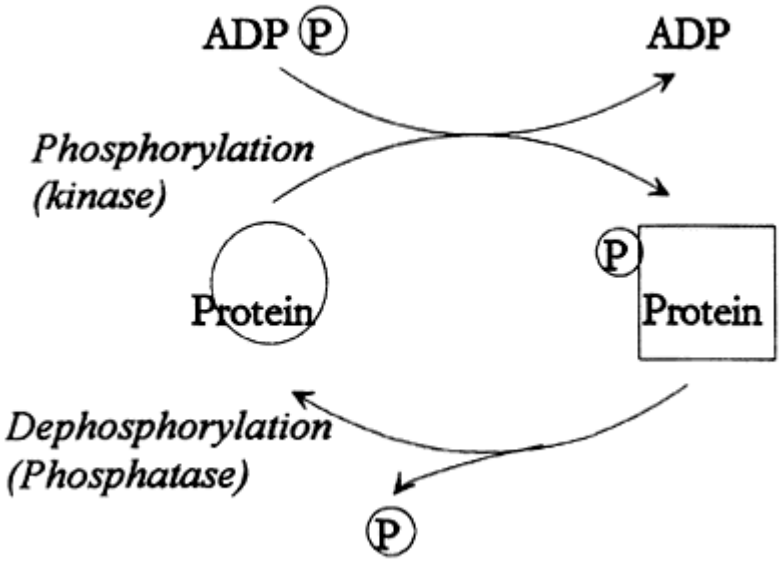


Figure 3-1: Reversible protein phosphorylation.
(http://nobelprize.org/nobel_prizes/medicine/laureates/1992/press.html) A protein kinase moves a phosphate group (P) from ATP (ADP (P)) to the protein. The biological properties of the protein are thereby altered. There is also a protein phosphatase that is able to remove the phosphate group. The amount of phosphate that is associated with the protein is thus determined by the relative activities of the kinase and the phosphatase.

Phosphorylation usually results in a functional change of the target protein (substrate) by changing enzyme activity, cellular location, or association with other proteins. Kinases are known to regulate the majority of cellular pathways, especially those known involved in signal transduction, the transmission of signal within the cell.

Protein kinase C belongs to serine/threonine specific protein kinases, which phosphorylates the OH group of serine or threonine. Activity of these protein kinases can be regulated by specific events (e.g. DNA damage), as well as numerous chemical signals, including cAMP/cGMP, diacylglycerol (DAG), and Ca^{2+}/calmodulin.

3.2 Protein Kinase C Super family

Currently, at least 12 PKC isozymes have been cloned (Hayashi *et al.*, 1999), and individual isozymes have been implicated in such diverse cellular responses as extra-cellular stimuli, modulate contractile properties, and promote cell growth and survival. The rapid growth of knowledge in the area of PKC signaling and PKC substrate interactions, may further our understanding on the muscle diseases such as Duchenne Muscular Dystrophy.

3.2.1 PKC Isotypes

The 12 isoforms of PKC are grouped in to 3 categories based on their sequences and enzyme properties: classical/conventional PKC (α, βI, βII, γ); novel PKC (δ, ε, θ, η/L), and atypical PKC (ζ, λ/ι) (Figure 3-2). Recently, a fourth subfamily, composed of two closely related isozymes (μ, ν), has been identified by cloning and sequence analysis (Hayashi *et al.*, 1999).

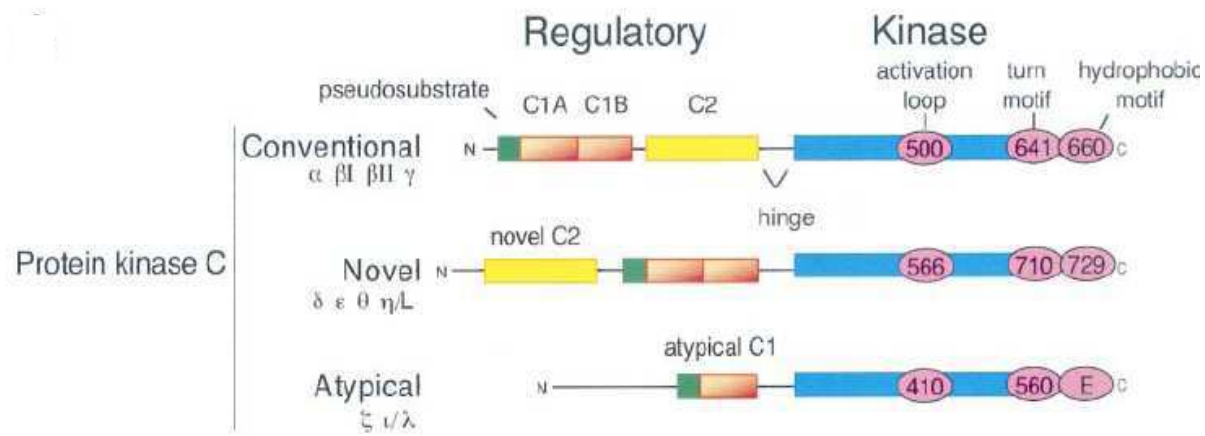


Figure 3-2: The primary structure of PKC isotypes. All kinases have a conserved kinase core (colored cyan) and C-terminal extension that contains two conserved phosphorylation sites: the turn motif and hydrophobic motif (shown by pink circles). The kinases vary in the nature of the regulatory moiety. PKC isoenzymes differ primarily in the composition of membrane targeting modules in the regulatory moiety. All isoenzymes have an autoinhibitory pseudosubstrate sequence (shown in green) that is N-terminal to the C1 domain (colored orange); the C1 domain is a tandem repeat for conventional and novel PKCs, and functions as a diacylglycerol sensor. Atypical PKCs have an impaired C1 domain that does not respond to diacylglycerol/phorbol esters. Conventional PKCs have a C2 domain (shown in yellow) that serves as a Ca^{2+}-regulated phospholipid-binding module ; the C2 domain in novel PKCs binds neither Ca^{2+} nor membrane phospholipids. (Newton, 2003)

All PKC consist of a regulatory domain and a catalytic domain (kinase domain) tethered together by a hinge. The kinase region is highly conserved among the different isoforms that contains two conserved phosphorylation sites: the turn motif and hydrophobic motif. The regulatory domain or the N-terminus of the PKCs contains several shared sub-regions: C1, C2, and pseudo-substrate region. The second messenger requirement differences in the isoforms are a result of the regulatory region.

Conventional PKC: Members of conventional PKC possess two NH2-terminal regulatory domains (C1/C2). The C1 domain is the diacylglycerol (DAG) or phorbol ester for activation sensor and the C2 domain is the Ca^{2+} sensor (Steinberg *et al.*, 1995).

Novel PKC: Novel PKCs contain a functional C1 domain, but not a non-ligand-binding C2 domain: these isozymes respond to DAG, but not Ca^{2+} signals.

Atypical PKC: Atypical PKCs contain a non-ligand-binding C1 domain and no C2 domain and, as a consequence, respond to neither DAG nor Ca^{2+}. The C1 domain is a small globular structure (approx. 8kDa) (Bogi *et al.*, 1999). Conventional and novel PKCs have two C1 domains. The ligand-binding pocket of the C1 domain is impaired in atypical PKCs, and these isoenzymes do not respond to either DAG or phorbal esters (Hurley *et al.*, 1997). The C1 domain also specifically binds phosphatidylserine (Johnson *et al.*, 2000).

3.2.2 The Mode of Regulation of PKC

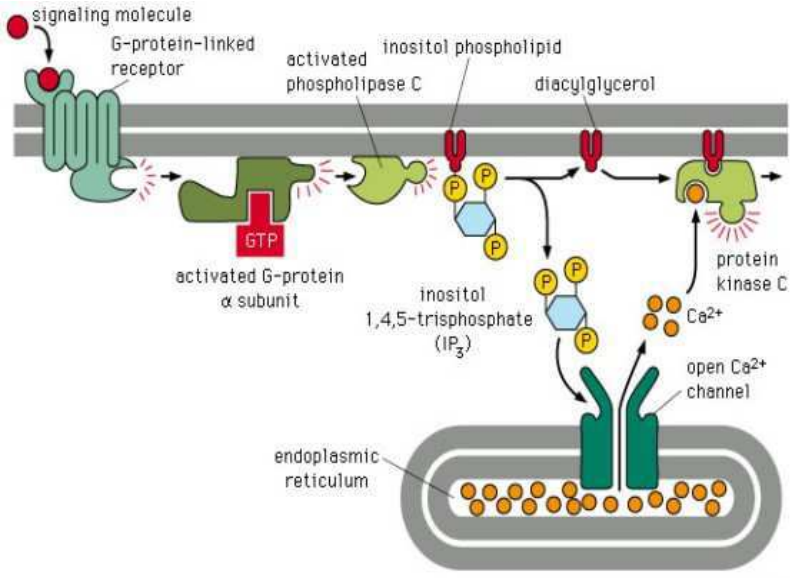


Figure 3-3: Pre-signal pathway before PKC activation process. The binding of a hormone or other effect molecules to the membrane receptor results in activation of phospholipase C (PLC) or phospholipase A2 (PLA2) via a G-protein-dependent phenomenon. The activated PLC hydrolyzes phosphatidylinositol-4, 5-bisphosphate (PIP2) to produce DAG and inositol-1, 4, 5-trisphosphate (IP3). The IP3 causes the release of endogenous Ca^{2+} that binds to the cytosolic PKC and exposes the phospholipid-binding site. The binding of Ca^{2+} translocates PKC to the membrane, where it interacts with DAG and is transformed into a fully active enzyme.

The activation of PKC occurs in three steps:

a. the phosphorylation at the activation loop of the kinase domain of the enzyme with 3-phosphoinositide-dependent protein kinase-1 (PDK-1) (Dutil *et al.*, 1998)

b. auto-phosphorylation after PDK-1 dependent phosphorylation, which is facilitated at two key COOH-terminus sites of the protein; a turn motif conserved among all PKC isozymes ($Thr^{500}Thr^{641}$) and a hydrophobic phosphorylation motif (Ser^{660}) conserved in conventional and novel PKC isozymes (Dutil *et al.*, 1998; Dempsey *et al.*, 2000). The atypical PKC isozymes differ by the insertion of an acidic Glu residue in place of Ser^{660}. The phosphorylated enzyme is then released into the cytosol where it is maintained in an inactive conformation by binding an auto-inhibitory sequence, the pseudo-substrate.

c. Membrane recruitment in response to DAG and phosphatidylserine (PS) provides the energy to expel the pseudo-substrate sequence from the substrate-binding cavity, thus activating PKC for downstream signaling (Dempsey *et al.*, 2000). On binding DAG and elevation of Ca^{2+}, the enzyme binds firmly to the membrane, the pseudo-substrate detaches from the catalytic site and the cell becomes both competent and accessible.

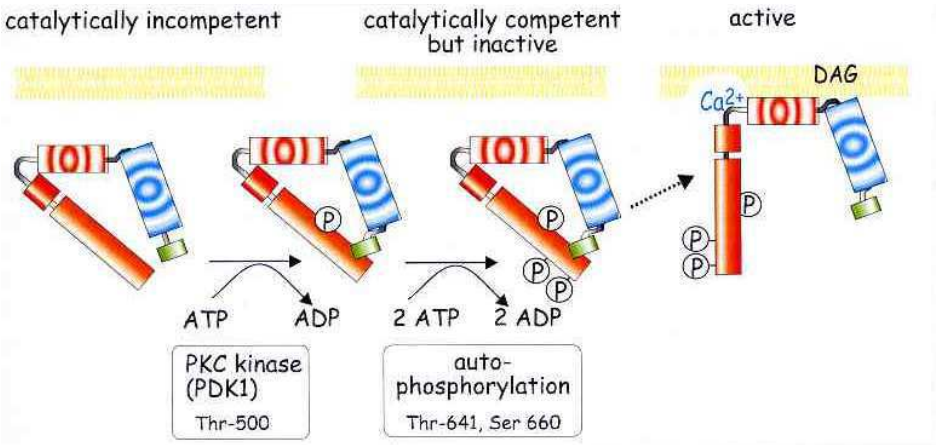


Figure3-4: Activation of PKC steps. The catalytic site is accessible to substrate but inactive. Three different phosphorylations follow, rendering the enzyme catalytically competent but still inactive. Substrate access is prevented by attachment of the pseudosubstrate to catalytic site. On binding DAG and Ca^{2+}, the enzyme binds firmly to the membrane, the pseudo-

substrate detaches from the catalytic site and the system becomes both competent and accessible.

3.3 Molecular Basis of PKC Signaling Pathway in Different Muscle Cell Types

PKC was originally identified as a phospholipids and calcium-dependent-kinase (Takai *et al.*, 1979). Consistent with their different biological functions, PKC isoforms differ in their structure, tissue distribution, sub-cellular localization, mode of activation and substrate specificity. Activation of PKC isozymes results in changes in their sub-cellular location following translocation to specific anchoring proteins. Activated PKC then phosphorylates and activates a range of "kinases". The downstream events following PKC activation are little understood, although researches in cell lines bring out some results, such as the MEK-ERK (mitogen activated protein kinase kinase-extra-cellular signal related kinase) pathway (Marshall, 1996; Cai *et al.*, 1997) and PI3K-Akt pathway (Balendran *et al.*, 2000). PKC isozymes probably form part of the multi¬protein complex that facilitates cellular signal transduction.

Within a number of pathways which PKC are involved, we will only attempt to discuss apoptotic regulatory mechanisms.

Apoptosis is mediated by 2 central pathways: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (Danial and Korsmeyer, 2004).

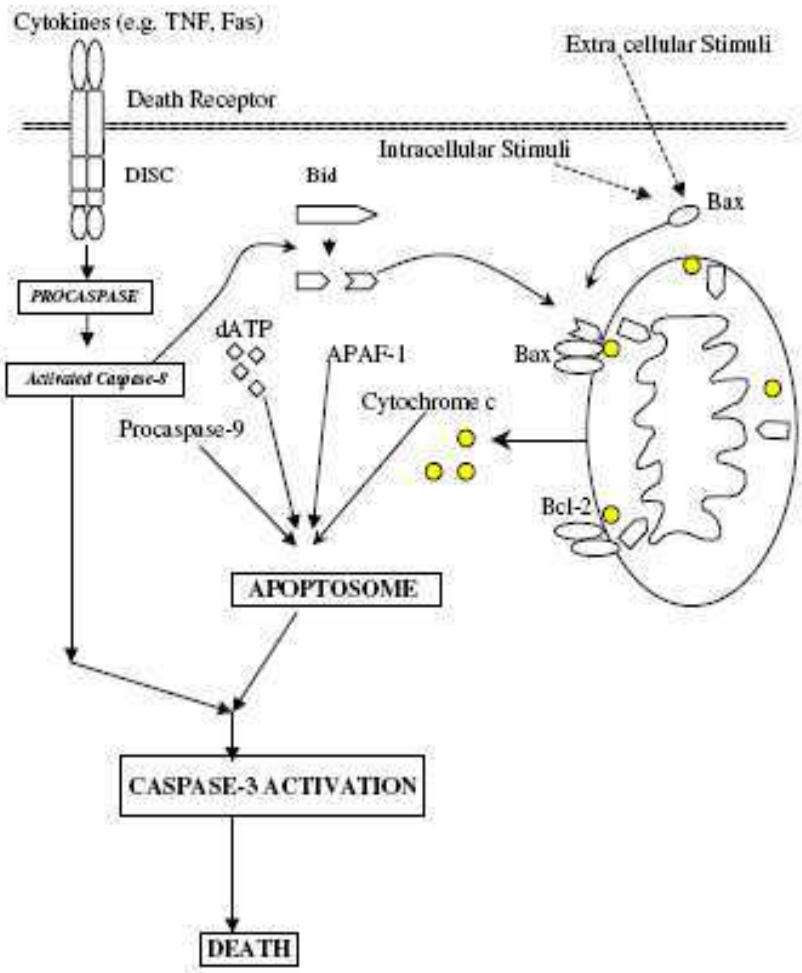


Figure 3-5: Apoptosis pathways. In the extrinsic pathway, activation of the death receptor stimulates the DISC assembly, leading to activation of procaspase-8. Activated caspase-8 then cleaves the downstream procaspase-3, resulting in proteolysis of cellular substrate and cell death. In the intrinsic or mitochondrial pathway, extra-cellular and intracellular stimuli are transimitted to the mitochondria via the Bh-3only proteins and Bax, which then translocate to the outer mitochondrial memebrane. The release of cytochrome c, Smac/DIABLO, and other apoptogens is stimulated by Bax and Bak. Cytochrome C, Apaf-1, dATP, and procaspas-9 together form the apoptosome, leading to activation of procaspase-3. Bid; a Bh3-only protein, is a direct substrate of caspase-8 and connects the extrinsic and intrinsic pathways. Cleavage of Bid releases the C-terminus end of Bid, which then translocates to and inserts into the outer mitochondrial membrane thus trigging the activation of Bax and Bak and release of cytochrome c.

Both the activation and the inhibition of PKC have been linked with the induction of apoptosis. PKC-α and PKC-βII generally seem to be anti-apoptotic (Goss *et al.*, 1994; Lee *et al.*, 1996). PKC-α phosphorylates the anti-apoptotic protein BCL-2, potentiating its anti-apoptotic function (Ruvolo *et al.*, 1998).

However, PKC-δ can be pro- or anti-apoptotic depending on the cell type or signal received (Cross *et al.*, 2000; Wert and Palfrey, 2000). Another mechanism by which the PKC family influences apoptosis is through the PI3K-Akt pathway. PKC-α, β, η directly phosphorylate Akt (Aeder *et al.*, 2004; Kawakami *et al.*, 2004; Partovian and Simons, 2004). Moreover, both PKC and Akt can phosphorylate a serine/threonine kinase involved in metabolism, development and apoptosis, glycogen synthase kinase 3β(GSK3β) (Goode *et al.*, 1992; Cross *et al.*, 1995; Fang *et al.*, 2002). Therefore, cross-talk between the PI3K and PKC pathway might influence apoptosis.

3.3.1 Roles of PKC in Cardiac Myocytes

PKC isoforms expression

Several PKC isozymes are present in the heart. The isozymes α, δ, and ε PKC are consistently detected in both neonatal and adult ventricular myocytes by many researches (Disatnik *et al.*, 1994; Puceat *et al.*, 1994; Rybin and Steinberg, 1997).η PKC is present in cultured neonatal ventricular myocytes and also in adult rat and rabbit heart extracts (Erdbrugger *et al.*, 1997; Ping *et al.*, 1997).ζ PKC has been detected in neomatal myocytes (Disatnik *et al.*, 1994), although it has been suggested that this immuno-reactivity may represent cross-reactivity with λ PKC (Rybin *et al.*, 1997). The most inconsistently detected isozyme is β PKC. The presence of βI and βII PKC has been demonstrated in neonatal rat ventricular myocytes by immuno-fluorescence (Disatnik *et al.*, 1994).

Identification of target proteins for activated cardiac PKCs isozymes

Although cardiac PKC isozymes may sub-serve different functional roles with respect to the phenotype and physiological properties expressed by muscle cells, the identity of the individual isozymes that control cardiac gene regulation and trigger diverse responses in myocytes is largely unknown. The availability of genetically engineered mice and the application of molecular pharmacology to study PKC substrate interactions have led to the identification of transcription factors (AP-1), genes (TGF-β1), and intra-cellular proteins (troponin I; TnI), that are substrates for activated PKC isozymes linking heart disease such as diabetes, myocardial fibrosis, hypertrophy, etc (Jideama *et al.*, 1996; Malhotra *et al.*, 1997; Takeishi *et al.*, 1998).

A growing body of evidence indicates that the L-type Ca^{2+} channel in cardiac muscle cells is also a target for PKC isozymes (Puri *et al.*, 1997; Kamp and Hell, 2000). Alteration in density or function of L-type Ca^{2+} channels have been implicated in a variety of cardiovascular diseases, including heart failure (Balke and Shorofsky, 1998; Richard *et al.*, 1998), and ischemic heart disease (Aggarwal and Boyden, 1995). In vitro studies have demonstrated that the α_{1c} and β_{2a} subunits of the L-type Ca^{2+} channel are substrates for PKC isozymes (Bourinet *et al.*, 1992). The ultimate effect of stimulation of PKC on I_{Ca} may be closely related to the isoforms of PKC activated by a particular signaling pathway or chemical molecules because the PKC isoforms are expressed in developmentally regulated, species-dependent, and disease-specific fashion in the heart (Puceat *et al.*, 1994; Cohen and Downey, 1996; Rybin and Steinberg, 1997; Rybin *et al.*, 1997).

In the last decade, there has been increasing evidence suggesting the importance of apoptosis in the development and progression of cardiovascular disease. The role of apoptosis in development of ischemic heart disease, hypertensive heart disease, and end-stage heart failure has been well documented (Chen *et al.*, 1994; Narula *et al.*, 1996). And there is now considerable evidence that a variety of PKC isoforms also act as major modulators of the myocytes death machinery, having both pro- and anti-apoptotic effects.

3.3.2 Roles of PKC in Smooth Muscle Cells

PKC isoforms expression

Smooth muscle cells (SMCs) express at least 8 isotypes of PKC (Itoh *et al.*, 2001). PKC-α is a universal isoforms that is expressed in almost all blood vessels tested. PKC-γ is mainly expressed in the neurons and vascular nerve ending. PKC-δ is mainly associated with the vascular cytoskeleton. PKC-ζ is a universal isoforms that has been found in many tissues. PKC-η/L has been found in the lung, skin, heart and brain while PKC-λ/ι is expressed in the ovary and testis (Kanashiro and Khalil, 1998).

Functions of PKC in Vascular Smooth Muscle (VSM)

Several studies suggest a role for PKC in VSM contraction (Nishizuka, 1992; Horowitz *et al.*, 1996; Kanashiro and Khalil, 1998; Dallas and Khalil, 2003; McNair *et al.*, 2004). PKC activation causes significant contraction in isolated vascular preparations while PKC inhibition causes significant inhibition of vascular contraction.

Increased expression/activity of PKC isoforms in VSM could cause excessive vasoconstriction as well as trophic vascular changes leading to increased vascular resistance and hypertension (Liou and Morgan, 1994; Horowitz *et al.*, 1996).

Apoptosis of VSM is a well established component of the remodeling that occurs during normal development of the circulatory system (Arends and Wyllie, 1991; Bauriedel *et al.*, 1998). The novel PKC isoforms, PKC-δ in SMCs led to accumulation as well as phosphorylation of p53, which is the tumor suppressor; this induction correlated with apoptosis (Ryer *et al.*, 2005). However, despite the importance of the SMCs apoptosis, the precise molecular mechanism underlying the regulation of apoptotic pathway in SMCs remains largely unknown.

3.3.3 Roles of PKC in Skeletal Myocytes

PKC isoforms expression

Two PKC isoforms, classical PKC-α and novel PKC-θ are enriched in skeletal muscle (Osada *et al.*, 1992). Other PKC isoforms also are confirmed in skeletal muscle, such as atypical PKC: PKC-ζ, λ, novel PKC:PKC-δ, ε(Perrini *et al.*, 2004).

Functions of PKC in skeletal muscle

According to the specific location of isozymes, the different functions are studied by the observation of their expression pattern. Novel PKC-θ is the most abundant PKC in skeletal muscle, which is localized to the neuron-muscular junction (Hilgenberg and Miles, 1995). The expression of PKC-θ is regulated by the innervation, thus here coming the hypothesis that PKC-θ plays role in maturation of neuron-muscular junction and/or the signal transduction in the synapse (Hilgenberg *et al.*, 1996).

Meanwhile, acute exercise influence the (de)phosphorylation of atypical PKC-ζ, -λ in membrane and cytosolic fraction but not novel PKC-θ, -ε(Perrini *et al.*, 2004). This finding of isoforms-specific effects on PKC shows that PKC isoforms are likely to have unique roles or abundant roles in cellular events.

Until now, little is known about what role PKC plays in skeletal muscle especially about the function that relates to skeletal muscle death, although some similar PKC involved signal pathways found in cardiac muscle perhaps mimic those in skeletal muscle, such as ERK/MAPK pathway (Schonwasser *et al.*, 1998); however, this is remains to be determined. But we have reasons to think that the mechanism of apoptosis in skeletal muscle may be distinct from the other muscle cell types. Skeletal muscle cells are multinucleated, which is called myonucleus (Cheek, 1985). And the destruction of the entire cell does not necessarily follow the elimination of a nucleus, as occurs in mononucleated cells (Rodrigues Ade and Schmalbruch, 1995).

Chapter4 *Caenorhabditis elegans* as an animal model for studying DMD

4.1 Introduction

Since Sydney Brenner introduced the soil nematode *Caenorhabditis elegans* as a model to study development and neurobiology in 1960s (Brenner, 1974), *C. elegans* is used to study a much larger variety of biological processes including apoptosis, cell signaling, cell cycle, gene regulation, metabolism, etc (http://www.wormbase.org). Many key discoveries were first made in worm. *C. elegans* has a number of features that make it a powerful tool for biological research:

First, it is easy to culture and is very low-cost.

Second, it reproduces rapidly and prolifically. Growing in 23°C, within 3 days it develops from egg to an adult worm of 1.3mm in length. Short generation time and about 300 progenies per self-fertilizing hermaphrodite enable the large-scale production of several million animals per day.

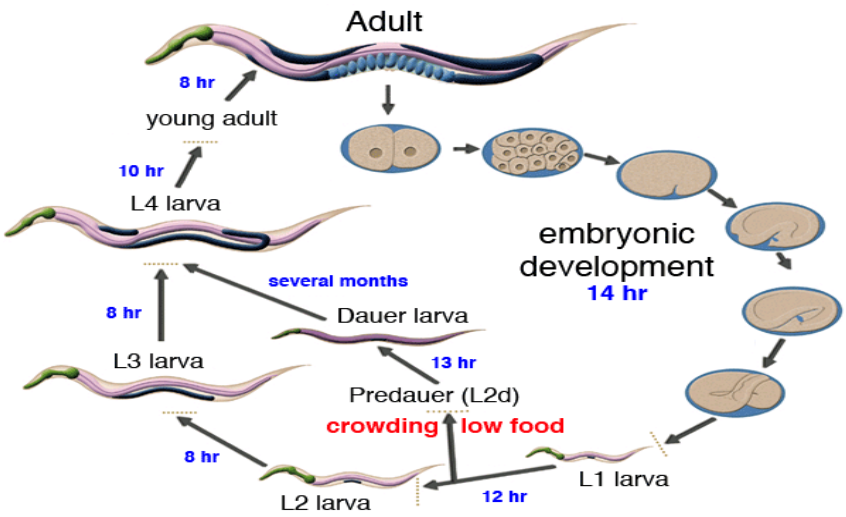


Figure 4-1: Life cycle of *C. elegans* in 23°C.
(http://www.wormatlas.org/handbook/bodyshape.htm)

Third, the worm is transparent and with the use of in vivo fluorescence markers, biological processes can be studied in living animals.

Fourth, it is a simple multi-cellular animal. Only 959 somatic cells in the adult hermaphrodite form many different organs and tissues including muscle, hypodermis intestine, reproductive system, glands, and a nervous system containing 302 neurons (Riddle, 1997).

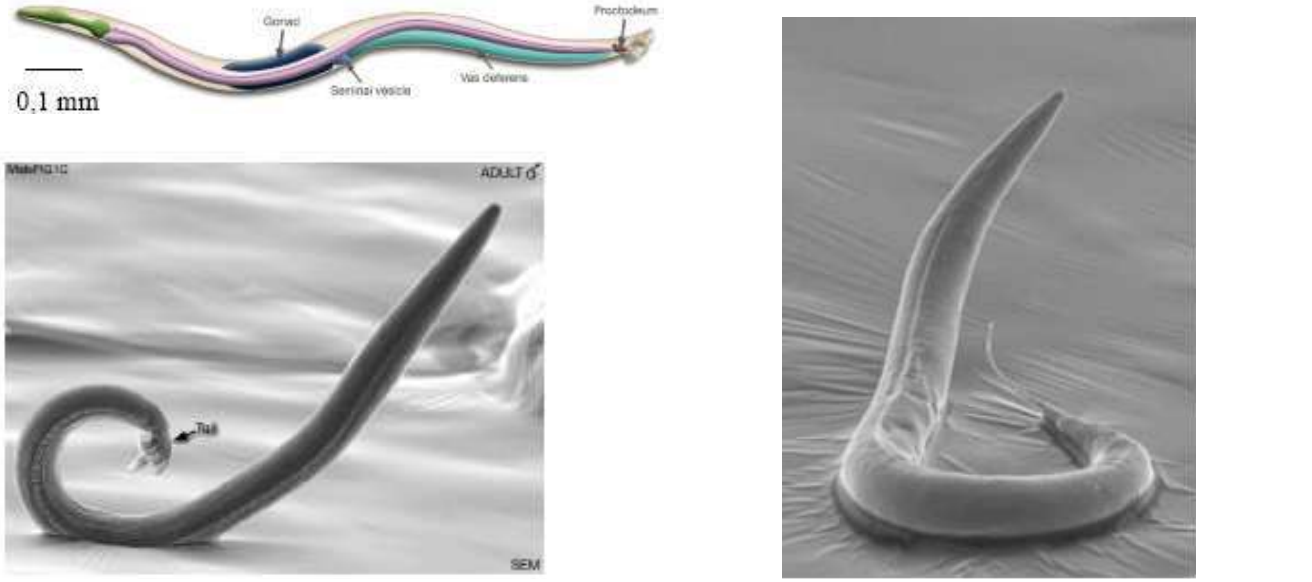


Figure 4-2: The anatomy of male and hermaphordite *C. elegans*. Figure left presents the male; figure right presents the hermaphrodite. (http://www.wormatlas.org/handbook/bodyshape.htm)

Fifth, the genome of *C. elegans* was completely sequenced at the end of 1998. It has 5 autosomes and an X chromosome. The genome is similar to that of human (40% homologous), thus *C. elegans* becoming an animal model in the study of human diseases.

All these make the nematode as an ideal model for scientific research.

4.2 The muscle structure in *C. elegans*

Here, we review the body-wall muscle musculature which relates to the myopathology of muscular dystrophy in *C. elegans*.

There are 95 body-wall muscle cells in *C. elegans*, which arrange into 4 quadrant musculaire. Each quadrant contains 24 mononucleate, diploid cells, except for the ventral left quadrant, which contains 23. Each muscle cell has three parts: the cell body contains the nucleus and cytoplasmic organelles; the arm extending from the cell body to the nerve cord; the spindle contains the contractile myofilament lattice itself.

The thick filaments containing myosin produce A band which is analogous to the M line of vertebrate striated muscle.

The thin filaments containing actin attach to dense body rather than Z line as in vertebrate muscle; produce I band.

The contractile unit of *C. elegans* is analogous to the sarcomere of vertebrate muscles (Figure 4-3).

Unlike mammalian, the muscle cells of *C. elegans* do not fuse and they are entirely post-mitotic; besides, there are no satellite cells in *C. elegans*. However, the protein composition of the sarcomere has been studied very well and it has revealed a high degree of similarity with mammalian sarcomere (Wood, 1988), thus allowing *C. elegans* be an ideal animal model to study DMD.

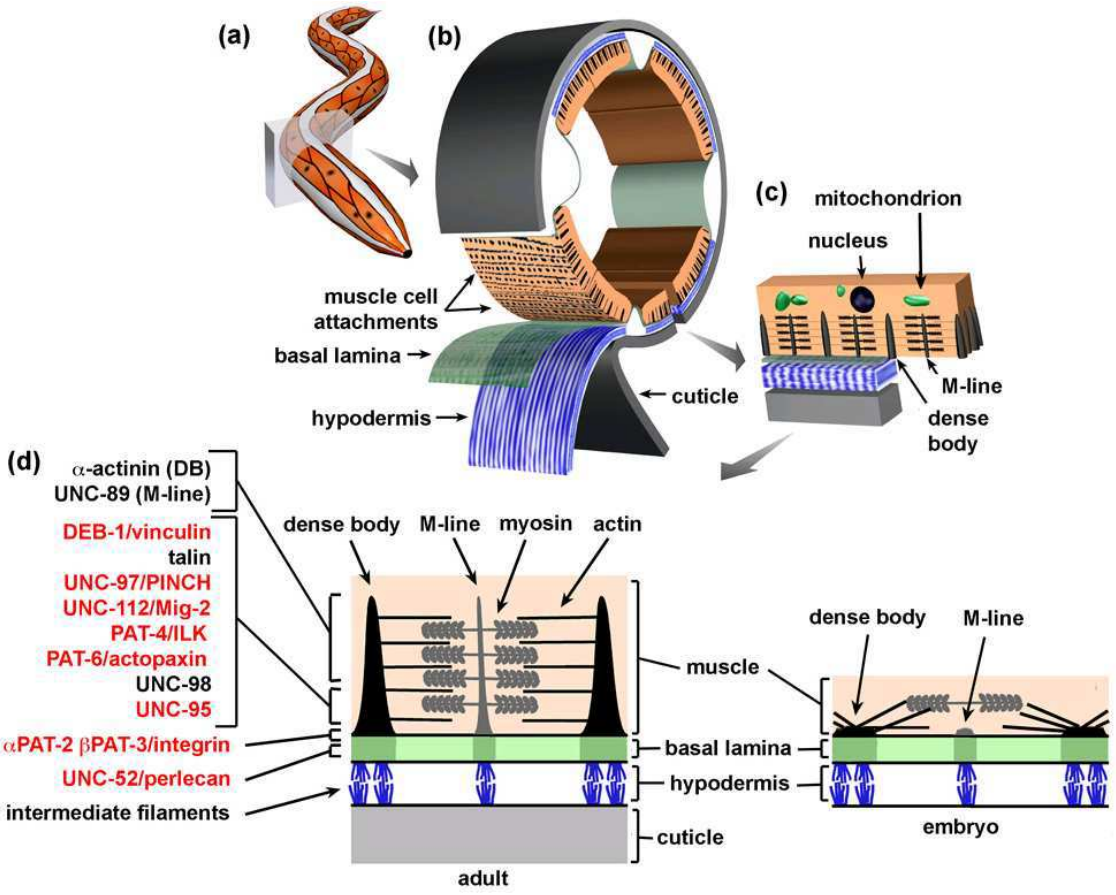


Figure 4-3: Schematic Diagram of the *C. elegans* Body-Wall Muscle Structure. (A).An adult worm with body-wall muscle quadrants visible (orange). (B). A body-wall cross-section with cuticle, hypodermis, and basal lamina peeled away to reveal the basal membrane of two body-wall muscle cells. (C). A longitudinal section through a body-wall muscle cell. Dense bodies and M-lines attach actin thin filaments and myosin thick filaments, respectively, to the basal sarcolemma. (D). Locations of several different muscle attachment proteins. Loss-of-function for proteins shown in red causes the Pat developmental arrest phenotype (Moerman and Williams, 2006).

4.3 The *C. elegans* model for studying DMD

4.3.1 Dystrophin gene and protein in *C. elegans*

There is only one homologue of mammalian dystrophin gene named *dys-1* in *C. elegans* (Bessou *et al.*, 1998). It contains 46 exons, and is about 3.1kb. Allele *cx26*, *cx18* and *cx35* introduce a stop codon in exon39, exon33 and exon1 respectively.

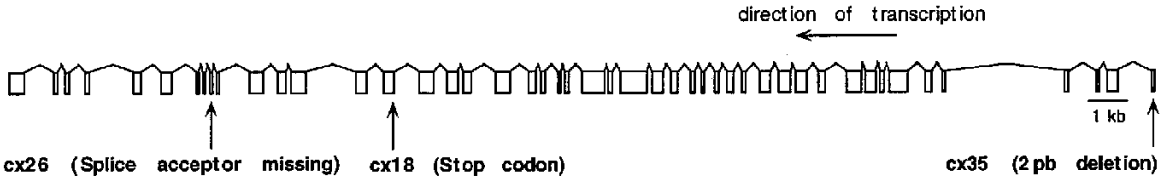


Figure 4-4: The structure of dystrophin gene in *C. elegans*. (Bessou *et al.*, 1998)

The nematode protein DYS-1 composes 3674 amino acids and presents 37% similar to that of human (Bessou *et al.*, 1998). It is characterized by three domains: the actin-binding N-terminal domain, the large central domain containing β-spectrin repeats, and the C-terminal domain.

4.3.2 Basic worm muscle degeneration: model *dys-1* (*cx18*); *hlh-1* (*cc561*)

Unlike mammalians who have a visible phenotype of muscle degeneration, *C. elegans* mutants *dys-1*(*cx18*) lacking DYS-1 (*dys-1* mutants) are hyperactive and show a tendency of hyper contraction. The inability to detect muscle degeneration in *dys-1(cx18)* mutants in C. *elegans* is probably due to the short life span of the animals. However, the double mutant *dys-1(cx18); hlh-1(cc561)* exhibits a severe progressive muscle degeneration and paralyze phenotype (Gieseler *et al.*, 2000).

hlh-1 is a homologue of the mammalian myogenic transcription factor *Myo-D,* that functions among others in inducing the majority of muscle cells. In mammalians, myogenin, *myf5* and MRF4 compose together with *Myo-D* make the *Myo-D* transcription factor family. All the four proteins have a common highly conserved bHLH sequence that is responsible for the regulation of muscle-specific gene expression. In *mdx* mice, lacking the dystrophin gene; the additional knock out of the *Myo-D* gene causes a severe muscle death (Sicinski *et al.*, 1989). In *C. elegans*, the mutant *dys-1(cx18); hlh-1(cc561)* exhibits reduced locomotion of adult

worms. Animals show abnormal egg-laying behavior and have up to 30% body-wall muscle cells are damaged compare to wild type animals.

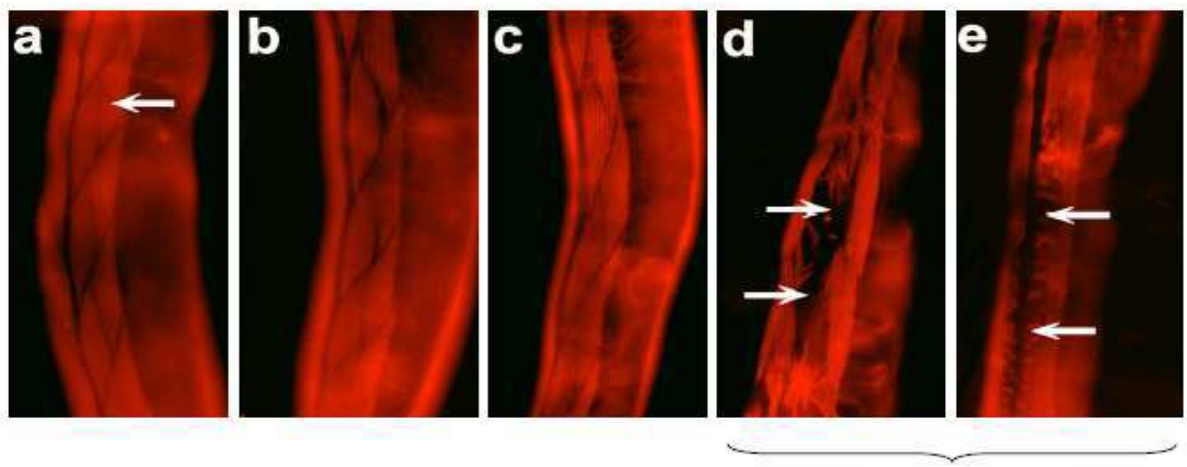


Figure 4-5: Muscle degeneration observed in 4 *C. elegans* strains. (a). N2. (b). Mutant *dys-1(cx18).* (c). Mutants *MyoD/hlh(cc561).* (d and e). Double mutant *dys-1*; MyoD/*hlh-1*.

4.3.3 Research progresses in *C. elegans* DMD model

Although the mechanism of DMD is very little understood, researches in *C. elegans* has shed light on the understanding of myopathy of DMD.

Genetic screen for *dys-1* related genes

Database search confirmed the conserved DAPC homologues in *C. elegans* (Grisoni *et al.*, 2002). DAPC binding partners were identified which display a behavioral phenotype similar to that of *dys-1*, such as *dyb-1* (dystrobrevin), *dyc-1*, *stn-1* (syntrophin), *dgn-1* (dystroglycan), *sgn-1* (sarcoglycan), *slo-1* (a potassium channel) and *snf-6* (an acetylcholine transporter) with classical genetic approach (or forward genetics) (Gieseler *et al.*, 2001; Grisoni *et al.*, 2002; Grisoni *et al.*, 2003; Kim *et al.*, 2004; Carre-Pierrat *et al.*, 2006b). In certain level, the *dys-1* related genes can compensate the absence of dystrophin (Figure 4-6).

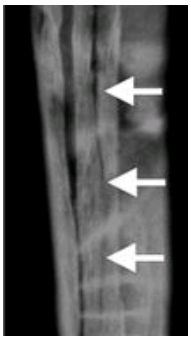


Figure 4-6: Over-expression of *dyc-1* in double mutant *dys-1; hlh-1*. Over-expression of *dyc-1* ameliorates muscle degeneration in *dys-1; hlh-1* (white arrows).

The function investigation of these DAPC binding partners helps us understand the function of *dys-1* in mechanism of DMD in *C. elegans*. Among them, the investigation of dyc-1 supposed that protein Dyc-1 has important role in maintaining the stability and normal function of dense-body, thus muscle degeneration might beginning in dense-body in *C. elegans* (Lecroisey *et al.*, 2008).

Drug screen

Model *dys-1*; *hlh-1* animal is used for drug screening. Drugs are added into the nematode growth medium and get inside of the worms either by feeding or transport through the cuticle. The purpose of drug screen is to find potential blockers of muscle degeneration. The advantage of *C. elegans* makes high through-put drug screen possible. Recently, a neuron-transmitter named serotonin (5-HT) showed its ability to reduce muscle degeneration (Carre-Pierrat *et al.*, 2006c), so besides its important role in invertebrate such as feeding, locomotion (Horvitz *et al.*, 1982), serotonin is beneficial to striated muscles and reduces muscle dystrophy.

Suppressor screen

A typical worm screen uses the chemical mutagen ethylmethanesulphonate (EMS), which induces point mutation and small deletions in DNA. Genetic screens in *C. elegans* are so efficient that it can be up-scaled to screen millions of genomes. Mutants with the desired phenotype are collected and used to identify and characterize genes involved in the process of interest. In parallel, an RNAi screen was performed to find gene suppressors that reduce muscle degeneration.

An interesting gene *pkc-2* was selected, which could ameliorate muscle degeneration in *dys-1*; *hlh-1* background by knocking out *pkc-2 (ok328)* (unpublished result).

Meanwhile, screen suppressors in *dys-1*; *hlh-1* background in presence of serotonin find a homologue IP_3 receptor in *C. elegans* named *itr-1*. It encodes a putative inositol (1, 4, 5) trisphosphate receptor, when mutated, could decrease the effect of serotonin on *dys-1(cx18); hlh-1(cc561),* which in turn increase *C. elegans* muscle degeneration (unpublished result).

4.4 Protein kinase C in *C. elegans*

4.4.1 Protein kinase C isotypes in *C. elegans*

There are four genes express PKC family (listed in table 4-1):

Table 4-1: Character of PKC isoforms in *C. elegans*.

Gene Name	Isoforms	Structure	Enzymatic Properties	Reference
tpa-1	*tpa-1*A *tpa-1*B	Novel PKC	Regulate the nAchR level	(Waggoner *et al.*, 2000)
pkc-1	*pkc-1*A *pkc-1*B	Novel PKC	ND Ca^{2+}-independent kinase activity	(Land *et al.*, 1994)
pkc-2	6 PKC isoforms	Conventional PKC	ND	ND
pkc-3	PKC-3	Atypical PKC	Ca^{2+}-and DAG/phorbol ester independent kinase activity	(Tabuse, 2002)

Except *pkc-2*, other PKC isoforms have been well studied in *C. elegans*. For example, *tpa-1* is found to play a role in nicotin-induced adaptation (Waggoner *et al.*, 2000), while *pkc-3* has been shown to play an essential role in establishing the polarity of the zygote (Tabuse *et al.*, 1998). Islas-Trejo et al. were the first to study *pkc-2* gene expression with a β-galactosidase reporter gene (*lacZ*) (Islas-Trejo *et al.*, 1997). They used three promoters drive *pkc-2* gene

transcription into *pkc-2a*, *pkc-2b* and *pkc-2c*, in which two specific isoforms were found in L1 larval nematode body-wall muscle cells: *pkc-2a* expressed in body-wall muscle cells (immediately below the tip of the head) and *pkc-2b* expressed in muscle cells of head. It has already been shown that in mammalian muscle cells phosphorylation of dystrophin by PKC inhibits effective binding to actin (Senter *et al.*, 1995). But the real function of PKC in muscle dystrophy remains unknown. In fact, how the phosphorylation and dephosphorylation of PKC triggers a downstream signaling pathway is not yet completely known in *C. elegans*, even in other cell types or organisms.

4.4.2 *pkc-2*: gene and protein

The *pkc-2* gene is located on the X chromosome of *C. elegans*. So far, Worm Base presents 4 confirmed isoforms of cDNA transcript from the single *pkc-2* gene:

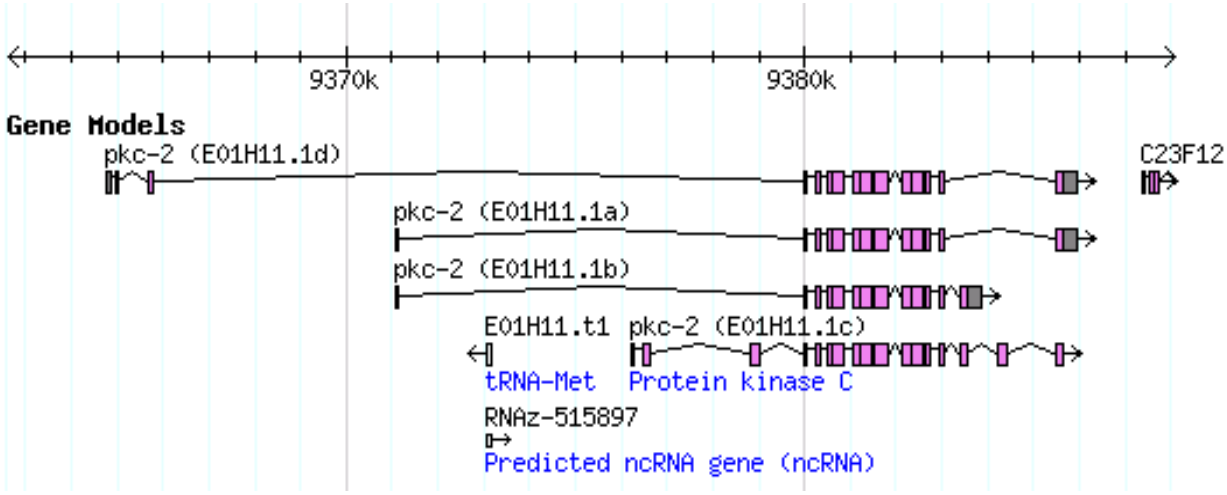


Figure 4-7: Gene model of *pkc-2*.

(http://www.wormbase.org/db/gene/gene?name=*pkc-2*;class=Gene)

Each isoforms was translated into PKC-2 protein with similar domains according to SMART analysis:

PKC-2a:

PKC-2b:

PKC-2c:

PKC-2d :

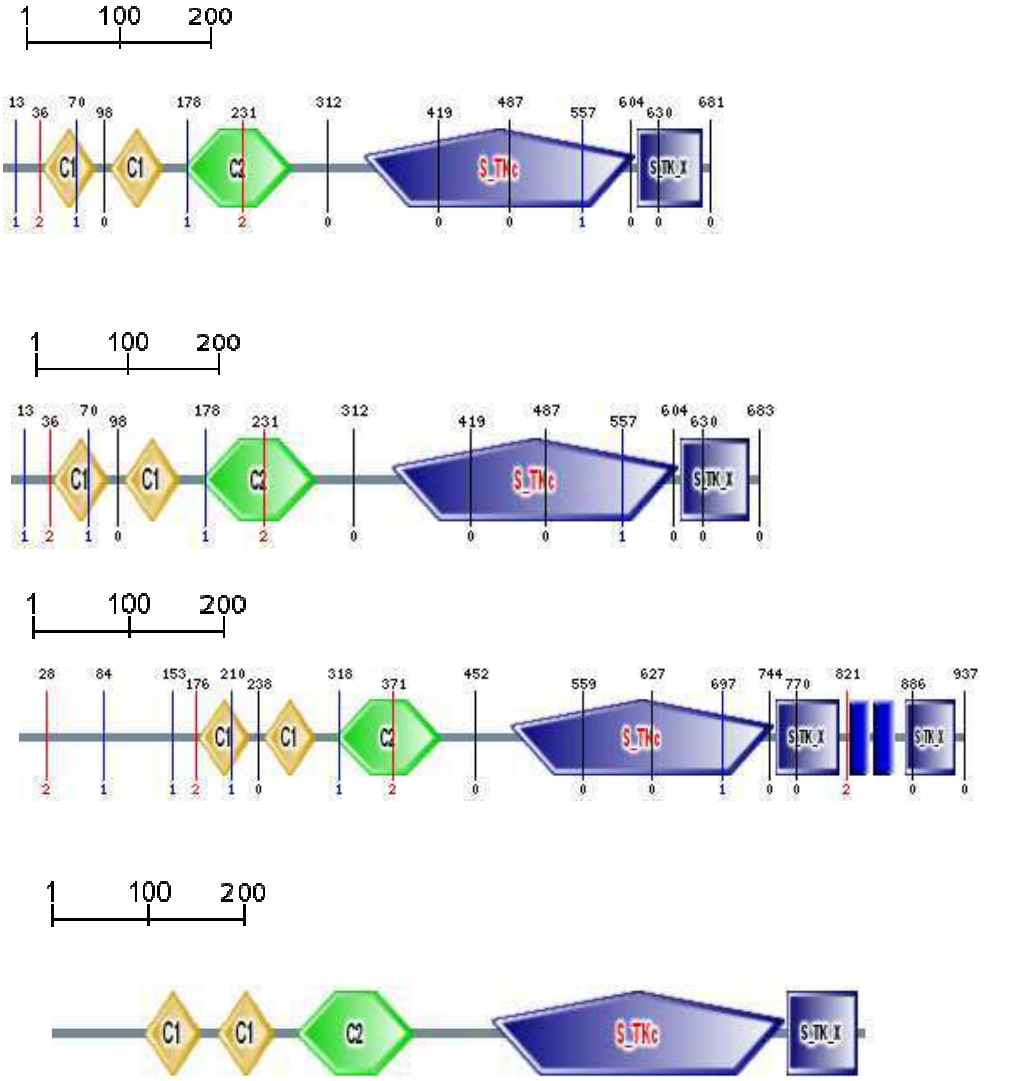


Figure 4-8: PKC-2 protein structure. The model shows different domain of protein PKC-2: C1 domain (yellow), C2 domain (green) and catalytic domain (blue). Figures indicate the number of amino acids.

A Phylip analysis of kinase domain of PKC isoforms, which is most highly conserved, showed the evolutionary relationship between PKC-2 in *C. elegans* and other species. It is clearly that PKC-2 belongs to classical PKCs family and is an orthologous of the human gene Protein Kinase C Alpha (PKC).

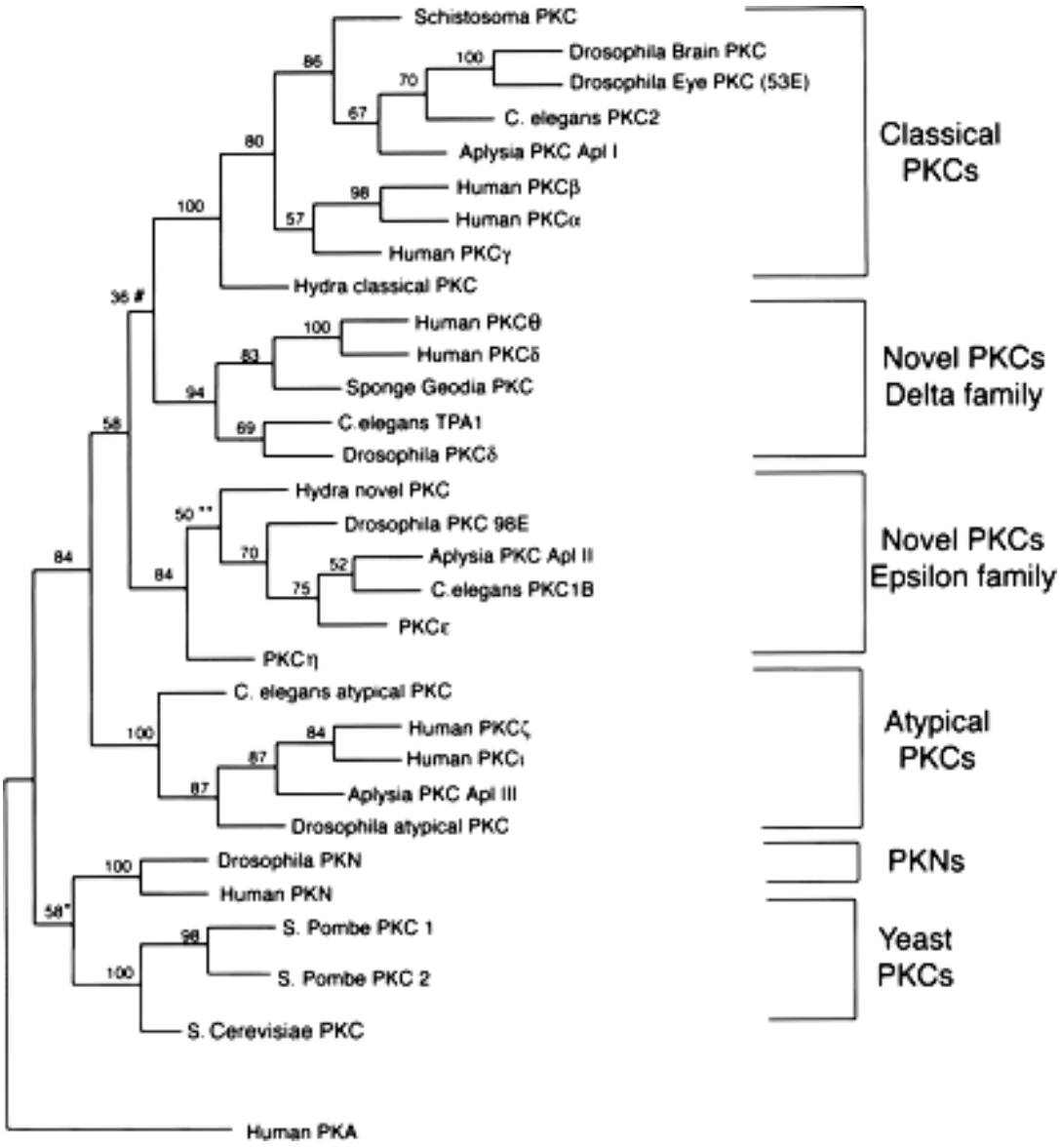


Figure 4-9: Phylogenies tree of PKC.(Sossin, 2007)

Chapter 5 Aim of Thesis

The aim of this thesis is to decipher the role of *pkc-2* in the mechanism of muscle degeneration of Duchenne Muscular Dystrophy in animal model *Caenorhabditis elegans*.

5.1 The expression pattern of *pkc-2* in *C. elegans*

Knock out the gene *pkc-2* in *C. elegans* could ameliorate the muscle degeneration in *C. elegans* double mutant *dys-1(cx18); hlh-1(cc651)* (unpublished result). Until now, the real function of *pkc-2* in *C. elegans* is little known especially that of in muscle. Therefore, it is essential for further researches to study the localization of PKC-2 in *C. elegans* adult animals. To determine the expression of *pkc-2* in muscles, our research group constructed different kinds of *pkc-2* expression recombinant vectors in order to generate transgene strains as well as a strain carrying a reporter gene gfp in the *pkc-2* genomic DNA, These transgene have also been placed in varies genetic background.

RNAi experiment was also designed to study the effect of separate domain of protein PKC-2 in muscle degeneration process.

5.2 Yeast two hybrid screen to identify PKC-2 function partner

It has been already shown that in mammalian muscle cells phosphorylation of Dystrophin by PKC inhibits effective binding to actin (Senter *et al.*, 1995). Since *pkc-2* is expressed in both muscle cells and neurons in *C. elegans* and as PKC can inhibit the expression of Ach, PKC might be involved in processing signal transduction pathways that regulate the growth of muscle cells. The DAPC and several important receptors of neurotransmitters could be also among these targets.

As a protein kinase which activates or inhibits the downstream signaling pathway by phosphorylating the substrates, it is helpful to understand the potential PKC-2 function by identifying the PKC-2 interaction partner. This has been done by the yeast two-hybrid method.

5.3 Suppressor screen of *pkc-2* mutant in presence of serotonin

As a wildly existed protein kinase, there are much many substrates involved or overlapped in PKC pathway. Hence it is difficult to isolate the molecule which has function in the process of muscle degeneration. Then screen for *pkc-2* suppressors in purpose is meaningful and more effective.

As mentioned in chapter4, the neurotransmitter serotonin (5-HT) was found by drug screening to be an active blocker of striated muscle degeneration (Carre-Pierrat *et al.*, 2006c). Serotonin pathway regulates several behaviors of *C. elegans* such as egg-laying, locomotion etc (Horvitz *et al.*, 1982; Carre-Pierrat *et al.*, 2006c) by interacting with different 5-HT receptors subtypes coupled to various second-messenger systems.

Three serotonin receptors are known in *C. elegans*: SER-1, SER-4 and MOD-1(Olde and McCombie, 1997; Hamdan *et al.*, 1999; Ranganathan *et al.*, 2000). They belong to the metabotropic G-protein-coupled receptors (GPCR) except for the 5-HT-gated chloride channel MOD-1 (Ranganathan *et al.*, 2000). Our group searched the *C. elegans* genome for additional 5-HT GPCR genes and isolated five further genes which encode putative 5-HT receptors: K02F2.6, M03F4.3, F16D3.7, T02E9.3, and C24A8.1 (Carre-Pierrat *et al.*, 2006a).

Second messenger systems involved in this regulation include protein kinase and phosphatases, of which protein kinase C has been the best characterized. Increasing evidence suggests that PKC is involved in serotonergic pathway, via the regulation of serotonin receptors (SERs) and transporters (SERTs) (Blakely *et al.*, 1991; Raymond, 1991; Bhattacharyya *et al.*, 2002; Sun *et al.*, 2003). The (de)phosphorylation of PKC triggers a downstream signaling pathway that is not yet completely known for each cell type or each organism.

We observed that *C. elegans* lacking *pkc-2(ok328)* displays a blister phenotype on exogenous 5-HT. This observation supports that *pkc-2* possibly plays a role in serotonergic pathway in regulating muscle degeneration, which could be the (de)phosphorylation of SERs or SERTs.

Then, performing a genetic screen the suppressors of *pkc-2* mutant in presence of serotonin is an efficient way to study the potential function of both *pkc-2* and serotonin in the process of

muscle degeneration in *C. elegans*, as well as their correlation between PKC-2 and serotonin. We tried to isolate and identify *C. elegans* mutation involved in these double responses.

Results

Chapter6 Analysis of the *Caenorhabditis elegans* PKC-2 gene

6.1 Material and Methods

6.1.1 *C. elegans* strains, Culture and Vectors

Techniques for growing, handling, and microscopy of *C. elegans* have been described previously (Brenner, 1974). N2 Bristol strain was used as wild-type control. Strains *pkc-2 (ok328), pkc-2(ok328)::gfp, dys-1(cx18); hlh-1(cc561), hlh-1(cc561), pkc-2(ok328); dys-1(cx18); hlh-1(cc561)* were kept in lab. Strains were grown at 15°C.

Plasmid pRM248 (6.5Kb) contains the *snb-1* promoter. Plasmid pPD118.20 (6Kb) contains a *gfp* gene and carries an antibiotic gene ampicilin. Plasmid pBSC (3Kb) carries an antibiotic gene ampicilin. Fosmid WRM0639dD11ΔMS (28Kb) was derived from the fosmid pCC1FOS (8Kb) which was inserted by *C. elegans pkc-2* genomic DNA (20Kb). It carries an antibiotic gene chloramphenicol.

6.1.2 DNA/ RNA extraction and cDNA production

Total RNA was isolated from a mixed-stage worm population (Trizol reagent, Invitrogen). To obtain mRNA of *pkc-2*, RT-PCR reverse-transcribed the total RNA into cDNA (MHLV reverse transcriptase, New England, Biolabs Inc) using random primer RT. Designed primers PKC-2-KG5 and PKC-2-KG6 were used to get isoforms *pkc-2b* and *pkc-2c*, respectively.

A 1993bp of cDNA fragment corresponding to *pkc-2b* was amplified using the primers PKC-2-KG1 and PKC-2-KG3. A 2811bp of cDNA fragment was amplified using primers PKC-2-KG2 and PKC-2-KG4 that encompass *pkc-2c*. Compare to sequence of *pkc-2c* in Worm Base, the identified *pkc-2* cDNA isoform named *pkc-2c.2* has a primary exon composed of exon 1C, intron 1C and exon 2C, but missing exon 3C, exon15 and exon16 (Figure 1).

All primers used were listed in Table 6-1.

Total DNA was isolated from individual adult animals.

RNA extraction Protocol:

- Wash worms with Tris-HCl pH8.0 10mM and keep the worm in -80°C
- Add 1ml Trizol into worms, vortex 10min
- Add 200µl chloroform, vortex 5min
- Centrifuge 10min in Vmax and keep supernatant, add same volume of isopropanol
- Centrifuge 10min in Vmax and eliminate supernatant, wash pellet with 500µl 70% ethanol
- Centrifuge 5min in 8000rpm, eliminate supernatant and air dry
- Add 50µl H_2O RNase free, vortex
- Centrifuge then keep in -20°C

DNA extraction Protocol:

- Pick 2 adult worms into 2.5µl Worm lysis buffer (10mM Tris-HCl pH8.2; 2.5mM $MgCl_2$; 50mM KCl; 0.5% Tween20; Proteinase K 200µg/ml).
- Keep worms in -80°C for 30min
- Incubated for 1H at 60°C
- Heat-inactivation of Proteinase K at 95°C for 10min
- PCR reaction: add 22.5µl PCR reaction mix (0.4µM forward/reverse primer, 0.4mM dNTPs, 1×PCR reaction buffer, 0.25U Taq polymerase); Cycling parameters were: 2min 95°C, 30sec 95°C, 30sec 95°C, 58°C 30sec, 72°C 1min, 30cycles

6.1.3 *pkc-2::gfp* and *myo-3::pkc- 2* Constructs

Reporter-gene constructs were made in green fluorescent protein (GFP)-encoding vectors (Chalfie *et al.*, 1994) pPD118.20 (kindly provided by A. Fire).

The GFP coding sequence was amplified by PCR on plasmid pPD118.20 and the resulting fragment inserted into different enzyme sites of fosmid WRM0639dD11ΔMS, which contains about 20Kb *pkc-2* genomic DNA, to generate genomic clones. Technique was followed standard procedures (Sambrook and Gething, 1989). All fosmid were transformed into ultra-competent cell C3230 (NewEngland Biolab),

pkc-2 cDNA isoforms were inserted into the pPD118.20 plasmids. This plasmid contains a *gfp* encoding sequence gene under the control of *myo-3* promoter. All plasmid were transformed into competent *E. coli* strain SURE (Strategy Gene).

***pkc-2::gfp* (XhoI) (pKG49).** A 900bp of *gfp* fragment modified by enzyme SalI was inserted into the site XhoI of fosmid WRM0639dD11ΔMS which located in exon 2C (Figure 6-1, 6-2). The recombinant fosmid was named pKG49. Used PCR screen primers were pPD118.20.2/PKC-2-KG12.

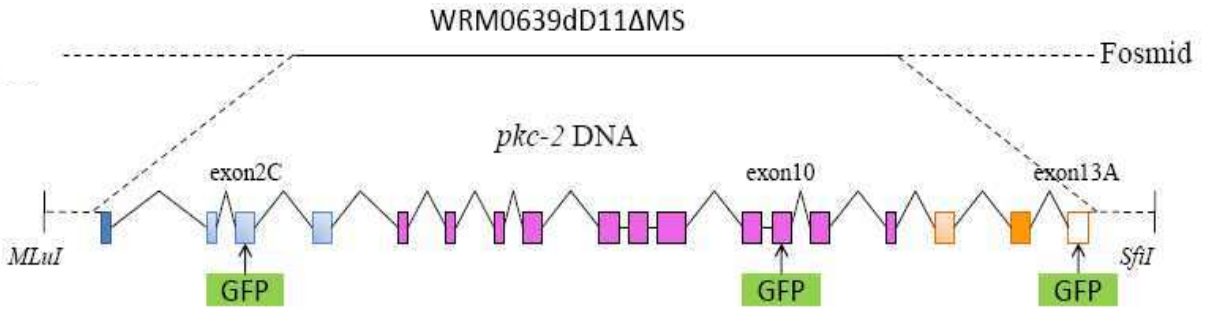


Figure 6-1: Construction of *pkc-2::gfp* protocol. Fosmid WRM0639dD11ΔMS contains the *pkc-2* genomic DNA. Middle, genomic organization of the *pkc-2* gene was showed. Rectangles indicate the specific exons and commune exons with different colors. Purple ones are commune exons of all cDNA isoforms; dark blue ones are exons of *pkc-2a* and *pkc-2b*, light blue ones are exons of *pkc-2*c; transparent yellow ones are exons of *pkc-2a*, *pkc-2*c, dark yellow ones are exons of *pkc-2*c, light yellow ones are exons of *pkc-2b* and *pkc-2*c. Bottom, GFP was inserted into exon 2C, exon 10 and exon 13A to generate pKG49, pKG57 and pKG55 respectively.

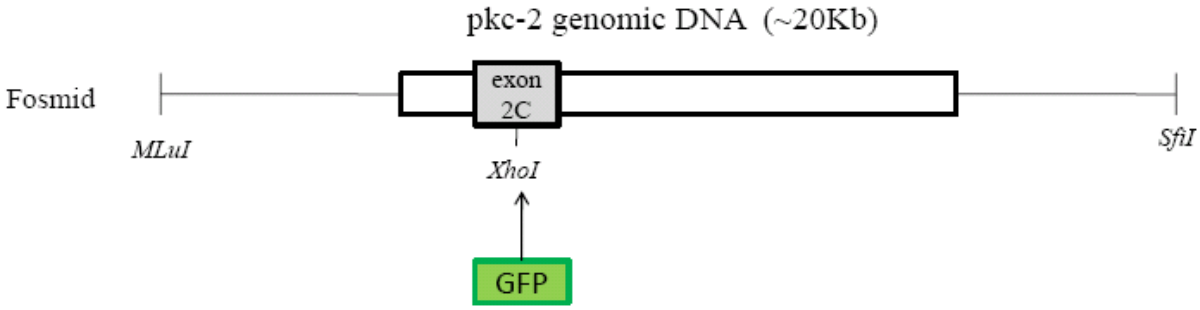


Figure 6-2: Construction of pKG49. *gfp* fragment was inserted into the exon 2C of *pkc-2* gene in fosmid WRM0639dD11ΔMS.

***pkc-2::gfp* (EcoRI) (pKG55).** The 5Kb genomic fragment spanning the AvrII site was excised from fosmid WRM0639dD11ΔMS. This fragment, which contains the region of *pkc-2* genomic DNA from exon 10 to exon 13A was then blunted and sub-cloned into the site SmaI of the modified plasmid pKG48, which was plasmid pBSC but its EcoRI site was removed, to generate pKG50. *gfp* fragment was amplified with primers GFP-KG3/GFP-KG4 and was then inserted into the unique site of EcoRI of pKG50 which located in *pkc-2* gene exon 13A

(Figure 6-1, 6-3). The recombinant plasmid was named pKG54. The AvrII fragment of fosmid WRM0639dD11ΔMS was then replaced by the *gfp* carrying AvrII fragment of pKG54. The recombinant fosmid was named pKG55.Used PCR screen primers were pPD118.20.2/PKC-2-KG16. The recombinant fosmid was named pKG55.

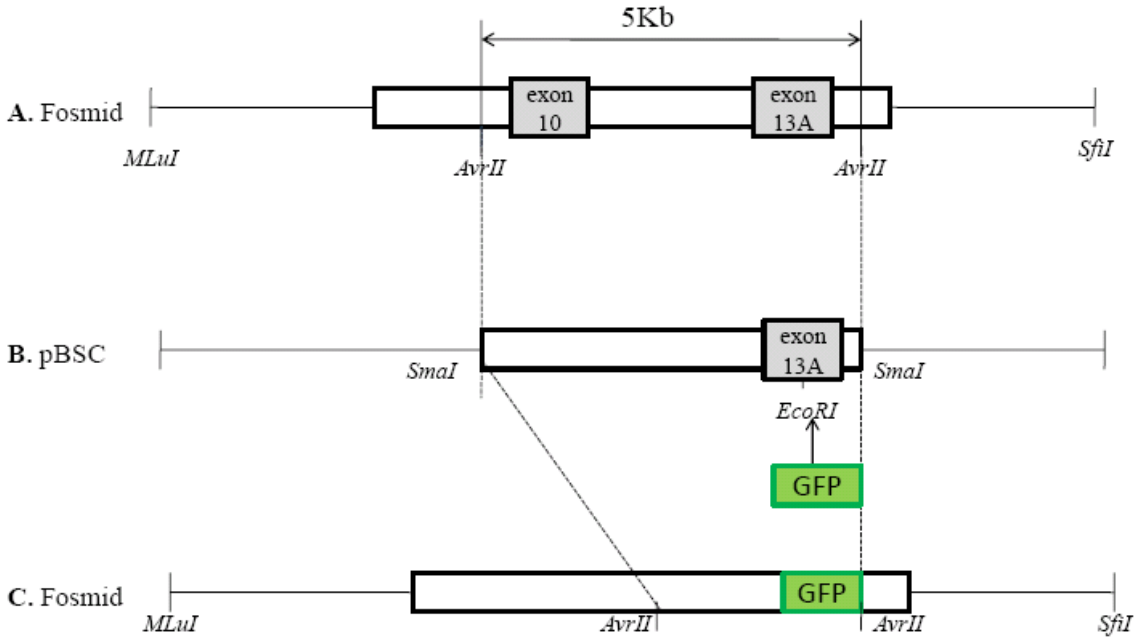


Figure 6-3: Construction of pKG55. (A). fosmid WRM0639dD11ΔMS contains the *pkc-2* gene. A fragment spanning 5Kb was excised by enzyme AvrII and was blunted to sub-clone into site SmaI of pBSC (B), whose site EcoRI was removed, and was named pKG50. (B). gfp was inserted into site EcoRI of pKG50 and named pKG54. (C). pKG54 was cut by AvrII and replaced the region spanning AvrII of fosmid WRM0639dD11ΔMS.

***pkc-2::gfp* (AccIII) (pKG57).** A 900bp of *gfp* fragment was inserted into the unique enzyme site AccIII of pKG50 which located in common exon 10 (Figure 6-1, 6-4), and was renamed pKG56. PCR screen with primers pPD118.20.2/PKC-2-KG13 was performed to confirm the *gfp* insertion in pKG56. Then the fragment excised by AvrII replaced the correspond region in the fosmid WRM0639dD11ΔMS and the consequent recombinant fosmid was named pKG57.

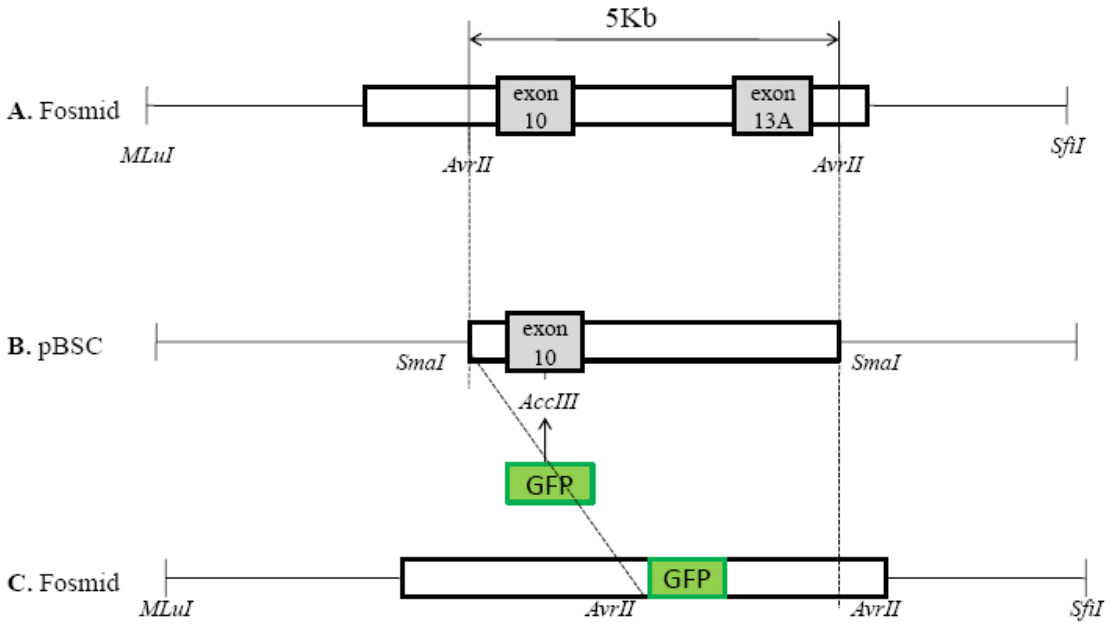


Figure 6-4: Construction of pKG57. (A). fosmid WRM0639dD11ΔMS contains the *pkc-2* gene. A fragment spanning 5Kb was excised by enzyme AvrII and was blunted to sub-clone into site SmaI of pBSC (B), whose site EcoRI was removed, and was named pKG50. (B). *gfp* was inserted into site AccIII of pKG50 and named pKG54. (C). pKG54 was cut by AvrII and replaced the region spanning AvrII of fosmid WRM0639dD11ΔMS.

***myo-3::pkc-2* (pPD118.20) (pKG52).** The cDNAs of *pkc-2c.2* was amplified with primers PKC-2-KG2 and PKC-2-KG4, and then cloned into the site NheI of the vector pPD118.20, in frame with the GFP encoding sequence. The recombinant vector was named pKG52 (≅8.4Kb). It was then sequenced and confirmed (Figure 6-5).

***myo-3::pkc-2* (pPD118.20) (pKG63).** A PCR reaction was performed by amplifying the insertion from the ATG start codon of exon 1C.2 in pKG52 using primers PKC-2-KG4 and PKC-2-KG18.The PCR fragment was then cloned into the sites EcoRI and NheI of the plasmid pPD118.20. The new recombinant plasmid was named pKG63 and was sequenced (Figure 6-5).

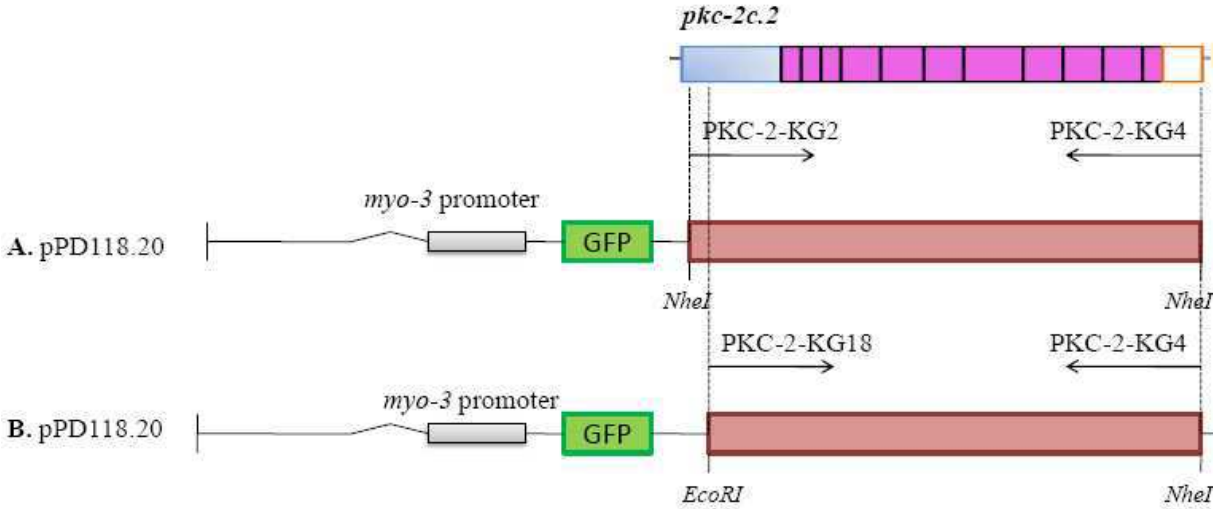


Figure 6-5: Construction of pKG52 and pKG63. (A). RT-PCR product *pkc-2c.2* was inserted into site NheI of pPD118.20 named pKG52. (B). PCR fragment started from ATG of exon 1C.2 was inserted into pPD118.20 named pKG63.

***myo-3::pkc-2* (pPD118.20) (pKG53).** The cDNA of *pkc-2b* that has been amplified with primers PKC-2-KG1 and PKC-2-KG3 was then cloned into the site NheI of the vector pPD118.20. The recombinant vector was named pKG53 (≒8.4Kb) (Figure 6-6).

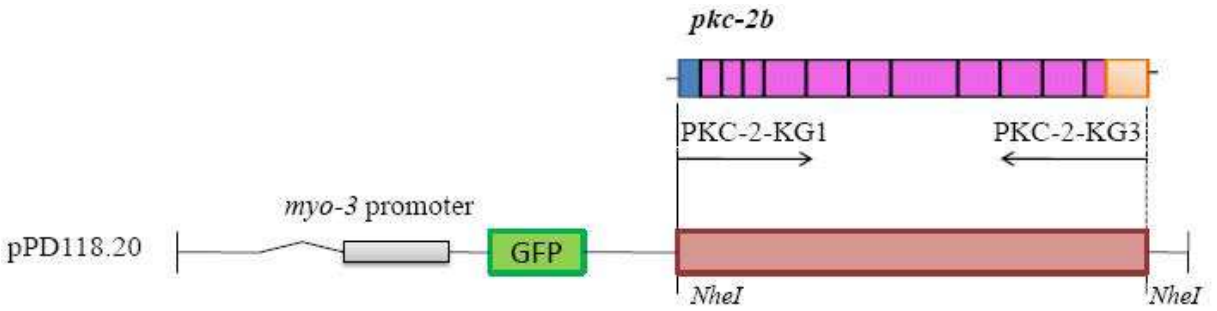


Figure 6-6: Construction of pKG53. RT-PCR product *pkc-2b* was inserted into site NheI of pPD118.20, which carries *gfp* and *myo-3* promoter, and named pKG53.

Table 6-1: Primer Sequences

Primer name	Sequence (5'→3' direction)
PKC-2-KG1	AATTGCTAGCTCGTTGAGCACGAACAGCTCC
PKC-2-KG2	AATTGCTAGCAGCCTACGACCAAAGCAAAAG
PKC-2-KG3	TTAAGCTAGCACTCGTCGCCCTTCAGATTTTCC
PKC-2-KG4	TTAAGCTAGCGGACCTCGTGAGTAAACTCTG
PKC-2-KG5	CGACTCTTGAACATATTCTGGG
PKC-2-KG6	TACATCTTTGACATAAAACGGG
PKC-2-KG12	AGGGATACAAAGGACTTGTG
PKC-2-KG13	GAGCGACTAAGTAGCAGAGG
PKC-2-KG16	AGCCACCGATCTGCATCTCG
PKC-2-KG17	TTAAGGATCCGGACCTCGTGAGTAAACTCTG
PKC-2-KG18	TTAACAATTGGATAGTGCAGAGCGTCGACG
GFP-KG1	AATTGTCGACATGAGTAAAGGAGAAGAAC
GFP-KG2	TTAAGTCGACGCCATGTGTAATCCCAGC
GFP-KG3	AATTGAATTCATGAGTAAAGGAGAAGAAC
GFP-KG4	TTAAGAATTCGCCATGTGTAATCCCAGC
GFP-KG5	AATTTCCGGACATGAGTAAAGGAGAAGAAC
GFP-KG6	TTAATCCGGAGTGCCATGTGTAATCCCAGC
pPD118.20.2	TGGAAGCGTTCAACTAGCAG
Random primer RT	a mixture of single-stranded random hexa-nucleotides with 5'- and 3'-hydroxyl ends

RT reaction:

- Ambion-DNA free treatment
- Reverse transcript reaction:

  Add 4µl Tp5×, 2µl dNTP 10mM, 0.5µl RNase inhibitor (40U/µl), 1µlRT

  Cycle parameters: 25°C 10min, 42°C 60min, 70°C 10min

RT-PCR reaction:

- Add H_2O 37.6µl, buffer Taq10× 5µl, dNTP10mM1µl, primer sets10µM 2µl respectively, product RT 2µl, Taq5U/µl 0.4µl
- Cycle parameters: 95°C 2min, 95°C 30sec, 53°C 30sec, 68°C 2min, 30 cycles

PCR GFP reaction:

- Final concentrations in 50µl PCR reaction were: 1×PCR reaction buffer (Promega), 2mM $MgCl_2$ (Promega), 0.4mM dNTPs, 0.5µg forward/reverse primer, GoTaq DNA polymerase (Promega) 0.25U, 10ng plasmid pPD118.20
- Cycle parameters: 95°C 5min, 95°C 30sec, 55°C 30sec, 72°C 1min, 30 cycles

Bacteria PCR screen reaction:

- 1×PCR reaction buffer (Promega), 2mM $MgCl_2$ (Promega), 0.4mM dNTPs, 0.4µM forward/reverse primer (Table 4), GoTaq DNA polymerase (Promega) 0.25U, 2µl bacteria mix;
- cycle parameters: 95°C 5min, 95°C 30sec, 55°C 30sec, 72°C 1min, 30 cycles

6.1.4 Transgenic strains and Microscopy

The recombinant constructions were injected at a concentration of 10ng/µl into the cytoplasm of the distal gonad of wide-type adult hermaphrodite (Figure 6-7). Procedure standard followed protocol of Mello et al (Mello and Fire, 1995). As marker, *myo-3::dsRed* or *unc-122::gfp* (kindly provided by V. Robert) was co-injected at a final concentration of 30ng/µl.

Observation of living animals under a fluorescence microscopy (Zeiss Axioplan, le Pecq, France) was done after immobilization of the animals on a 2% agarose pad containing Dakocytomation fluorescent mounting medium (Interchim).

Figure 6-7: Generate transgenic worm. Injection into cytoplasm of the distal gonad of wide-type adult hermaphrodite (arrows presented area)(Mello and Fire, 1995).

6.1.5 RT-PCR of transgenic worms

9 transgenic pKG53::GFP strains were extracted DNA and RNA respectively followed protocol mentioned. N2 and pKG53 were used as negative and positive control respectively. PCR amplify primers used were PKC-2-KG8 (located in *pkc-2* gene exon6) and pPD118.20.2 (located in the end of GFP in plasmid pPD118.20) (Table 6-2).

Table 6-2: Primers used in PCR

PKC-2-KG8	CAGACAATCCATTGGGGTCC (5'-3' direction)
pPD118.20.2	TGGAAGCGTTCAACTAGCAG (5'-3' direction)

6.1.6 Immunocytochemistry and Microscopy

Worms were analyzed by immunocytochemistry on whole-mount preparations as described by Benian et al (Benian *et al.*, 1996). Monoclonal antibodies MH24 (anti-DEB-1/vinculin which recognizes the DEB-1/vinculin protein, one of the main components of dense bodies (Francis and Waterston, 1985), mouse anti-GFP(Santa Cruz Biotechnology) and rabbit anti-GFP (Interchim) were used at a dilution of 1:100,1:100 and 1:1000 respectively. Secondary mouse anti-rabbit (FluoroProbes 488, green, Interchim, Montluçon, France), goat anti-mouse (FluoroProbes 586, red, Interchim) and goat anti-mouse (green, Santa Cruz Biotechnology) antibodies were used at a dilution of 1:200. Images were captured with a Zeiss ZI Imager using either 63× or 100× oil immersion objective lenses using the Metaview software. Images were processed and annotated with Adobe Photoshop CS2 (San Jose, CA).

Immunocytochemistry Protocol:

- Wash 20 plates of worm with cold M9 into 15ml tube
- Add 4ml buffer fixation into pellet, freezing quickly the worms with liquefied nitrogenize
- Incubate the tube in 4°C for 1H
- Remove the supernatant and wash the pellet with 1ml Tris Triton Buffer(TTB) 2 times in 1.5ml silicon Eppendorf tube
- Add 10µl 10% β-mercapto-ethanol and incubate in 37°C for 2H
- Centrifuge in 300rpm for 2min and wash the pellet with 1ml buffer BO3 2 times
- Add 1ml BO3-10% 0.1M DTT, rotate in room temperature for 15min
- Centrifuge in 300rpm for 2min and add 1ml buffer AbB to pellet, incubate in room temperature for 20min, rotating
- Centrifuge and add 1ml buffer AbA to pellet, keep in 4°C
- The next day add 170µl buffer AbA to 30µl worm mixture and 170µl antibodyI (rabbit anti GFP at a dilution of 1/1000 and mouse anti DEB-1 at a dilution of 1/100, respectively); incubate the worms in room-temperature for 2H
- Centrifuge in 300rpm for 2min, wash the pellet with 1ml buffer AbB in room-temperature for 30min 4 times, rotating
- Centrifuge in 300rpm for 2min, add 170µl antibody II (goat anti rabbit and goat anti mouse, respectively) at a dilution of 1/250; incubate the worms in room-temperature for 2H
- Wash worms with buffer AbB for 15min 3times
- Mounting samples, observation

6.1.7 RNA Interference

RNAi was performed by feeding N2 wild-type, *MosTIC pkc-2, dys-1(cx18); hlh-1(cc561), and pkc-2(ok328); dys-1(cx18); hlh-1(cc561)* with double-strand (dsRNA)-producing *E. coli* (Timmons *et al.*, 2001).

Two fragments were recovered after digesting of pKG52 with HindIII, which excised a shorter fragment carrying exon 1C.2 (≅450bp) and a larger fragment spanning common exon 2 to exon 8 (≅750bp) (Figure 6-8). Then they were cloned into the site HindIII of RNAi

feeding vector L4440 named pKG65 and pKG66 respectively. These two plasmids were transformed into competent cell HT115 *E. coli* cells. Transformed bacteria were cultivated over night in Luria-Bertani broth medium added with ampicilin (100µg/ml), tetracycline (15µg/ml) (LBAT). Nematode growth medium (NGM) Petri plate (□90mm) containing carbenicillin (25mg/ml final) and IPTG (1mM final) were inoculated with 0,5ml of bacteria culture and grown one week at room temperature. RNAi was performed by feeding N2 wild-type or PKC-2-GFP-expressing transgenic worms with those double-strand (dsRNA)-producing *E. coli* (Timmons *et al.*, 2001). Empty vector L4440 was used as control.

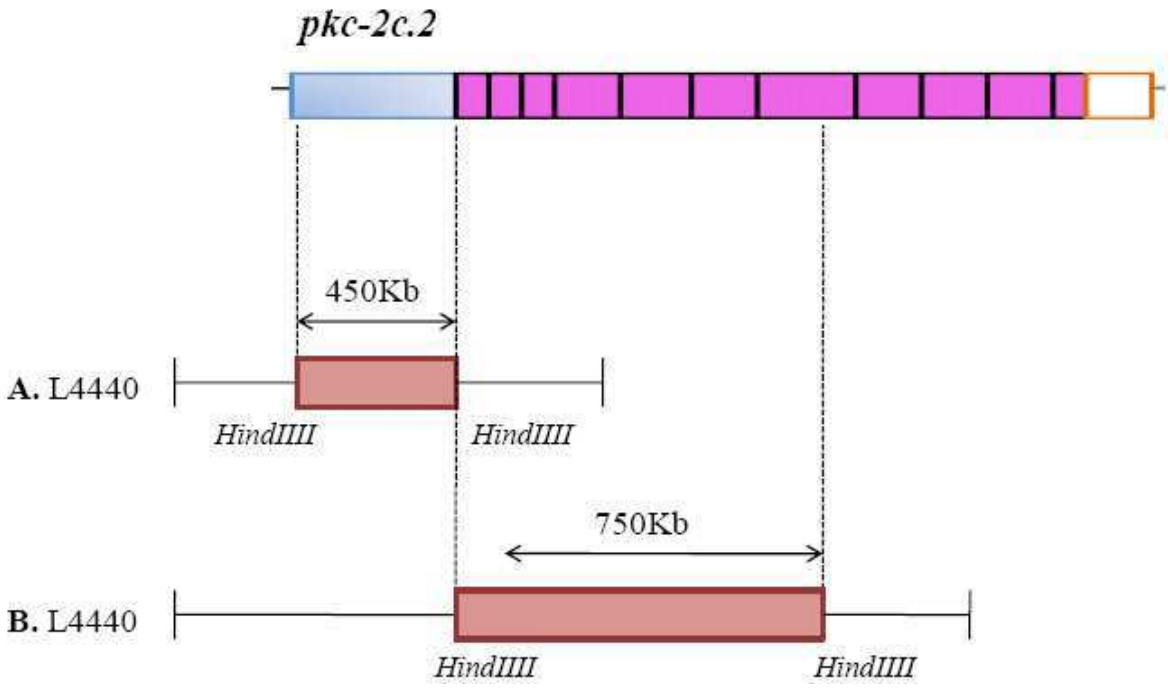


Figure 6-8: Construction of pKG65 and pKG66. (A). A 450Kb fragment of pKG52 was cut by HindIII and was inserted into L4440 named pKG65. (B). A 750Kb fragment of pKG52 was cut by HindIII and was inserted into L4440 named pKG66.

RNAi Protocol:

- Prepare NGM Petri plate (□90mm) containing carbelicillin (25mg/ml final) and IPTG (1mM final)

- Pre-culture bacteria HT115 containing 5 plasmids: pKG65, pKG66, L4440, *pos-1*, *dys-1* respectively, in LBA (ampicilin 100µg/ml, tetracycline 15µg/ml) in 37°C overnight. Add 1ml of this pre-culture into 15ml LBA and let grow 6 hours minimum until the bacteria reaches the exponential growth phase. Depose 1.5ml of culture into the NGM Petri plate, air dry.
- Day1: Pick the gravis animals (F0) onto plate RNAi, the number of worm depends on the strain
- Day2: Remove the F0
- Day6: Check the phenotype of worms of RNAi
- Day7: Mark the muscular with phalloidine-rhodamine; observe and measure body-wall muscle cells

6.1.8 Quantification of Muscle Degeneration

All the worms were grown at 15°C. Animals were fixed and stained 3 days after they reached the L4 stage. Fixation and phalloidin-rhodamine staining (Fluoroprobes, Interchim) were performed as described (Waterston *et al.*, 1984). Stained body-wall muscles were observed using a Zeiss Axioscop microscope. Only one of the two most visible quadrants of body-wall muscles in each animal was quantified (20 cells per animal). Twenty animals were scored for each genotype.

Mark Phalloidin-Rhodamine Protocol:

- Wash worms with PBS 1×, collect adults into Eppendorf silicone 1.5ml
- Centrifuge the tubes 1min in 8000 rpm
- Add 1ml PBS 1× and 30µl 37% formaldehyde into the pellet, incubate rolling in room

temperature for 1H20min

- Centrifuge the tubes 1min in 8000 rpm
- Add 1ml PBS 1× in to pellet, inverse 6 times
- Centrifuge the tubes 1min in 8000 rpm
- Add 1ml acetone glacial into pellet, agitation, incubate the tube in -20°C for 2min
- Centrifuge the tubes 1min in 8000 rpm
- Add 1ml PBS 1× into pellet, inverse
- Centrifuge the tubes 1min in 8000rpm
- Add 500µl PBS 1× into pellet
- Add 1µl 300U phalloidin (Interchim), inverse
- Incubate rolling the tubes in room temperature for at least 2H
- Centrifuge the tubes 1min in 8000 rpm
- Add 1ml PBS 1× into pellet, incubate rolling the tubes in room temperature for 1H
- Keep the tubes in 4°C, overnight
- Remove supernatant and add 40µl DAKO into 40µl pellet, mix
- Mounting the samples, observation

6.1.9 Yeast Two Hybrid Screen

All procedures of yeast two-hybrid were followed the protocol of Fromont-Racine M (Fromont-Racine *et al.*, 1997).

pKG64: a PCR was settled by amplifying pKG52 from the ATG start codon of exon 1C.2 using primers PKC-2-KG17 and PKC-2-KG18. The PCR fragment was then cloned in to the site EcoRI and BamHI of the vector pAS2-1. The new recombinant plasmid was named pKG64 and was sequenced to be sure no errors.

pKG67: The fragment containing two C1 domains (residues 74-188) and a C2 domain (residues 210-303) were obtained by digesting pKG64 with EcoRI and PstI; eliminate the PKC-2 kinase domain of the insert.

The recombinant plasmid expressed a fusion protein composed of PKC-2 and a DNA binding domain (BD) of the yeast gal-4 transcription factor. Yeast strain CG1945 was then transformed by pKG67.

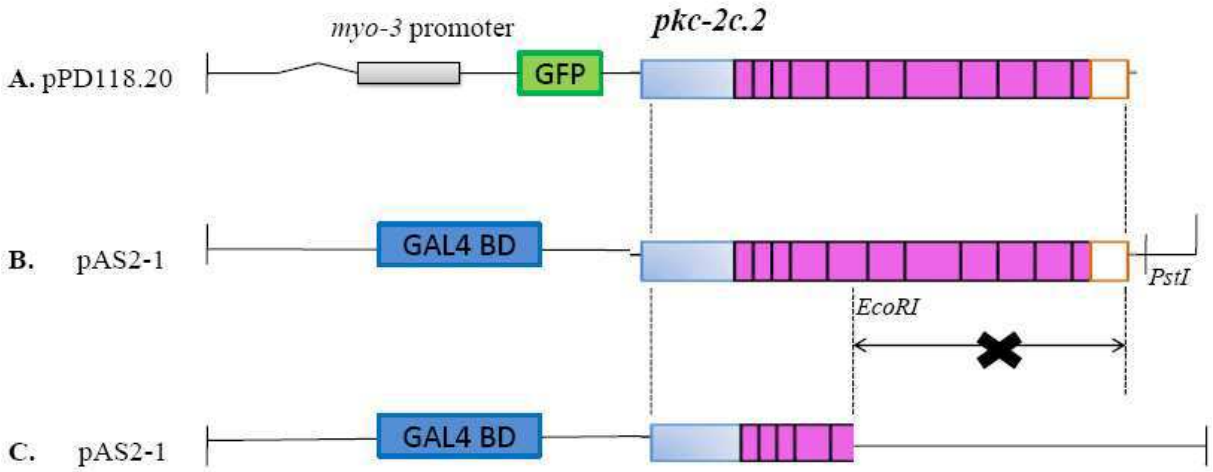


Figure 6-9: Construction of pKG64 and pKG67. (A). pKG52. (B). PCR fragment from pKG52 was cloned into plasmid pAS2-1, renamed pKG64. (C). PKC-2 kinase domain was deleted by EcoRI and PstI. Plasmid carrying DNA binding domain (BD) and regulatory domain of PKC-2 was named pKG67.

Construct pKG67 Protocol:

- Digest the product of PCR pKG52 (started with second ATG codon) with MfeI and BamHI
- Digest plasmid pAS2-1 with EcoRI and BamHI
- Clone into pAS2-1
- Transform into competent cell E.coli strain SURE, get recombinant plasmid named pKG64
- Digest pKG64 with EcoRI and PstI, construct pKG67 containing commune two C1 domains and a C2 domain of PKC-2 family
- Transform pKG67 into yeast cell CG1945

Protocol of Transform plasmid in Yeast CG1945:

- Incubate 5ml YPD + clones CG1945 in 30°C, shaking overnight (220rpm)
- Dilute 10 times with 40ml YPD, incubate in 30°C for 3H~4H
- Centrifuge the culture 5min in 3000 rpm
- Pick the pellet in 20ml TE1× pH7.5
- Centrifuge for 5min in 3000 rpm
- Pick the pellet in 200µl 100mM LiAC/TE1×
- Denature 100µg (10µl) SSDNA in 100°C for 5min, keep it in glace immediately and centrifuge a few sec
- Add 1µg plasmid
- Add 100µl yeast culture
- Add 600µl 100mM LiAC/40%PEG/TE1× , vortex, incubate in 30°C for 30min
- Add 70µl DMSO, mix gentle, incubate in 42°C for 15min
- Put the tube in glace for 1min at most
- Centrifuge in Vmax for 10sec
- Pick the pellet in 200µl YPD
- Spread out 50µl in SD/Trp-
- Incubate in 30°C for 2~3 days

An EcoRI- PstI cDNA fragment from pKG52 encoding the PKC-2 2 C1 domains (residues 74-188) and a C2 domain (residues 210-303) was sub-cloned into the pAS2-1bait vector (Clontech, Palo Alto, CA). The resulting construct, which called pKG67, encodes a fusion protein composed of PKC-2 regulatory domain (C1, C1, and C2) and the DNA-binding domain (BD) of the yeast gal4 transcription factor. Yeast cells of the CG1945 strain were transformed with pKG67.

A two-hybrid screen was performed as described (Fromont-Racine *et al.*, 1997) by mating yeast cell CG1945 transformed with pKG67 with yeast cell Y187 transformed with a random primed *C. elegans* cDNA activation domain library (RB2) sub-cloned into the prey vector pACT2 (R.Barstead, Oklahoma Medical Research Foundation, Oklahoma City). Ten micro liters of mating cultures were deposed on minimal medium lacking Trp, Leu, and His and

minimal medium lacking Leu and Trp as growth control. The cells were incubated at 30°C for 3 days. Diploid cells, 3.67×10^5, containing the pKG67 bait plasmid and one prey-pACT2 plasmid (from the library) were analyzed for their ability to grow on minimal medium lacking Leu, Trp, and His, after 3 days incubation at 30°C. Growth can occur only if the bait and the prey proteins interact and the *HIS3* reporter gene is trans-activated. One positive clone Y59A8A.3 was obtained and prey cDNA was sequenced. Sequence analysis was performed by BLASTn searches.

Mating Protocol:

- Day 1: culture yeast strain CG1945 containing pKG67 in 10ml SD/Trp- in 30°C shaking in 230 rpm overnight
- Day 2: culture the 10ml pre-culture in 200ml SD/Trp-, 30°C shaking in 230 rpm overnight
- Day 3: keep the yeast growing until DO (600nm) =1 (1 DO=1×10^7 cell/ml)
- Centrifuge 80 units of DO in 1000g for 10min
- Pick the pellet in 100ml YPD
- Culture 2ml yeast cell Y187 containing the bank RB2 in 60ml YPD in 30°C for 10min
- Mix two yeast strains, incubate in 30°C for 5H, shaking every 30min
- Centrifuge the mixture in 1000g for 10min
- Pick the pellet in 20ml SD/Trp-Leu-His-
- Dilute the mating culture to 1/100, 1/1000, 1/10000 respectively, spread out 100µl into plate SD/Trp-, SD/Leu-, SD/Trp-Leu- respectively to calculate mating efficiency
- Spread out 500µl mating culture into plate SD/Trp-Leu-His- □140
- Incubate the plates in 30°C for 3 days

Recover cDNA from yeast clones protocol (Analyze the clones)**:**

- Pick a clone into 400µl glass bills and 200µl extraction buffer in a 1.5ml Eppendorf tube
- Add 200µl phenol/chloroform/alcohol isoamylique (25:24:1) , mix
- Vortex 10min
- Centrifuge in Vmax for 10min
- Transfer supernagent into new Eppendorf tube and add 500µl ethanol/acetate ammonium (6:1)
- Centrifuge in Vmax for 15min
- Wash the pellet with 250µl 70% ethanol and centrifuge in Vmax for 5min
- Dry out the pellet and then add 10µl H_2O UP, keep in -20°C
- Transformer the DNA into competent cell SURE

Screen positive clone by PCR clone and Sequence analyze

6.2 Results

All clones used in this study were confirmed below (Figure 6-10):

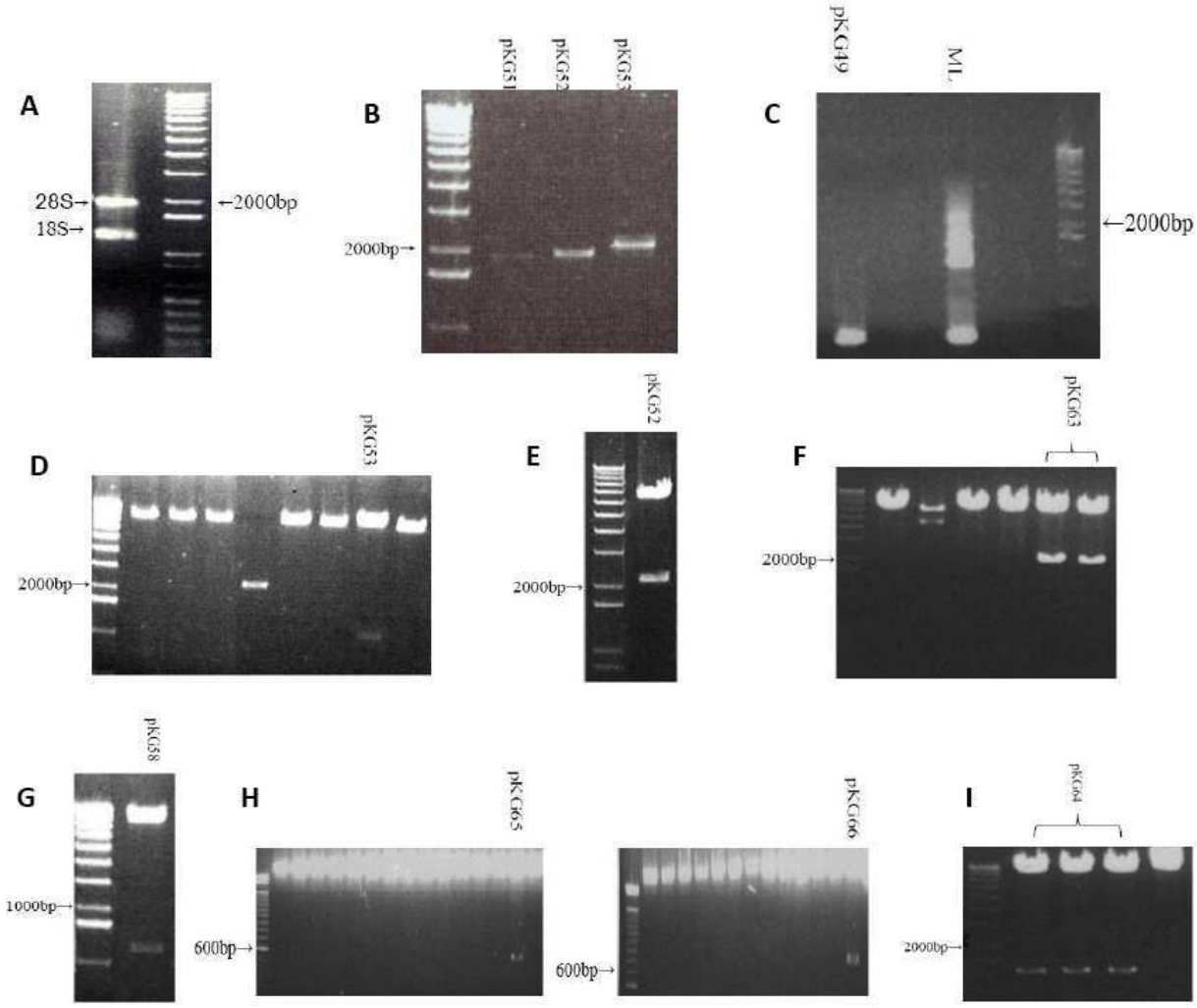


Figure 6-10: Gel electrophoresis. (A). Total RNA (D260/D280=2.09, 206.5ng/µl). (B). RT-PCR 3 *pkc-2* cDNA isoforms. (C). pKG49: PCR screen (D). pKG53: digest the plasmid with NheI. (E). pKG52: digest the plasmid with NheI. (F). pKG63: digest the plasmid with NcoI. (G). pKG58: digest plasmid with NheI. (H). pKG65 and pKG66: digest the plasmid with HindIII. (I). pKG64: digest plasmid with MfeI and BamHI.

6.2.1 Identification of a new isoform of *pkc-2*

Different *pkc-2* isoforms are described on the Worm Base database. All isoforms possess a common region that encodes the functional domains: regulatory domain and kinase domain. Isoforms *pkc-2a* and *pkc-2b* differ by only one 3' exon, while isoform *pkc-2c* exhibits three alternative 5' exons. We performed RT-PCR experiments in order to produce cDNAs of isoform b and isoform c. During this process, we identified a previously undocumented form of *pkc-2*.

Sequence analysis revealed that the new *pkc-2* cDNA isoform resembles isoform *pkc-2*-c but missing exon 3C, exon 15 and exon 16 (Figure 6-11). The first exon 1C.2 was composed of exon 1C, intron 1C and exon 2C, in which located a kozak sequence carrying an ATG start codon (Kozak, 1991) (Figure 6-12). The Kozak consensus sequence plays a major role in the initiation of translation process (De Angioletti *et al.*, 2004). The translation of *pkc-2c.2* started from the ATG start codon of exon 1C.2. Therefore, we called it *pkc-2c.2*.

The predicted protein sequence of *pkc-2c.2* consists of 682 amino acids. Domains were predicted by computational analysis of this protein sequence using the SMART bioinformatics tool package (SMART http://smart.embl-heidelberg.de) (Letunic *et al.*, 2006) (Figure 6-14). There are two C1 domains (green, red respectively), a C2 domain (blue) and a protein kinase domain (grey) (Figure 6-13). The translated protein of isoform *pkc-2b* displayed the same protein domains (Figure 6-15).

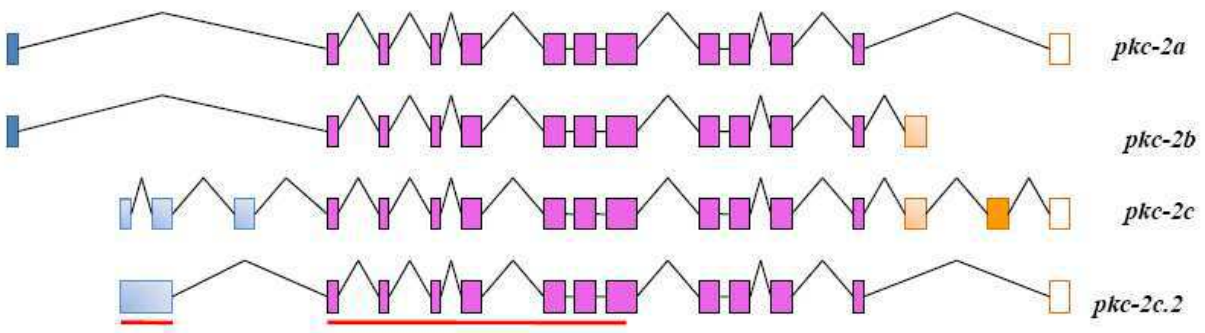
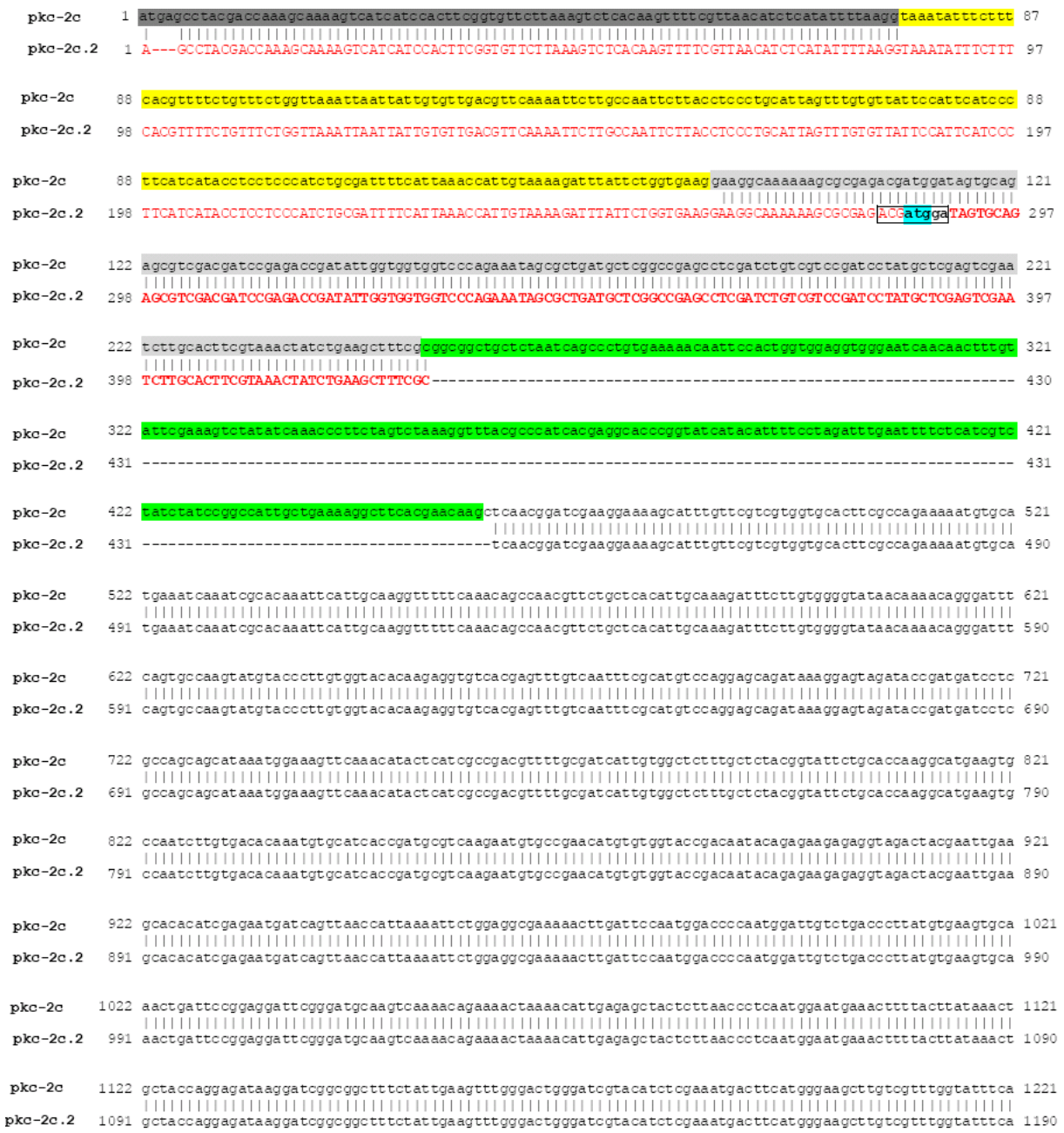


Figure 6-11: Comparison of *pkc-2c.2* to other *pkc-2* cDNA isoforms. *pkc-2* gene cDNA isoforms structure comparison. Comparing to other cDNA isoforms, the first exon of *pkc-2*c.2 included exon 1C, intron 1C and exon 2C of *pkc-2c*, but missing exon 3C, exon 15, and exon16. Isoform-specific regions used in the RNAi experiments are shown as red bars.

Results


pkc-2c 1 atgagcctacgaccaaagcaaaagtcatcatccacttcggtgttcttaaagtctcacaagttttcgttaacatctcatattttaaggtaaatatttctttt 87
pkc-2c.2 1 A---GCCTACGACCAAAGCAAAAGTCATCATCCACTTCGGTGTTCTTAAAGTCTCACAAGTTTTCGTTAACATCTCATATTTTAAGGTAAATATTTCTTT 97
pkc-2c 88 cacgttttctgtttctggttaaattaattattgtgttgacgttcaaaattcttgccaattcttacctccctgcattagtttgtgttattccattcatccc 88
pkc-2c.2 98 CACGTTTTCTGTTTCTGGTTAAATTAATTATTGTGTTGACGTTCAAAATTCTTGCCAATTCTTACCTCCCTGCATTAGTTTGTGTTATTCCATTCATCCC 197
pkc-2c 88 ttcatcatacctcctcccatctgcgattttcattaaaccattgtaaaagatttattctggtgaaggaaggcaaaaaagcgcgagacgatggatagtgcag 121
pkc-2c.2 198 TTCATCATACCTCCTCCCATCTGCGATTTTCATTAAACCATTGTAAAAGATTTATTCTGGTGAAGGAAGGCAAAAAAGCGCGAGACGatggaTAGTGCAG 297
pkc-2c 122 agcgtcgacgatccgagaccgatattggtggtggtcccagaaatagcgctgatgctcggccgagcctcgatctgtcgtccgatcctatgctcgagtcgaa 221
pkc-2c.2 298 AGCGTCGACGATCCGAGACCGATATTGGTGGTGGTCCCAGAAATAGCGCTGATGCTCGGCCGAGCCTCGATCTGTCGTCCGATCCTATGCTCGAGTCGAA 397
pkc-2c 222 tcttgcacttcgtaaactatctgaagctttcgcggcggctgctctaatcagccctgtgaaaaacaattccactggtggaggtgggaatcaacaactttgt 321
pkc-2c.2 398 TCTTGCACTTCGTAAACTATCTGAAGCTTTCGC---------- 430
pkc-2c 322 attcgaaagtctatatcaaacccttctagtctaaaggtttacgcccatcacgaggcacccggtatcatacattttcctagatttgaatttttctcatcgtc 421
pkc-2c.2 431 ---------- 431
pkc-2c 422 tatctatccggccattgctgaaaaggcttcacgaacaagctcaacggatcgaaggaaaagcatttgttcgtcgtggtgcacttcgccagaaaaatgtgca 521
pkc-2c.2 431 ----------tcaacggatcgaaggaaaagcatttgttcgtcgtggtgcacttcgccagaaaaatgtgca 490
pkc-2c 522 tgaaatcaaatcgcacaaattcattgcaaggtttttcaaacagccaacgttctgctcacattgcaaagatttcttgtggggtataacaaaacagggattt 621
pkc-2c.2 491 tgaaatcaaatcgcacaaattcattgcaaggtttttcaaacagccaacgttctgctcacattgcaaagatttcttgtggggtataacaaaacagggattt 590
pkc-2c 622 cagtgccaagtatgtacccttgtggtacacaagaggtgtcacgagtttgtcaatttcgcatgtccaggagcagataaaggagtagataccgatgatcctc 721
pkc-2c.2 591 cagtgccaagtatgtaccctttgtggtacacaagaggtgtcacgagtttgtcaatttcgcatgtccaggagcagataaaggagtagataccgatgatcctc 690
pkc-2c 722 gccagcagcataaatggaaagttcaaacatactcatcgccgacgttttgcgatcattgtggctctttgctctacggtattctgcaccaaggcatgaagtg 821
pkc-2c.2 691 gccagcagcataaatggaaagttcaaacatactcatcgccgacgttttgcgatcattgtggctctttgctctacggtattctgcaccaaggcatgaagtg 790
pkc-2c 822 ccaatcttgtgacacaaatgtgcatcaccgatgcgtcaagaatgtgccgaacatgtgtggtaccgacaatacagagaagagaggtagactacgaattgaa 921
pkc-2c.2 791 ccaatcttgtgacacaaatgtgcatcaccgatgcgtcaagaatgtgccgaacatgtgtggtaccgacaatacagagaagagaggtagactacgaattgaa 890
pkc-2c 922 gcacacatcgagaatgatcagttaaccattaaaattctggaggcgaaaaacttgattccaatggaccccaatggattgtctgacccttatgtgaagtgca 1021
pkc-2c.2 891 gcacacatcgagaatgatcagttaaccattaaaattctggaggcgaaaaacttgattccaatggaccccaatggattgtctgacccttatgtgaagtgca 990
pkc-2c 1022 aactgattccggaggattcgggatgcaagtcaaaacagaaaactaaaacattgagagctactcttaaccctcaatggaatgaaacttttacttataaact 1121
pkc-2c.2 991 aactgattccggaggattcgggatgcaagtcaaaacagaaaactaaaacattgagagctactcttaaccctcaatggaatgaaacttttacttataaact 1090
pkc-2c 1122 gctaccaggagataaggatcggcggctttctattgaagtttgggactgggatcgtacatctcgaaatgacttcatgggaagcttgtcgtttggtatttca 1221
pkc-2c.2 1091 gctaccaggagataaggatcggcggctttctattgaagtttgggactgggatcgtacatctcgaaatgacttcatgggaagcttgtcgtttggtatttca 1190

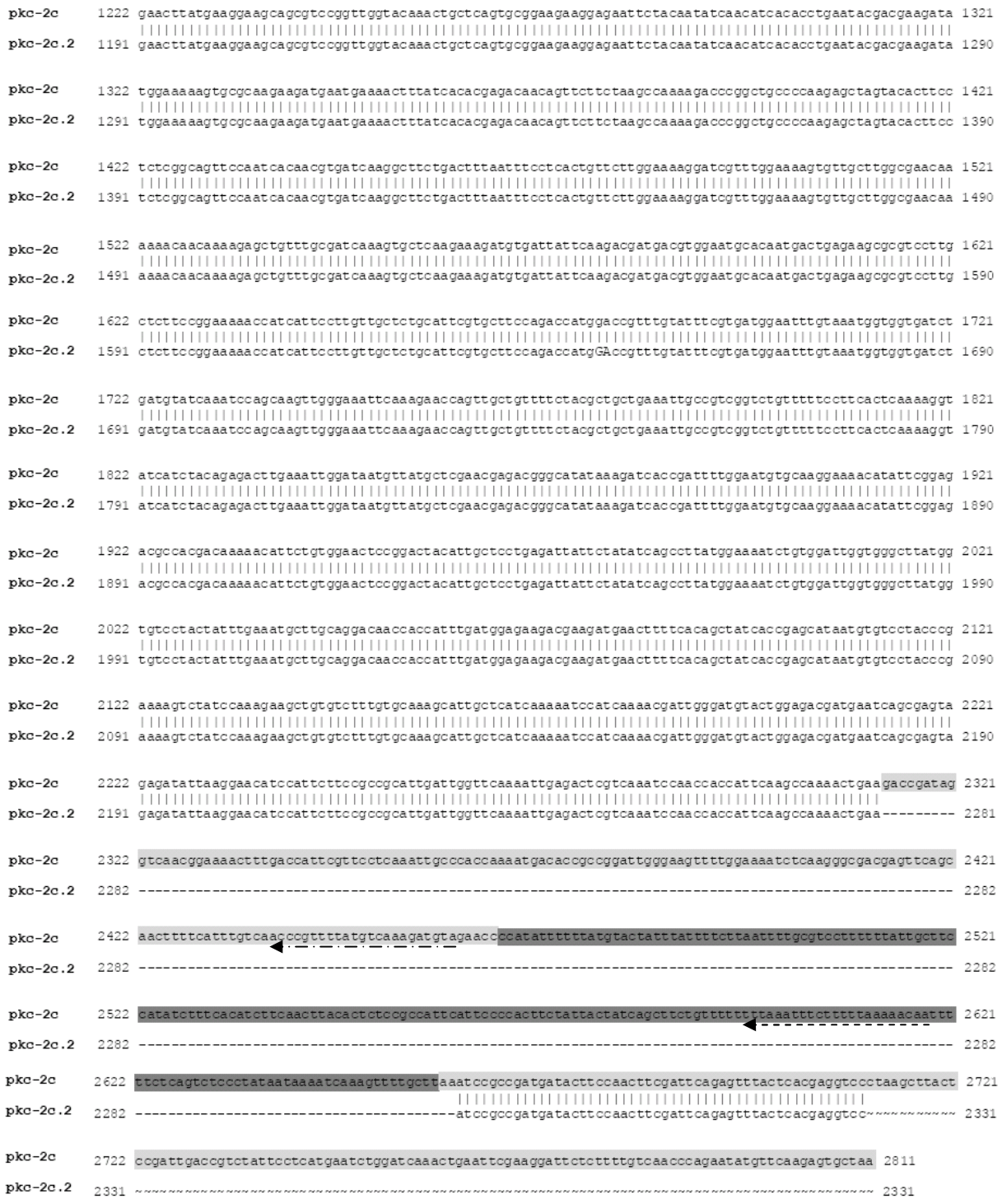


Figure 6-12: Total sequence alignment of *pkc-2c.2* comparing to *pkc-2*-c. Total sequence alignment of *pkc-2c.2* comparing to *pkc-2*c. Intron 1C was marked yellow. Red line presents the primary exon 1C.2 of *pkc-2c.2*, including exon 1C (dark grey in line1), intron 1C and exon 2C (followed light grey line). A Kozak sequence (framed with rectangle) located in exon

1C.2, carrying an ATG start codon (blue). *pkc-2c.2* exhibits a highly homology with *pkc-2c* but missing exon 3C, exon 15, exon 16 and having an incomplete of exon 17 (marked light grey and dark grey lines in turn in the end). The primer positions for RT-PCR were shown by black arrows (PKC-2-KG5: dash line; PKC-2-KG6: “dot-dash-dot” line).

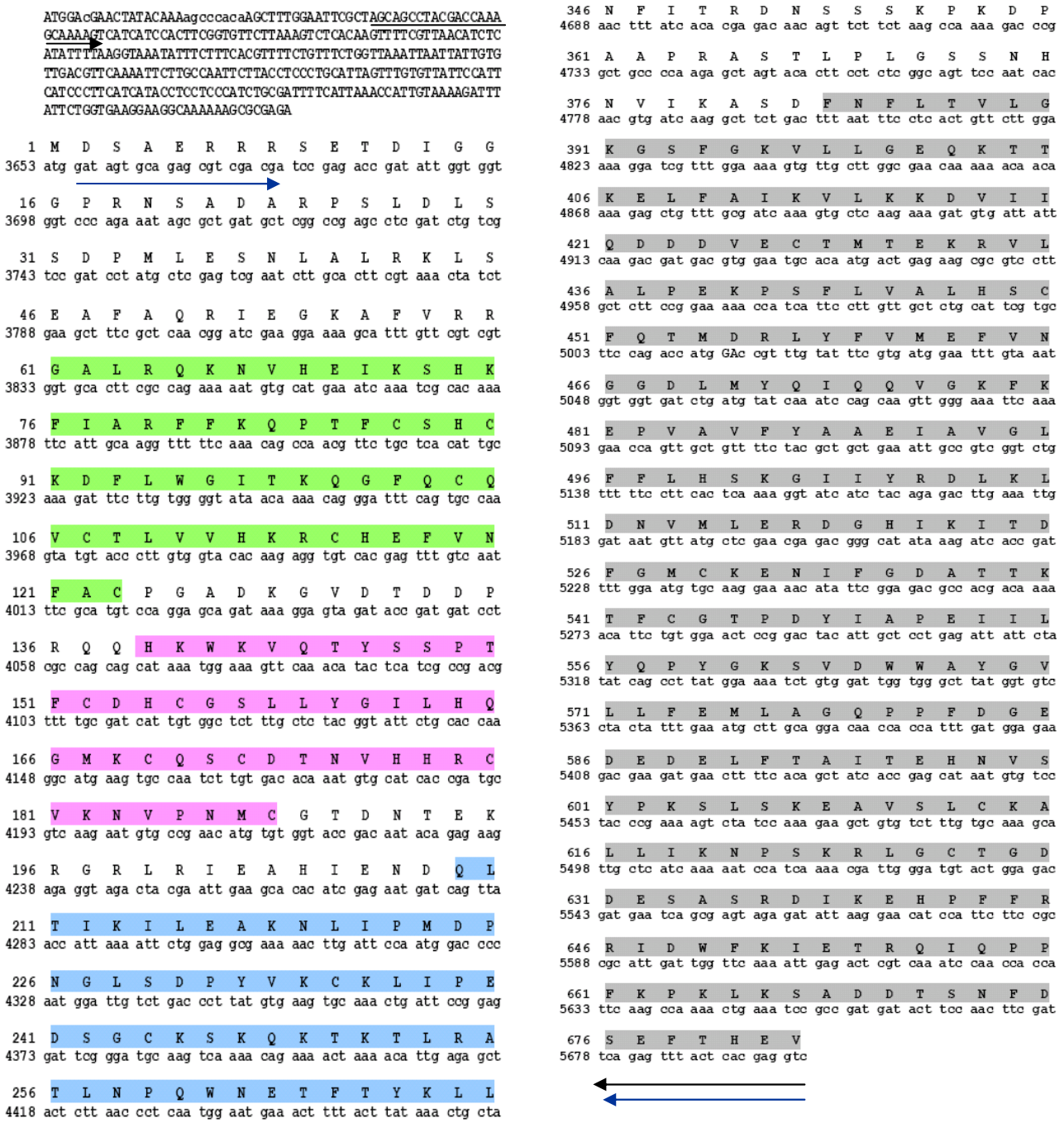


Figure 6-13: *pkc-2c.2* DNA and protein sequence with predicted domain architecture. Two C1 domains (green and red lines in the text), a C2 domain (blue line) and a protein kinase domain (grey line). Primer pairs were shown by black (KG2/KG4) and blue arrows (KG17/KG18) (full line).

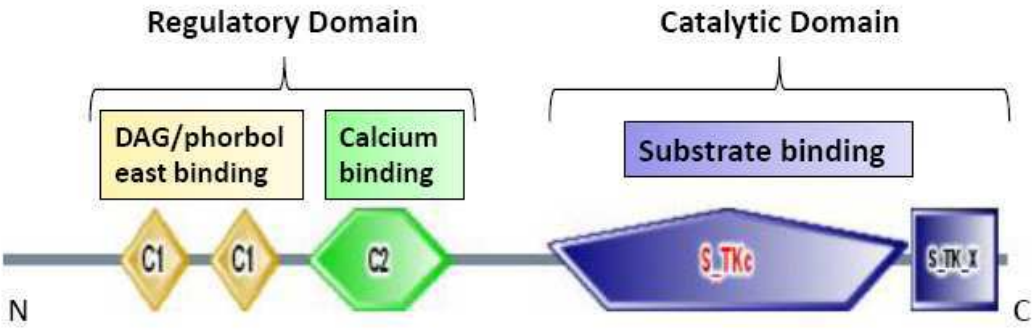


Figure 6-14: SMART analysis of predict *pkc-2c.2* translation product structure. The regulatory domain is composed of Two C1 domains (yellow) which bind DAG/phorbol east, and a C2 domain (green) which binds Ca^{2+}. The catalytic domain binding substrate is marked blue.

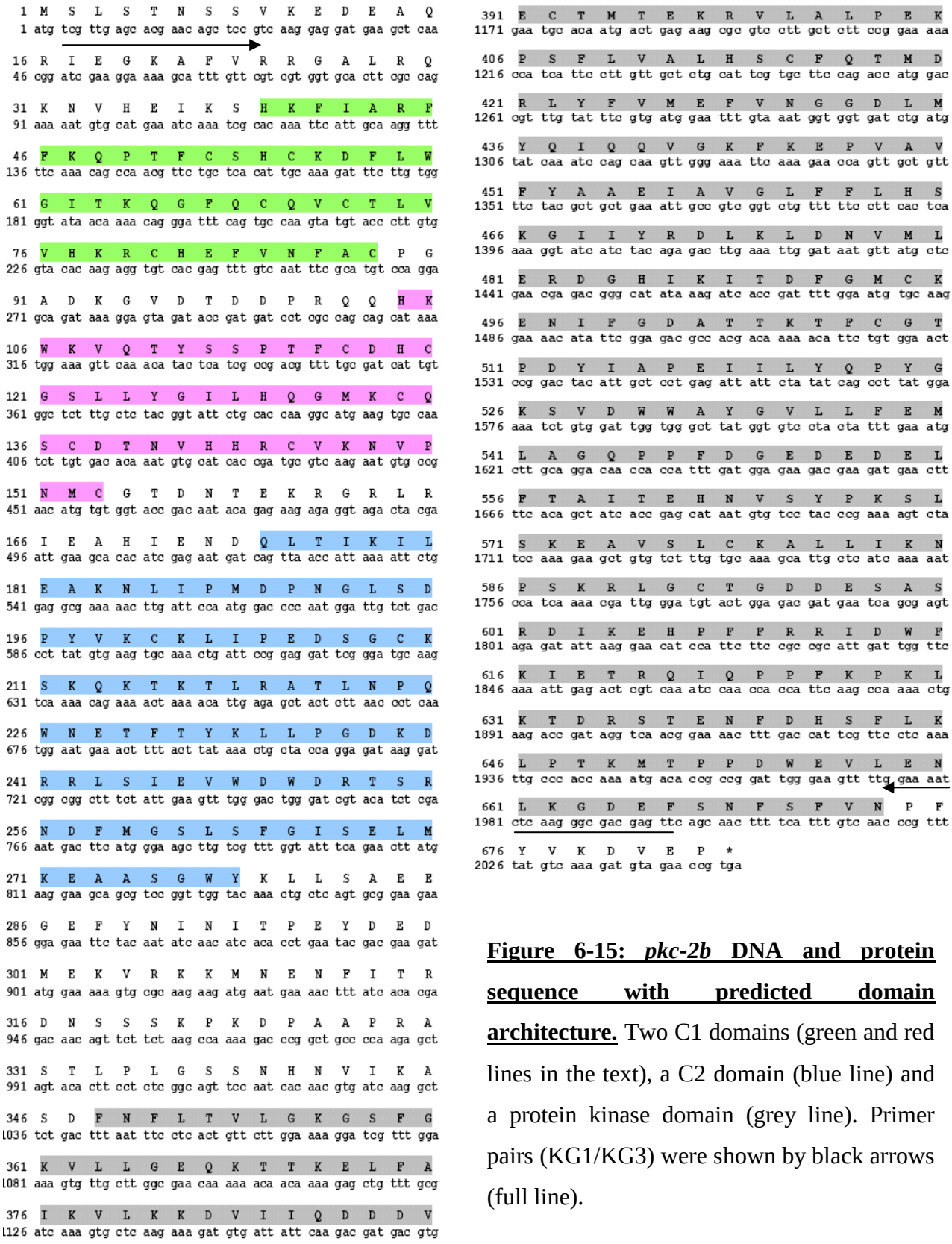


Figure 6-15: *pkc-2b* DNA and protein sequence with predicted domain architecture. Two C1 domains (green and red lines in the text), a C2 domain (blue line) and a protein kinase domain (grey line). Primer pairs (KG1/KG3) were shown by black arrows (full line).

6.2.2 PKC-2 is expressed in Pharynx and Neurons

In order to investigate the expression pattern of the *pkc-2* gene, we analysed various reporter gene constructs in *C. elegans* adult worms. To detect the expression of every isoform in *C. elegans*, the report gene *gfp* was inserted into three positions of *pkc-2* gene: the 5' exon 2C, the 3' exon 13A, and exon 10 in common region to be sure that every isoform of *pkc-2* cDNA driven by its own promoter will carry *gfp*. We observed that all three constructs exhibited the similar expression pattern. GFP was intensely expressed in pharynx and neurons. In the head of adult animal, the nerve ring surrounding the pharynx and the ventral nerve cord (VNC) were clearly seen (Figure 6-16). No GFP signal was found in posterior half of adult worms. And we did not observed GFP signal in body-wall muscle cells in adult animals, but only DEB-1/vinculin expressed in dense-body in muscle cell (Figure 6-16, H).However, in muscles of head, a very weak GFP signal was detected, where the muscle was more compact than body wall muscles (Figure 8-3, E).

Considering the expression of *pkc-2* might be too weak to be observed in muscle cells, we tried to over-express PKC-2 specifically in muscles. The two constructs *myo-3::pkc-2* carried the potent muscle promoter *myo-3*, and contained cDNA isoforms *pkc-2b* and *pkc-2c.2* respectively fused to the GFP encoding sequence (Figure 6-5A, 6-6). We also performed double-staining experiments with the MH24/anti-DEB-1 antibody, which recognizes the DEB-1/vinculin protein, one of the main components of dense bodies (Francis and Waterston, 1985; Barstead and Waterston, 1991), and mouse anti GFP antibody. However, no GFP signal could be detected in body-wall muscles. RT-PCR detected weak *pkc-2b* mRNA expression in transgenic animals (Figure 6-17). It is possibly because of RNA or protein instability.

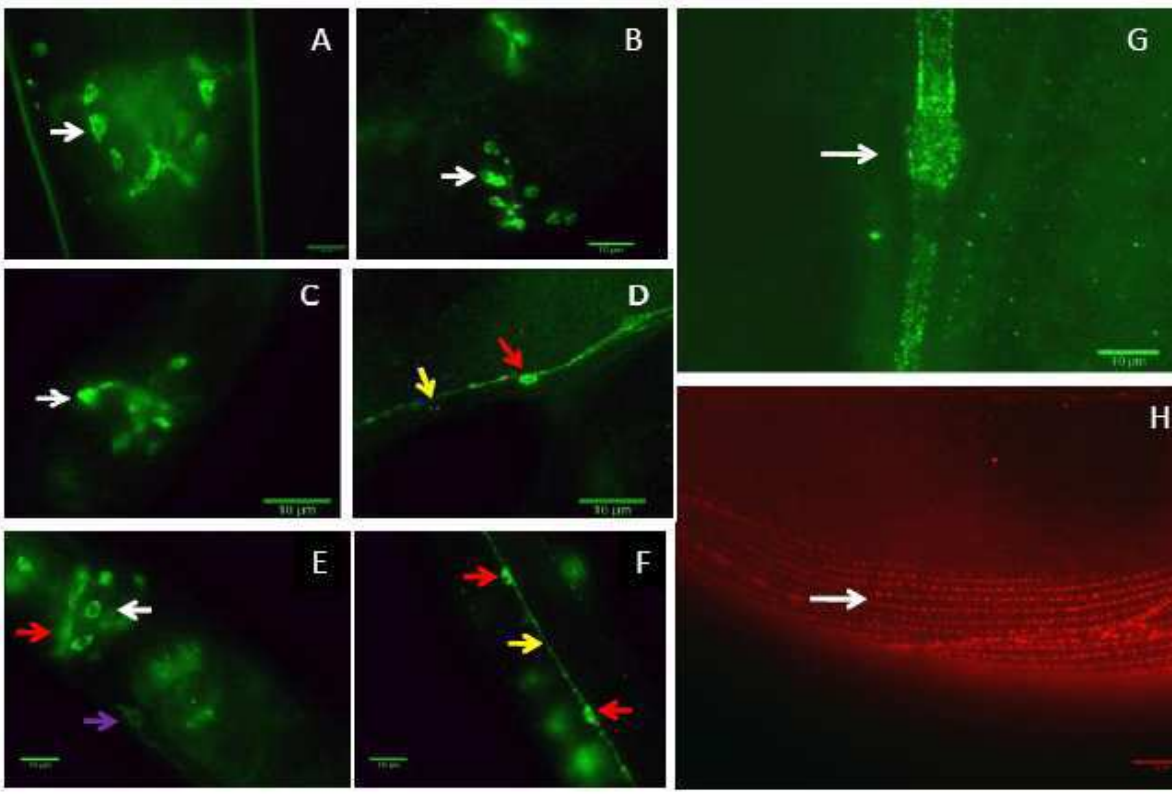


Figure 6-16: Cellular localization of PKC-2 protein in *C. elegans.* (A and B). *pkc-2::gfp* with *gfp* inserted into exon 2C of *pkc-2* gene expressed in head ring ganglia (white arrows). (C and D). *pkc-2::gfp* with *gfp* inserted into common exon 10 of *pkc-2* gene expressed in head ring ganglia (white arrow), ventral nerve cord (yellow arrow) with a cell body of a neuron (red arrow). (E and F). *pkc-2::gfp* with *gfp* inserted into exon 13C of *pkc-2* gene expression. (E). *gfp* was detected in head ring ganglia (white arrow), nerve ring (red arrow) and ventral process (purple arrow). (F). *gfp* located in ventral nerve cord (yellow arrow) and two neuronal cell bodies were seen (red arrows). (G). PKC-2 expressed in pharynx. (H). Dense body localization of the DEB-1/vinculin protein labeled with MH24 antibody (red) (white arrow). Scale bar=10µm.

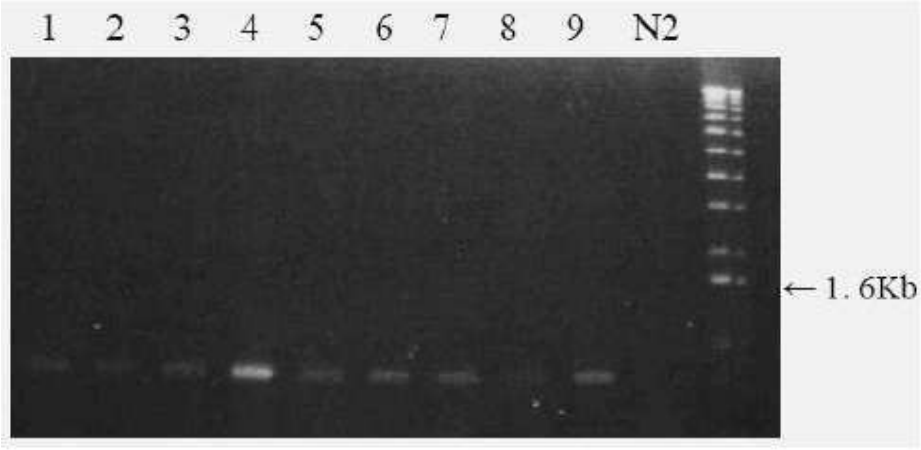


Figure 6-17: RT-PCR of transgenic *C. elegans* pKG53. Transgenic worm lines 1 to 9 expressed a weak *pkc-2b* mRNA. N2 was negative control.

6.2.3 Investigation of PKC-2 function

In order to investigate the function of *C. elegans pkc-2*, we knocked down its expression by RNAi: a larger fragment corresponding to common region of *pkc-2* cDNA isoforms (pKG66); a smaller fragment corresponding to particular 5' exon of *pkc-2c.2* (pKG65) (Figure 6-11, red bars) which helped us study the putative function of 5' exon of *pkc-2c.2*.

In particular, since our laboratory is interested in muscle biology and muscle degeneration, we looked at the potential effect of reducing *pkc-2* function in muscles. *C. elegans* mutants *dys-1(cx18)* lacking DYS-1 (*dys-1* mutants) are hyperactive and show a tendency of hyper contraction (Bessou *et al.*, 1998). The inability to detect muscle degeneration in *dys-1(cx18)* mutants in *C. elegans* is probably due to the short life span of the animals. However, the double mutant *dys-1(cx18); hlh-1(cc561)* exhibits a severe progressive muscle degeneration, up to 30% body-wall muscle cells are damaged compare to wild type animals, and paralyze phenotype(Gieseler *et al.*, 2000). Therefore, it is an ideal working model to study the mechanism of muscle degeneration.

First, we tested the efficiency of our RNAi construct by feeding strain *MosTIC pkc-2*, which carried the *gfp* reporter gene in the endogenous *pkc-2* gene (strain established in laboratory). Strain *MosTIC pkc-2* displays a *gfp* expression in neurons and spermatheca (Figure 6-18A). Results showed that worms fed with *pkc-2* RNAi had a greatly diminished *gfp* expression in

the spermatheca (Figure 6-18 C). However, the fragment corresponding to 5' exon of *pkc-2c.2* had no effect on worms (Figure 6-19 B). Although it showed no specific function in 5' exon of *pkc-2c.2*, there was possibility that RNAi experiment of this smaller fragment did not work.

Then we fed wild-type N2 worms with the same construct. No morphological defects were observed and muscles appeared normal. *pkc-2* RNAi seemed to make worms more sluggish and bloated with more eggs (data not shown).

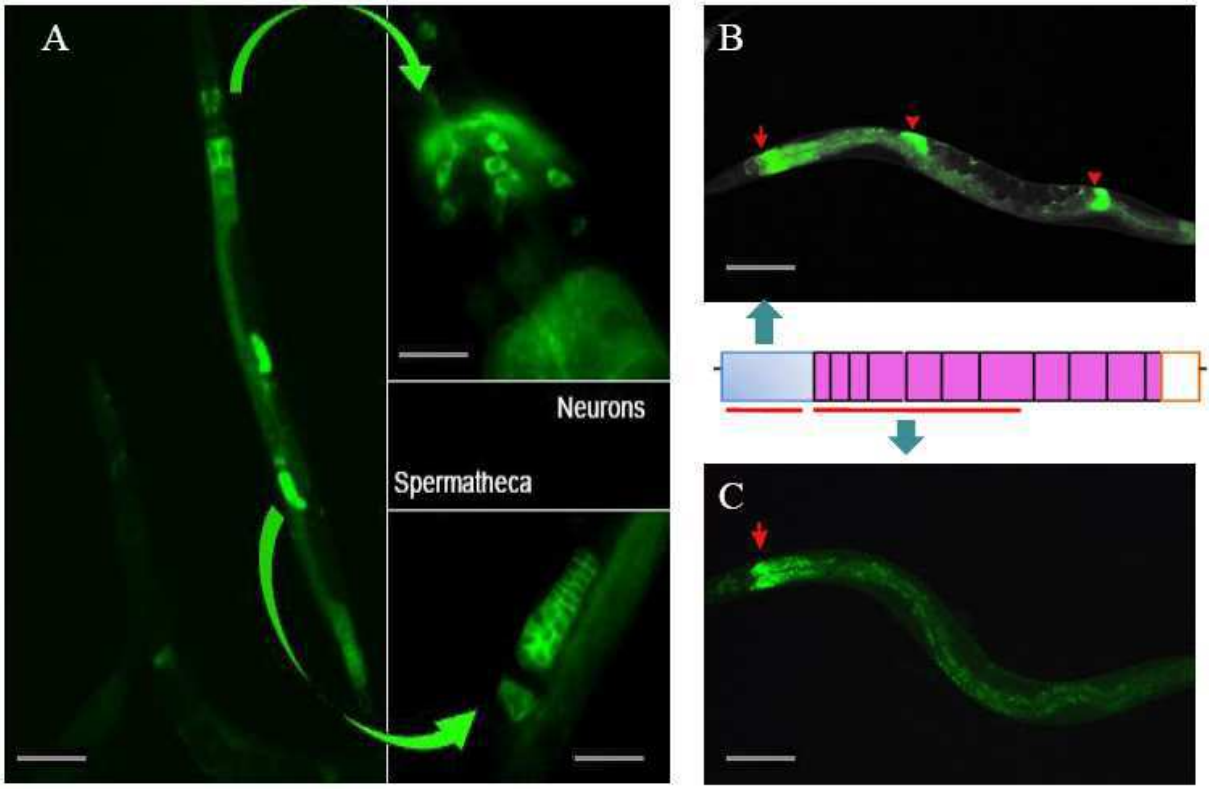


Figure 6-18: *pkc-2* isoform-specific RNAi control experiments. (A). GFP expressed in nerve ring and spermatheca in transgenic worms. (B). Adult animals were fed with dsRNA specific to particular domain of *pkc-2c.2* (shorter red bar). GFP signal was found in neurons in head (red arrow) and in spermatheca (red arrow head). (C). Adult animals were fed with dsRNA specific to commune domain of *pkc-2* cDNA isoforms (longer red bar). GFP expression was only detected in head neurons (red arrow).

To investigate the RNAi effect of *pkc-2* on *dys-1(cx18); hlh-1(cc561)*, we used *pkc-2(ok328); dys-1(cx18); hlh-1(cc561)* as control strain. The *dys-1(cx18); hlh-1(cc561)* and *pkc-2(ok328); dys-1(cx18); hlh-1(cc561)* were fed with bacteria containing empty RNAi feeding vector L4440 as control, and bacteria producing dsRNA. Knocking out *pkc-2(ok328)* could ameliorate muscle degeneration in double mutant *dys-1; hlh-1*, comparing 3.95 to 6 muscle cells missing (Table 6-3). However, RNAi experiment showed that neither dsRNA specific to particular domain of *pkc-2c.2* nor commune domain of *pkc-2* cDNA isoforms could affect muscle degeneration in *dys-1; hlh-1* comparing to triple mutant *pkc-2(ok328); dys-1(cx18); hlh-1(cc561)*. Unlike other tissues in *C. elegans*, RNAi effect is less effective in neuron system. The results probably indicate that *pkc-2* levels are not essential to muscle physiology.

Table 6-3: Muscle defects in *dys-1; hlh-1* by feeding dsRNA

Genotype	Number of Worms scored	Number of abnormal muscle cells (per line)	Standard deviation
dys-1(cx18) ; hlh-1(cc561) on RNAi **L4440**	20	6	1
dys-1(cx18) ; hlh-1(cc561) on RNAi **pKG65**	20	5.5	1.1
dys-1(cx18) ; hlh-1(cc561) on RNAi **pKG66**	20	5.2	1
pkc-2(ok328);dys-1(cx18) ;hlh-1(cc561) on RNAi **L4440**	20	3.95	1.2

Muscle cells were observed after phalloidin-rhodamin staining of animals fixed 3 days after the L4 stage.

6.2.4 Identification of PKC-2 binding protein by Yeast two-hybrid Screen

In order to identify potential molecular partners of PKC-2 in *C. elegans*, we performed a yeast two-hybrid screen in order to find potential functional partner of PKC-2. A candidate gene emerged from the screen: Y59A8A.3 (Figure 6-19). When the Y59A8A.3 was used as a query against Swisspro, protein database presented homologues including vertebrate filamin A interacting protein 1 isoform 3, shown in the partial alignments in Figure 6-20. In addition, SMART analysis indicated that the conserved sequences correspond to coiled-coil domain.

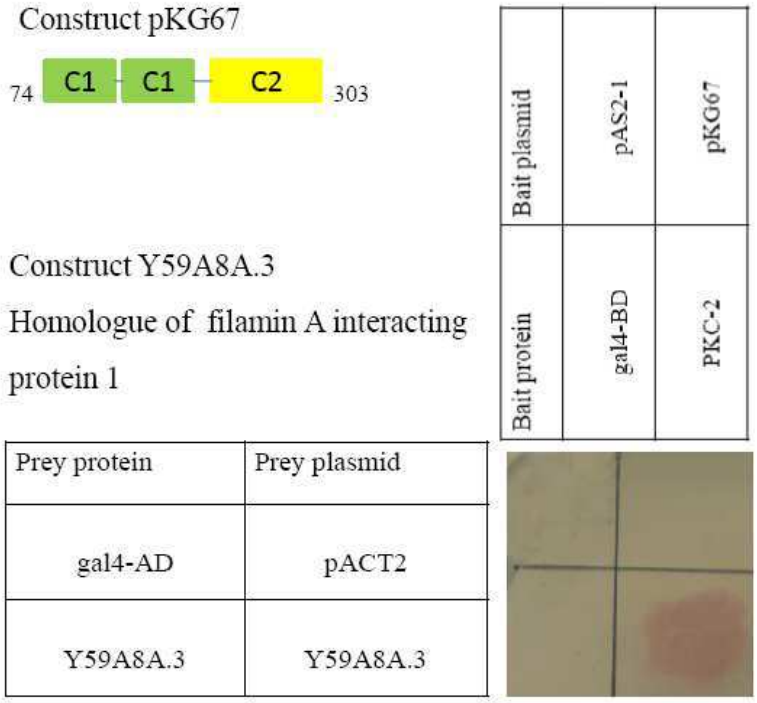


Figure 6-19: PKC-2 interacts with Y59A8A.3 in diploid yeast cell. The table indicate the different plasmids used in this screen. Bait and prey protein proteins are schematically indicated (top left). The cells grow in medium without His, Leu, and Trp. pACT2 and pAS2-1were used as control. Only interaction between Y59A8A.3 and PKC-2 regulatory domain (aa 74-303) allows cells to grow in the selective medium.

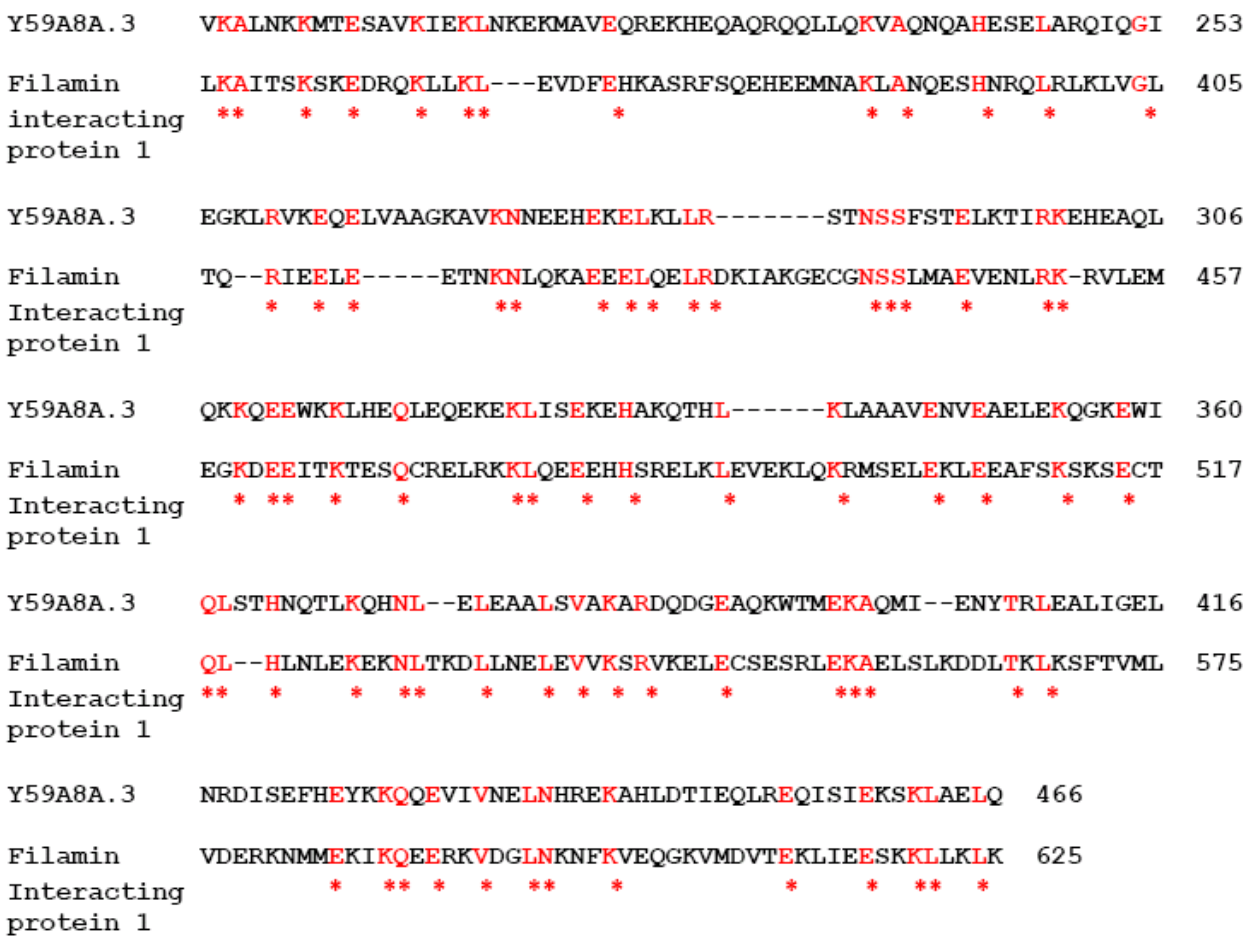


Figure 6-20: Partial alignment of Y59A8A.3 to vertebrate filamin interacting protein 1. Amino acids which conserved in protein Y59A8A.3 and filamin interacting protein 1 are marked with red asterisks.

In vertebrate, filamin A promotes orthogonal branching of actin filaments and links actin filaments to membrane glycoproteins (Gorlin *et al.*, 1990). It anchors various trans-membrane proteins to the actin cytoskeleton and because many filamin-interacting proteins (FILIP) are membrane receptors for cell signaling molecules, filamins may also function as an important signaling scaffold by connecting and coordinating a large variety of cellular processes to the dynamic regulation of actin cytoskeleton (Stossel *et al.*, 2001; Beekman *et al.*, 2008). Although abundant evidences link filamins to cell signaling pathways, it is unclear how the

signaling reactions affect filamin function. Several serine/threonine protein kinases phosphorylate filamins, including protein kinase C (Kawamoto and Hidaka, 1984).

Therefore, Ca^{2+} and phospholipid-dependent phosphorylation by protein kinase C may modulate the function of actin-binding proteins has to be considered. To study the potential interaction between PKC-2 and filamin A, we should verify the filamin A in neuron, and a test of *pkc-2* dependent filamin A phosphorylation is also needed.

6.3 Conclusion

We have generated different kinds of recombinant constructions, including cDNA clones and genomic clones, to examine the expression pattern of PKC-2 in *C. elegans* adult animals.

Our analysis showed that PKC-2 is undoubtedly expressed intensely in pharynx and neurons. A nerve ring and head ganglion, where very strong expression of PKC-2 was detected, has a characteristic structure. Ventral cord neurons also expressed PKC-2. These neurons are cholinergic motor neurons required for locomotion (Wood, 1988). No PKC-2 expressed in tail neurons. RNAi experiments suggest that PKC-2 expressing level is not essential to muscle physiology of *C. elegans*. However, although our control experiments showed that the RNAi constructs were active, we did not quantitate the level of *pkc-2* extinction, which may also vary from tissue to tissue.

Chapter7 Screen for 5-HT resistant mutant in a *pkc-2(ok328)* background

7.1 Material and Methods

7.1.1 *C. elegans* strains, alleles, and culturing

The Bristol strain N2 was used as the standard wild-type strain (Brenner, 1974). *pkc-2 (ok328)* strain has a null allele of gene *pkc-2* in X chromosome. Hawaii CB4856 strain was used for single-nucleotide polymorphism mapping as described by Wicks et al (Wicks *et al.*, 2001). The strains were obtained from the Caenorhabditis Genetics Center (CGC). All worms grow in 15°C. Animals were maintained using standard procedures (Brenner, 1974).

EMS (ethane methyl sulfonate) (Sigma #M-0880) was used as mutagen to treat L4 stage *C. elegans*.

The *C. elegans* genetic markers used were: *lin-31(n301), unc-4 (e120),* LGII.

EMS Mutagenesis Protocol:

- Collect 100 L4 stage *pkc-2* worms in a NGM plate □5.5cm
- Wash plate with M9 buffer
- Add EMS solution (at a dilution of 1:100 in M9) into 2ml worms
- Keep worms in room-temperature for 4H
- Centrifuge in 1000rpm for 1min
- Wash worms with 10ml M9 buffer for 2 times
- Centrifuge
- Collect worms into new fresh NGM plate
- Worms grow in 15°C for 6 days

7.1.2 Genetic Crosses

All cross experiments were done in 15°C.

The males were generated following a mild heat shock (34°C for 3-4 hours). Males are diploid for autosomes but haploid for X chromosome (X/□).

To construct *pkc-2/Hawaii* strain (LS944), Hawaii CB4856 males were crossed to *pkc-2* hermaphrodite 5 times to get 15/16 of Hawaii genome sequence.

To generate candidate mapping strain, LS944 males were crossed to candidate hermaphrodite. The m/m animals were selected from heterozygote F1 by 4mg/ml serotonin.

To generate three-factor mapping strain *lin-31 unc-4*/m *pkc-2*/*pkc-2*, N2 males were crossed to *lin-31 unc-4* hermaphrodite first. Then I picked *lin-31 unc-4*/+ males to cross to candidate hermaphrodite. The genotype *lin-31 unc-4/m pkc-2*/+ was identified from the cross-progeny according to their recombinant off-spring Lin Non Unc and Unc Non Lin. The genotype *lin-31 unc-4/m pkc-2*/*pkc-2* was identified by testing with 4mg/ml serotonin.

7.1.3 Single worm DNA extraction

Two adult worms were collected in 2.5µl lysis buffer (10mM Tris-HCl pH8.2; 2.5mM $MgCl_2$; 50mM KCl; 0.5% Tween20; Proteinase K 200µg/ml). Keep worms in -80°C for 30min and then incubated for 1H at 60°C followed by heat-inactivation of Proteinase K at 95°C for 10min.

7.1.4 PCR and RFLP fragment analysis

Single-worm lysates (2.5µl samples) were added to 22.5µl PCR reaction mix. Final concentrations in PCR reaction were: 0.4µM forward/reverse primer, 0.4mM dNTPs, 1× PCR reaction buffer, 0.25U Taq polymerase. PCR reaction setup was done in 96-well plates. Cycling parameters were: 2min 95°C, 30sec 95°C, 30sec 95°C, 58°C 30sec, 72°C 1min,

30cycles. Following PCR, 5µl reactions were digested with 0.5U enzyme, 1× buffer in V_{TT}10µl. 1.5% gel electrophoresis analyzes.

7.1.5 RNAi screen

Worm growth culture, bacteria culture, clones and vector

Regular Nematode Growth Medium final concentrations were: 4mg/ml serotonin, 1mM IPTG, and 25mg/ml carbenicillin.

The bacteria strain fed on worm is competent cell HT115 *E. coli*. The bacteria culture is Luria-Bertani broth with 100µg/ml Ampicillin (final) and 15µg/ml tetracycline.

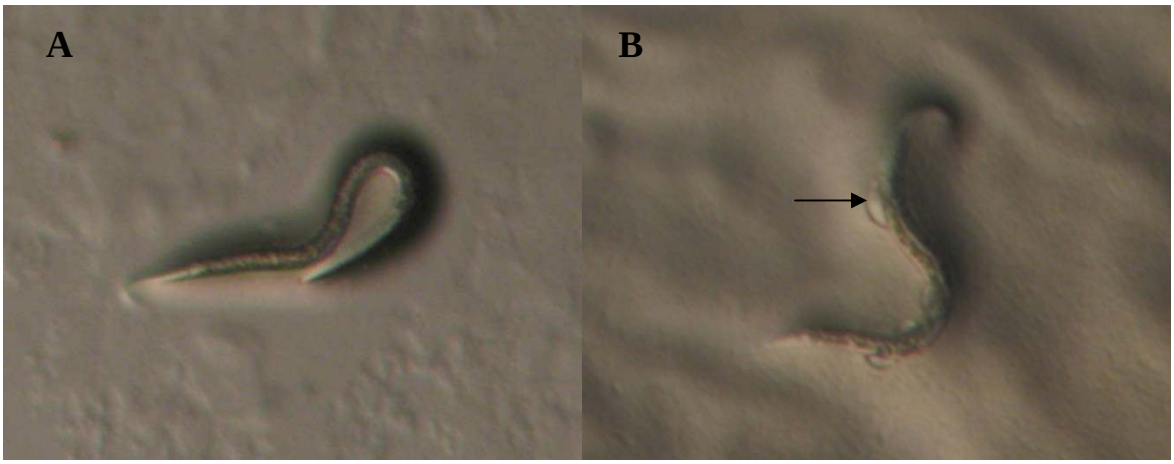
Used plasmid is L4440, and control clones are *pos-1*(embryonic lethal), *dys-1*(hyperactive).

7.2 Results

7.2.1 The *pkc-2* Recessive Suppressor Screen

The *pkc-2*(*ok328*) mutants are sensitive to serotonin. We observed that the *pkc-2* parent adult animal has increased egg-laying and has decreased locomotion in the first 30min when put on 4mg/ml serotonin compare to N2. In addition, 75% of *pkc-2* larvae animals display a severe blister phenotype in presence of 4mg/ml exogenous serotonin and died before L4 stage. The blister mutants have fluid-filled blebs between the two layers forming the cuticle (Figure 7-1, B, and black arrow).

In order to understand the potential function of both *pkc-2* and serotonin in the process of muscle degeneration in *C.elegans*, as well as their correlation between PKC-2 and serotonin, I performed an EMS screen (10,000 haploid genomes) for recessive suppressors of the blister phenotype of *pkc-2*(*ok328*) mutants in presence of 4mg/ml exogenous serotonin (5-HT). The chemical mutagenesis (EMS) protocol was described in “material and methods”.



N2 *pkc-2*

Figure 7-1: *pkc-2* blister phenotype in 4mg/ml serotonin plate. (A). *C. elegans* in NGM petri plate. (B). *C. elegans* in 4mg/ml 5-HT NGM petri plate presents blister in cuticle (black arrow).

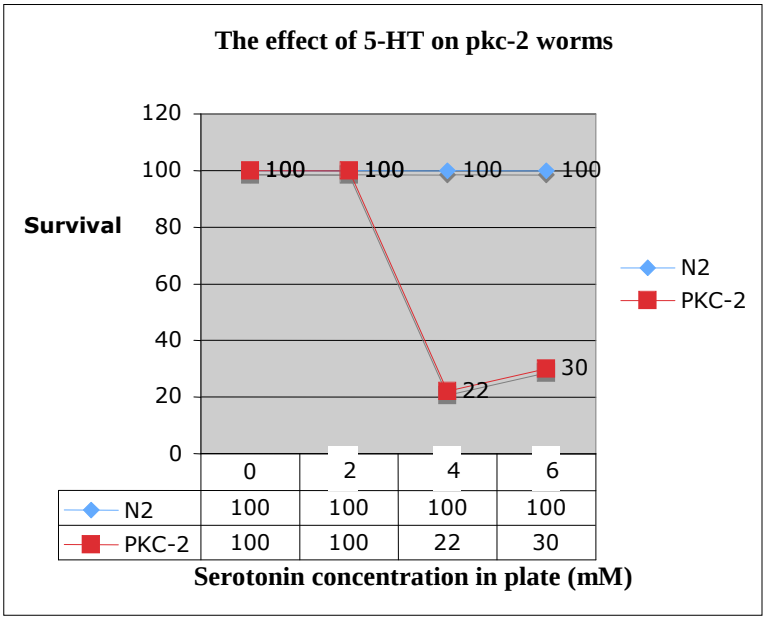


Figure 7-2: *pkc-2* response to different concentration of serotonin. The concentration of serotonin is 0mg/ml, 2mg/ml, 4mg/ml, 6mg/ml. The N2 is not affected by all 4 concentrations of serotonin but *pkc-2* survival is extremely decreased in 4mg/ml serotonin.

The L4 stage worms have many meiosis cells in the distal gonad. When EMS induces a mutation in the chromosome, that most commonly causes single base pair mutations G/C to A/T point mutations or small deletions/insertions, we can isolate the homozygote mutant after several generation.

I treated 5000 *pkc-2* L4 stage worms (F1) with EMS, then transfer a single worm into a new NGM plate to let it grow in 15°C for 7 days to get the self-progeny F2. Based on the Mendel Law, there will be ¼ of F2 homozygote containing the mutation, ¼ of F2 homozygote wild-type, and ½ of F2 are heterozygote. The 4 F2 generation worms were picked randomly, when selected the homozygote worm by 4mg/ml serotonin.

However, if the mutated allele is low penetrance, then there will only sometimes produce the symptom or trait with which it has been associated at a detectable level. Penetrance is a term used in genetic, describing the proportion of individuals carrying a particular variation of a gene (an allele or genotype) that also express an associated phenotype. Penetrance only considers whether individuals express the phenotype or not. In the case of low penetrance it is difficult to distinguish environmental from genetic factors. Considering only few progeny carrying the mutation will possess the blister phenotype, the candidate was not confirmed until the sixth generation (F6).

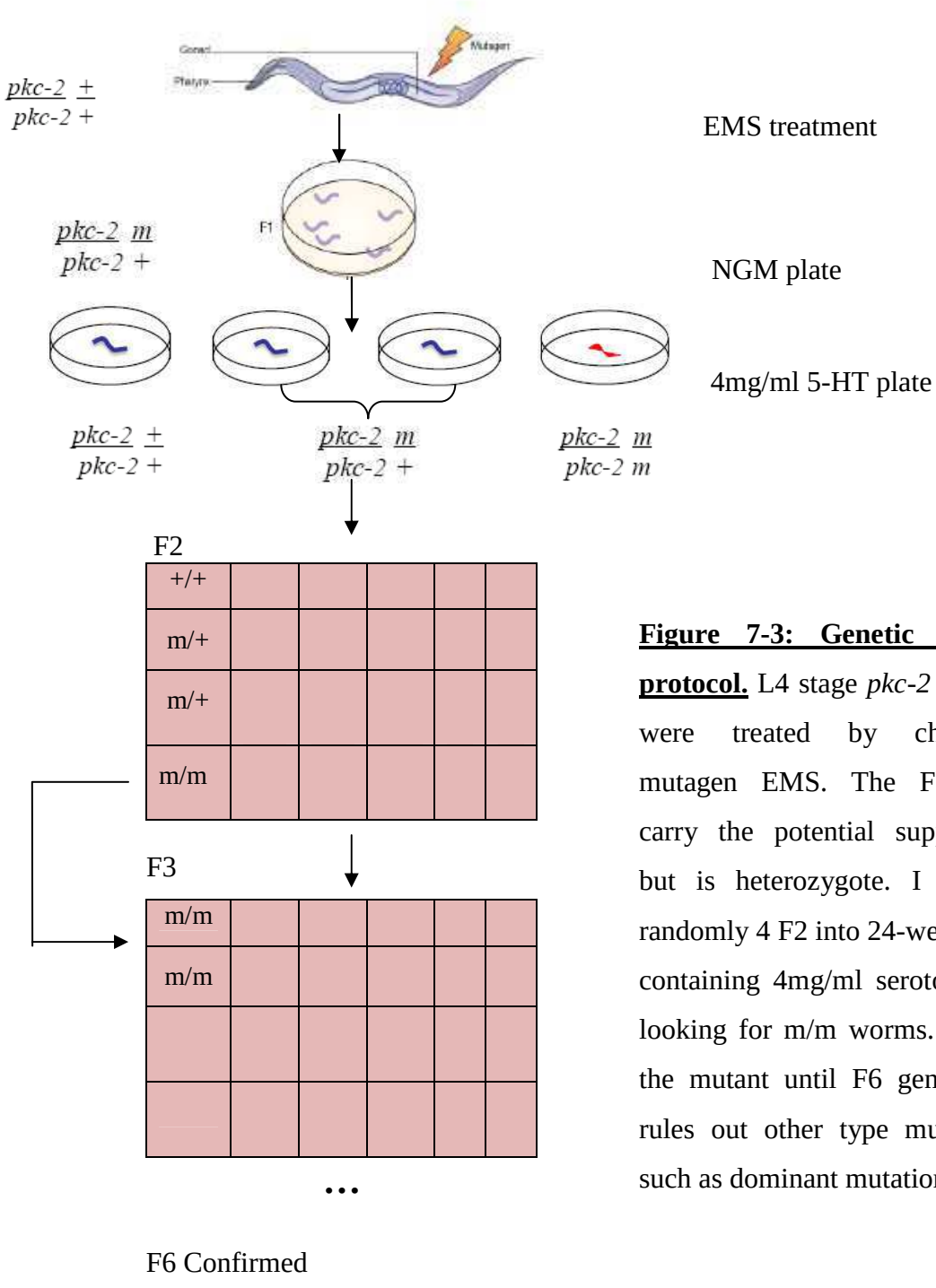


Figure 7-3: Genetic screen protocol. L4 stage *pkc-2* worms were treated by chemical mutagen EMS. The F1 will carry the potential suppressor but is heterozygote. I picked randomly 4 F2 into 24-well plate containing 4mg/ml serotonin to looking for m/m worms. Retest the mutant until F6 generation rules out other type mutations such as dominant mutation.

When screen was completed, in total, 26 candidates were emerged, named *cx251* to *cx276*.

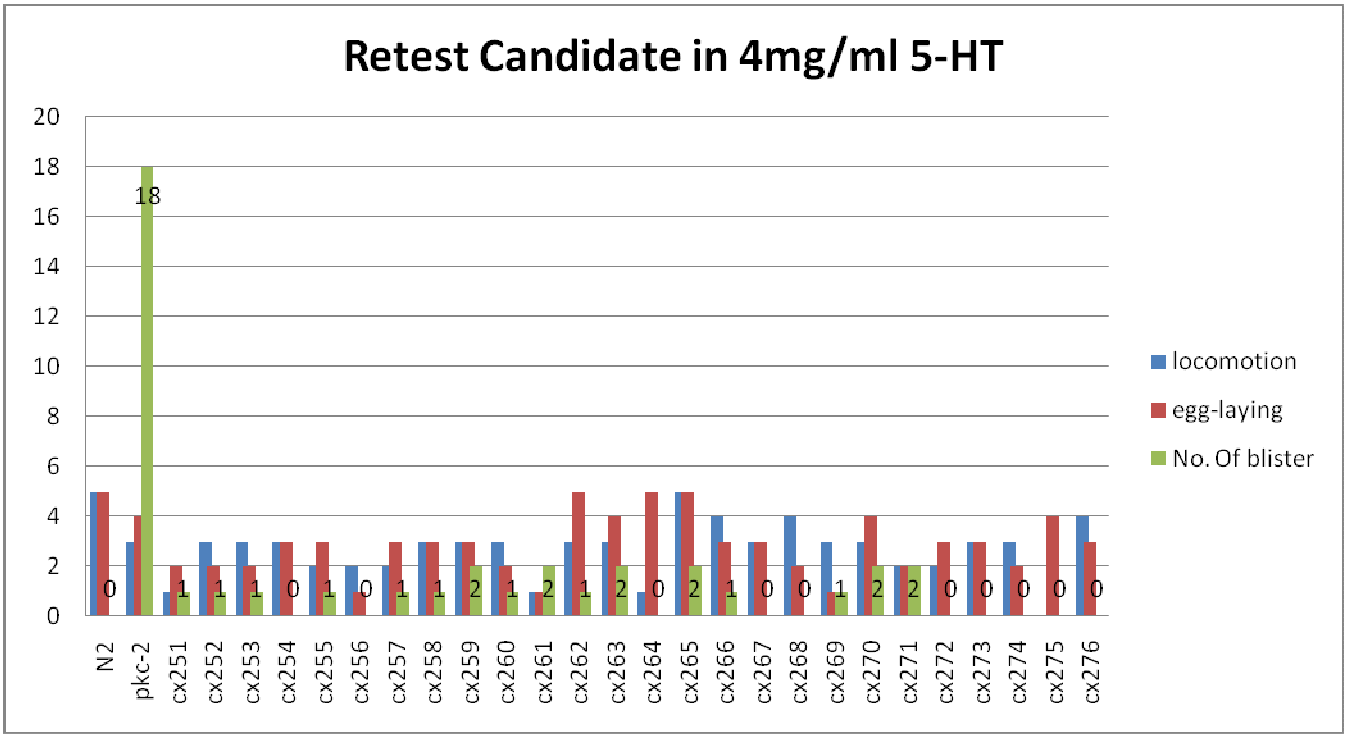


Figure 7-4: Confirmed candidates by retesting with 4mg/ml serotonin. Each strain was tested 2 adult worms and checked the progeny in the following week. N2 and *pkc-2* were used as negative control and positive control, respectively. Green bars indicate the average number of blister animals; the ability of locomotion blue bar) and egg-laying (red bar) shows no significant differences. The *pkc-2* displays a severe blister phenotype compare to N2 and the candidates.

7.2.2 Genetic Mapping

To identify the location of potential mutation, I narrowed down the mutated region into the specific chromosome by single nucleotide polymorphism (SNP) mapping, and then performed three-point mapping for the precise location of mutation. Considering the difficulty of identifying the specific mutation in a very small region in chromosome by three-factor mapping, RNAi screen helps to point the mutation into a particular gene in the region on the chromosome.

In SNP mapping, DNA sequence polymorphisms between the wild-type N2 strain and a closely related strain CB4856 Hawaii are used as silent genetic markers, with applications

ranging from classical two- and three-factor mapping to measuring recombination across whole chromosome.

SNP mapping is done in two phases. The first phase; chromosome mapping, seeks to identify the relevant chromosome and rough position of the mutation. The second phase, interval mapping, seeks to place the mutation in an interval between two SNPs and can be used to fine map the mutation. SNP detection in both phases is typically performed by using RFLP-type SNP which alter a restriction site.

Chromosome mapping:

All used SNP markers covered the 6 chromosomes are RFLP-type SNP of CB4856 strain. In this case, I generated the strain LS944 which has been enriched 15/16 of Hawaii genetic background, and is *pkc-2* homozygote as well, to cross with the 26 candidates (Figure 7-5, 7-6). SNP test showed the LS944 is highly homology to Hawaii strain in SNP character, except SNP marker 7, 8 and 9 that are homology to N2 (Table 7-1).

The expected genotype of succeed cross between LS944 and the candidates is *pkc-2*(*ok328*)/*pkc-2*(*ok328*) m/m containing 7/8 of Hawaii sequence, and is resistant to 4mg/ml exogenous serotonin. After the genotype get confirmed, 20 separated progeny of one cross were kept to extract genomic DNA, and then tested the SNP linkage with all markers listed in table below except markers 7, 8 and 9. When mapping a recessive mutant in the N2 background against the Hawaii strain, linked SNPs will display an enrichment of N2 bands in the mutant DNA, approaching 100% N2 for tight linkage. The closer the mutation lies to the given SNP being tested, the more that there will be a significant over-representation of N2 homozygous loci among mutant animals.

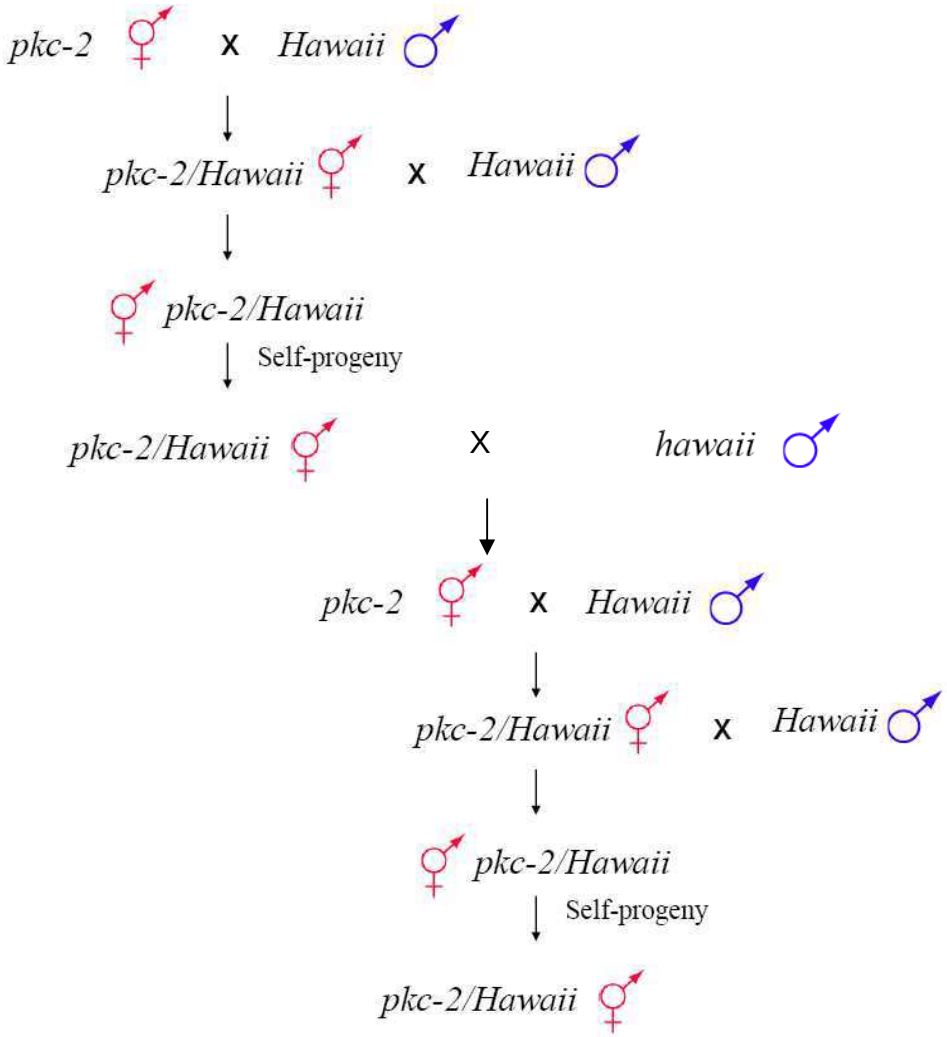


Figure 7-5: Protocol of LS944 strain generation. 7 young adult Hawaii males were crossed to 3 L4 state *pkc-2* hermaphrodites. I picked cross-progeny hermaphrodite to cross to Hawaii males for 5 times until I got 15/16 of Hawaii genome sequence.

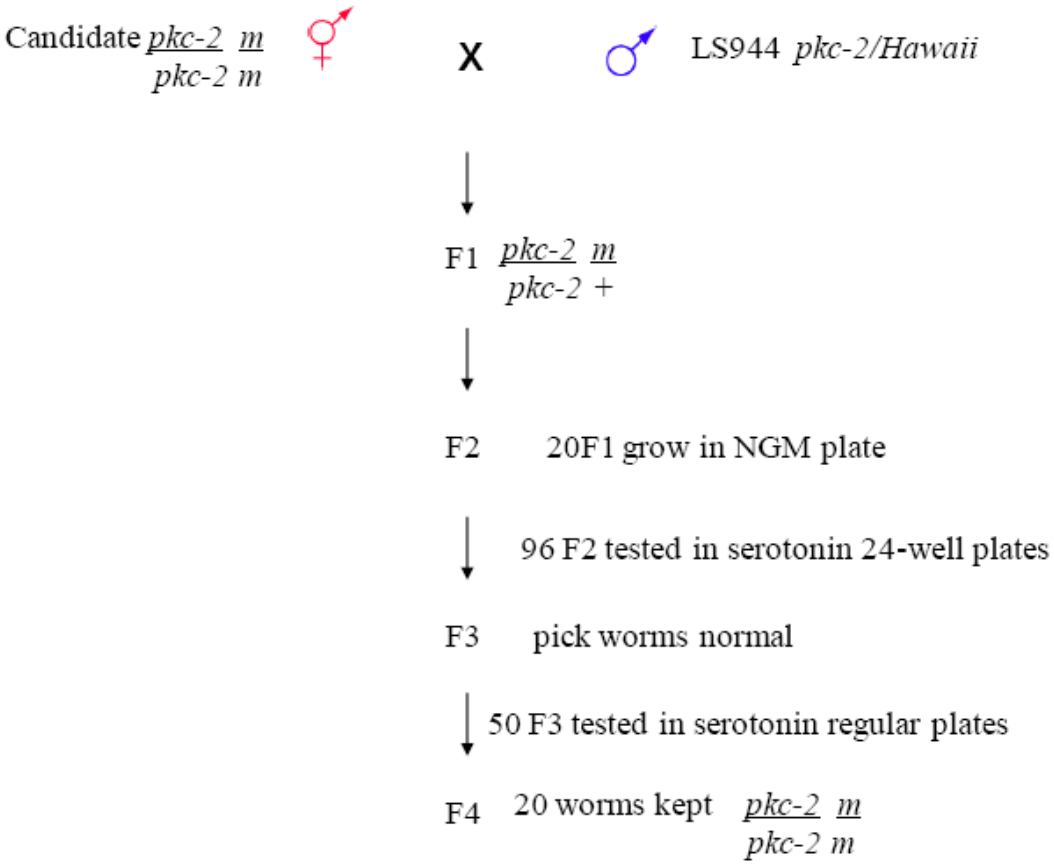


Figure 7-6: Protocol of cross LS944 to candidate. 7 young adult males of LS944 were crossed to 3 L4 stage candidate hermaphrodites. The cross progeny is *pkc-2* homozygote but suppressor heterozygote. The m/m self-progeny were selected by 4mg/ml serotonin and got confirmed until F3 generation.

Table 7-1: List of all SNP markers used for mapping and the test for N2/Hawaii/LS944

SNP marker	chromosome	clone	Genetic map	enzyme	Observed band size (bp)			allele
					N2 digest	Hawaii digest	LS944 digest	
1		ZC123	-17.9	*SspI*	ND	ND	ND	P1051
2		K04F10	0.91	*NdeI*	594	310	310	P1057
3	I	C37A5	22.46	*EcoRI*	310/280	560	560	P1071
4BIS		C07B3	-15.98	/	ND	ND	ND	P4050
4		Y66H1A	-24.93	/	ND	ND	ND	P4050
5		R105	1	*HincII*	600	300	300	P4034
5A		D2096	3.7	*EcoRI*	650/230	850	850	P4039
6A		Y45F10A	8.95	*AluI*	210/130	310	310	P4059
6		M199	12.5	*AvaI*	850	450/400	450/400	P4045
6E		Y73F8A	13.35	*DraI*	220/120	380	380	P4098
6C		Y105C5	14	*BstBi*	800	400/380	400/380	P4046
6F		Y51H4A	15.14	*EcoRI*	210	320	320	P4100
6D	IV	C35D6	14.92	*SspI*	700	380	380	P4024
7		F28C10	-19	*BspHI*	220/180	400	180/220	P6100
8		F45E1	-0.76	*EcoRI*	550/300	800	550/300	P6110
9	X	F23D12	16.77	*SspI*	280	200/100	280	P6124
10		F36H9	-18.9	*DraI*	330/80	420/80	420/80	P5076
11		F20D6	0.88	*AluI*	240/93	350	350	P5062
12	V	Y51A2	17.16	*DraI*	400	300/120	300/120	P5082
13		T01D1	-18	*DraI*	410/200/44	600/44	600/44	P2101
13B		F29A7	-9.2	*AluI*	700	420/300	420/300	P2114
13A		F54D10	-5.27	*DraI*	450	210/280	280/210	P2103
14B		C01F1	-4.51	*HaeII*				P2051
14		T13C2	0.12	*DraI*	310/140/70	400/140	400/140	P2107
14A		C50E10	5.5	*EcoRI*	680	400/290	400/290	P2111
14C		F15D4	15.74	*DraI*	500	300	300	P2116
15	II	Y48E1b	16.01	*SspI*	520	280/237	280/237	P2117
16B		T17H7	-26.33	*SspI*	950	560/350	620/380	P3002
16ter		C39B5	-15.08	*BstUI*	210/150	350	350	P3055
17		F10E9	-0.31	*AccI*	600/280	854	854	P3049
18	III	Y75B8A	15.77	*HindIII*	330	260/70	280/70	P3075

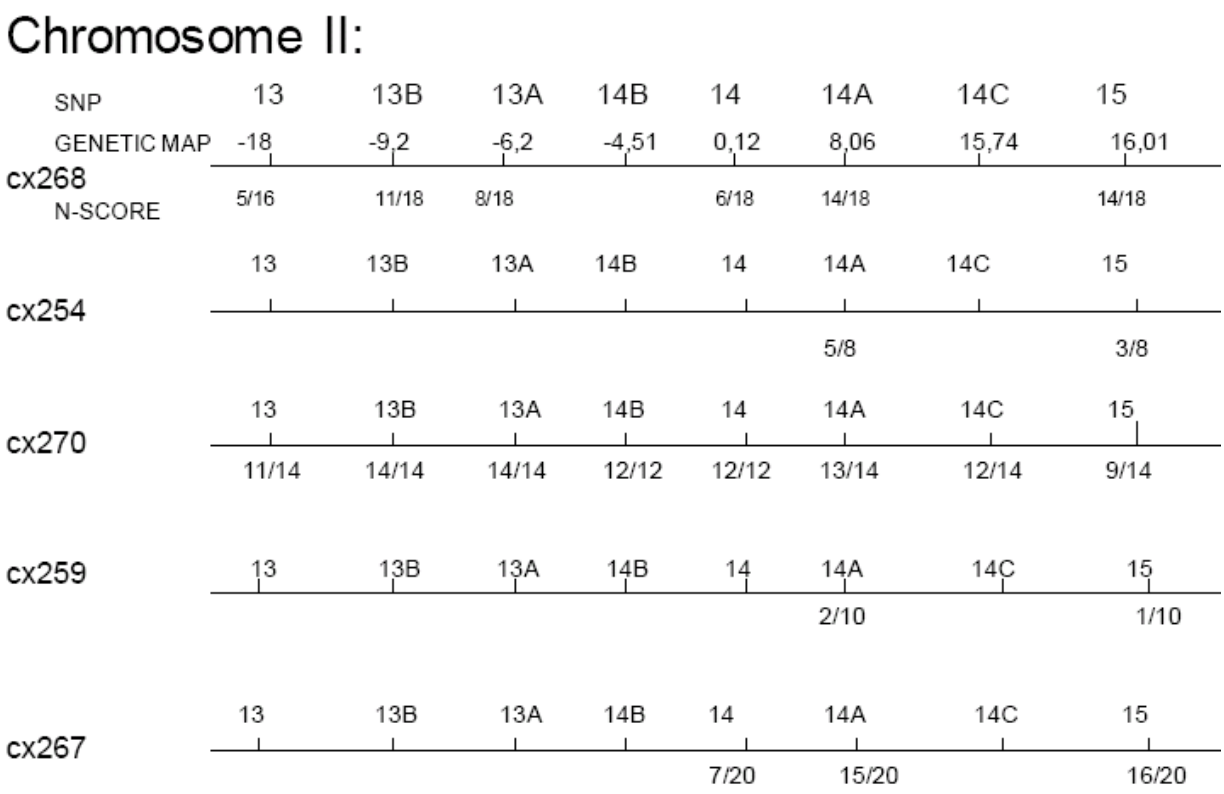
I chose 16 better candidates from 26 to perform a first round SNP mapping by PCR and counted the N2-like digest band for each SNP marker. As diploid organism, for one candidate, more than 32 N2-like bands from 20 samples (≥80%) are thought to be linked with the related SNP. In the 16 candidates, 8 showed the strong linkage with SNP marker and concerted in chromosome II, III, IV (Table 7-2).

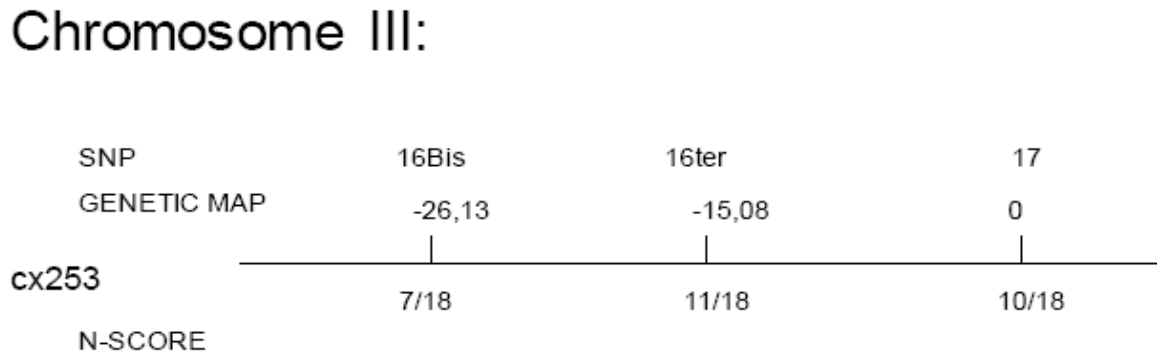
Table 7-2: Results of chromosome mapping

candidate	Linked SNP	N2 score	Chromosome region	
cx253	16B	27/32	LGIII	-26.33
cx254	5	30/36	LGIV	1
	6	30/36		12.5
	15	31/36	LGII	16.01
cx259	6	30/40	LGIV	12.5
	15	29/38	LGII	16.01
cx263	5	29/40	LGIV	1
	6	27/38		12.5
cx267	14	26/36	LGII	0.12
	15	34/40		16.01
cx268	13	27/38	LGII	-18
	14	26/38		0.12
cx270	13	25/28	LGII	-18
	14	28/28		0.12
cx276	5	35/40	LGIV	1
	6	37/40		12.5

Interval mapping:

To narrow down the region around the mutation in correspond chromosome, I performed a second round of SNP mapping, counted N2-like digest band for each marker. The strongest linkage was located in *cx270*, 100% N2 between 13B and 14 indicated a highly possibility of mutation in this region (Figure 7-7).


Chromosome II:
SNP
GENETIC MAP
13
13B
13A
14B
14
14A
14C
15
-18
-9,2
-6,2
-4,51
0,12
8,06
15,74
16,01
cx268
N-SCORE
5/16
11/18
8/18
6/18
14/18
14/18
cx254
5/8
3/8
cx270
11/14
14/14
14/14
12/12
12/12
13/14
12/14
9/14
cx259
2/10
1/10
cx267
7/20
15/20
16/20


Chromosome III:
SNP
GENETIC MAP
16Bis
16ter
17
-26,13
-15,08
0
cx253
N-SCORE
7/18
11/18
10/18

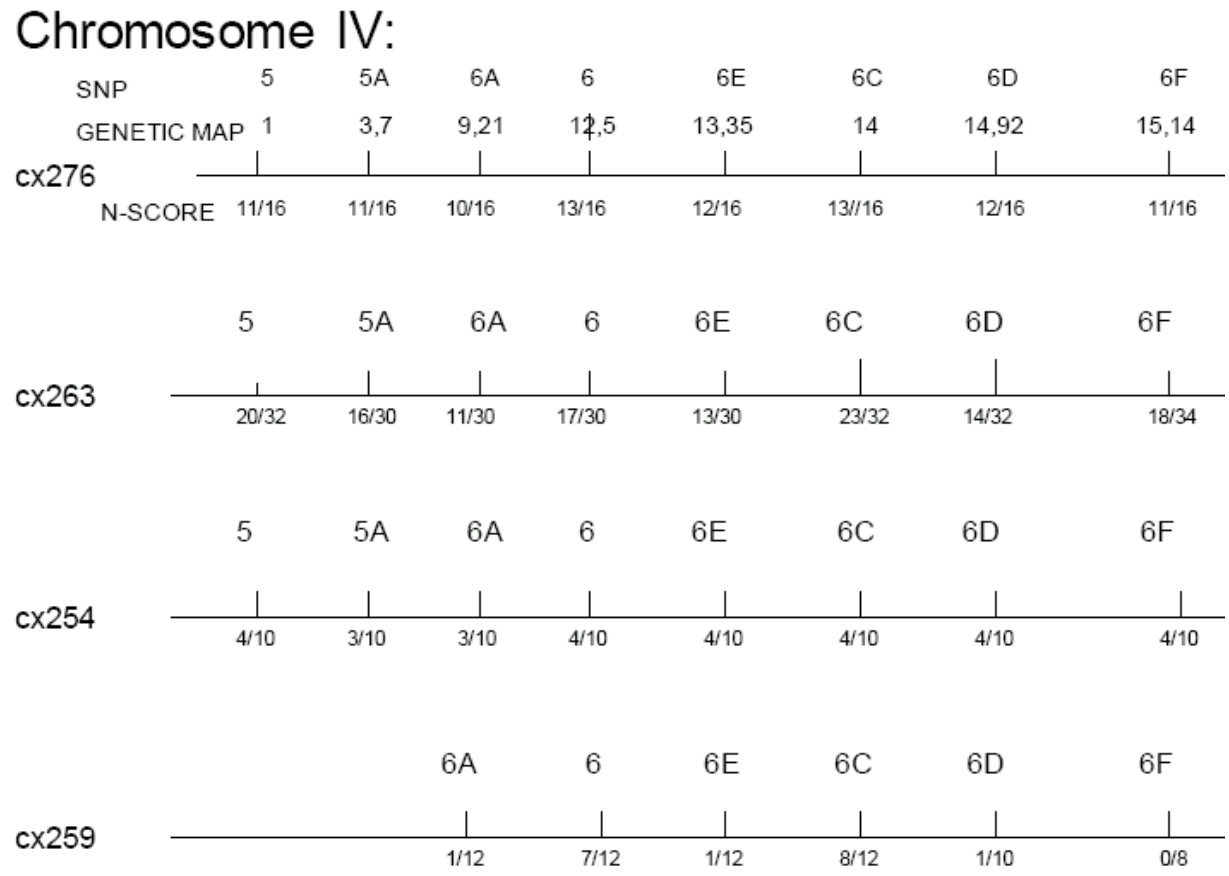


Figure 7-7: Results of fine mapping. The coordinate suggests the genetic map in chromosome. In all candidates, *cx270* demonstrates the strongest linkage with SNP markers 13B, 13A, 14B, 14, 14A in chromosome II.

Then I did three-factor mapping (Fay, 2006) with genetic markers *lin-31(n301)* (genetic position: II:-5.89cM) and *unc-4(e120)* (genetic position: II: 1.76cM). The basic idea is to cross the mutation into a strain with two linked morphological markers *lin-31* and *unc-4* that are on the same chromosome as mutation to generate *m/lin-31 unc-4* heterozygote. Followed two classes of progeny Lin Non Unc and Unc Non Lin, to see which of these two classes also produce the mutant type in the subsequent generation, we can determine whether the mutation lies to the left, right, or between set of markers. Furthermore, when the mutation lies in between, we may determine the approximate distance from each marker (Fay, 2006).

Mapping results showed both Lin Non Unc and Unc Non Lin carried the mutation, which indicates the mutation is between *lin-31* and *unc-4* (Figure 7-8).

I tested recombinant strain *lin-31 cx270/pkc-2*, *lin-31* and *lin-31 pkc-2* as control strain; *unc-4 cx270*/*pkc-2*, *unc-4* and *unc-4/pkc-2* as control strain; in 4mg/ml 5-HT. 4 from 10 *unc-4 cx270*/*pkc-2* display a blister phenotype, that mutation is thought not to be in these recombinant, while 6 are normal. However, only 1 from 11 *lin-31 cx270/pkc-2* seems to not carry mutation according to their blister phenotype. The results raised the question that in this case it is difficult to map the mutation with three-factor mapping since the mutated region was too small to isolate.

RNAi screen allows us to looking for the potential gene carrying the mutation. The principle rule is to feed *pkc-2*(*ok328*) strain with bacteria carrying plasmid L4440 which express a double chain RNA of every clone of *C. elegans* gene, in the presence of 4mg/ml serotonin, and look for their phenotype. If a certain clone could suppress the blister phenotype of *pkc-2* in serotonin, then the clone correspond to the gene could be candidate mutation.

The RNAi bank has more than 87% of *C. elegans* genes and conserved in 50 384-well plates (Figure 7-9). Knowing rough region in chromosome II of *cx270*, I screened RNAi clone from genetic position -8.48, cDNA T10D4.7 to 0.66, cDNA C41C4.2, that correspond to 384-well plate No.3, No.4, No.5, respectively.

After the screen, the coming out candidate gene would be retested for sure.

Besides *cx270*, other candidates are need to mapping.

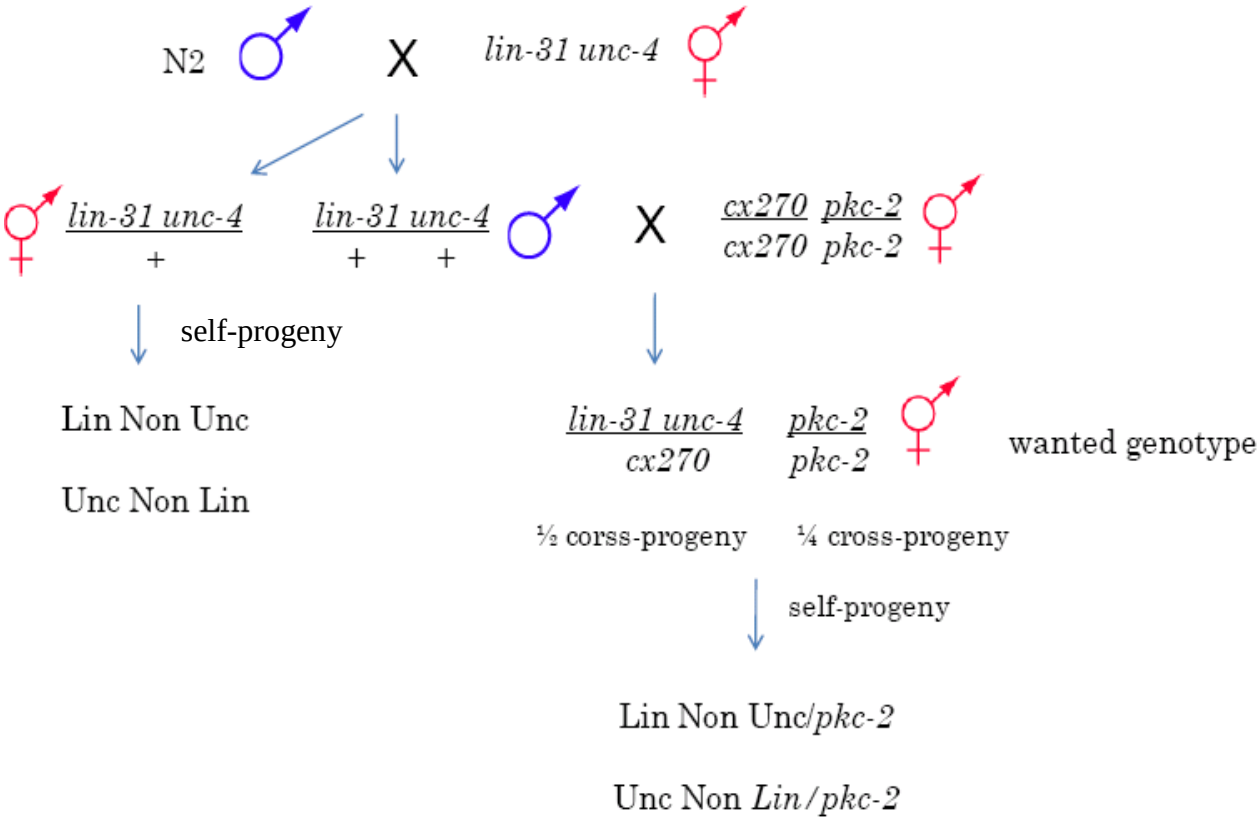


Figure 7-8: Three-factor mapping protocol. 8 N2 young adult males were crossed to 4 L4 stage *lin-31 unc-4* hermaphrodites. From the cross-progeny, I picked males to cross to candidate *cx270* hermaphrodite. Meanwhile, I picked hermaphrodite to generate *lin-31* and *unc-4* strain as control strain. The second cross-progeny included several genotypes. First, I identified *lin-31 unc-4/m* heterozygote by the self-progeny; second, from these heterozygote worms I identified those have *pkc-2/pkc-2* homozygote in chromosome X by selecting worms in 4mg/ml serotonin. Confirmed the genotype *lin-31 unc-4/cx270 pkc-2/pkc-2*, I picked the recombinant worms from the self-progeny, whose genotype are: *lin-31/cx270 pkc-2/pkc-2* and *unc-4/cx270 pkc-2/pkc-2*.

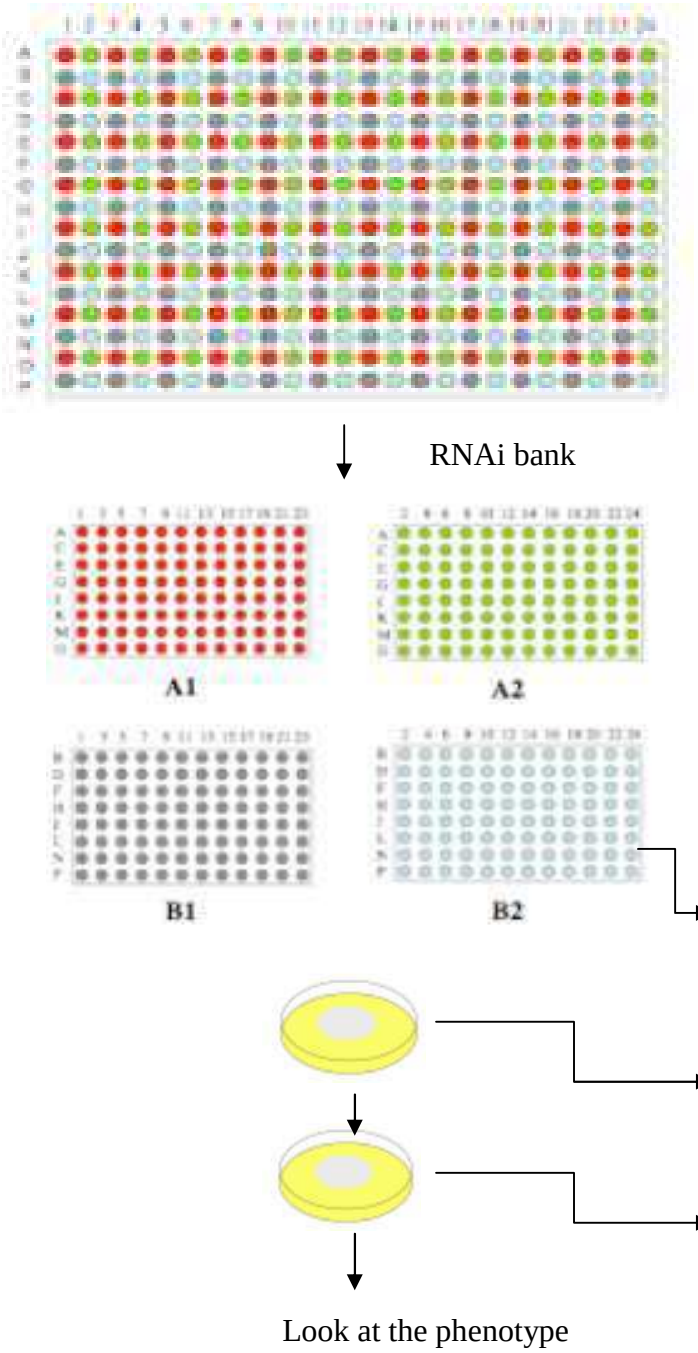


Figure 7-9: RNAi screen protocol. Pre-cultured clones in 50µl LB containing ampicilin (100µg/ml final) and tetracycline (15µg/ml final) in 37°C overnight. Expended this pre-culture into 300µl LBA and let grow 6 hours minimum until the bacteria reaches the exponential growth phase. Depose the culture into the NGM Petri plate (□90mm) and wait for 7 days, air dry. Put 2 pkc-2 adult worms into RNAi plate and check the phenotype of the progeny.

7.3 Conclusion

The work presented in this chapter was aimed at identifying suppressor genes involved in the serotonin signal pathway in *pkc-2(ok328)* background.

The candidates identified in the screen imply that the method was successful in finding serotonin resistant mutant. Of all mutants, mutated genes are located in chromosome II, III, IV, in where we know where the precise region is. We also identified the best mutant: *cx270*, which shows strong linkage with genetic markers.

There are many ways to find a gene; here we used RNAi screen method because it is low cost and much easier as the whole genome of *C. elegans* was known. However, the RNAi effect on wild type worms in the presence of exogenous serotonin was different from the mutant phenotype, which means in this case RNAi method was limited to identify the mutation. There is possibility that RNAi effect is not effective in all cell types in *C. elegans*, for example, it is not effective in neurons. Therefore, other mapping methods should be used, such as sequence every gene in the mutated region. Considering that is very expensive and takes much more time, we are looking for better ways to map the position of suppressor gene.

In a word, the screen presented candidates with knowing chromosome and mutated regions, which suggested there are several genes are related in serotonin signal pathway in *pkc-2(ok328)* background.

Discussion

Discussion

Duchenne/Becker Muscular Dystrophy (DMD/BMD) is X-linked recessive progressive muscle wasting disease. The gene responsible to the disease was identified decades years ago, which named dystrophin gene. Since the discovery of dystrophin gene, the scientists never stop studying the mechanism of the disease. So far, the gene character, different types of mutation, the gene expression pattern in muscles, and dystrophin associated proteins were studied thoroughly. Meanwhile, there are researches that focus on the therapies to ameliorate or even cure the malady, such as gene therapy. On the other hand, there are also researches looking for effective pharmacological treatment. Until now, however, we still know little about the mechanism of DMD and there is no curable treatment for the disease.

Based on our observations, the *pkc-2* gene has effect on progressive muscle degeneration in *C. elegans*. That raises a question: **What the potential role of *pkc-2* gene in muscle degeneration process in *C. elegans*?**

Therefore, it is essential for further researches to study the localization of PKC-2 in *C. elegans* adult animals and screen for molecular interactor of PKC-2. My thesis analyzed the *pkc-2* gene to confirm its expression pattern, and performed yeast two hybrid screen to identify PKC-2 binding partner. Considering the worm lacking *pkc-2* gene has a severe blister phenotype in the presence of endogenous neurotransmitter serotonin (5-HT), it is meaningful of studying interaction of PKC-2 and serotonin pathways (Figure 8-1).

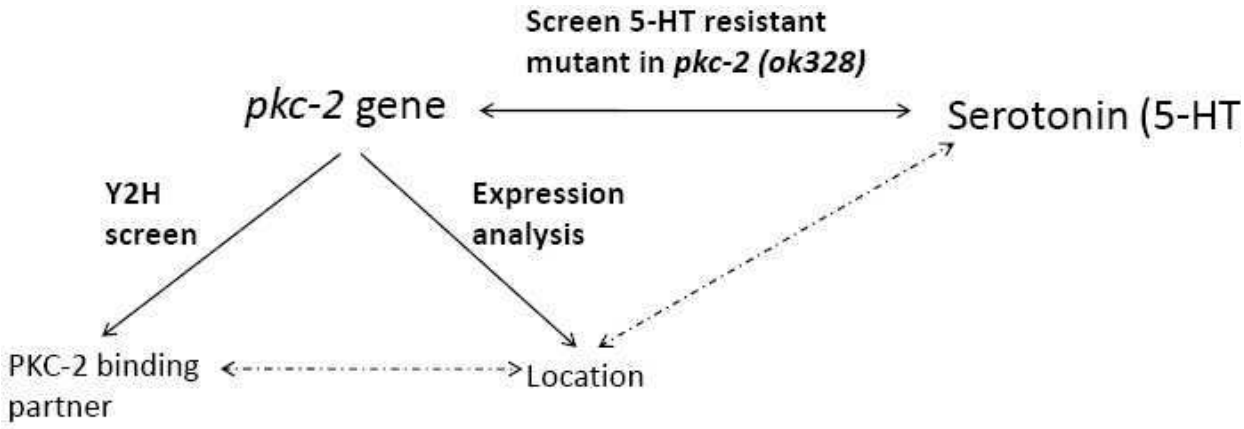


Figure 8-1: Strategy of thesis to study the role of *pkc-2* gene in *C. elegans*. We analyzed the expression pattern of *pkc-2* gene and performed yeast two hybrid screen to see in what place PKC-2 protein located, in order to reveal the function of PKC-2 pathway in regulating muscle degeneration in *C. elegans*; and that would help us understanding the role of serotonin pathway which has effect on muscle degeneration in *C. elegans* too (dash arrow). We also screen serotonin resistant mutant in *pkc-2 (ok328)* background to study the possible interaction between PKC-2 pathway and serotonin pathway.

1 PKC-2 expressed in neurons and pharynx, possible expressed in body-wall muscle cells

The results presented in chapter 6 clearly confirmed that PKC-2 is expressed in neurons and pharynx. However, we still question that PKC-2 expressed ONLY in neurons and pharynx in *C. elegans* adult animals. Although *MosTIC pkc-2* did not show expression of *pkc-2* in body-wall muscles, that could due to the low copies of *pkc-2* gene.

Islas-Trejo et al. reported *pkc-2a* and *pkc-2b* expressed in compact muscle of head in L1 larval nematode with *lacZ* (Islas-Trejo *et al.*, 1997). Their results did not show *pkc-2* expression in body-wall muscle cells.

In parallel, when we used rabbit anti-GFP/goat anti rabbit other than mouse anti-GFP in immunocytochemistry experiment, we got the speculative results. First, a construct pKG63 which containing *pkc-2* cDNA isoform *pkc-2b* (Figure 6-2) seemed expressed in body-wall muscles (Figure 8-2, A-C), while using mouse anti-GFP only *myo-3::gfp* was detected (image not shown). Although the control experiment with *gfp* only was also found in the same place with *myo-3::pkc-2b* (Figure 8-2, D-F), there is possibility that GFP and PKC-2 located in the same place coincidently.

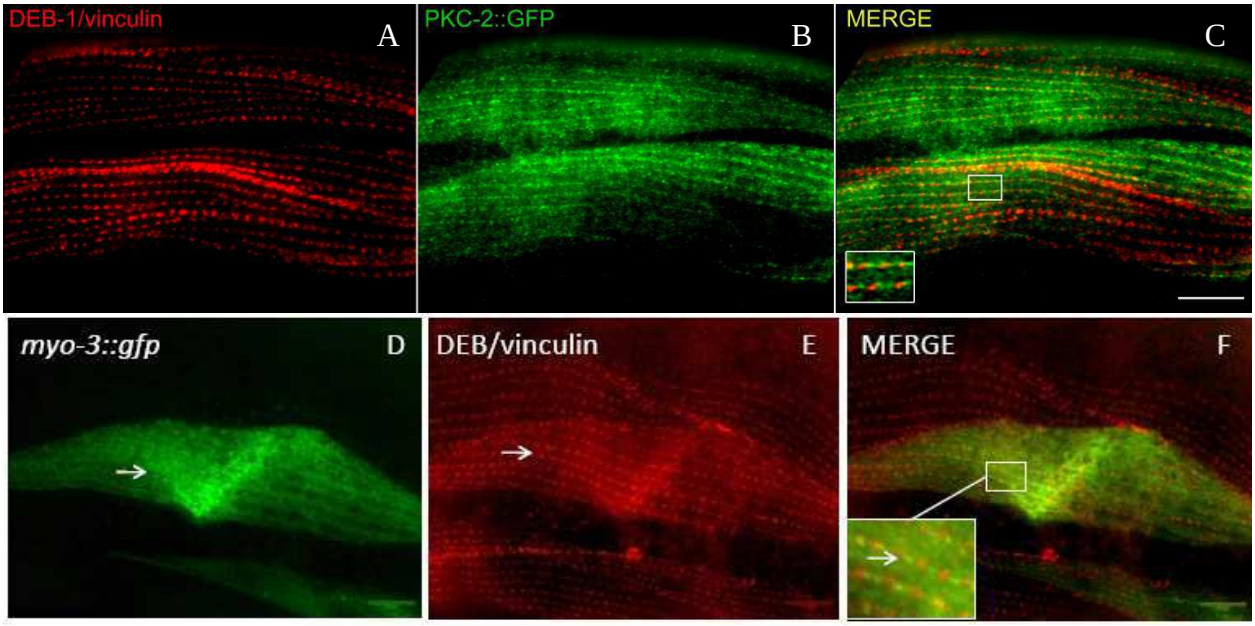


Figure 8-2: Sub-cellular localization of *myo-3::pkc-2* in N2 with rabbit anti-GFP. Scale bar=10µm. (A). Transgenic animals expressing a *myo-3::pkc-2* construct. Body-wall muscles of animals labeled with anti-DEB-1 MH24 antibody (red dot-lines in A and C). (B).The muscle localization of PKC-2-GFP protein (green dot-lines, the GFP signal was amplified with a rabbit anti-GFP antibody). (C). Merge images of A and B showed PKC-2-GFP localizes next to dense bodies. (D). *myo-3::gfp* was expressed in a body wall muscle cell (white arrow). (E) DEB-1/vinculin expression in body wall muscle cell (red dot-line, white arrow). (F) Merge images of a and b showed *myo-3::gfp* localizes next to dense bodies (white arrow).

Second, signal of some *pkc-2::gfp* expression were also detected in body-wall muscles (Figure 8-3). The construct contained *pkc-2* gene with *gfp* inserted in commune exon 10,

which has the strongest expression in three genomic clones. In more compact muscles in head, with mouse anti-GFP, we can observe PKC-2-GFP expression (Figure 8-3, D-F).

Comparing to research of Islas-Trejo et al, the expressed *pkc-2* isoform in muscle cells of head could be *pkc-2a* and *pkc-2b*. However, with mouse anti-GFP antibody, we could not detect the GFP signal in body-wall muscles. Considering the background noise of antibody rabbit anti-GFP, the results from antibody mouse anti-GFP is probably more convincing.

There is also a possibility that PKC-2 express too low to be detected in body-wall muscles. Detection of weak transgene *myo-3::pkc-2* mRNA supported this in a way, or that could due to instable transgene or RNA level.

On the other hand, we observed the adult animals but not larvae. Then we can not rule out the possibility that *pkc-2* could express in larvae such as L1 stage worms.

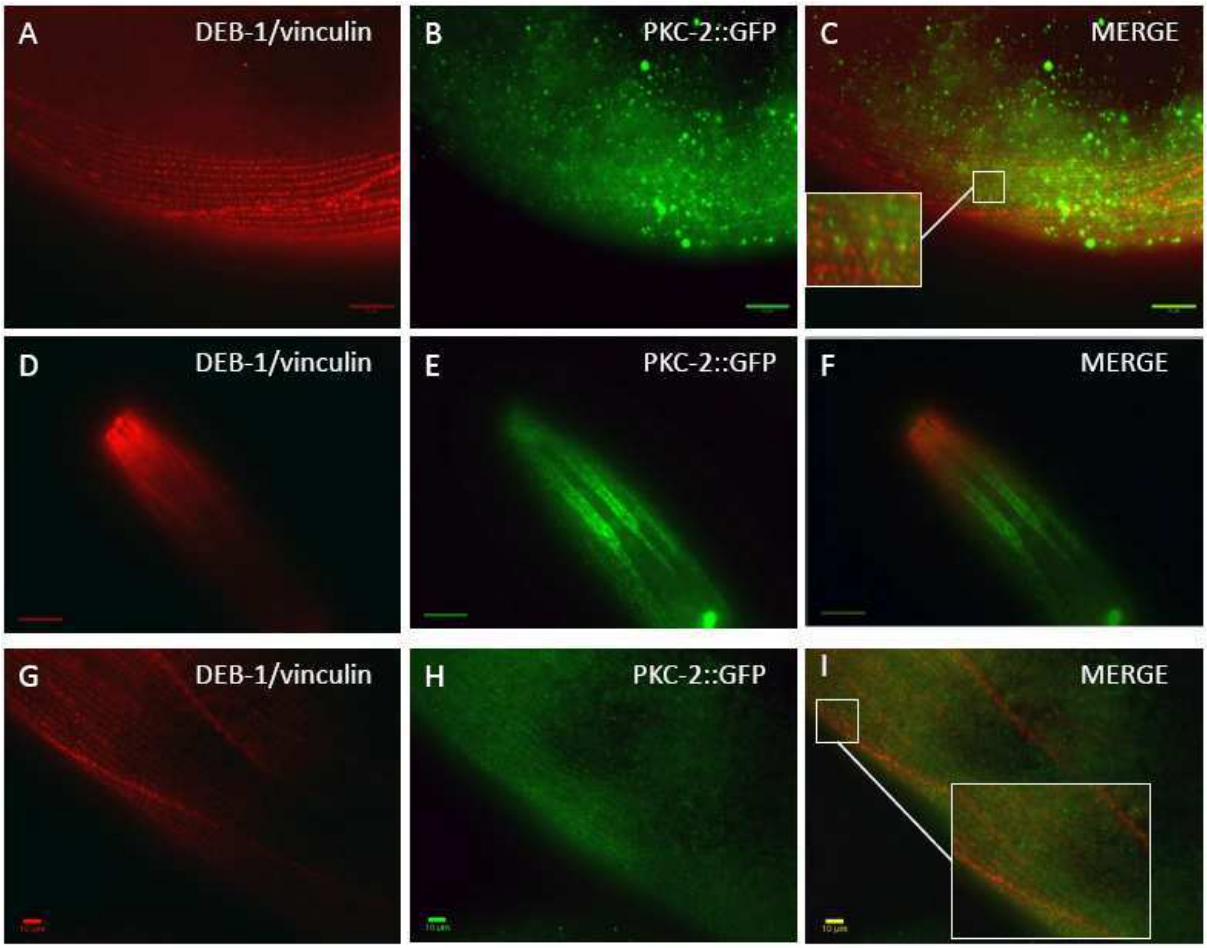


Figure 8-3: Sub-cellular localization of PKC-2 protein in muscle cells. Immunofluorescence images of wild-type animals (A-I). Scale bar=10µm. Body-wall muscles and head muscles of animals labeled with anti-DEB-1 MH24 antibody (red) and rabbit anti-GFP (green). (A, B, C) *pkc-2::gfp* (XhoI) (pKG49). (C, D, E) *pkc-2::gfp* (AccIII) (pKG57). (F, G, H) *pkc-2::gfp* (EcoRI) (pKG55).

Yeast two hybrid screen identified the unknown gene Y59A8A.3, a homologue to vertebrate filamin A interacting protein 1. Filamin A is one of the cytoskeleton proteins and maintains the normal cellular function (Stossel *et al.*, 2001; Beekman *et al.*, 2008). More experiments such as expression of Y59A8A.3 in *C. elegans* would help us to confirm the location of PKC-2.

In addition, the location of PKC-2 protein in wild-type *C. elegans* would help us understanding its possible function in the process of muscle degeneration.

2 The potential interaction between PKC-2 and serotonin

We have observed that knocking out *pkc-2* in *dys-1; hlh-1* could decrease muscle degeneration in *C. elegans* (Table 6-3). Since PKC-2 expressed intensely in neuron system, it may has function in neuronal level. PKC might be involved in processing signal transduction pathways that regulate the growth of muscle cells. The DAPC and several important receptors of neurotransmitters could be also among these targets.

The neurotransmitter serotonin (5-HT) could ameliorate muscle degeneration when its concentration augmented (Carre-Pierrat et al., 2006c). It is formed by two different enzymes called L-tryptophan hydroxylases (TPH), TPH1 and TPH2 respectively, which determine the rate at which serotonin is made (Nakamura and Hasegawa, 2007). Serotonin regulates several behaviors of *C. elegans* such as egg-laying, locomotion, etc (Horvitz et al., 1982; Carre-Pierrat et al., 2006c) by interacting with different 5-HT receptor or transporter subtypes coupled to various second-messenger systems. Second messenger systems involved in this regulation include protein kinases and phosphatases, of which protein kinase C has been the best characterized.

The *pkc-2* gene expression pattern analysis showed that some PKC-2 expressing neurons are also serotonin-containing neurons of *C. elegans*: (Figure 8-4).

NSMs: neurosecretory motor neuron, NSM Right and Left (bilaterally symmetric), somas located within the anterior bulb of the pharynx (part of pharyngeal nervous system) with a bifurcating processes extending into the isthmus and posterior bulb of the pharynx, covered with varicosities. These cells are among the strongest, most reliably staining cells using anti-serotonin.

ADFs: amphid sensory neurons, ADF Right and Left (bilaterally symmetric), somas located ventrolaterally. Each extends a process into the nerve ring and a dendrite to the tip of the nose with each amphid (sensory structure containing endings of many sensory neurons).

RIH: unpaired interneuron, soma located ventral to isthmus of pharynx.

AIMs: interneuron, AIM Right and Left (bilaterally symmetric), somas located near ventral midline below posterior bulb of pharynx.

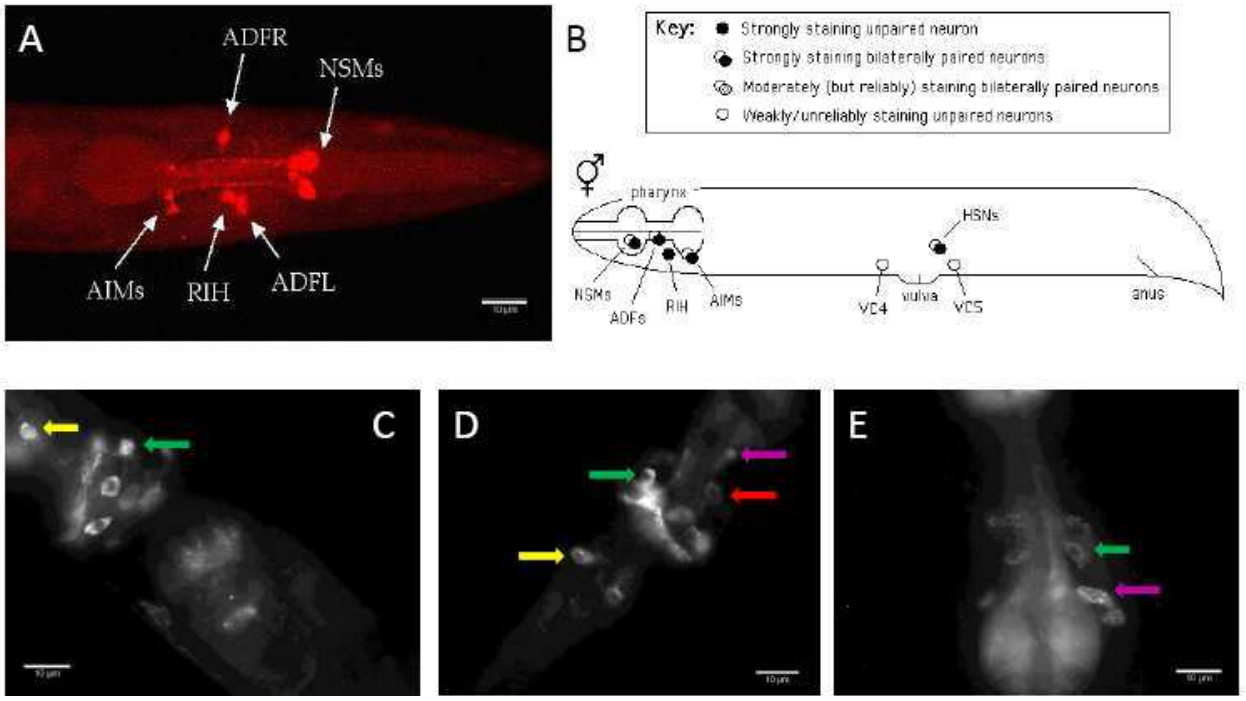


Figure 8-4: Comparison of serotonin cells in the head and PKC-2 expressing cells. (A). Hermaphrodite (same in male) anterior to the right; ventral (slightly lateral) view (http://home.sandiego.edu/~cloer/loerlab/5hthead.jpg). (B). Schematic of Serotonin-containing (serotonin immunoreactive) cells in hermaphrodite. (C, D, E). Transgenic animals expressing *pkc-2::gfp*. NSM (yellow arrows), ADF (green arrows), RIH (red arrow) and AIM (purple arrows) are presented in figure.

Increasing evidences suggest that PKC is involved in serotonergic pathway, via the regulation of serotonin receptors (SERs) and transporters (SERTs). In mammalian, discoveries suggest that there are not only direct interaction between PKC and receptors, but also identified the interaction sites at molecular level (Lembo and Albert, 1995; Bhattacharyya *et al.*, 2002). There are also studies suggested the indirect phosphorylation but through an F-actin-dependent mechanism (Sun *et al.*, 2003). Activation of PKC is proposed to disorder the dynamics of the actin filament network by removing a barrier to vesicle trafficking and

docking, thereby promoting transport of 5-HT_{3A} receptors to the cell membrane. In invertebrate animals, not many studies reveal the interaction between PKC and the 5-HT.

The nematode *C. elegans* is particularly well suited because highly conserved synaptic proteins identified in *C. elegans* offer an opportunity to study the signaling pathway. Furthermore, the great advantage of *C. elegans* comparing to other organisms is that most mutants affecting synaptic transmission are viable and can be propagated by self-fertilization (Richmond, 2005).

Mutant *pkc-2 (ok328)* exhibits a severe blister phenotype in presence of endogenous serotonin (described in chapter 7). Then screen serotonin (5-HT) resistant mutant in strain *pkc-2 (ok328)* would help to understand the link of PKC-2 and serotonin pathways in *C. elegans*. Due to the unexpected genetic screen techniques, it is difficult to isolate specific mutation but the best candidate knowing the mutated region in chromosome. The region was: chromosome II (-6.2~0.12). More effective and cheaper methods need to be studied to identify mutation in these emerged candidates. However, the screen presented candidates with knowing chromosome and mutated regions, which suggested there are several genes are related in serotonin signal pathway in *pkc-2(ok328)* background.

3 Hypotheses of PKC-2 working models

Identification of a homologue of filamin A interacting protein 1, Y59A8A.3, was by far the most represented clone in our two-hybrid screen. It resembles to vertebrate filamin A interacting protein 1. In COS-7 cells, filamin A interacting protein (FILIP) negatively regulated the function of Filamin A and induce degradation of Filamin A (Nagano *et al.*, 2002). Filamin A is one of the cytoskeleton structural proteins, attaching actin filaments to trans-membrane receptors. Filamin can also serve as scaffold protein for formation of signal proteins complexes. We suggested that PKC-2 might modulate filamin A activity through the filamin A interacting protein 1 in neurons, therefore has effect on secondary message system (Figure 8-5).

Then based on the speculative *pkc-2* expression in body wall muscle cells, we also proposed a working model in muscle (Figure 8-6). The PKC-2 protein interacts FILIP and to participate muscle cell metabolism in serotonin pathway. In addition, it has also been shown that in mammalian muscle cells phosphorylation of dystrophin by PKC inhibits effective binding to actin (Senter *et al.*, 1995). We presumed the interaction in the model; however, no such researches were documented previously in *C. elegans*.

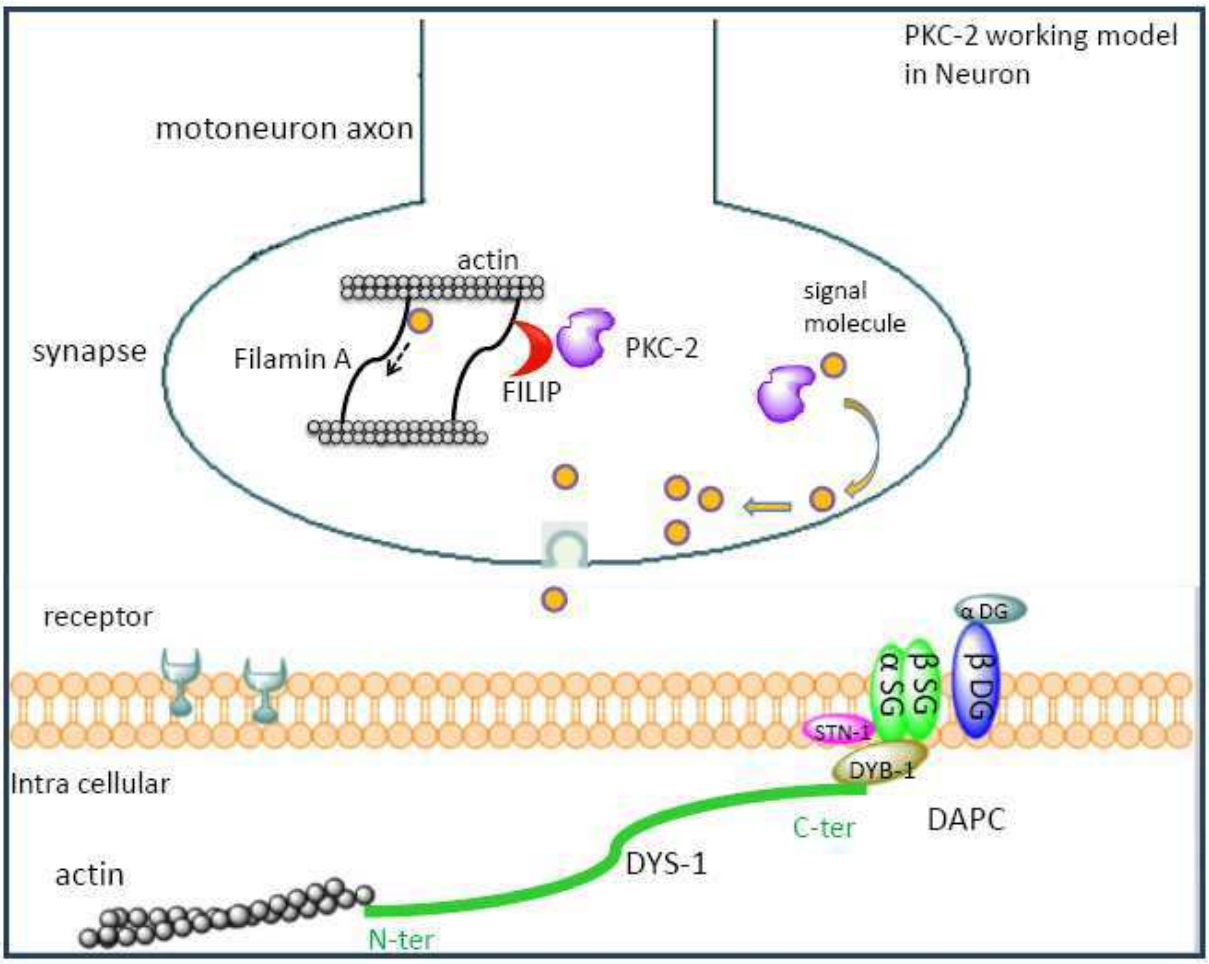


Figure 8-5: Working model of PKC-2 function in neuron in regulating muscle activity in *C. elegans*. Filamin A (black filament) maintains the structure of neuron by binding actin (grey). It has related function to filamin A interacting protein 1 (FILIP, red), which is a homologue of PKC-2 (purple) interactor in two yeast hybrid, and keeps normal function by PKC-2 modulation. Signal molecule (eg. 5-HT) was transducted in synapse along with filamin or cytoskeleton structure (brown beads with arrows) to pre-synapse membrane. There are some receptors in muscle cell membrane; dystrophin (green) interacts with actin through N-terminal while binding Dystroglycan (DG)-Sarcoglycan (SG) complex through C-terminal.

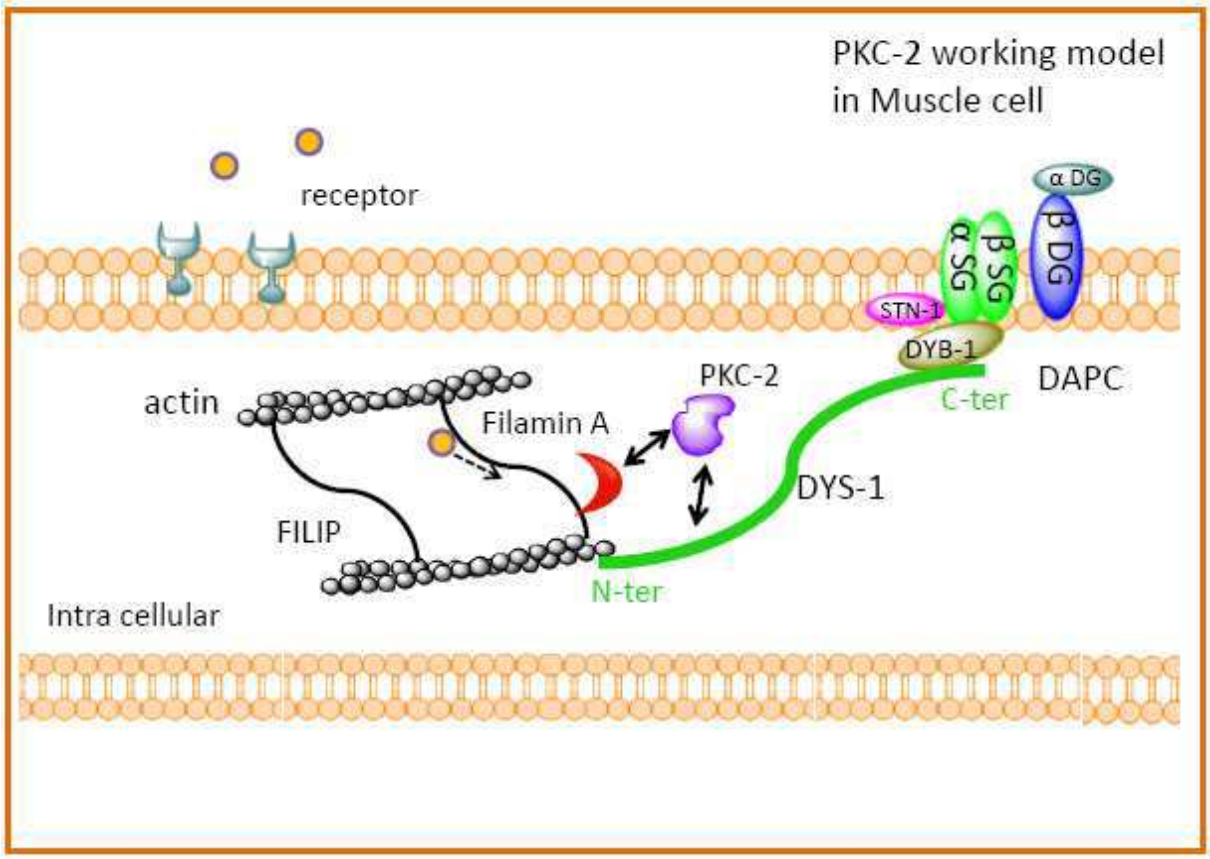


Figure 8-6: Working model of PKC-2 function in muscle in regulating muscle activity in *C. elegans*. Filamin A (black filament) maintains the structure of muscle cell by binding actin (grey). It has related function to filamin A interacting protein 1 (FILIP, red), which is a homologue of PKC-2 (purple) interactor in two yeast hybrid, and keeps normal function by PKC-2 modulation. Signal molecule (eg. 5-HT) was transfered into cell along with filamin or cytoskeleton structure (brown beads with arrows). There are some receptors in muscle cell membrane; dystrophin (green) interacts with actin through N-terminal while binding Dystroglycan (DG)-Sarcoglycan (SG) complex through C-terminal. Presumed interactions between PKC-2 and FILIP, DYS-1 were presented with dark bidirectional arrows.

Further researches such as studying the interaction between PKC-2 and Y59A8A.3 *in vivo* and generating construct containing gene Y59A8A.3 to study the expression will be helpful. Based on the fact that yeast two hybrid is not very effective in finding PKC-2 functional partner, other methods such as proteomics should be considered.

4 Detailed analyses of *pkc-2* gene and genetic screen

In this study, we identified a new *pkc-2* cDNA isoform: *pkc-2c.2* by RT-PCR. Compared to isoform *pkc-2c*, the exon 1C.2 included exon 1C, intron 1C and exon 2C of *pkc-2c*. The reserved intron 1C supposed that *pkc-2c.2* was pre-mRNA. However, sequence analysis of exon 1C.2 suggested it as a new isoform of *pkc-2* because of a kozark sequence inserted in. Besides, an ATG start codon found in exon 1C.2 in the kozark sequence suggested a 5'UTR existed in this isoform. Untranslated regions (UTRs) are sections of the mRNA before the start codon and after the stop codon that are not translated, termed the five prime untranslated region (5' UTR) and three prime untranslated region (3' UTR), respectively. These regions are transcribed with the coding region and thus are exonic as they are present in the mature mRNA. Several roles in gene expression have been attributed to the untranslated regions, including mRNA stability, mRNA localization, and translational efficiency. The ability of a UTR to perform these functions depends on the sequence of the UTR and can differ between mRNAs.

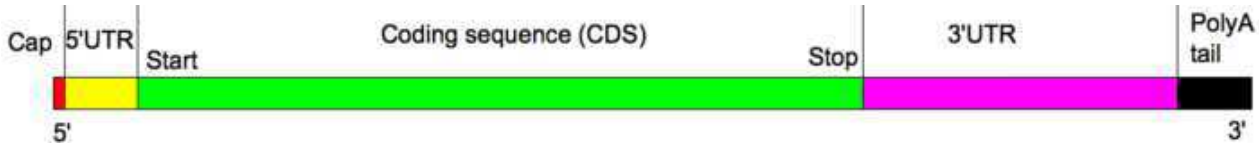


Figure 8-7: The structure of a typical human protein coding mRNA including the untranslated regions (UTRs). (http://de.wikipedia.org/wiki/Untranslatierter_Bereich)

The genetic screen identified the candidates implied that the method we used here was successful to screen serotonin resistant mutant. During the mapping, we used SNP mapping, three point mapping and RNAi screen.

In SNP mapping, DNA sequence polymorphisms between the wild-type *C. elegans* strain (N2 Bristol) and a closely related strain (CB4856 Hawaiian) were used as genetic markers. Compared to other markers that have been used for genetic mapping, SNPs have two distinct advantages. First, unlike conventional marker mutations that cause visible phenotypes, SNPs have no associated phenotype in general. Therefore, mutant phenotypes that are masked by

conventional marker mutations, such as those with subtle behavioral defects, can be mapped using SNPs. Second, SNPs are far denser than other markers, including both visible markers and DNA polymorphisms such as Tc1 insertions. Because SNPs are approximately as dense as genes, SNP mapping can provide single-gene resolution (Jakubowski and Kornfeld, 1999). Taken together, these two advantages have made SNP mapping the technique of choice for many *C. elegans* researchers.

When SNP markers are too closer, it is difficult to isolate mutation by RFLP analysis. In this case, three point mapping seems better than SNP mapping. But as we have mentioned above, one of the mutations normally masks the other. For example, the Blister phenotype is often masked (suppressed) by *dpy* and *rol* mutations; or where penetrance is an issue. Furthermore, regional variation in recombination frequency along the chromosome will lead to discrepancies between the actual locations and those predicted using these methods. RNAi screen could help us to identify mutations in a specific knowing region in chromosome. However, RNAi effect is not effective in all cell types in *C. elegans* and reduces the probability of finding mutation.

For these unexpected conditions in the experiment, we are still looking for the better ways to map the mutations.

5 Conclusions/Perspectives

In this thesis, we analyzed *pkc-2* gene in *Caenorhabditis elegans* adult animals and showed the exact locations where PKC-2 protein expresses. We also ran the yeast two hybrid screens and identified a candidate gene Y59A8A.3 which homologue to vertebrate filamin A interacting protein 1, that suggested the possible function of PKC-2 in *C. elegans*. Furthermore, we performed a genetic screen to search serotonin resistant mutant in strain *pkc-2 (ok328)*, in order to understand the link of *pkc-2* and serotonin pathways. Taken together the two aspects of the study, we presented the hypotheses of PKC-2 working models both in neurons and in muscles.

The work provides a starting point for the analysis of PKC-2 function. In addition, we raised several questions about the results and the methods which are uncertain at present. We are questioned about the *pkc-2* expression in body-wall muscles; the effectiveness of RNAi experiment. To further study the function of PKC-2 in muscle degeneration in *C. elegans*, more important researches are needed in the future. For example, where Y59A8A.3 expresses *in vivo* and what is interaction with PKC-2? What links *pkc-2* and serotonin pathways? At the moment, the analysis of Y59A8A.3 is still in process. And fortunately, we have obtained the candidates from genetic screen with confirmed mutated regions in chromosome. We have generate *snb-1::pkc-2* (Figure 8-8) which was under a neuronal promoter *snb-1*. Then plus *myo-3::pkc-2*, the two kinds of construct will be injected into various genetic background, such as *pkc-2; dys-1; hlh-1* to find out the effect of *pkc-2* on muscle degeneration in *C. elegans*.

These answers would help us to complete or correct the hypotheses.

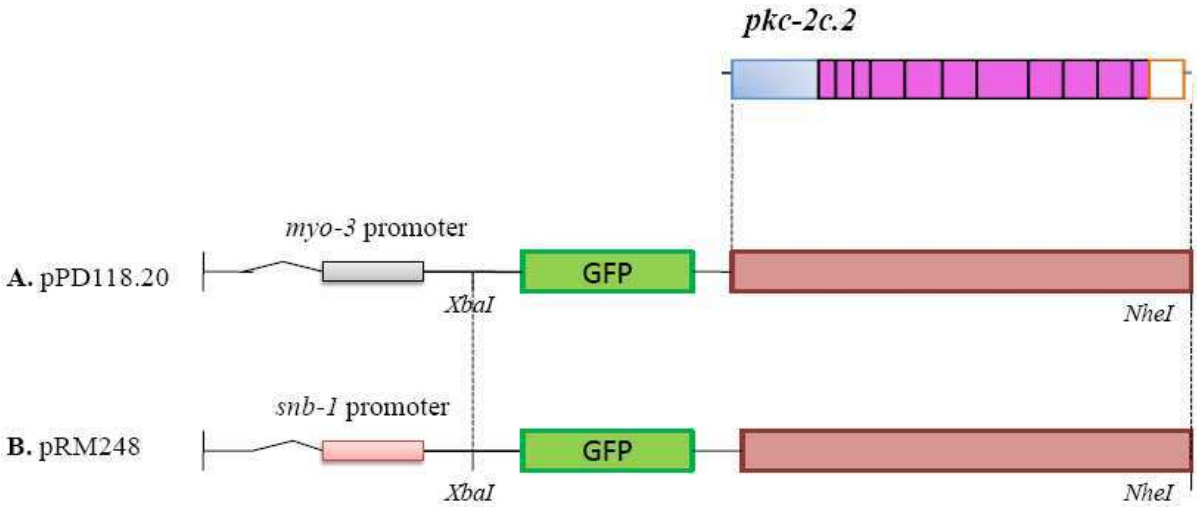


Figure 8-8: Construction of *snb-1::pkc-2* (pKG69). (A). pKG63 was cut by XbaI and NheI and cloned into pRM248 (B), which carries a neuronal promoter *snb-1*(pink box in the beginning). The recombinant plasmid was named pKG69.

Bibliography

(220 Publications)

Bibliography

Abu-Baker, A., and Rouleau, G.A. (2007). Oculopharyngeal muscular dystrophy: recent advances in the understanding of the molecular pathogenic mechanisms and treatment strategies. Biochimica et biophysica acta *1772*, 173-185.

Acharyya, S., Butchbach, M.E., Sahenk, Z., Wang, H., Saji, M., Carathers, M., Ringel, M.D., Skipworth, R.J., Fearon, K.C., Hollingsworth, M.A., Muscarella, P., Burghes, A.H., Rafael-Fortney, J.A., and Guttridge, D.C. (2005). Dystrophin glycoprotein complex dysfunction: a regulatory link between muscular dystrophy and cancer cachexia. Cancer Cell *8*, 421-432.

Adams, M.E., Butler, M.H., Dwyer, T.M., Peters, M.F., Murnane, A.A., and Froehner, S.C. (1993). Two forms of mouse syntrophin, a 58 kd dystrophin-associated protein, differ in primary structure and tissue distribution. Neuron *11*, 531-540.

Aeder, S.E., Martin, P.M., Soh, J.W., and Hussaini, I.M. (2004). PKC-eta mediates glioblastoma cell proliferation through the Akt and mTOR signaling pathways. Oncogene *23*, 9062-9069.

Aggarwal, R., and Boyden, P.A. (1995). Diminished Ca2+ and Ba2+ currents in myocytes surviving in the epicardial border zone of the 5-day infarcted canine heart. Circulation research *77*, 1180-1191.

Ahn, A.H., Freener, C.A., Gussoni, E., Yoshida, M., Ozawa, E., and Kunkel, L.M. (1996). The three human syntrophin genes are expressed in diverse tissues, have distinct chromosomal locations, and each bind to dystrophin and its relatives. The Journal of biological chemistry *271*, 2724-2730.

Ahn, A.H., Yoshida, M., Anderson, M.S., Feener, C.A., Selig, S., Hagiwara, Y., Ozawa, E., and Kunkel, L.M. (1994). Cloning of human basic A1, a distinct 59-kDa dystrophin-associated protein encoded on chromosome 8q23-24. Proceedings of the National Academy of Sciences of the United States of America *91*, 4446-4450.

Amann, K.J., Guo, A.W., and Ervasti, J.M. (1999). Utrophin lacks the rod domain actin binding activity of dystrophin. The Journal of biological chemistry *274*, 35375-35380.

Anderson, J.T., Rogers, R.P., and Jarrett, H.W. (1996). Ca2+-calmodulin binds to the carboxyl-terminal domain of dystrophin. The Journal of biological chemistry *271*, 6605-6610.

Apel, E.D., Roberds, S.L., Campbell, K.P., and Merlie, J.P. (1995). Rapsyn may function as a link between the acetylcholine receptor and the agrin-binding dystrophin-associated glycoprotein complex. Neuron *15*, 115-126.

Bibliography

Arends, M.J., and Wyllie, A.H. (1991). Apoptosis: mechanisms and roles in pathology. International review of experimental pathology *32*, 223-254.

Bachinski, L.L., Udd, B., Meola, G., Sansone, V., Bassez, G., Eymard, B., Thornton, C.A., Moxley, R.T., Harper, P.S., Rogers, M.T., Jurkat-Rott, K., Lehmann-Horn, F., Wieser, T., Gamez, J., Navarro, C., Bottani, A., Kohler, A., Shriver, M.D., Sallinen, R., Wessman, M., Zhang, S., Wright, F.A., and Krahe, R. (2003). Confirmation of the type 2 myotonic dystrophy (CCTG)n expansion mutation in patients with proximal myotonic myopathy/proximal myotonic dystrophy of different European origins: a single shared haplotype indicates an ancestral founder effect. Am J Hum Genet *73*, 835-848.

Balasubramanian, S., Fung, E.T., and Huganir, R.L. (1998). Characterization of the tyrosine phosphorylation and distribution of dystrobrevin isoforms. FEBS letters *432*, 133-140.

Balendran, A., Biondi, R.M., Cheung, P.C., Casamayor, A., Deak, M., and Alessi, D.R. (2000). A 3-phosphoinositide-dependent protein kinase-1 (PDK1) docking site is required for the phosphorylation of protein kinase Czeta (PKCzeta) and PKC-related kinase 2 by PDK1. The Journal of biological chemistry *275*, 20806-20813.

Balke, C.W., and Shorofsky, S.R. (1998). Alterations in calcium handling in cardiac hypertrophy and heart failure. Cardiovascular research *37*, 290-299.

Barstead, R.J., and Waterston, R.H. (1991). Vinculin is essential for muscle function in the nematode. The Journal of cell biology *114*, 715-724.

Bashir, R., Strachan, T., Keers, S., Stephenson, A., Mahjneh, I., Marconi, G., Nashef, L., and Bushby, K.M. (1994). A gene for autosomal recessive limb-girdle muscular dystrophy maps to chromosome 2p. Human molecular genetics *3*, 455-457.

Bauriedel, G., Schluckebier, S., Hutter, R., Welsch, U., Kandolf, R., Luderitz, B., and Prescott, M.F. (1998). Apoptosis in restenosis versus stable-angina atherosclerosis: implications for the pathogenesis of restenosis. Arteriosclerosis, thrombosis, and vascular biology *18*, 1132-1139.

Beekman, J.M., van der Poel, C.E., van der Linden, J.A., van den Berg, D.L., van den Berghe, P.V., van de Winkel, J.G., and Leusen, J.H. (2008). Filamin A stabilizes Fc gamma RI surface expression and prevents its lysosomal routing. J Immunol *180*, 3938-3945.

Bibliography

Belkin, A.M., and Burridge, K. (1994). Expression and localization of the phosphoglucomutase-related cytoskeletal protein, aciculin, in skeletal muscle. Journal of cell science *107 (Pt 7)*, 1993-2003.

Benian, G.M., Tinley, T.L., Tang, X., and Borodovsky, M. (1996). The Caenorhabditis elegans gene unc-89, required fpr muscle M-line assembly, encodes a giant modular protein composed of Ig and signal transduction domains. The Journal of cell biology *132*, 835-848.

Benson, M.A., Newey, S.E., Martin-Rendon, E., Hawkes, R., and Blake, D.J. (2001). Dysbindin, a novel coiled-coil-containing protein that interacts with the dystrobrevins in muscle and brain. The Journal of biological chemistry *276*, 24232-24241.

Bessou, C., Giugia, J.B., Franks, C.J., Holden-Dye, L., and Segalat, L. (1998). Mutations in the Caenorhabditis elegans dystrophin-like gene dys-1 lead to hyperactivity and suggest a link with cholinergic transmission. Neurogenetics *2*, 61-72.

Bhattacharyya, S., Puri, S., Miledi, R., and Panicker, M.M. (2002). Internalization and recycling of 5-HT2A receptors activated by serotonin and protein kinase C-mediated mechanisms. Proceedings of the National Academy of Sciences of the United States of America *99*, 14470-14475.

Birchmeier, C., and Brohmann, H. (2000). Genes that control the development of migrating muscle precursor cells. Current opinion in cell biology *12*, 725-730.

Blake, D.J., Nawrotzki, R., Peters, M.F., Froehner, S.C., and Davies, K.E. (1996). Isoform diversity of dystrobrevin, the murine 87-kDa postsynaptic protein. The Journal of biological chemistry *271*, 7802-7810.

Blake, D.J., Weir, A., Newey, S.E., and Davies, K.E. (2002). Function and genetics of dystrophin and dystrophin-related proteins in muscle. Physiological reviews *82*, 291-329.

Blakely, R.D., Berson, H.E., Fremeau, R.T., Jr., Caron, M.G., Peek, M.M., Prince, H.K., and Bradley, C.C. (1991). Cloning and expression of a functional serotonin transporter from rat brain. Nature *354*, 66-70.

Bogi, K., Lorenzo, P.S., Acs, P., Szallasi, Z., Wagner, G.S., and Blumberg, P.M. (1999). Comparison of the roles of the C1a and C1b domains of protein kinase C alpha in ligand induced translocation in NIH 3T3 cells. FEBS letters *456*, 27-30.

Borycki, A.G., Li, J., Jin, F., Emerson, C.P., and Epstein, J.A. (1999). Pax3 functions in cell survival and in pax7 regulation. Development *126*, 1665-1674.

Bibliography

Bourinet, E., Fournier, F., Nargeot, J., and Charnet, P. (1992). Endogenous Xenopus-oocyte Ca-channels are regulated by protein kinases A and C. FEBS letters *299*, 5-9.

Boyd, Y., and Buckle, V.J. (1986). Cytogenetic heterogeneity of translocations associated with Duchenne muscular dystrophy. Clinical genetics *29*, 108-115.

Brenner, S. (1974). The genetics of Caenorhabditis elegans. Genetics *77*, 71-94.

Brune, B., von Knethen, A., and Sandau, K.B. (1999). Nitric oxide (NO): an effector of apoptosis. Cell death and differentiation *6*, 969-975.

Buckle, V.J., Guenet, J.L., Simon-Chazottes, D., Love, D.R., and Davies, K.E. (1990). Localisation of a dystrophin-related autosomal gene to 6q24 in man, and to mouse chromosome 10 in the region of the dystrophia muscularis (dy) locus. Human genetics *85*, 324-326.

Cai, H., Smola, U., Wixler, V., Eisenmann-Tappe, I., Diaz-Meco, M.T., Moscat, J., Rapp, U., and Cooper, G.M. (1997). Role of diacylglycerol-regulated protein kinase C isotypes in growth factor activation of the Raf-1 protein kinase. Molecular and cellular biology *17*, 732-741.

Carre-Pierrat, M., Baillie, D., Johnsen, R., Hyde, R., Hart, A., Granger, L., and Segalat, L. (2006a). Characterization of the Caenorhabditis elegans G protein-coupled serotonin receptors. Invert Neurosci *6*, 189-205.

Carre-Pierrat, M., Grisoni, K., Gieseler, K., Mariol, M.C., Martin, E., Jospin, M., Allard, B., and Segalat, L. (2006b). The SLO-1 BK channel of Caenorhabditis elegans is critical for muscle function and is involved in dystrophin-dependent muscle dystrophy. Journal of molecular biology *358*, 387-395.

Carre-Pierrat, M., Mariol, M.C., Chambonnier, L., Laugraud, A., Heskia, F., Giacomotto, J., and Segalat, L. (2006c). Blocking of striated muscle degeneration by serotonin in C. elegans. J Muscle Res Cell Motil *27*, 253-258.

Cartaud, A., Coutant, S., Petrucci, T.C., and Cartaud, J. (1998). Evidence for in situ and in vitro association between beta-dystroglycan and the subsynaptic 43K rapsyn protein. Consequence for acetylcholine receptor clustering at the synapse. The Journal of biological chemistry *273*, 11321-11326.

Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994). Green fluorescent protein as a marker for gene expression. Science (New York, N.Y *263*, 802-805.

Bibliography

Chamberlain, J. (1999). The dynamics of dystroglycan. Nature genetics *23*, 256-258.

Chan, Y.M., Bonnemann, C.G., Lidov, H.G., and Kunkel, L.M. (1998). Molecular organization of sarcoglycan complex in mouse myotubes in culture. The Journal of cell biology *143*, 2033-2044.

Chardin, P., Cussac, D., Maignan, S., and Ducruix, A. (1995). The Grb2 adaptor. FEBS letters *369*, 47-51.

Cheek, D.B. (1985). The control of cell mass and replication. The DNA unit--a personal 20-year study. Early human development *12*, 211-239.

Chen, F., Qian, L., Yang, Z.H., Huang, Y., Ngo, S.T., Ruan, N.J., Wang, J., Schneider, C., Noakes, P.G., Ding, Y.Q., Mei, L., and Luo, Z.G. (2007). Rapsyn interaction with calpain stabilizes AChR clusters at the neuromuscular junction. Neuron *55*, 247-260.

Chen, Z., Faaberg, K.S., and Plagemann, P.G. (1994). Determination of the 5' end of the lactate dehydrogenase-elevating virus genome by two independent approaches. The Journal of general virology *75 (Pt 4)*, 925-930.

Christ, B., and Ordahl, C.P. (1995). Early stages of chick somite development. Anat Embryol (Berl) *191*, 381-396.

Cohen, M.V., and Downey, J.M. (1996). Myocardial preconditioning promises to be a novel approach to the treatment of ischemic heart disease. Annual review of medicine *47*, 21-29.

Cossu, G., and Biressi, S. (2005). Satellite cells, myoblasts and other occasional myogenic progenitors: possible origin, phenotypic features and role in muscle regeneration. Semin Cell Dev Biol *16*, 623-631.

Cossu, G., Kelly, R., Tajbakhsh, S., Di Donna, S., Vivarelli, E., and Buckingham, M. (1996a). Activation of different myogenic pathways: myf-5 is induced by the neural tube and MyoD by the dorsal ectoderm in mouse paraxial mesoderm. Development (Cambridge, England) *122*, 429-437.

Cossu, G., Tajbakhsh, S., and Buckingham, M. (1996b). How is myogenesis initiated in the embryo? Trends Genet *12*, 218-223.

Crawford, G.E., Faulkner, J.A., Crosbie, R.H., Campbell, K.P., Froehner, S.C., and Chamberlain, J.S. (2000). Assembly of the dystrophin-associated protein complex does not require the dystrophin COOH-terminal domain. The Journal of cell biology *150*, 1399-1410.

Bibliography

Crosbie, R.H., Lim, L.E., Moore, S.A., Hirano, M., Hays, A.P., Maybaum, S.W., Collin, H., Dovico, S.A., Stolle, C.A., Fardeau, M., Tome, F.M., and Campbell, K.P. (2000). Molecular and genetic characterization of sarcospan: insights into sarcoglycan-sarcospan interactions. Human molecular genetics *9*, 2019-2027.

Cross, D.A., Alessi, D.R., Cohen, P., Andjelkovich, M., and Hemmings, B.A. (1995). Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature *378*, 785-789.

Cross, T., Griffiths, G., Deacon, E., Sallis, R., Gough, M., Watters, D., and Lord, J.M. (2000). PKC-delta is an apoptotic lamin kinase. Oncogene *19*, 2331-2337.

Dallas, A., and Khalil, R.A. (2003). Ca2+ antagonist-insensitive coronary smooth muscle contraction involves activation of epsilon-protein kinase C-dependent pathway. Am J Physiol Cell Physiol *285*, C1454-1463.

Danial, N.N., and Korsmeyer, S.J. (2004). Cell death: critical control points. Cell *116*, 205-219.

De Angioletti, M., Lacerra, G., Sabato, V., and Carestia, C. (2004). Beta+45 G --> C: a novel silent beta-thalassaemia mutation, the first in the Kozak sequence. Br J Haematol *124*, 224-231.

Dempsey, E.C., Newton, A.C., Mochly-Rosen, D., Fields, A.P., Reyland, M.E., Insel, P.A., and Messing, R.O. (2000). Protein kinase C isozymes and the regulation of diverse cell responses. American journal of physiology *279*, L429-438.

Dent, K.M., Dunn, D.M., von Niederhausern, A.C., Aoyagi, A.T., Kerr, L., Bromberg, M.B., Hart, K.J., Tuohy, T., White, S., den Dunnen, J.T., Weiss, R.B., and Flanigan, K.M. (2005). Improved molecular diagnosis of dystrophinopathies in an unselected clinical cohort. American journal of medical genetics *134*, 295-298.

Disatnik, M.H., Buraggi, G., and Mochly-Rosen, D. (1994). Localization of protein kinase C isozymes in cardiac myocytes. Experimental cell research *210*, 287-297.

Duncan, C.J. (1978). Role of intracellular calcium in promoting muscle damage: a strategy for controlling the dystrophic condition. Experientia *34*, 1531-1535.

Durbeej, M., and Campbell, K.P. (1999). Biochemical characterization of the epithelial dystroglycan complex. The Journal of biological chemistry *274*, 26609-26616.

Bibliography

Dutil, E.M., Toker, A., and Newton, A.C. (1998). Regulation of conventional protein kinase C isozymes by phosphoinositide-dependent kinase 1 (PDK-1). Curr Biol *8*, 1366-1375.

Elbrink, J., and Malhotra, S.K. (1985). The pathogenesis of Duchenne muscular dystrophy: significance of experimental observations. Med Hypotheses *17*, 375-385.

Emery, A.E. (1993). Duchenne muscular dystrophy--Meryon's disease. Neuromuscul Disord *3*, 263-266.

Emery, A.E. (2002). The muscular dystrophies. Lancet *359*, 687-695.

Engel, A., and Franzini-Armstrong, C. (1994). Myology : basic and clinical. McGraw-Hill: New York ; London.

Erdbrugger, W., Keffel, J., Knocks, M., Otto, T., Philipp, T., and Michel, M.C. (1997). Protein kinase C isoenzymes in rat and human cardiovascular tissues. British journal of pharmacology *120*, 177-186.

Fang, X., Yu, S., Tanyi, J.L., Lu, Y., Woodgett, J.R., and Mills, G.B. (2002). Convergence of multiple signaling cascades at glycogen synthase kinase 3: Edg receptor-mediated phosphorylation and inactivation by lysophosphatidic acid through a protein kinase C-dependent intracellular pathway. Molecular and cellular biology *22*, 2099-2110.

Fay, D. (2006). Genetic mapping and manipulation: chapter 3--Three-point mapping with genetic markers. WormBook, 1-7.

Francis, G.R., and Waterston, R.H. (1985). Muscle organization in Caenorhabditis elegans: localization of proteins implicated in thin filament attachment and I-band organization. The Journal of cell biology *101*, 1532-1549.

Franklin, R.A., and McCubrey, J.A. (2000). Kinases: positive and negative regulators of apoptosis. Leukemia *14*, 2019-2034.

Fromont-Racine, M., Rain, J.C., and Legrain, P. (1997). Toward a functional analysis of the yeast genome through exhaustive two-hybrid screens. Nature genetics *16*, 277-282.

Garcia, R.A., Forde, C.E., and Godwin, H.A. (2000). Calcium triggers an intramolecular association of the C2 domains in synaptotagmin. Proceedings of the National Academy of Sciences of the United States of America *97*, 5883-5888.

Gee, S.H., Madhavan, R., Levinson, S.R., Caldwell, J.H., Sealock, R., and Froehner, S.C. (1998). Interaction of muscle and brain sodium channels with multiple members of the syntrophin family of dystrophin-associated proteins. J Neurosci *18*, 128-137.

Bibliography

Gieseler, K., Grisoni, K., and Segalat, L. (2000). Genetic suppression of phenotypes arising from mutations in dystrophin-related genes in Caenorhabditis elegans. Curr Biol *10*, 1092-1097.

Gieseler, K., Mariol, M.C., Bessou, C., Migaud, M., Franks, C.J., Holden-Dye, L., and Segalat, L. (2001). Molecular, genetic and physiological characterisation of dystrobrevin-like (dyb-1) mutants of Caenorhabditis elegans. Journal of molecular biology *307*, 107-117.

Goode, N., Hughes, K., Woodgett, J.R., and Parker, P.J. (1992). Differential regulation of glycogen synthase kinase-3 beta by protein kinase C isotypes. The Journal of biological chemistry *267*, 16878-16882.

Gorlin, J.B., Yamin, R., Egan, S., Stewart, M., Stossel, T.P., Kwiatkowski, D.J., and Hartwig, J.H. (1990). Human endothelial actin-binding protein (ABP-280, nonmuscle filamin): a molecular leaf spring. The Journal of cell biology *111*, 1089-1105.

Goss, V.L., Hocevar, B.A., Thompson, L.J., Stratton, C.A., Burns, D.J., and Fields, A.P. (1994). Identification of nuclear beta II protein kinase C as a mitotic lamin kinase. The Journal of biological chemistry *269*, 19074-19080.

Goulding, M., Lumsden, A., and Paquette, A.J. (1994). Regulation of Pax-3 expression in the dermomyotome and its role in muscle development. Development (Cambridge, England) *120*, 957-971.

Grisoni, K., Gieseler, K., Mariol, M.C., Martin, E., Carre-Pierrat, M., Moulder, G., Barstead, R., and Segalat, L. (2003). The stn-1 syntrophin gene of C.elegans is functionally related to dystrophin and dystrobrevin. Journal of molecular biology *332*, 1037-1046.

Grisoni, K., Martin, E., Gieseler, K., Mariol, M.C., and Segalat, L. (2002). Genetic evidence for a dystrophin-glycoprotein complex (DGC) in Caenorhabditis elegans. Gene *294*, 77-86.

Grozdanovic, Z., and Baumgarten, H.G. (1999). Nitric oxide synthase in skeletal muscle fibers: a signaling component of the dystrophin-glycoprotein complex. Histology and histopathology *14*, 243-256.

Guglieri, M., Straub, V., Bushby, K., and Lochmuller, H. (2008). Limb-girdle muscular dystrophies. Current opinion in neurology *21*, 576-584.

Hack, A.A., Ly, C.T., Jiang, F., Clendenin, C.J., Sigrist, K.S., Wollmann, R.L., and McNally, E.M. (1998). Gamma-sarcoglycan deficiency leads to muscle membrane defects and apoptosis independent of dystrophin. The Journal of cell biology *142*, 1279-1287.

Hamdan, F.F., Ungrin, M.D., Abramovitz, M., and Ribeiro, P. (1999). Characterization of a novel serotonin receptor from Caenorhabditis elegans: cloning and expression of two splice variants. J Neurochem *72*, 1372-1383.

Hance, J.E., Fu, S.Y., Watkins, S.C., Beggs, A.H., and Michalak, M. (1999). alpha-actinin-2 is a new component of the dystrophin-glycoprotein complex. Arch Biochem Biophys *365*, 216-222.

Hasegawa, M., Cuenda, A., Spillantini, M.G., Thomas, G.M., Buee-Scherrer, V., Cohen, P., and Goedert, M. (1999). Stress-activated protein kinase-3 interacts with the PDZ domain of alpha1-syntrophin. A mechanism for specific substrate recognition. The Journal of biological chemistry *274*, 12626-12631.

Hayashi, A., Seki, N., Hattori, A., Kozuma, S., and Saito, T. (1999). PKCnu, a new member of the protein kinase C family, composes a fourth subfamily with PKCmu. Biochimica et biophysica acta *1450*, 99-106.

Henry, M.D., and Campbell, K.P. (1999). Dystroglycan inside and out. Current opinion in cell biology *11*, 602-607.

Hilgenberg, L., and Miles, K. (1995). Developmental regulation of a protein kinase C isoform localized in the neuromuscular junction. Journal of cell science *108 (Pt 1)*, 51-61.

Hilgenberg, L., Yearwood, S., Milstein, S., and Miles, K. (1996). Neural influence on protein kinase C isoform expression in skeletal muscle. J Neurosci *16*, 4994-5003.

Hillier, B.J., Christopherson, K.S., Prehoda, K.E., Bredt, D.S., and Lim, W.A. (1999). Unexpected modes of PDZ domain scaffolding revealed by structure of nNOS-syntrophin complex. Science (New York, N.Y *284*, 812-815.

Hohenester, E., Tisi, D., Talts, J.F., and Timpl, R. (1999). The crystal structure of a laminin G-like module reveals the molecular basis of alpha-dystroglycan binding to laminins, perlecan, and agrin. Molecular cell *4*, 783-792.

Holt, K.H., and Campbell, K.P. (1998). Assembly of the sarcoglycan complex. Insights for muscular dystrophy. The Journal of biological chemistry *273*, 34667-34670.

Horowitz, A., Menice, C.B., Laporte, R., and Morgan, K.G. (1996). Mechanisms of smooth muscle contraction. Physiological reviews *76*, 967-1003.

Bibliography

Horvitz, H.R., Chalfie, M., Trent, C., Sulston, J.E., and Evans, P.D. (1982). Serotonin and octopamine in the nematode Caenorhabditis elegans. Science (New York, N.Y *216*, 1012-1014.

Hurley, J.H., Newton, A.C., Parker, P.J., Blumberg, P.M., and Nishizuka, Y. (1997). Taxonomy and function of C1 protein kinase C homology domains. Protein Sci *6*, 477-480.

Islas-Trejo, A., Land, M., Tcherepanova, I., Freedman, J.H., and Rubin, C.S. (1997). Structure and expression of the Caenorhabditis elegans protein kinase C2 gene. Origins and regulated expression of a family of Ca2+-activated protein kinase C isoforms. The Journal of biological chemistry *272*, 6629-6640.

Itoh, H., Yamamura, S., Ware, J.A., Zhuang, S., Mii, S., Liu, B., and Kent, K.C. (2001). Differential effects of protein kinase C on human vascular smooth muscle cell proliferation and migration. American journal of physiology *281*, H359-370.

Iwata, Y., Nakamura, H., Fujiwara, K., and Shigekawa, M. (1993). Altered membrane-dystrophin association in the cardiomyopathic hamster heart muscle. Biochemical and biophysical research communications *190*, 589-595.

Iwata, Y., Pan, Y., Yoshida, T., Hanada, H., and Shigekawa, M. (1998). Alpha1-syntrophin has distinct binding sites for actin and calmodulin. FEBS letters *423*, 173-177.

Jacobson, C., Cote, P.D., Rossi, S.G., Rotundo, R.L., and Carbonetto, S. (2001). The dystroglycan complex is necessary for stabilization of acetylcholine receptor clusters at neuromuscular junctions and formation of the synaptic basement membrane. The Journal of cell biology *152*, 435-450.

Jakubowski, J., and Kornfeld, K. (1999). A local, high-density, single-nucleotide polymorphism map used to clone Caenorhabditis elegans cdf-1. Genetics *153*, 743-752.

Jarrett, H.W., and Foster, J.L. (1995). Alternate binding of actin and calmodulin to multiple sites on dystrophin. The Journal of biological chemistry *270*, 5578-5586.

Jideama, N.M., Noland, T.A., Jr., Raynor, R.L., Blobe, G.C., Fabbro, D., Kazanietz, M.G., Blumberg, P.M., Hannun, Y.A., and Kuo, J.F. (1996). Phosphorylation specificities of protein kinase C isozymes for bovine cardiac troponin I and troponin T and sites within these proteins and regulation of myofilament properties. The Journal of biological chemistry *271*, 23277-23283.

Johnson, J.E., Giorgione, J., and Newton, A.C. (2000). The C1 and C2 domains of protein kinase C are independent membrane targeting modules, with specificity for phosphatidylserine conferred by the C1 domain. Biochemistry *39*, 11360-11369.

Jung, D., Yang, B., Meyer, J., Chamberlain, J.S., and Campbell, K.P. (1995). Identification and characterization of the dystrophin anchoring site on beta-dystroglycan. The Journal of biological chemistry *270*, 27305-27310.

Kachinsky, A.M., Froehner, S.C., and Milgram, S.L. (1999). A PDZ-containing scaffold related to the dystrophin complex at the basolateral membrane of epithelial cells. The Journal of cell biology *145*, 391-402.

Kamp, T.J., and Hell, J.W. (2000). Regulation of cardiac L-type calcium channels by protein kinase A and protein kinase C. Circulation research *87*, 1095-1102.

Kanashiro, C.A., and Khalil, R.A. (1998). Signal transduction by protein kinase C in mammalian cells. Clinical and experimental pharmacology & physiology *25*, 974-985.

Kawakami, Y., Nishimoto, H., Kitaura, J., Maeda-Yamamoto, M., Kato, R.M., Littman, D.R., Leitges, M., Rawlings, D.J., and Kawakami, T. (2004). Protein kinase C betaII regulates Akt phosphorylation on Ser-473 in a cell type- and stimulus-specific fashion. The Journal of biological chemistry *279*, 47720-47725.

Kawamoto, S., and Hidaka, H. (1984). Ca2+-activated, phospholipid-dependent protein kinase catalyzes the phosphorylation of actin-binding proteins. Biochemical and biophysical research communications *118*, 736-742.

Kim, H., Rogers, M.J., Richmond, J.E., and McIntire, S.L. (2004). SNF-6 is an acetylcholine transporter interacting with the dystrophin complex in Caenorhabditis elegans. Nature *430*, 891-896.

Koenig, M., Monaco, A.P., and Kunkel, L.M. (1988). The complete sequence of dystrophin predicts a rod-shaped cytoskeletal protein. Cell *53*, 219-228.

Kozak, M. (1991). Structural features in eukaryotic mRNAs that modulate the initiation of translation. The Journal of biological chemistry *266*, 19867-19870.

Land, M., Islas-Trejo, A., Freedman, J.H., and Rubin, C.S. (1994). Structure and expression of a novel, neuronal protein kinase C (PKC1B) from Caenorhabditis elegans. PKC1B is expressed selectively in neurons that receive, transmit, and process environmental signals. The Journal of biological chemistry *269*, 9234-9244.

Le Grand, F., and Rudnicki, M.A. (2007). Skeletal muscle satellite cells and adult myogenesis. Current opinion in cell biology *19*, 628-633.

Lebakken, C.S., Venzke, D.P., Hrstka, R.F., Consolino, C.M., Faulkner, J.A., Williamson, R.A., and Campbell, K.P. (2000). Sarcospan-deficient mice maintain normal muscle function. Molecular and cellular biology *20*, 1669-1677.

Lecroisey, C., Martin, E., Mariol, M.C., Granger, L., Schwab, Y., Labouesse, M., Segalat, L., and Gieseler, K. (2008). DYC-1, a Protein Functionally Linked to Dystrophin in Caenorhabditis elegans Is Associated with the Dense Body, Where It Interacts with the Muscle LIM Domain Protein ZYX-1. Mol Biol Cell *19*, 785-796.

Lee, J.Y., Hannun, Y.A., and Obeid, L.M. (1996). Ceramide inactivates cellular protein kinase Calpha. The Journal of biological chemistry *271*, 13169-13174.

Lembo, P.M., and Albert, P.R. (1995). Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxytryptamine1A receptor by protein kinase C. Mol Pharmacol *48*, 1024-1029.

Letunic, I., Copley, R.R., Pils, B., Pinkert, S., Schultz, J., and Bork, P. (2006). SMART 5: domains in the context of genomes and networks. Nucleic Acids Res *34*, D257-260.

Lim, L.E., and Campbell, K.P. (1998). The sarcoglycan complex in limb-girdle muscular dystrophy. Current opinion in neurology *11*, 443-452.

Liou, Y.M., and Morgan, K.G. (1994). Redistribution of protein kinase C isoforms in association with vascular hypertrophy of rat aorta. The American journal of physiology *267*, C980-989.

Lowenstein, E.J., Daly, R.J., Batzer, A.G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D., and Schlessinger, J. (1992). The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell *70*, 431-442.

Luise, M., Presotto, C., Senter, L., Betto, R., Ceoldo, S., Furlan, S., Salvatori, S., Sabbadini, R.A., and Salviati, G. (1993). Dystrophin is phosphorylated by endogenous protein kinases. The Biochemical journal *293 (Pt 1)*, 243-247.

Lumeng, C., Phelps, S., Crawford, G.E., Walden, P.D., Barald, K., and Chamberlain, J.S. (1999). Interactions between beta 2-syntrophin and a family of microtubule-associated serine/threonine kinases. Nature neuroscience *2*, 611-617.

Madhavan, R., and Jarrett, H.W. (1999). Phosphorylation of dystrophin and alpha-syntrophin by Ca(2+)-calmodulin dependent protein kinase II. Biochimica et biophysica acta *1434*, 260-274.

Malhotra, A., Reich, D., Reich, D., Nakouzi, A., Sanghi, V., Geenen, D.L., and Buttrick, P.M. (1997). Experimental diabetes is associated with functional activation of protein kinase C epsilon and phosphorylation of troponin I in the heart, which are prevented by angiotensin II receptor blockade. Circulation research *81*, 1027-1033.

Mariol, M.C., and Segalat, L. (2001). Muscular degeneration in the absence of dystrophin is a calcium-dependent process. Curr Biol *11*, 1691-1694.

Marshall, C.J. (1996). Cell signalling. Raf gets it together. Nature *383*, 127-128.

McNair, L.L., Salamanca, D.A., and Khalil, R.A. (2004). Endothelin-1 promotes Ca2+ antagonist-insensitive coronary smooth muscle contraction via activation of epsilon-protein kinase C. Hypertension *43*, 897-904.

Mello, C., and Fire, A. (1995). DNA transformation. Methods Cell Biol *48*, 451-482.

Metzinger, L., Blake, D.J., Squier, M.V., Anderson, L.V., Deconinck, A.E., Nawrotzki, R., Hilton-Jones, D., and Davies, K.E. (1997). Dystrobrevin deficiency at the sarcolemma of patients with muscular dystrophy. Human molecular genetics *6*, 1185-1191.

Milner, R.E., Busaan, J.L., Holmes, C.F., Wang, J.H., and Michalak, M. (1993). Phosphorylation of dystrophin. The carboxyl-terminal region of dystrophin is a substrate for in vitro phosphorylation by p34cdc2 protein kinase. The Journal of biological chemistry *268*, 21901-21905.

Mizuno, Y., Thompson, T.G., Guyon, J.R., Lidov, H.G., Brosius, M., Imamura, M., Ozawa, E., Watkins, S.C., and Kunkel, L.M. (2001). Desmuslin, an intermediate filament protein that interacts with alpha -dystrobrevin and desmin. Proceedings of the National Academy of Sciences of the United States of America *98*, 6156-6161.

Moerman, D.G., and Williams, B.D. (2006). Sarcomere assembly in C. elegans muscle. WormBook, 1-16.

Monaco, A.P., Bertelson, C.J., Liechti-Gallati, S., Moser, H., and Kunkel, L.M. (1988). An explanation for the phenotypic differences between patients bearing partial deletions of the DMD locus. Genomics *2*, 90-95.

Moores, C.A., and Kendrick-Jones, J. (2000). Biochemical characterisation of the actin-binding properties of utrophin. Cell motility and the cytoskeleton *46*, 116-128.

Nagano, T., Yoneda, T., Hatanaka, Y., Kubota, C., Murakami, F., and Sato, M. (2002). Filamin A-interacting protein (FILIP) regulates cortical cell migration out of the ventricular zone. Nat Cell Biol *4*, 495-501.

Nakamura, K., and Hasegawa, H. (2007). Developmental role of tryptophan hydroxylase in the nervous system. Mol Neurobiol *35*, 45-54.

Narula, J., Haider, N., Virmani, R., DiSalvo, T.G., Kolodgie, F.D., Hajjar, R.J., Schmidt, U., Semigran, M.J., Dec, G.W., and Khaw, B.A. (1996). Apoptosis in myocytes in end-stage heart failure. The New England journal of medicine *335*, 1182-1189.

Nawrotzki, R., Loh, N.Y., Ruegg, M.A., Davies, K.E., and Blake, D.J. (1998). Characterisation of alpha-dystrobrevin in muscle. Journal of cell science *111 (Pt 17)*, 2595-2605.

Newbell, B.J., Anderson, J.T., and Jarrett, H.W. (1997). Ca2+-calmodulin binding to mouse alpha1 syntrophin: syntrophin is also a Ca2+-binding protein. Biochemistry *36*, 1295-1305.

Newey, S.E., Benson, M.A., Ponting, C.P., Davies, K.E., and Blake, D.J. (2000). Alternative splicing of dystrobrevin regulates the stoichiometry of syntrophin binding to the dystrophin protein complex. Curr Biol *10*, 1295-1298.

Newey, S.E., Howman, E.V., Ponting, C.P., Benson, M.A., Nawrotzki, R., Loh, N.Y., Davies, K.E., and Blake, D.J. (2001). Syncoilin, a novel member of the intermediate filament superfamily that interacts with alpha-dystrobrevin in skeletal muscle. The Journal of biological chemistry *276*, 6645-6655.

Newton, A.C. (2003). Regulation of the ABC kinases by phosphorylation: protein kinase C as a paradigm. The Biochemical journal *370*, 361-371.

Nishizuka, Y. (1992). Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science (New York, N.Y *258*, 607-614.

Olde, B., and McCombie, W.R. (1997). Molecular cloning and functional expression of a serotonin receptor from Caenorhabditis elegans. J Mol Neurosci *8*, 53-62.

Ort, T., Maksimova, E., Dirkx, R., Kachinsky, A.M., Berghs, S., Froehner, S.C., and Solimena, M. (2000). The receptor tyrosine phosphatase-like protein ICA512 binds the PDZ

domains of beta2-syntrophin and nNOS in pancreatic beta-cells. European journal of cell biology *79*, 621-630.

Osada, S., Mizuno, K., Saido, T.C., Suzuki, K., Kuroki, T., and Ohno, S. (1992). A new member of the protein kinase C family, nPKC theta, predominantly expressed in skeletal muscle. Molecular and cellular biology *12*, 3930-3938.

Park, D.S., Woodman, S.E., Schubert, W., Cohen, A.W., Frank, P.G., Chandra, M., Shirani, J., Razani, B., Tang, B., Jelicks, L.A., Factor, S.M., Weiss, L.M., Tanowitz, H.B., and Lisanti, M.P. (2002). Caveolin-1/3 double-knockout mice are viable, but lack both muscle and non-muscle caveolae, and develop a severe cardiomyopathic phenotype. The American journal of pathology *160*, 2207-2217.

Partovian, C., and Simons, M. (2004). Regulation of protein kinase B/Akt activity and Ser473 phosphorylation by protein kinase Calpha in endothelial cells. Cellular signalling *16*, 951-957.

Pearce, M., Blake, D.J., Tinsley, J.M., Byth, B.C., Campbell, L., Monaco, A.P., and Davies, K.E. (1993). The utrophin and dystrophin genes share similarities in genomic structure. Human molecular genetics *2*, 1765-1772.

Perrini, S., Henriksson, J., Zierath, J.R., and Widegren, U. (2004). Exercise-induced protein kinase C isoform-specific activation in human skeletal muscle. Diabetes *53*, 21-24.

Peters, M.F., Sadoulet-Puccio, H.M., Grady, M.R., Kramarcy, N.R., Kunkel, L.M., Sanes, J.R., Sealock, R., and Froehner, S.C. (1998). Differential membrane localization and intermolecular associations of alpha-dystrobrevin isoforms in skeletal muscle. The Journal of cell biology *142*, 1269-1278.

Piluso, G., Mirabella, M., Ricci, E., Belsito, A., Abbondanza, C., Servidei, S., Puca, A.A., Tonali, P., Puca, G.A., and Nigro, V. (2000). Gamma1- and gamma2-syntrophins, two novel dystrophin-binding proteins localized in neuronal cells. The Journal of biological chemistry *275*, 15851-15860.

Ping, P., Zhang, J., Qiu, Y., Tang, X.L., Manchikalapudi, S., Cao, X., and Bolli, R. (1997). Ischemic preconditioning induces selective translocation of protein kinase C isoforms epsilon and eta in the heart of conscious rabbits without subcellular redistribution of total protein kinase C activity. Circulation research *81*, 404-414.

Puceat, M., Hilal-Dandan, R., Strulovici, B., Brunton, L.L., and Brown, J.H. (1994). Differential regulation of protein kinase C isoforms in isolated neonatal and adult rat cardiomyocytes. The Journal of biological chemistry *269*, 16938-16944.

Puri, T.S., Gerhardstein, B.L., Zhao, X.L., Ladner, M.B., and Hosey, M.M. (1997). Differential effects of subunit interactions on protein kinase A- and C-mediated phosphorylation of L-type calcium channels. Biochemistry *36*, 9605-9615.

Rando, T.A. (2001). The dystrophin-glycoprotein complex, cellular signaling, and the regulation of cell survival in the muscular dystrophies. Muscle & nerve *24*, 1575-1594.

Ranganathan, R., Cannon, S.C., and Horvitz, H.R. (2000). MOD-1 is a serotonin-gated chloride channel that modulates locomotory behaviour in C. elegans. Nature *408*, 470-475.

Raymond, J.R. (1991). Protein kinase C induces phosphorylation and desensitization of the human 5-HT1A receptor. The Journal of biological chemistry *266*, 14747-14753.

Rentschler, S., Linn, H., Deininger, K., Bedford, M.T., Espanel, X., and Sudol, M. (1999). The WW domain of dystrophin requires EF-hands region to interact with beta-dystroglycan. Biological chemistry *380*, 431-442.

Richard, S., Leclercq, F., Lemaire, S., Piot, C., and Nargeot, J. (1998). Ca2+ currents in compensated hypertrophy and heart failure. Cardiovascular research *37*, 300-311.

Richmond, J. (2005). Synaptic function. WormBook, 1-14.

Riddle, D.L. (1997). C. elegans II. Cold Spring Harbor Laboratory Press: Plainview, N.Y.

Roberds, S.L., Ervasti, J.M., Anderson, R.D., Ohlendieck, K., Kahl, S.D., Zoloto, D., and Campbell, K.P. (1993). Disruption of the dystrophin-glycoprotein complex in the cardiomyopathic hamster. The Journal of biological chemistry *268*, 11496-11499.

Rodrigues Ade, C., and Schmalbruch, H. (1995). Satellite cells and myonuclei in long-term denervated rat muscles. The Anatomical record *243*, 430-437.

Ruvolo, P.P., Deng, X., Carr, B.K., and May, W.S. (1998). A functional role for mitochondrial protein kinase Calpha in Bcl2 phosphorylation and suppression of apoptosis. The Journal of biological chemistry *273*, 25436-25442.

Rybakova, I.N., Patel, J.R., and Ervasti, J.M. (2000). The dystrophin complex forms a mechanically strong link between the sarcolemma and costameric actin. The Journal of cell biology *150*, 1209-1214.

Rybin, V., and Steinberg, S.F. (1997). Do adult rat ventricular myocytes express protein kinase C-alpha? The American journal of physiology *272*, H2485-2491.

Rybin, V.O., Buttrick, P.M., and Steinberg, S.F. (1997). PKC-lambda is the atypical protein kinase C isoform expressed by immature ventricle. The American journal of physiology *272*, H1636-1642.

Ryer, E.J., Sakakibara, K., Wang, C., Sarkar, D., Fisher, P.B., Faries, P.L., Kent, K.C., and Liu, B. (2005). Protein kinase C delta induces apoptosis of vascular smooth muscle cells through induction of the tumor suppressor p53 by both p38-dependent and p38-independent mechanisms. The Journal of biological chemistry *280*, 35310-35317.

Sadoulet-Puccio, H.M., Khurana, T.S., Cohen, J.B., and Kunkel, L.M. (1996). Cloning and characterization of the human homologue of a dystrophin related phosphoprotein found at the Torpedo electric organ post-synaptic membrane. Human molecular genetics *5*, 489-496.

Sambrook, J., and Gething, M.J. (1989). Protein structure. Chaperones, paperones. Nature *342*, 224-225.

Sander, M., Chavoshan, B., Harris, S.A., Iannaccone, S.T., Stull, J.T., Thomas, G.D., and Victor, R.G. (2000). Functional muscle ischemia in neuronal nitric oxide synthase-deficient skeletal muscle of children with Duchenne muscular dystrophy. Proceedings of the National Academy of Sciences of the United States of America *97*, 13818-13823.

Schachter, H., Vajsar, J., and Zhang, W. (2004). The role of defective glycosylation in congenital muscular dystrophy. Glycoconj J *20*, 291-300.

Schonwasser, D.C., Marais, R.M., Marshall, C.J., and Parker, P.J. (1998). Activation of the mitogen-activated protein kinase/extracellular signal-regulated kinase pathway by conventional, novel, and atypical protein kinase C isotypes. Molecular and cellular biology *18*, 790-798.

Sciandra, F., Bozzi, M., Bianchi, M., Pavoni, E., Giardina, B., and Brancaccio, A. (2003). Dystroglycan and muscular dystrophies related to the dystrophin-glycoprotein complex. Annali dell'Istituto superiore di sanita *39*, 173-181.

Senter, L., Ceoldo, S., Petrusa, M.M., and Salviati, G. (1995). Phosphorylation of dystrophin:effects on actin binding. Biochemical and biophysical research communications *206*, 57-63.

Sicinski, P., Geng, Y., Ryder-Cook, A.S., Barnard, E.A., Darlison, M.G., and Barnard, P.J. (1989). The molecular basis of muscular dystrophy in the mdx mouse: a point mutation. Science (New York, N.Y *244*, 1578-1580.

Smythe, G.M., Eby, J.C., Disatnik, M.H., and Rando, T.A. (2003). A caveolin-3 mutant that causes limb girdle muscular dystrophy type 1C disrupts Src localization and activity and induces apoptosis in skeletal myotubes. Journal of cell science *116*, 4739-4749.

Sossin, W.S. (2007). Isoform specificity of protein kinase Cs in synaptic plasticity. Learning & memory (Cold Spring Harbor, N.Y *14*, 236-246.

Steinberg, S.F., Goldberg, M., and Rybin, V.O. (1995). Protein kinase C isoform diversity in the heart. Journal of molecular and cellular cardiology *27*, 141-153.

Stossel, T.P., Condeelis, J., Cooley, L., Hartwig, J.H., Noegel, A., Schleicher, M., and Shapiro, S.S. (2001). Filamins as integrators of cell mechanics and signalling. Nat Rev Mol Cell Biol *2*, 138-145.

Straub, V., Duclos, F., Venzke, D.P., Lee, J.C., Cutshall, S., Leveille, C.J., and Campbell, K.P. (1998). Molecular pathogenesis of muscle degeneration in the delta-sarcoglycan-deficient hamster. The American journal of pathology *153*, 1623-1630.

Sun, H., Hu, X.Q., Moradel, E.M., Weight, F.F., and Zhang, L. (2003). Modulation of 5-HT3 receptor-mediated response and trafficking by activation of protein kinase C. The Journal of biological chemistry *278*, 34150-34157.

Suzuki, A., Yoshida, M., Yamamoto, H., and Ozawa, E. (1992). Glycoprotein-binding site of dystrophin is confined to the cysteine-rich domain and the first half of the carboxy-terminal domain. FEBS letters *308*, 154-160.

Tabuse, Y. (2002). Protein kinase C isotypes in C. elegans. J Biochem *132*, 519-522.

Tabuse, Y., Izumi, Y., Piano, F., Kemphues, K.J., Miwa, J., and Ohno, S. (1998). Atypical protein kinase C cooperates with PAR-3 to establish embryonic polarity in Caenorhabditis elegans. Development (Cambridge, England) *125*, 3607-3614.

Takai, Y., Kishimoto, A., Iwasa, Y., Kawahara, Y., Mori, T., and Nishizuka, Y. (1979). Calcium-dependent activation of a multifunctional protein kinase by membrane phospholipids. The Journal of biological chemistry *254*, 3692-3695.

Takeishi, Y., Chu, G., Kirkpatrick, D.M., Li, Z., Wakasaki, H., Kranias, E.G., King, G.L., and Walsh, R.A. (1998). In vivo phosphorylation of cardiac troponin I by protein kinase Cbeta2

decreases cardiomyocyte calcium responsiveness and contractility in transgenic mouse hearts. The Journal of clinical investigation *102*, 72-78.

Tang, Z., Scherer, P.E., Okamoto, T., Song, K., Chu, C., Kohtz, D.S., Nishimoto, I., Lodish, H.F., and Lisanti, M.P. (1996). Molecular cloning of caveolin-3, a novel member of the caveolin gene family expressed predominantly in muscle. The Journal of biological chemistry *271*, 2255-2261.

Tews, D.S., and Goebel, H.H. (1997). Apoptosis-related proteins in skeletal muscle fibers of spinal muscular atrophy. Journal of neuropathology and experimental neurology *56*, 150-156.

Thomas, G.D., and Victor, R.G. (1998). Nitric oxide mediates contraction-induced attenuation of sympathetic vasoconstriction in rat skeletal muscle. The Journal of physiology *506 (Pt 3)*, 817-826.

Tidball, J.G., Albrecht, D.E., Lokensgard, B.E., and Spencer, M.J. (1995). Apoptosis precedes necrosis of dystrophin-deficient muscle. Journal of cell science *108 (Pt 6)*, 2197-2204.

Timmons, L., Court, D.L., and Fire, A. (2001). Ingestion of bacterially expressed dsRNAs can produce specific and potent genetic interference in Caenorhabditis elegans. Gene *263*, 103-112.

Tinsley, J., Deconinck, N., Fisher, R., Kahn, D., Phelps, S., Gillis, J.M., and Davies, K. (1998). Expression of full-length utrophin prevents muscular dystrophy in mdx mice. Nature medicine *4*, 1441-1444.

Tinsley, J.M., Blake, D.J., Roche, A., Fairbrother, U., Riss, J., Byth, B.C., Knight, A.E., Kendrick-Jones, J., Suthers, G.K., Love, D.R., and et al. (1992). Primary structure of dystrophin-related protein. Nature *360*, 591-593.

Tinsley, J.M., Potter, A.C., Phelps, S.R., Fisher, R., Trickett, J.I., and Davies, K.E. (1996). Amelioration of the dystrophic phenotype of mdx mice using a truncated utrophin transgene. Nature *384*, 349-353.

Tochio, H., Zhang, Q., Mandal, P., Li, M., and Zhang, M. (1999). Solution structure of the extended neuronal nitric oxide synthase PDZ domain complexed with an associated peptide. Nature structural biology *6*, 417-421.

Turner, P.R., Westwood, T., Regen, C.M., and Steinhardt, R.A. (1988). Increased protein degradation results from elevated free calcium levels found in muscle from mdx mice. Nature *335*, 735-738.

Waggoner, L.E., Dickinson, K.A., Poole, D.S., Tabuse, Y., Miwa, J., and Schafer, W.R. (2000). Long-term nicotine adaptation in Caenorhabditis elegans involves PKC-dependent changes in nicotinic receptor abundance. J Neurosci *20*, 8802-8811.

Walsh, M.P., Busaan, J.L., Fraser, E.D., Fu, S.Y., Pato, M.D., and Michalak, M. (1995). Characterization of the recombinant C-terminal domain of dystrophin: phosphorylation by calmodulin-dependent protein kinase II and dephosphorylation by type 2B protein phosphatase. Biochemistry *34*, 5561-5568.

Waterston, R.H., Hirsh, D., and Lane, T.R. (1984). Dominant mutations affecting muscle structure in Caenorhabditis elegans that map near the actin gene cluster. Journal of molecular biology *180*, 473-496.

Wert, M.M., and Palfrey, H.C. (2000). Divergence in the anti-apoptotic signalling pathways used by nerve growth factor and basic fibroblast growth factor (bFGF) in PC12 cells: rescue by bFGF involves protein kinase C delta. The Biochemical journal *352 Pt 1*, 175-182.

Wicks, S.R., Yeh, R.T., Gish, W.R., Waterston, R.H., and Plasterk, R.H. (2001). Rapid gene mapping in Caenorhabditis elegans using a high density polymorphism map. Nature genetics *28*, 160-164.

Wood, W.B. (1988). The nematode Caenorhabditis elegans. Cold Spring Harbor Laboratory: Cold Spring Harbor, N.Y.

Yang, B., Jung, D., Motto, D., Meyer, J., Koretzky, G., and Campbell, K.P. (1995). SH3 domain-mediated interaction of dystroglycan and Grb2. The Journal of biological chemistry *270*, 11711-11714.

Yoshida, M., Hama, H., Ishikawa-Sakurai, M., Imamura, M., Mizuno, Y., Araishi, K., Wakabayashi-Takai, E., Noguchi, S., Sasaoka, T., and Ozawa, E. (2000). Biochemical evidence for association of dystrobrevin with the sarcoglycan-sarcospan complex as a basis for understanding sarcoglycanopathy. Human molecular genetics *9*, 1033-1040.

Zhu, X., Hadhazy, M., Groh, M.E., Wheeler, M.T., Wollmann, R., and McNally, E.M. (2001). Overexpression of gamma-sarcoglycan induces severe muscular dystrophy. Implications for the regulation of Sarcoglycan assembly. The Journal of biological chemistry *276*, 21785-21790.

Acknowledgements

The work presented in this thesis was done at the Centre de Génétique Moléculaire et Cellulaire, Université Lyon 1 under the supervision of Dr. Laurent SEGALAT.
I would like to thank my supervisor for creating a challenging and inspiring scientific environment and for providing endless support throughout my studies in France. In particular, I am grateful to Prof. Kathrin GIESELER for her comments on this thesis and her helpful suggestions and guides throughout the project. I would also like to thank my dear colleagues Stefan JÜNEMANN, Marie Christine MARIOL, Maïté CARRE PIERRAT, Lucie CHAMBONNIER, Claire LECROISEY, Edwige MARTIN, Laure GRANGER, Jean GIACOMOTTO, Hannah KAHOS, Mohamed TRIBA, Elodie VALLIN, Guillaume TANNIOU, Caroline BORREL, Chi WANG and Stefanie PETITJEAN for their kindly help and scientific discussions.

I would thank Prof. GU Fukang and Mr. LI Haisheng for their advices and help in my study in France.

And my biggest thanks to my parents and my husband for their understandings and supports that keep me going.