ABSTRACT

The Functional Analysis of NPXY Motif in β Integrin *in vivo*

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NPXY (Asn-Pro-X-Tyr) is a conserved tyrosine phosphorylation motif that binds to PTB (phospho-tyrosine binding) domain of other protein. Integrins, a heterodimeric cell surface receptor for extracellular matrix (ECM), include two NPXY motifs in tandem on the cytoplasmic tails of β subunits. I generated a tyrosine to glutamate mutation mimicking constitutive phosphorylation of NPXY in β*pat-3* integrin of *Caenorhabditis elegans*. The transgenic animals displayed disorganized muscle actin and abnormal gonad migration and tail morphology, suggesting that the phosphorylation of tyrosine causes defective phenotypes. In addition, the transgenic animals produced the high number of males, implying that the transgenic animals are similar to *him-4*/hemicentin alleles and that the lack of *him-4* may cause the phosphorylation of NPXY. Genetic analyses revealed that tyrosine to phenylalanine mutations in both positions, β*pat-3(YYFF)*, was able to suppress high incidence in male, mating ability, and egg-laying phenotypes of *him-4* mutant, suggesting that a function of *him-4* is to prevent the phosphorylation of β*pat-3* NPXY. Taken together, our data suggest that changes in the ECM regulate the phosphorylation of the integrin NPXY.

The Functional Analysis of NPXY Motif in β Integrin *in vivo*

by

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CHAPTER ONE

Introduction

Extracellular Matrix

Extracellular matrix (ECM) is a network of secreted proteins and carbohydrates interwoven together to assist various cellular processes such as cell adhesion, migration, growth, survival, and differentiation (Boudreau and Bissell, 1998; Huang and Ingber, 1999; Howe et al., 1998). Absence or disruption of ECM molecules often leads to pathological conditions such as congenital muscular dystrophy, proteinuria, and junctional epidermolysis bullosa (Besse et al, 2003; Arcangelis and Georges-Labouesse, 2000; Gorski and Olsen, 1998). Studies on the major functions of ECM were more focused on its physical property rather than biologically dynamic roles. However, many studies revealed the involvement of ECM molecules in cellular processes. In connective tissues, fibroblasts are the main source of ECM molecules (Zhang et al., 2006). There are two major types of ECM molecules; proteoglycans, complex of core protein and a polysaccharide glycosaminoglycans (GAGs), and glycoproteins such as collagen, elastin, fibronectin, and laminin (Geiger et al., 2001).

Proteoglycans

GAGs are negatively charged polysaccharide chains that prefer extended form rather than globular form whose negative charge attracts positive ions such as Na^+ favoring osmotically active state, which hydrates extracellular space (Iozzo, 1998). This hydrated space created by GAGs can function as a reservoir for hormones or other secreted proteins as well as buffering machinery against compressive forces generated

onto connective tissue from outside (Iozzo, 1998). In addition, this space allows other fibrous proteins to build a network among them. Especially, this provides a ground for any migrating cells to attach and move directionally.

Hyaluronan is composed of long chain of repeating non-sulfated disaccharides. This is the only type that is not in the form of proteoglycan among GAGs. The rest are covalently attached to other core proteins forming proteoglycans. Types of sugars, the length of sugar chain, and the presence of sulfate groups can classify these. Proteoglycans serve as a selective filter to control the trafficking of molecules in the extracellular space (Iozzo, 1998). It regulates the secreted proteins by using bound enzymes such as protease to release them in an appropriate space. Some are incorporated into plasma membrane and function as co-receptors to enhance the binding of cell to ECM or sensitivity of cell to signaling molecules in extracellular space.

ECM Glycoproteins

Among several fibrous proteins, collagens are the most abundant proteins in mammal. They are the major components of skin and bones and secreted by connective tissue and other various cells. It is composed of three collagen polypeptide chains called α chain, which has long, stiff and helical structure. Different combinations of 25 distinct α chains can form many collagen molecules. Depending on their functions, collagens are classified as fibrillar collagens, fibril-associated collagens, and network-forming collagens (Adachi et al., 1997). All these different types of collagens are thought to organize the ECM.

Elastin is the main component of elastic fibers. It gives a fiber resilience to recoil after stretch. For this characteristic, elastin is found largely in arteries. It is

composed of hydrophobic segment giving elastic property and alanine (A) and lysine (K) rich alpha-helical segment that enables cross-linking with other molecules (Debelle and Tamburro, 1999). The secreted precursors of elastin in extracellular space are cross-linked to one another forming networks of fibers and sheet-like structure. These assembled elastic fibers adopt "random coil" conformation within its network to behave like rubber bands (Eyre, 1984).

Fibronectin is a large glycoprotein made up of two large subunits that contain several functionally distinct domains and that are connected by disulfide bonds at the C-terminal end (Dallas et al., 2006). Each domain within subunit is composed of repeating modules. Through these modules, fibronectin can interact with cells as well as other ECM molecules or themselves. The most common module found in many vertebrate proteins is type III repeat. Especially, the arg-gly-asp (RGD) sequence within type III repeats is a well-known binding site for integrin, a cell surface receptor for ECM (Baneyx et al., 2002). Finbronectin exists in two distinct forms, soluble and fibrillar forms (Hocking and Chang, 2003). Soluble forms circulate in the blood or other bodily fluids and interact with the target molecules when it is triggered. The fibrillar forms are only deposited on the surface of certain cells. This fibrillar fibronectin formation is mainly controlled by intracellular actin filaments across the plasma membrane through integrin receptors, so it can contribute to organization of extracellular space (Schwarzbauer and Sechler, 1999). This explains the tight interaction between the ECM and cytoskeleton in migrating cells. Based on ECM organization, migrating cells can regulate cell shapes through many intracellular signaling pathway controlling cytoskeleton networks.

Basement Memberane ECM

There is a specialized extracellular structure called basal lamina, which is a thin layer of sheet-like structure composed of several ECM molecules. Main components of basal lamina are type IV collagen, laminin, nidogen, and perlecan, a heparin sulfate preteoglycan (Lebleu et al., 2007). Unlike other collagen fibrils, Type IV collagens contain uncleaved terminal domains that are able to form flexible networks. Laminin is another ECM protein composed of α, β, and γ chains assembled into a shape of a cross, which contains several binding domains for other molecules such as perlecan, nidogens, and other laminins (Huang et al., 2003).

Basal lamina, basement membrane (BM), has multiple functions. It can function as a filter to prevent a passage of certain molecules. Especially in kidneys, it prevents large proteins transferring from blood into the urine (Lebleu et al. 2007). It can also act as a selective barrier affecting the points of contacts. It is also known to facilitate tissue regeneration by providing a ground for directed cell migration (Lebleu et al. 2007).

Cell migration is an important process during normal development as well as certain pathogenesis like tumor metastasis. ECM can contribute to the regulation of cell migration by remodeling the ECM architecture. Some cells are able to release proteolytic enzymes called matrix proteases for ECM remodeling. Matrix metalloproteases (MMP) are major species that degrade major components of ECM such as collagens to promote cell migration by clearing a path for cells or by exposing hidden active sites of ECM molecules, which in turn facilitates the migration process (Vu and Werb, 2000).

Integrins

Integrins are heterodimeric molecules containing alpha and beta subunit. In mammals, there are 18 α and 8 β chains comprising 24 functional αβ heterodimers (Hynes 2002). Integrins are a type of cell surface receptors that links ECM to the inside of the cell (cytoskeleton) to regulate various cellular events. Each subunit possesses large extracellular, transmembrane, and a short cytoplasmic tail domains with its distinct roles. Some integrins such as α5β1 recognize the RGD tripeptide sequence of fibronectin and vitronectin, and α2β1 recognizes collagens in vertebrates (Hynes 2002).

From the targeted deletion experiments, most of integrin subunits showed severe phenotypic defects in various stages during development (Hynes 2002). The extracellular domains interact with multiple ECM ligands, whereas the intracellular cytoplasmic domains interact with cytoskeletal proteins. This physical link enables bidirectional signaling between interior and exterior of the cell through plasma membrane and leads to robust regulation of cellular behaviors such as adhesion, migration, and differentiation (Qin et al, 2004). Integrin-mediated cell growth is an example of cell regulations by the outside of the cell, whereas intracellular signaling events can also influence the ECM distributions as well as affinity of integrins for ECM ligands (Ginsberg et al., 2005). These two major events are termed as “outside-in” and “inside-out” signaling of integrins, respectively (Ginsberg et al., 2005).

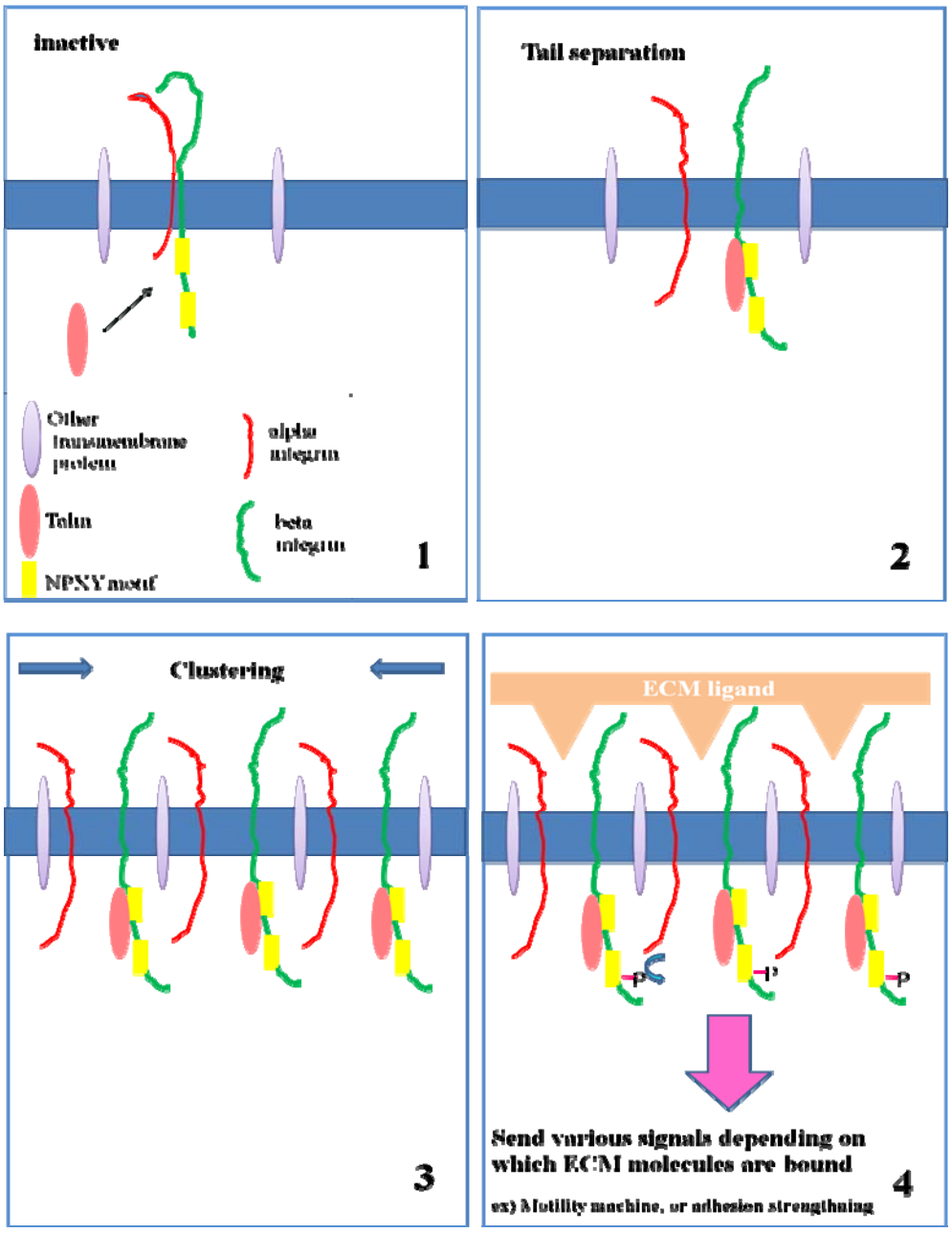


Figure 1. Integrin activation model. This model depicts plausible sequential mechanism of integrin activation upon talin binding to β integrin subunits. Panel 1 is an integrin in resting state where integrin subunits are put close together. Panel 2 shows the separation of integrin subunits upon binding of talin. Panel 3 shows the sequential clustering of integrin once activated by separation of subunits. Panel 4 shows the interaction of integrins with ECM molecules upon clustering. This interaction may transduce outside information to the inside of the cell through various signaling molecules.

Inside-out signaling. There are several proposed potential mechanisms of how "inside-out" signaling works. A supportive model from structural, biochemical, and biophysical studies is "clasp and unclasp" model, which are similar to Figure 1 (Qin et al, 2004). The study of αIIbβ3 integrin suggested the existence of a salt-bridge between arginine (R) 995 of α subunit and aspartic acid (D) 723 of β subunit in the membrane proxy regions of cytoplasmic tails (Campbell and Ginsberg, 2004). Deletion mutants of either α or β membrane proximal regions in the cytoplasmic tails of various integrins lead to constitutively active state of an integrin heterodimer (Campbell and Ginsberg, 2004). This supports the existence of salt bridge between α and β subunits during inactive or resting state of integrin.

The next question is how the salt-bridge or association between α and β subunits is interrupted. Although there are some debates over how it actually happens, Ginsberg's group suggested that talin is likely an intracellular molecule directly binding to β integrin cytoplasmic tail and that interrupts the interaction between the two subunits (Campbell and Ginsberg, 2004).

The overexpression of talin fragments containing a phospho-tyrosine binding (PTB) domain activated integrins (Ginsberg et al., 2005). The binding of talin to cytoplasmic tail of β integrin may initiate conformational change of α and β subunits causing the dissociation of α and β cytoplasmic domains, which then will cause shifting of the extracellular domain of integrin into more favorable conformation for the ligand binding (Ginsberg et al., 2005). However, a recent study in mouse model disagrees with the concurrent idea of integrin activation by dissociation of salt bridge between α and β subunits. In this gene replacement study, the point mutation (D759A) to disrupt the salt-

bridge showed normal development without any noticeable phenotypes from detailed histological analysis of mutant skin (Czuchra et al., 2006). There has been a good deal of discrepancies between animal model and cell culture studies, because it is almost impossible to reproduce the naturally occurring cellular environment in any cell culture experiments.

Outside-in signaling. Unlike the inside-out signaling, much information about outside-in signaling has not been described. There are two major events supposedly involved in this signaling: ligand binding to extracellular domain and receptor (integrin) clustering. Outside-in signals are supposedly transduced upon the binding of ECM ligands to integrin, which may cause conformational change leading to multiple intracellular signaling events depending on the type of ligands that are bound. Structural analysis of the extracellular domain of αvβ3 integrin revealed that the conformation of active and inactive states of integrin can be 'stalk-like' and 'bent' respectively (Qin et al, 2004).

The outside-in signaling pathway leads to the formation of focal adhesion (FA) (Burridge and Chrzanowska-Wodnicka, 1996). Although there are other types of transmembrane protein such as syndecan IV, integrins are the major adhesion receptors found in FA (Burridge and Chrzanowska-Wodnicka, 1996). Many different types of cytoplasmic proteins are also known to be co-localized in FA. Vinculin, talin, FAK, and paxillin are some examples (Johnson and Craig, 1995; Burridge and Chrzanowska-Wodnicka, 1996; Turner et al., 1990). The detail mechanisms of how each component is recruited to FA are yet to be understood. However, the recruitments of some components have been proposed (Burridge and Chrzanowska-Wodnicka, 1996). Among them, some

has structural role and the other has signaling role. Recently, evidences for the direct interaction of protein kinase with integrin have been reported to support the signaling events mediated through integrin receptors (Ginsberg et al., 2005). Src family tyrosine kinases (SFK) are one of the best studied molecules in signaling events. The study of interaction between c-Src and β3 integrin subunit revealed direct interaction of c-Src SH3 domain and the cytoplasmic tail region of β3 integrin subunit (Arias-Salgado *et al.,* 2003). Another important cytoplasmic molecule reported is the integrin-linked kinase (ILK). Direct interaction of ILK with β1 or β3 cytoplasmic domain in adherent cells has been confirmed by co-immunoprecipitation (co-IP) (Ginsberg et al., 2005). This finding revealed the role of integrins in regulation of multiple cell behaviors such as cell adhesion, motility, proliferation and survival by recruitment of other signaling proteins (Ginsberg et al., 2005).

Caenorhabditis elegans

Caenorhabditis elegans is a free-living soil nematode usually growing found in soil and feeding soil bacteria. A fully grown adult is about 1mm in length and possesses transparent body which make it possible to see the inside structure with a simple optical microscope without dissection. It is composed of 1000 somatic cells that form many tissues such as muscles, pharynx, intestine, epidermis, gonad and neurons (Brenner 1974). It became available for study on the late 1940s and started to use for animal genetics model in early 1960s. *C. elegans* is hermaphrodite that usually produces 300 progeny by self-fertilization. Rare number of male is also available for genetic crosses. In the laboratory, it grows on an agar plate seeded with bacterial species at room temperature. Genetic analysis of *C. elegans* revealed that it contains 6 chromosomes,

100 Mega bases genome (43% similar to human), and about 19,000 genes. For several decades, extensive genetic and molecular studies analyzed gene functions, which generated about 3000 genetically defined genes containing at least one allele of mutations. In addition, the whole genome was sequenced in 1990s and RNA-mediated interference of gene expression (RNAi) techniques paved the way to analyze the function of gene by reverse genetics in the genome-wide level.

C. elegans contains extracellular matrix (ECM) which wraps around many tissues such as muscles, pharynx, and gonad. Genetic analyses revealed that mutations in ECM genes cause very severe consequences, demonstrating that the cell-matrix interaction is crucial for the development and function of the tissue cells in *C. elegans*. Basement membrane that wraps around many tissues contains glycoproteins and proteoglycans such as *emb-9*, *let-2* (collagen type IV), *epi-1*, *lam-1*, *lam-3* (laminins), *nid-1* (nidogen), and *unc-52* (perlecans) (Williams and Waterston, 1994). Except for *nid-1*, null mutations in BM components display embryonic lethality, demonstrating the necessity of BM during tissue formation (Williams and Waterston, 1994).

C. elegans Integrin

C. elegans has three integrin genes, two α chains (*ina-1* and *pat-2*) and one β subunit genes (*pat-3*), which forms two distinct sets of integrins. Null alleles in *C. elegans* integrin genes cause embryonic (*pat-2* and *pat-3*) or larval lethality (*ina-1*) (Baum and Garriga, 1997; Williams and Waterston, 1994).

INA-1 is generally involved in cell migration and morphogenesis and is expressed ubiquitously at gastrulation with more highlighted in pharynx. The dynamic regulation of *ina-1* expression was evident in most migrating cells or cells undergoing morphogenesis such as the hermaphrodite uterus, vulva and the male tail. In *ina-1* mutants, axons migrate normally to its target but fail to bundle into nerve fascicles (Baum and Garriga, 1997). In the same study, *ina-1* mutant showed additional morphological defects in the head, pharynx, vulva, gonad, uterus, and male tail. During distal tip cell (DTC) migration of hermaphrodite, *ina-1* is downregulated by *vab-3,* a gene encoding for a transcription factor similar to mouse *pax6*, to stop migration at proper developmental stage. Most recently, *ina-1* is reported to disrupt muscle cell migration acting from juxtaposed epidermal tissue (Tucker and Han, 2008).

PAT-2 is another α integrin subunit encoded by *pat-2* gene (Williams and Waterston, 1994). The expression pattern of *pat-2* is distinct from *ina-1*; it is highly expressed in body-wall muscle cells and vulva tissues. *pat-2* is also expressed in DTC immediately after L2 stage where DTC starts dorsal migration (Meighan and Schwarzbauer, 2007). This study uncovered unique role of *pat-2* in pathfinding during hermaphrodite gonad morphogenesis. The similarity of localization patterns and mutant phenotypes among *pat-2*, *pat-3*, and *unc-52* suggests important role of *pat-2* in body-wall muscle cell integrity. PAT-2 co-localized at dense bodies and M-line with *pat-3* and *unc-52* indicating possible interaction of *pat-2/pat-3* integrin with *unc-52* in the basement membrane (Francis and Waterson, 1991).

There is only one β subunit encoded by *pat-3* gene in *C. elegans*. It is widely expressed in most tissues throughout development. Especially within each muscle cell,

pat-3 was localized to the dense bodies, M-line, and borders between cells. It is also localized in anal depressor muscle, along the intestinal tract, sphincter muscle, vulva muscles, spermatheca, uterus, and coelomocytes. Although *pat-3* is reported to be absent in pharynx, *ina-1* is strongly expressed in pharynx (Baum and Garriga, 1997). Since, integrin is known to form heterodimer, it is reasonable to assume the presence of *pat-3* in pharynx.

Recent study also showed the localization of *pat-3* in myoepithelial sheath cells of the proximal ovary in hermaphrodites (Ono et al., 2007). However, the role of *pat-3* as an adhesive structure in this myoepithelial sheath cells showed somewhat different from body-wall muscle cells. PAT-3 normally colocalized with MYO-3 myosin heavy chain in M-line of body-wall muscle cells, but PAT-3 and MYO-3 did not colocalized at M-spot in myoepithelial sheath cells suggesting different mechanism for organization of muscle architect in this tissue (Ono et al., 2007). *pat-3* mutant shows Pat (Paralyzed at two-fold) phenotype due to failure of dense body formation in sarcomeres of muscle cell (Williams and Waterston, 1994). Integrin β tail has been reported to regulate integrin signaling by recruiting several signaling molecules. The over-expression of PAT-3 β tail shows multiple integrin related phenotypes such as Pat, Unc, and Egl (Lee et al., 2001). This result may be due to the competitive recruitment of cytoplasmic signaling molecules to extra sets of β tail, which inhibits the normal function of endogenous β tails. Although the exact mechanism of recruitment or modulation of signaling through integrin is still under intense debate, two well-conserved NPXY motifs in β tail are the most likely spots for regulating signaling events.

C. elegans Extracellular Matix

In *C. elegans*, there are two major types of extracellular matix (ECM). One is the basement membranes (BM). *C. elegans* BM are thin sheet-like structure composed of several ECM molecules similar to vertebrate. In *C. elegans* BM, the identified molecules are Collagen type IV and type XVIII, laminins, perlecan, nidogen, SPARC/Osteonectin, fibulin, hemicentin, and integrin. Unlike vertebrate, the variety of each molecule is limited to smaller set. The differential and tissue specific expression of each molecule have also been observed. The *C. elegans* BMs enclose surfaces of all internal tissues including body wall muscles, intestine, pharynx, gonad, and the pseudocoelomic sides of the hypodermis.

Collagen Type IV composed of two α1-like and one α2-like chain folded into a triple-helical structure in *C. elegans* (Gupta et al., 1997). This molecule is divided into 3 distinct regions; short non-helical N-terminal domain containing four conserved cysteines, a long Gly-X-Y repeat domain with multiple interruptions, and C- terminal globular NC1 domain containing 12 conserved cysteines (Graham et al., 1997). Type IV collagen molecules are assembled into a complex polygonal network stabilized by disulfide bonds formed between NC1 domains or N-terminal domains (Timple, 1989). Unlike mammalian genes, the *C. elegans* type IV collagen genes; *emb-9* and *let-2* coding for α1 and α2 chains respectively, are found in different chromosomes (Gupta et al., 1997). The splicing of the α2 chain gene *let-2* increases the diversity of type IV collagens. Interestingly, splice variants are unevenly present throughout the developments. Several mutations in either *emb-9 or let-2* gene have been identified. The most frequent mutations occur at Gly-X-Y repeat leading to temperature-sensitive phenotypes (Isnenghi et al., 1983). At restrictive temperature, worms are arrested at

twofold stage in embryo. At permissive temperature, worms show larval arrest or sterility in adult. This indicates that the normal functions of collagen Type IV genes are involved in fertility and development process of worms. The expression pattern of the *emb-9* and *let-2* are identical. The antibody staining showed co-localization of both genes in mesodermal cells such as body wall muscle and somatic gonadal cells (Graham et al., 1997). However, the presence of type IV collagens in other tissues suggests that different mechanisms for controlling the assembly of type IV collagens may exist (Graham et al., 1997).

A collagen type XV and XVIII homologue in *C. elegans* is *cle-1*. It is composed of C-terminal domain termed endostatin (ES), collagenous Gly-X-Y repeat domain, and N-terminal domain (Ackley et al., 2003). The most conserved domain between vertebrate and *C. elegans* is ES domain sharing 55% similarity with mouse ES (Ackley et al., 2001). ES domain has been reported to inhibit angiogenesis, cell migration, and proliferation (Ackley et al., 2001). *C. elegans* produce 3 isoforms of *cle-1; cle-1 A-C,* using three promoter and alternative splicing (Ackley et al., 2001). *cle-1* is expressed widely throughout the development. Although it is expressed in basement membrane surrounding muscle cells and somatic gonad and other tissues, the major expression is concentrated in nervous systems (Ackley et al., 2001). Several mutant studies revealed that *cle-1* is involved in directing cell migration and axon guidance.

Nidogen is a widely expressed basement membrane associated glycoprotein encoded by *nid-1* in *C. elegans*. This protein is consisted of three globular domains, G1, G2, G3, connected by rod-like domain primarily composed of epidermal growth factor (EGF) modules. Each globular domain shows affinity for other basement membrane

proteins such as collagen type IV and perlecan (Kim and Wadsworth, 2000). This association indicates its structural function in basement memebrane as a linker to enhance stability of the network. In vertebrate studies, nidogen facilitates cell migrations possibly by forming proper linkages among other basement membrane proteins (Kim and Wadsworth, 2000).

The *C. elegans* ortholog of SPARC/osteonectin gene is *ost-1*. OST-1 is localized along the body wall muscles concentrated at the boundaries rather than dense bodies (Fitzgerald and Schwarzbauer, 1998). It is also localized in gonad, sex muscles, and pharynx (Fitzgerald and Schwarzbauer, 1998). However, the expression of *ost-1* is not reported in pharynx. The distribution pattern is similar to other BM proteins such as type IV collagen. Like type IV collagen, *ost-1* may be subsequently localized to pharyngeal muscle basement membrane after expression by body wall muscle cells. It is also plausible that localization of *ost-1* may be related to the presence of type IV collagen since OST-1 has been reported to have collagen binding activity.

Laminins are large glycoproteins required for basement membrane assembly. Each laminin is a heterotrimer assembled from α, β, and γ subunits. In *C. elegans*, *lam-3* and *epi-1* code for αA and αB respectively. *lam-1* and *lam-2* each code for β, and γ subunits (Kao et al., 2006). Therefore, there are two isoforms of laminin. These two isoforms are localized in distinct regions as well as overlapping regions. Both α units are expressed in pharynx and intestine (Huang et al., 2003). However, *lam-3*(αA) is also expressed in excretory cell, spermatheca, nerve ring and nerve cords when *epi-1*(αB) is expressed in body wall muscles, hypodermis, gonad, accessory muscles, and coelomocytes (Huang et al., 2003). In *lam-3* mutant worm, pharyngeal basement

membrane was most defective when other tissues were relatively normal (Huang et al., 2003). In *epi-1* mutant worm, it did not show defective phenotype in pharynx (Huang et al., 2003). However, *epi-1* mutant showed multiple defects in other tissues including failures of cellular polarization, adhesion and organization (Huang et al., 2003). These studies indicate the structural roles of laminin over broad range of basement membrane organization during development.

The *unc-52* gene in *C. elegans* encodes for mammalian perlecan homologue, which is heparin sulfate proteoglycan found in basement membrane. The *unc-52* contains a unique signal sequence and four additional domains containing sequences similar to low-density lipoprotein (LDL) receptor, laminin, and the neural cell-adhesion molecule (NCAM) (Rogalski et al., 2001). Although it can produce multiple isoforms, only three major forms of Unc-52 has been well characterized (Rogalski et al., 2001). A short form contains the first domain containing signal sequence and LDL receptor-like sequence, but the long NCAM region and C-terminal agrin-like region is absent. A medium form contains the first domain and long NCAM region, but it lacks C-terminal agrin-like region. A long form contains all five domains that are identified in mammalian perlecan. From cell culture studies, perlecan has binding activities for other perlecans as well as other ECM molecules, laminin, nidogen, collagen, and fibronectin (Rogalski et al., 2001). In *C. elegans*, medium form is sufficient for normal myofilament lattice assembly in body wall muscle (Mullen et al., 1998). UNC-52 is only localized in the basement membrane where body wall muscle cells and hypodermis are interfacing. *unc-52* mutants shows paralyzed and arrested at two-fold stage (Pat) phenotypes (Hresko et al., 1994; Rogalski et al., 1995; Williams and Waterston, 1994). Within each muscle cell,

the myofilament network in individual muscle cell is linked to intermediate filament arrays in hypodermis through integrin-containing structure called dense bodies. The overlapping expression pattern in body wall muscle cell and several mutant studies support the idea that *unc-52* and *pat-3* are linked directly or indirectly through other possible linker proteins. When *unc-52* and βpat-3 mutants were analyzed, they both showed Pat phenotype and disruptions in myofilament networks (Rogalski et al., 2001). In addition, *unc-52* null mutant showed disorganized *pat-3* localization (Rogalski et al., 2001). Further study will be necessary to uncover more detail mechanisms on how *unc-52* affects the localization of integrin.

C. elegans has a single fibulin gene, *fbl-1*, which is highly conserved homologue of vertebrate fibulin-1. It contains N-terminal signal sequence, three anaphylatoxin repeats, nine EFG-like repeats and C-terminal that are alternatively spliced resulting in two isoforms, *fbl-1C* and *fbl-1D* (Hesselson and Kimble, 2006; Muriel et al., 2005). These two isoforms show distinct localization patterns. FBL-1C localizes to the gonad, phrynx, intestine, body-wall muscle, GLR cells, uterine attachement, and mechanosensory neurons (Muriel et al., 2005). FIB-1D localized on ALM/PLM mechanosensory neuron attachements, body-wall muscle, GLR cells, uterine attachements, and flexible tracks linking pharyngeal and body-wall muscle basement membranes (Muriel et al., 2005). However, the synthesis of *fbl-1* is from intestinal cells, body-wall muscle, spermatheca, and hypodermal syncytium (Muriel et al., 2005). *fbl-1* mutants show smaller body size and slower growth rate than wildtype (Hesselson and Kimble, 2006). The loss of function of *fbl-1* shows multiple defects (Muriel et al., 2005). In gonad, the distal tip cells does not migrate in proper trajectory and seem to stop during

dorsal migration causing the proximal gonad to rupture possibly due to proliferating germ cells in the gonad. This results in sterility or very few numbers of progeny. In pharynx, asymmetric development of pharynx is observed. This may explain the small size of the worm due to inefficient feeding. In muscle cell, general structure seems normal except junctions between muscle cells. The shape is also a bit deformed. The intestine is shriveled compared to wild type. An assembly of hemidesmosomes and intermediate filaments, and epidermal squamification show defects due to failure of neuronal migration (Muriel et al., 2005). In addition, *fbl-1* is also reported to antagonize *gon-1* function in directing distal tip cell migration (Muriel et al., 2005).

Hemicentin is extracellular member of the immunoglobulin superfamily encoded by *him-4* gene in *C. elegans*. Hemicentin contains von Willebrand A (VWA) domain in N-terminal, a long stretch of immunoglobulin(Ig) domains, several epidermal growth factors (EGFs), and a single fibulin-like C-terminal domain (Dong et al., 2006). It is synthesized by body-wall muscle and gonadal leader cells of both male and hermaphrodite. It is diffusely localized to multiple locations and forms long flexible tracks. From the study of structural modules of hemicentin, the localization of hemicentin is mediated by VWA domain, which contains a cell binding domain (Vogel and Hedgecock, 2001). Once hemicentin is localized to the target site, assembly of hemicentins or possibly with other ECM molecules were mediated through EGF/fibulin-like C-terminal domain (Dong et al., 2006). In anterior body-wall muscle cells, hemicentins assemble into a multiple lines of flexible track connecting pharyngeal basement membrane and muscle cells. In male gonad, hemicentin is expressed along the path of linker cell (Vogel and Hedgecock, 2001). In hermaphrodite gonad, hemicentin is

concentrated on the distal regions of the gonad and regulates cellularization of the germline syncytium (Vogel and Hedgecock, 2001). In *him-4* mutant, the major defects are tissue fagility, linker cell migration defects, and high incidence in male (Him) phenotypes (Vogel and Hedgecock, 2001). As described above, all defects can be explained by the absence or disruption of hemicentin in proper tissues. The exact mechanism of how hemicentin interact with other molecules or which cells contain receptors for hemicentin is unclear. However, transcription factor *unc-30* has been identified to recruit hemicentin to specific cell type, and RGD-binding integrins have been mentioned as a possible receptor for hemicentin (Vogel and Hedgecock, 2001).

Conserved NPXY Motif in βpat-3 integrin cytoplasmic tail

There are also two NPXY (asp-pro-X-tyr) motifs, conserved tyrosine phosphorylation and binding site for proteins with phosphotyrosine binding (PTB) motif, conserved in βpat-3 tail (Calderwood 2004). Tyrosine phosphorylation in NPXY motif will activate integrin and promote interactions with downstream signaling molecules (Oxley et al., 2008; Vignoud et al., 1997). Functional studies on NPXY motifs in mouse β3 integrin showed that platelets displayed defective chemotaxis when one or both Y was changed to F (Phillips et al, 2001). The Y to alanine (A) mutation in mouse β1 or β3 integrin abolishes integrin functions (Czuchra et al., 2006). Disrupting the two NPXY motifs impaired the proliferation and survival of mouse embryonic fibroblast (MEF). The defect in MEF resulted from the impaired activation of FAK and a Rho family GTPase (Rac) (Brown et al., 2005), suggesting the indispensible role of the conserved amino acid residues in the survival of the cell. Phosphorylation of the tyrosine of NPXY

provides a binding site for phosphotyrosine binding (PTB) domains such as talin and ICAP-1 (Calderwood et al., 2003).

In a previous study, Lee *et al.* (2001) determined the role of conserved NPXY motifs in the cytoplasmic domain of βpat-3. Three rescued lines, βpat-3(Y792F), βpat-3(Y804F) and βpat-3(YYFF) rescued the embryonic lethality of *pat-3*(*st564)* and generated viable and fertile progeny (Lee 2001). These transgenic animals exhibited mild defects in distal tip cell migration. Most commonly, the DTC turned away from the dorsal surface and returned to the ventral side, because of defective adhesion to the dorsal body wall basement membrane (Lee 2001). Immunofluorescence staining of rescued animals with MH25, a monoclonal antibody against βpat-3, demonstrated normal localization and distribution of dense bodies (Lee 2001). Muscle filamentous actin staining in the rescued NPXY lines was also indistinguishable from βpat-3(+) animals (Lee 2001). These data suggest that NPXY motif plays an important role in cell migration but little in muscle cell organization.

Significance in Studying C. elegans β integrin

C. elegans βPAT-3 integrin has striking sequence similarity to human β1 integrin, 41.2% identity in the overall protein sequence (Gettner et al., 1995). Furthermore, many members of the mammalian focal adhesion complex have closely related to *C. elegans* counterpart, dense bodies, which is attachment structures anchoring muscles to body wall (Hresko et al., 1994). The highly conserved structure and its signaling components of *C. elegans* βPAT-3 to mammalian counterparts lead me to investigate the conserved signaling pathways relevant to human development and/or disease progression.

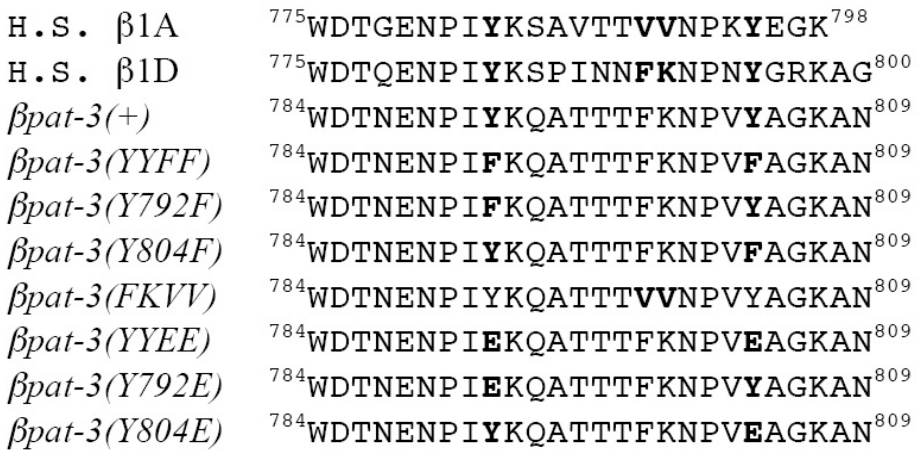


Figure 2. β integrin cytoplasmic tails. Cytoplasmic tails from *Homo sapiens* (H. S.) β1A and β1D integrins and *C. elegans* β*pat-3* were compared. For mutant *bpat-3*, one or both tyrosines were mutated to phenylalanine (F) or glutamate (E). Protein sequences from downstream of a unique tryptophan (W^{784}) are aligned and compared.

Vertebrate β1 and β3 integrins have two NPXY tyrosine (Y) phosphorylation motifs that are conserved in the *C. elegans* βPAT-3 cytoplasmic tail at Y792 and Y804 (Figure 2). Studies have suggested that mutation of the tyrosines to either phenylalanine (F) or alanine (A) abolishes integrin activity. Y to F changes at both positions (YYFF) impair cell migration of transfected β1 null mouse fibroblasts without affecting cell adhesion or matrix assembly (Sakai et al., 1998), whereas β1-deficient mouse lymphoma cells transfected with β1 YYFF had reduced adhesive properties (Stroeken et al., 2000). In NIH 3T3 cells plated on fibronectin, Y to A mutation reduces localization to focal adhesions (Reszka et al., 1992). Mice expressing integrin β3 YYFF had a tendency to rebleed and platelets from these mice were defective in retracting clots (Law et al., 1999). These studies indicated that conserved tyrosine motifs mediate diverse integrin activities.

Studies on conserved tyrosines in β integrin tail revealed that either Y to F or A mutations reduce or abolish activity of integrins. Y to E mutation which gives negatively charged residues in one of NPXY motifs failed to localize to focal adhesions, suggesting

that the activating mutations may result in the similar consequences to the inactivating mutations such as Y to F or A (Reszka et al., 1992).

To characterize the role of cell-matrix interaction during development, I used the nematode *C. elegans* for genetic and molecular experiments. I have created constitutively active mutations, Y to E, in cytoplamic tail of *pat-3* integrin and underwent molecular approaches to analyze the effects of modulating integrin functions and genetic interactions by over-expressing in the wild-type or rescuing null allele of *pat-3*. Our results show that integrins are involved in post-embryonic body wall muscle functions as well as DTC migration and male tail formation.

Because no mutations have been identified within the region encoding the βpat-3 cytoplasmic domain, previous studies have developed an approach to introduce such mutations by rescue of *pat-3* null animals and established rescued lines with a wild type βpat-3 gene or with βpat-3 genes containing Y to F or E cytoplasmic tail mutations (Lee et al., 2001). Through detailed phenotypic analyses, I used these lines to define the roles of these conserved tyrosines and other residues in integrin functions during morphogenesis. These approaches provide us with deeper understandings on the mechanisms of disease engaged in cell-matrix interaction such as tumor metastasis and inflammation and advance our knowledge on the biology of cell behavior further.

Research Rationale

In multicellular organisms, some cells migrate or differentiate from the origin to other areas of the body and differentiate into proper tissues and organs. Cell behavior is important in development such as morphogenesis, blood formation, and axonal outgrowth

as well as pathological conditions such as inflammation and tumor metastasis. Studies have shown that migrating and differentiating cells interact with extracellular matrix (ECM) to find their proper paths and to determine their fates. Deregulation of cell-ECM interaction perturbs cell migration and morphogenesis. For example, invasive tumor cells often lack integrin, a family of αβ heterodimeric receptors to ECM. Mutations in integrin molecules also destroy migration of fibroblasts or cause embryonic lethality, suggesting that interaction between cell and ECM plays an essential role in cell migration and that integrin is a key player to make the animal alive for proper development. Analysis of *in vivo* function of mammalian integrin is complicated by the numerous possible α and β heterodimers, and the severe defects of integrin null mice.

The nematode *C. elegans*, possessing only two α integrin subunits (PAT-2 and INA-1) and one β subunit (βPAT-3), provides an excellent model system for genetic and molecular analysis of integrin function in cell behavior. We have shown that integrin controls distal tip cell (DTC) migration and cell contraction (Lee et al, 2001), suggesting that DTC is a useful marker to study cell behaviors. My research interest is to understand key molecular interactions underlying morphogenesis.

Specific Aim: Construction and Phenotypic Analysis of βpat-3 rescued nematodes

In *C. elegans*, the *pat-3* null phenotype is embryonic lethal. Previously we were able to rescue the lethal phenotype with constructs encoding wild type βPAT-3 or βPAT-3 with Y to E cytoplasmic tail mutations (Lee et al., 2001). Several rescued lines have been established and used to investigate the role of the cytoplasmic domain in integrin signaling. Phenotypic analysis of these lines indicates that animals rescued with mutant

alleles of βPAT-3 integrin display defects in cell migration, cytoskeleton, and male tail generation. I performed detailed phenotypic analyses of muscle structure and cell migration as well as fertility of the transgenic animals to fully define the role of the βPAT-3 tail in integrin function.

CHAPTER TWO

Materials and Methods

Animals and Culture Condition

All *C. elegans* strains were cultured on NGM plates seeded with *E. coli* OP50 at room temperature (RT), otherwise specified. Mutant animals were obtained from Caenorhabditis Genetics Center at St. Paul, MN. The table 1 lists *C. elegans* mutant strains and transgenic lines used in this study.

Table 1. List of strains used in this research

Strains	Mutation and gene product	Reference
N2	Wild-type	Brenner (1974)
RW3600 *pat-3(st564)/ qC1dpy-19(e1259) glp-1(q339)*	Pat-3 null, β integrin	Williams and Waterston (1994)
lon-2(e678) him-4(e1267)	Loss of function *him-4*	Vogel and Hedgcock (2001)
unc-54(e190)	Loss of function myosin B	Brenner (1974)
mwEx35[βpat-3(Y792F)]	Transgenic rescue of *pat-3(st564)*	This study
mwEx31[βpat-3(Y804F)]	Transgenic rescue of *pat-3(st564)*	Lee et al. (2001)
mwEx32[βpat-3(YYFF)]	Transgenic rescue of *pat-3(st564)*	Lee et al. (2001)
mwEx30[βpat-3(+)]	Transgenic rescue of *pat-3(st564)*	Lee et al. (2001)
kqEx804[βpat-3(Y804E)]	Transgenic rescue of *pat-3(st564)*	This study
mwEx36[βpat-3(FKVV)]	Transgenic rescue of *pat-3(st564)*	This study

pPAT-3(Y804E) Construct Generation

Previously, pPAT-3(+), pPB12K containing 11799 bp of *pat-3* genomic DNA in pSP73 plasmid (Promega Corp., Madison, WI), was described in Lee et al. (2001). I subcloned PB12K using a unique MscI (9908-9913) restriction site and KpnI at the end of pPB12K into pSP73 between PvuII and KpnI, designated as pPAT-3(BK). Primers were designed to make a mutation (TAC to GAG) from tyrosine to glutamate as the following:

PAT-3 Y2EFW3: 5'- CCA GTA **GAG** GCT GGA AAA GCC -3'

PAT-3 Y2ERV3: 5'- GGC TTT TCC AGC **CTC** TAC TGG -3'

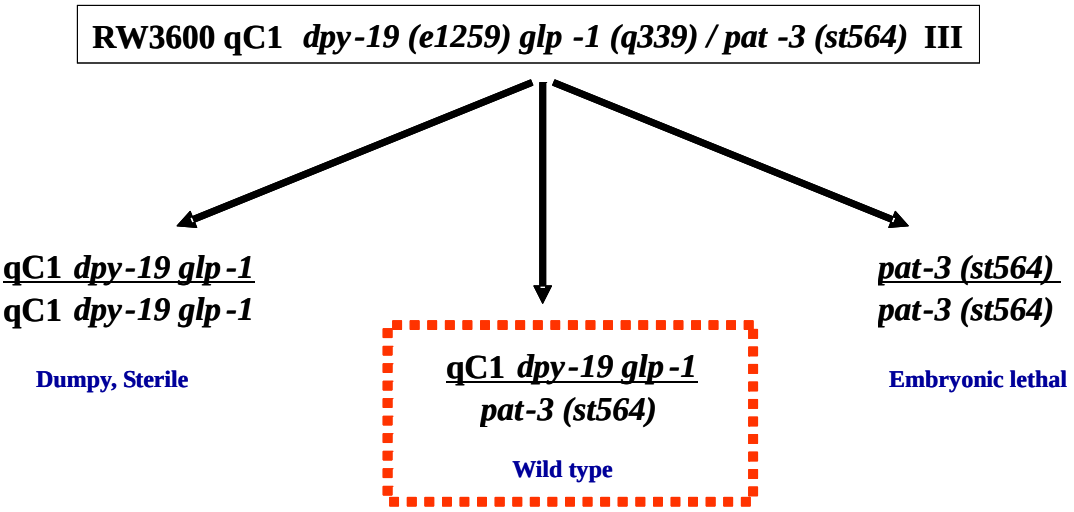
To perform overlap extension, two polymerase chain reactions (PCR), pat3FW3 and Y2ERV3 (pair 1) and Y2EFW3 and pat3RV5 (pair2), were prepared by using pPAT-3(+) as a template. PCR was performed by adding Taq polymerase (New England Biolab, Bedford, MA; 1U per reaction) with 5mM $MgCl_2$ PCR buffer at 52^{o}C annealing temperature (30 cycles). Amplified DNA was precipitated with phenol-chloroform (5:1) solution and alcohol. Two amplified resultant DNA molecules were then mixed into the second round PCR reaction as an overlap template with pat3FW3 and pat3RV5 as primers. The amplified DNA was digested with *EcoRI* and cloned into a pSP73 vector for sequencing to confirm mutation and subcloning. Confirmed DNA was digested with MscI and EcoRI, and the digested fragment was cloned back into pPB12K linearized with MscI and KpnI ligating two DNA fragments, MscI-EcoRI from PCR products and a EcoRI-KpnI fragment of pPAT-3(BK). Completed clones transformed *E. coli* DH5α strain for the large scale isolation of a plamid, pPAT-3(Y804E). Plasmid DNA was isolated by Sigma Miniprep kits (Sigma, St. Louis, MO, Cat# PLN-70). For injections,

50 mg/ml of pPAT-3(Y804E) was mixed with 50mg/ml of TG96 sug-5::GFP DNA in TE buffer (pH=7.5, 50mM Tris-HCl, 1mM EDTA). Other Y to E constructs, pPAT-3(Y792E) and pPAT-3(YYEE), were generated by Dr. Xiaojian Xu as a class project in BIO5V90, Fall 2003.

Microinjection and Rescue of pat-3 null

To rescue the embryonic lethal phenotype of *pat-3(st564)*, I used a balanced strain of *pat-3,* because *pat-3(st564)* can not survive more than embryonic stages. RW3600 *pat-3(st564)* / qC1 *dpy-19(e1259) glp-1(q339)* III is a well-balanced strain that prevents gene conversion by disabling crossover between the two homologous chromosomes (Williams and Waterston, 1994). As depicted in the Figure 3-A, RW3600 segregates homozygous lethal (Pat embryos), wild-type heterozygotes, and qC1 homozygotes (Dpy and Sterile). We used wild-type heterozygote segregates for microinjections; wild-type appearance worms were mounted on a mineral oil (Mallinckrodt Inc., Hazelwood, MO Cat# 6358) covered injection pads that were prepared on a 60x24 mm cover slip with dried 4% agarose (VWR Scientific, Cat# 48393 252). Injection pad was placed onto a Nikon TE-2000U microscope and focused onto distal gonad arms under Nomarski microscopy. Injection needle was prepared by using a microcapillary pipette (Narishige Ltd., Japan, Cat# GD-1) forged with a Narishige pipette puller (model #PC-10). Tapered capillaty pipette was filled with DNA solution using p10 Gilson micropipetter. Loaded injection needle was mounted on a standard needle holder (Narishige, HI-7), which are connected to Nitrogen gas controller.

A. A balanced strain of pat-3 null allele with genetic markers in the balanced chromosome III.



B. Rescue of *pat-3* null

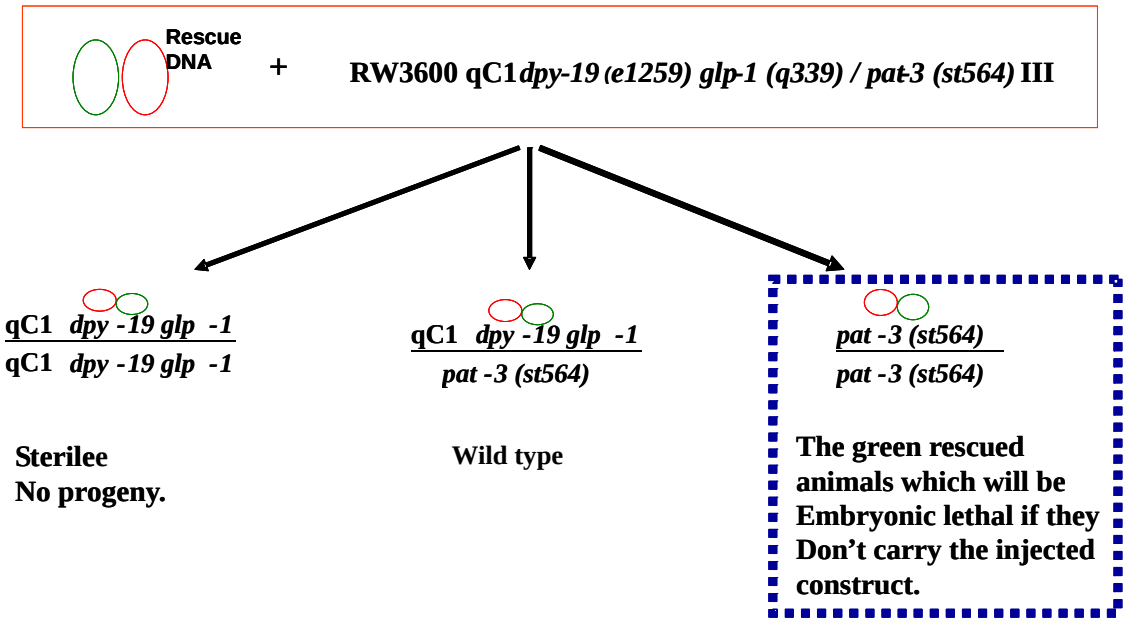


Figure 3. Rescue of pat-3 (st564) null animals with pat-3 rescue constructs. SUR-5::GFP was used as a co-injection marker. A. RW3600 segregates F1s: Dpy–Sterile (25%), wild-type (50%), and embryonic lethal (25%). B. Heterozygote (wild-type) was injected with rescue constructs, transmitted to all segregates. When *st564* homozygote (F1) carries a high enough level of rescue constructs, the homozygotes are no longer embryonic lethal and become viable. F1 heterozygote will carry rescue constructs as a transgene and may rescue *st564* homozygote segregates in the later generations.

Prior to injection, gonad and the tip of the needle were placed on the same focal plane to insure the correct insertion of the needle. Loaded DNA was introduced into *C. elegans* gonad by syringe action of nitrogen gas pressure at 20 psi. Injected worms were recovered with a drop of M9 buffer (pH=7.4, KH_2PO_4, Na_2HPO_4, NaCl, 1 M $MgSO_4$) and transferred to a NGM plate seeded with OP50 for a full recovery. Green transgenic progeny (F1) was separated onto a NGM plate and examined for segregation; if it segregates 100% green progeny with no Dpy/Sterile worms, I defined those as a rescued line (Figure 3).

Nematode Genetics and Phenotype Analysis

Four double mutants, *mwEx35[βpat-3 (Y792F)]; him-4(e1267)*, *mwEx31[βpat-3 (Y804F)]; him-4(e1267)*, and *mwEx32[βpat-3 (YYFF)]; him-4(e1267)*and *mwEx36[βpat-3 (FKVV)]*; *him-4(e1267),* were generated by crossing males from each transgenic lines to *lon-2(e678) him-4(e1267)* hermaphrodites. From the F2 generation, LonHim worms with 100% green and laying dead eggs were isolated for further studies. For DTC defects, worms were anesthetized with 1% NaN_3 solution on 3% agarose pads. Distal arms with abnormal trajectories such as no dorsal turns, looping back, and irregular turns were scored as gonad migration abnormal. For Him phenotypes, worms were self-fertilized and the number of males were scored. Abnormal tail was also scored for protrusions in the posterior end of the body and irregular folding of tail shafts. Male gonads were also scored for irregular turns. Retracted fan, fused rays, and protruded spicules were scored for male tail abnormal. To measure mating efficiency, two or three males and one hermaphrodite were placed on an NGM plate. Hermaphrodites of *unc-54(e190)* or *unc-119(ed3)* were used to promote successful mating. Increased number of

males or production of green eggs in F1 progeny was a criterion for the mating success and was scored as (+). Egg-laying defective (Egl) phenotype was determined by scoring hermaphrodites filled with fertilized eggs in the uterus; Egl animals appeared bloated and lethargic and sometimes displayed slow movement.

Muscle Staining and Fluorescence and DIC microscopy

To examine the muscle cytoskeleton, animals were collected and placed on the poly-L-lysine (Sigma, St. Louis, MO. Cat# P2636) coated slides and fixed with methanol and acetone for 5 min. each at -20^{o}C. Fixed worms were treated with rhodamine-conjugated phalloidin (0.2 U/ml, Sigma Chem. Co. Cat# A1951) for 2 to 3 hours at room temperature. Then samples were washed with M9 buffer for 3 times at 10 min each. Washed samples were mounted on the Nikon TE2000-U epifluorescence microscope. For immunofluorescence staining, worms were decapitated by using 26 gauge needles to extrude guts and gonads and to facilitate penetration of chemicals in the worm body. Dissected worms were fixed with 4% paraformaldehyde and ice-cold 100% methanol. Prepared sample were treated with 10% goat serum for 1 hr at 37^{o}C for blocking. Then worms were treated with MH25 antibody at 1:500 dilution with 1% goat serum for 4 hours at RT. Samples were washed three times with M9 buffer then treated with goat anti-mouse IgG antibody conjugated with TRITC as a 2^{o} antibody. The 2^{o} antibody was diluted in 1% goat serum and samples were incubated for overnight at 4^{o}C. After the 2^{o} antibody incubation, samples were washed three times and mounted on a microscopic slide and covered with Fluoguard (Bio-Rad, Hercules, CA) mounting solutions.

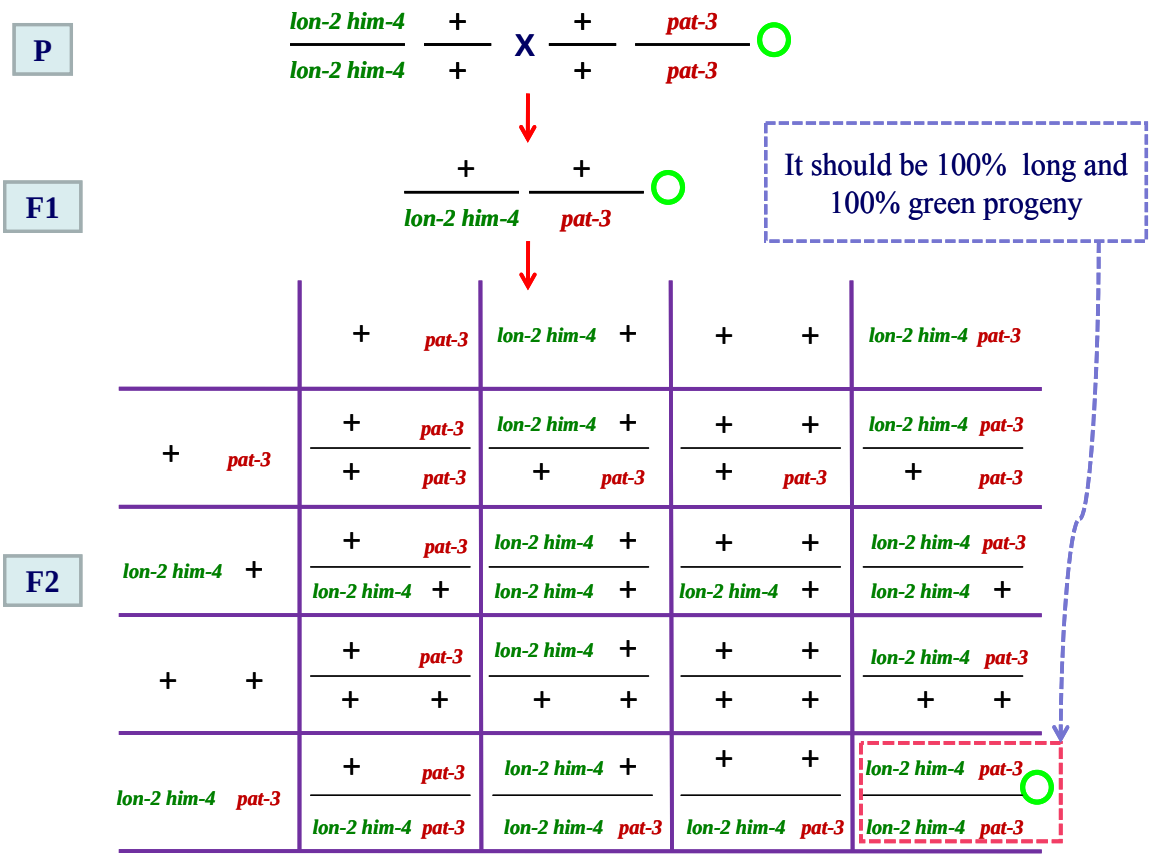


Figure 4. Punnett square scheme of *him-4* and *pat-3* mutant cross. This figure depicts the general scheme of *pat-3* mutant lines crossed with *him-4* mutant based on the Punnett square. 1 out of 16 progenies should be the double homozygotes for both *him-4* and *pat-3* highlighted by pink dotted box. This is the line used for phenotypic analysis.

Prepared sample was placed on a Nikon TE-2000U epifluorescence microscope with fluorescence filters. Images were captured using *CoolSnapcf* monochrome camera (Roper Scientific, Tucson, AZ) and analyzed with *Metavue* software (version 7, Molecular Devices, Downingtown, PA). For DIC microscopy, animals were fed with 2% NaN_3 solution on agarose pads and mounted on Nikon TE2000-U microscope. Samples were examined and analyzed for defects using 40X Plan Fluor objective lens. Images were obtained and analyzed using *CoolSnap* camera and *Metavue* software as well.

CHAPTER THREE

Results

In order to define the role of β integrin cytoplasmic tail in morphogenesis and tissue functions, we created such three mutations mimicking constitutively active NPXY, a conserved tyrosine phosphorylation site, as *βpat-3(Y792E),βpat-3(Y804E),* and*βpat-3(YYEE)*in the β integrin cytoplasmic tail. The Y to E mutagenesis was performed using the wild-type *C. elegans pat-3* genomic DNA (Lee et al., 2001). Three constructs were injected into a null allele, *pat-3(st564)*, to generate viable transgenic rescue lines with mutant phenotypes (Figure 3). Among the three constructs, *βpat-3 (Y804E)* rescued *pat-3(st564)* animals, while *βpat-3 (Y792E)* and *βpat-3 (YYEE)* constructs failed to rescue the *pat-3(st564)* null. Attempts to generate rescued line with *βpat-3 (Y792E)* and *βpat-3 (YYEE)* constructs were made repeatedly through the project. Although some transgenic lines in RW3600 background were generated, I was unable to generate viable rescued lines, non-Dpy100% green worms. The rescued animals showed arrested at larval stages of 18$\pm$3% (n=177) mostly at L2 or L3. The *βpat-3(Y804E)* also showed gonad migration defects. During the gonad morphogenesis, two distal tips cells (DTC) at the polar ends of hermaphroditic gonad direct the formation of the mirror image U-shaped tubular gonad (Montell, 1999). The 48$\pm$4% (n=130) of gonad arms in *βpat-3(Y804E)* displayed abnormal turn and irregular trajectory of distal arms (Figure 5). This transgenic line also showed tail morphology defects in 79$\pm$5% (n=65) hermaphrodites, presumably due to abnormal migration of tail hypodermal cells generating various forms of defective tail morphology (Figure 6H). Adult animals also showed mild dumpy appearance with

sluggish movements, which led us to examine the muscle organization and *βpat-3* localization. Actin cytoskeletons in the body wall muscles appeared abnormal where actin filaments were disorganized and irregularly arranged (Figure 6B). Expression of *βpat-3(Y804E*) also appeared abnormal; monoclonal antibody to *βpat-3*, MH25, follows general dense bodies and M-lines staining patterns, but shows uneven clustering of *βpat-3(Y804E)* (Figure 6D). In addition, we noticed the unusual number of males among the transgenic animals, of 6.7±3% (n=84). Collected males were also tested for mating efficiency; we found no sign of mating from the mated hermaphrodite, among the 30 males tested (Table 2, see Methods section).

Table 2. Comparing phenotypes of transgenic *him-4 (e1267)*, N2, pat-3(+)

Genotype	% Him (n)	Mating (n)	% Egl (n)
lon-2 (e678) him-4 (e1267)	1.9±1.0 (257)	- (21)	56.4±3.0 (257)
mwEx35(Y792F)	0(85)	+ (15)	4.7± 2.0(85)
mwEx31(Y804F)	0.3±0.0 (355)	- (30)	0 (355)
mwEx32(YYFF)	0.5±0.0 (219)	+ (14)	0.5±0.0 (219)
kqEx804(Y804E)	6.7 ±3.0 (84)	- (30)	ND

n = number of animals scored. Each data entry represents the sum of at least 2-3 phenotypic scorings. The standard error of the mean is indicated.

In contrast to the hermaphrodite counterpart, male gonad has an asymmetric arm formed by the asymmetric migration of anterior linker cell while the posterior linker, DTC, becomes static at the early larval stages (Sulston and Horvitz, 1977). Migration of *kqEx804[βpat-3(Y804E)]* male gonad was examined under DIC microscopy.

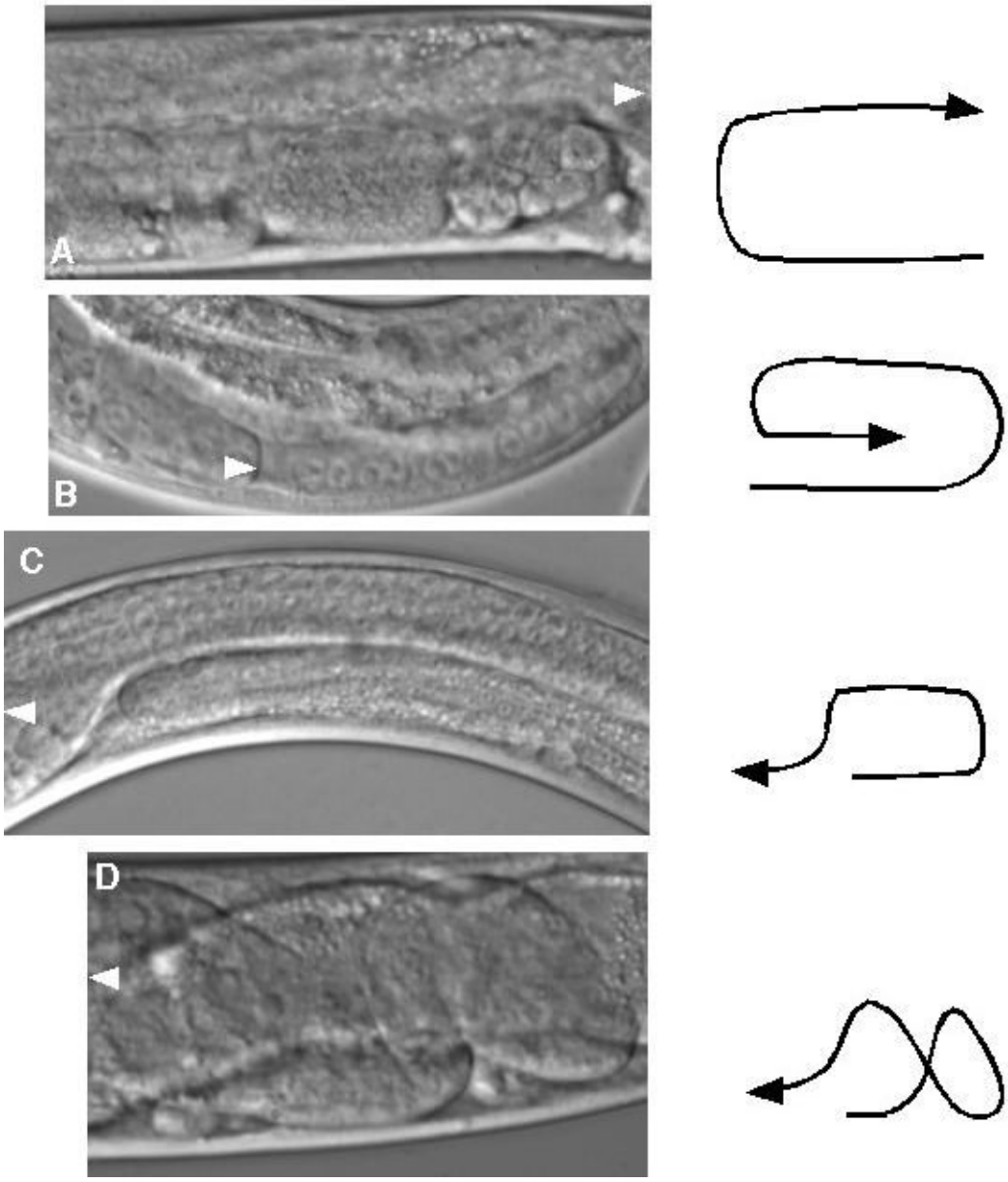


Figure 5. Gonad morphology of male and hermaphrodite transgenic animals
Gonad structures of control and *βpat-3(Y804E)* animals were examined under DIC microscopy. Panel A, control *βpat-3(+)* hermaphrodite; panel B, *βpat-3(Y804E)* hermaphrodite; panel C, *βpat-3(+)* male; panel D, *βpat-3(Y804E)* male. Arrowhead indicates the location of DTC.

Anterior linker cell migration appeared abnormal (Figure 5D), and gonads were displaced where the posterior tip was often anchored in various positions (data not shown). In addition, *βpat-3(Y804E)* transgenic male tails showed abnormal formation of rays and spicules, which further confirmed their mating inefficiency (Figure 6F).

The high incidence in male (Him) phenotype of *βpat-3(Y804E)* transgenic suggested that *βpat-3(Y804E)* parallels the phenotype of *him-4*/hemicentin alleles. *him-4* encodes hemicentin, an ECM protein similar to mammalian fibulin-5 (Dong et al., 2006); its alleles share many phenotypic similarities with *βpat-3(Y804E)* lines (Table 2). We examined the *him-4(e1267)* and found that Egl (56.4$\pm$3.0%, n=257) and Him (1.9$\pm$0.13%, n=257) were similar to that of *βpat-3(Y804E)*. The similarity of *him-4* and *βpat-3(Y804E)* implied that the absence of *him-4* may induce phosphorylation of integrin NPXY motifs to modulate normal integrin function. In order to investigate this possibility further, we created double mutants to assess the genetic interaction between *him-4* and *pat-3*. In previous studies, we have generated *βpat-3* transgenic rescue lines with Y to phenylalanine (F) mutations, *βpat-3(Y792F), βpat-3(Y804F),* and *βpat-3(YYFF)* (Lee et al, 2001). These mutants displayed mild gonad migration defects but no other phenotypes such as Him, abnormal tail, or muscle defects were observed. Therefore, we reasoned that Y to F mutations may fully or partially suppress the phenotypes of *him-4* by disabling potential phosphorylation of *βpat-3* NPXY in the *him-4* background. Thus, the double mutants, *mwEx35[βpat-3(Y792F)]; him-4(e1267)*, *mwEx31[βpat-3(Y804F)]; him-4(e1267)*, and *mwEx32[βpat-3(YYFF)]; him-4(e1267)* were generated.

Phenotypic analysis of the double mutants revealed that only *βpat-3(YYFF); him-4(e1267)* was able to suppress *him-4* phenotypes such as Egl, Him, and mating inefficiency (Table 3). Males were collected and tested for mating ability.

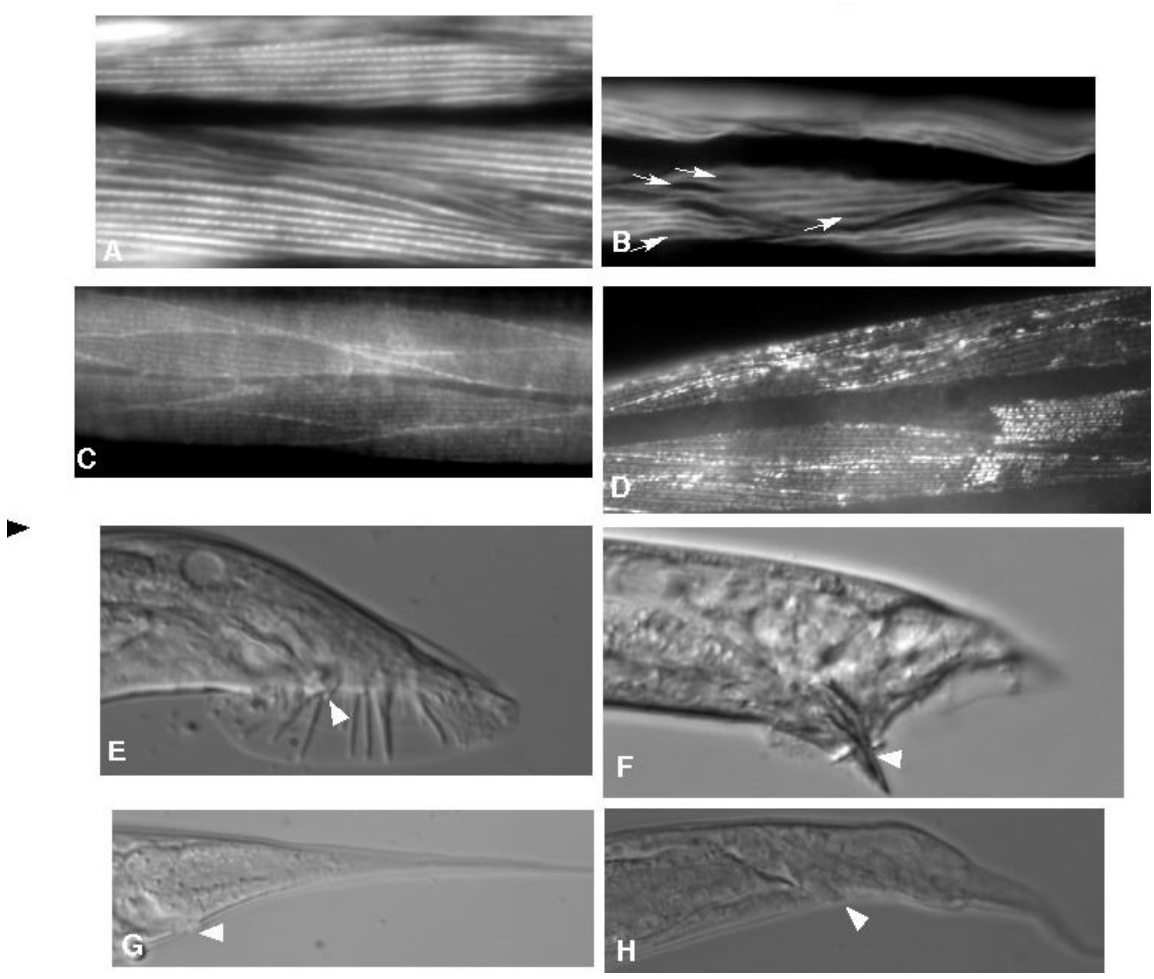


Figure 6. Phenotypes of *bpat-3(Y804E)* transgenic animal
Panel A, *βpat-3(+)* body wall muscle stained with rhodamine-phalloidin shows normal muscle filaments. Panel B, *βpat-3(Y804E)* transgenic animal has disorganized muscle filament patterns; each filament failed to position in straight lines and ectopic spaces (arrows) appeared between the filaments. Muscle cell shape was also deformed showing irregular wavy patterns. PAT-3 protein was not properly localized in the muscles; AlthoughMH25 stained *βpat-3(+)* and *βpat-3(Y804E)* muscles (panel C and D, respectively) showed regular dense bodies and M-lines patterns, the clustering of *βpat-3(Y804E*) integrin seems uneven. Panel E, *βpat-3(+)* male tail with normal spicule indicated (arrowhead). Panel F, *βpat-3(Y804E)* male tail appears abnormal with fused rays and protruding spicule (arrowhead). Panel G, *βpat-3(+)* hermaphrodite tail. Panel H, hermaphrodite tail has abnormal shape and a protrusion in the base of the tail posterior to the anus (arrowhead).

Only *βpat-3(YYFF); him-4(e1267)* males was able to mate with hermaphrodites and to produce progeny from mating (Table 3), revealing that *βpat-3(YYFF)* had fully suppressed *him-4* phenotypes. To examine the specificity of Y to F mutations for *him-4* suppression, a double mutant, *mwEx36[βpat-3(FKVV)]*; *him-4(e1267)* was also generated. The homologous *βpat-3* sequence (^{799}FKNPXY^{804}) was found in the integrin β1D, a muscle specific isoform, whereas the sequence was VVNPXY in β1A, a classical form of β1 integrin. A FK to VV mutation was introduced, and *βpat-3(FKVV)* rescued line was also generated. *βpat-3(FKVV); him-4(e1267)* double mutant showed identical phenotypes as *him-4(e1267)* (Table 3), demonstrating that the suppression of *him-4* by *βpat-3(YYFF)* was the Y specific event.

Table 3. Genetic interaction of βpat-3 transgenic and *him-4 (e1267)*

Genotype	% Him (n)	Mating (n)	% Egl (n)
mwEx35(Y792F); lon-2 (e678) him-4 (e1267)	1.6±1.0 (315)	- (28)	58.4±3.0 (315)
mwEx31(Y804F); lon-2 (e678) him-4 (e1267)	3.9±2.0 (154)	- (35)	61.6±4.0 (154)
mwEx32(YYFF); lon-2 (e678)him-4 (e1267)	0.2±0.0 (1728)	+ (4)	0.5±0.0 (1728)
mwEx33(FKVV); lon-2 (e678) him-4 (e1267)	1.4±1.0 (74)	- (27)	83.8±5.0 (74)
N2; lon-2 (e678) him-4 (e1267)	1.9±1.0 (257)	- (15)	56.4±3.0 (257)

n = number of animals scored. Each data entry represents the sum of at least 2-3 phenotypic scorings. The standard error of the mean is indicated.

Our studies have shown that only *βpat-3(Y804E)* construct was able to rescue *pat-3* null allele. Failure to rescue *pat-3(st564)* by the two constructs, *βpat-3(Y792E)* and *βpat-3(YYEE)*, prompted me to look at the dominant defects caused by the constructs. Three constructs were injected into N2 background to generate transgenic lines with dominant phenotypes (Table 4).

Table 4. Comparison of transgenic mutants in wildtype background

Transgenic lines	% DTC Mig (n)	% Emo (n)	% Pvl (n)	% Tail Morph. (n)
mwEx792(Y792E;N2)	79.9±2 (289)	52.1±3 (284)	80.2±3 (131)	20.4±3 (142)
mwEx51(Y804E;N2)	49.1±4 (161)	1.3±1 (152)	9.2±3 (87)	30.1±5 (83)
mwEx52(Y792/804E;N2)	55.6±3 (205)	11.0±2 (191)	54.5±5 (99)	33.3±5 (105)
mwEx804(Y804E)	54.8±4 (188)	13.2±2 (189)	71.1±5 (97)	81.4±4 (97)

n = number of animals scored. Each data entry represents the sum of at least 2-3 phenotypic scorings. The standard error of the mean is indicated.

Transgenic animals displayed several developmental defects. All three constructs caused significantly high percentage of cell migration and morphogenesis defects. In *C. elegans*, U-shaped gonad arm is formed by the proper migration of DTC along the ECM path over the bodywall. Typical defects found in three transgenics were irregular turns of dorsal arms or no dorsal turns.

Three constructs also showed tail abnormal phenotypes; all three transgenic lines showed protrusions in the ventral or dorsal side of tail bases. There were differences in the degree of severity of phenotypes but most of them were not significantly different from others. However, *βpat-3(Y804E)* construct showed less severe Emo and Pvl defects than other transgenes. In summary, *βpat-3(Y972E)*; N2 lines showed severer than others, suggesting that this constructs are more toxic than others and that the reason why this construct failed to rescue *pat-3(st564)* null allele.

CHAPTER FOUR

Discussion

In this study, the function of NPXY motif in β integrin cytoplasmic tail was analyzed. Y to E mutations mimicking constitutive NPXY phosphorylation were introduced to the first and/or second NPXY motif and caused defects in gonad, vulva and tail morphology in N2 background, demonstrating that they caused dominant phenotypes (Table 4). Among the three constructs, only *βpat-3(Y804E)* rescues the *pat-3(st564)* null allele. This rescued mutant *βpat-3*integrin also showed Him phenotypes uncharacterized in the previous studies (Table 2). *βpat-3(Y804E)* males had defective gonad migration, tails, and mating ability that resembled *him-4* alleles (Figures 5 and 6). Double mutant analysis revealed that the lack of phosphorylation in both NPXY, *βpat-3(YYFF)*, suppressed *him-4* phenotypes, whereas *βpat-3(Y792F)* or *βpat-3(Y804F)* was not able to suppress *him-4*, signifying that the absence of an ECM molecule such as *him-4* triggered the phosphorylation of NPXY in *βpat-3* (Table 3).

The role of *him-4* in integrin signaling still remains unexplained, although there are several reports on the interaction of integrins to fibulins in cell culture. Nevertheless, we would like to suggest that the role of *him-4* is to inhibit the phosphorylation of *βpat-3* integrin in particular tissues. Mutations in *him-4* may create an ECM environment that signals cells to modulate cell behavior possibly through integrin NPXY phosphorylation.

The plausible mechanism has been proposed in Figure 7. The loss of *him-4* may create a defective ECM environment, which triggers the phosphrylation of NPXY motifs of β integrin. Only *βpat-3(YYFF)* was able to suppress *him-4* Egl, Him, and mating

defects, while the single NPXY mutation, *βpat-3(Y792F)* or *βpat-3(Y804F)*, failed to complement *him-4* defects, suggesting that the defective ECM caused the phosphorylation on both NPXY. In addition, we also showed that *βpat-3(Y804E)* was able to rescue a *pat-3* null and that the transgenic animals have defective phenotypes, while *βpat-3(Y792E)* failed to rescue (data not shown).

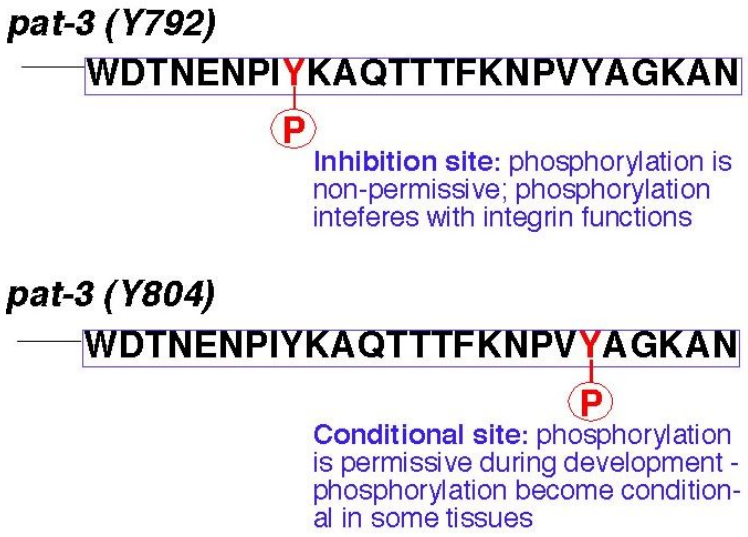


Figure 7. Putative model for Phosphorylation of β integrin NPXY motif

Based on our findings, we propose that Y804 might be permissive for phosphorylation; tissues defective in *βpat-3(Y804E)* such as tails, DTC, and muscles were subject to regulation through NPXY phosphorylation for their functional needs.

In contrast, we suggest that the phosphorylation in Y792 might be non-permissive or normally interfere with regular integrin functions; the phosphomimetic mutation on this Y failed to rescue *pat-3* null.

Our new findings provide valuable information on the function of NPXY motifs in β integrin regulation. This information will help us to interpret integrin signaling in other species.

REFERENCES

Ackley, B. D., Jennifer R. Crew, Harri Elamaa, Tania Pihlajaniemi, Calvin J. Kuo, and James M. Kramer. 2001. The NC1/Endostatin Domain of *Caenorhabditis elegans* Type XVIII Collagen Affects Cell Migration and Axon Guidance. *J. Cell Biol.*152(6): 1219-1232.

Ackley, B. D., Seong Hoon Kang, Jennifer R. Crew, Chris Suh, Yishi Jin, and James M. Kramer. 2003. The Basement Membrane Components Nidogen and Type XVIII Collagen Regulate Organization of Neuromuscular Junctions in *Caenorhabditis elegans. J. Neurosci.* 23(9):3577-3587.

Adachi, E., I. Hopkinson, and T. Hayashi. 1997. Basement-membrane stromal relationships: interactions between collagen fibrils and the lamina densa. *Int'l. Rev. Cytol.* 173**:** 73-225.

Arcangelis, A. D., and Elisabeth Georges-Labouesse. 2000. Integrin and ECM functions roles in vertebrate development. *TIG*. 16(9): 389-395.

Arias-Salgado, Elena G., Sergio Lizano, Sugata Sarkar, Joan S. Brugge, Mark H. Ginsberg, and Sanford J. Shattil. 2003. Src kinase activation by direct interaction with the integrin β cytoplasmic domain. *Proc Natl Acad Sci U S A*. 100(23): 13298-13302.

Baneyx, Gretchen, Loren Baugh, and Viola Vogel. 2002. Fibronectin extension and unfolding within cell matrix fibrils controlled by cytoskeletal tension. *Proc Natl Acad Sci U S A*. 99(8): 5139-5143.

Baum, P. D., and Gian Garriga. 1997. Neuronal Migrations and Axon Fasciculation Are Disrupted in *ina-1* Integrin Mutants. *Neuron*. 19: 51-62.

Bessea, Sylvie, Vale´rie Allamanda, Jean-Thomas Vilquina, Zhenlin Lib, Christophe Poirierc, Nicolas Vigniera, Hisae Horid, Jean-Louis Gue´netb, Pascale Guicheney. 2003. Spontaneous muscular dystrophy caused by a retrotransposal insertion in the mouse laminin a2 chain gene. *Neuromuscular Disorders*. 13: 216-222.

Boudreau, Nancy, and Mina J. Bissell. 1998. Extracellular matrix signaling: integration of form and function in normal and malignant cells. *Curr. Opin. Cell Biol.* 10: 640–646.

Brenner, Sydney. 1974. The genetics of *Caenorhabditis elegans*. Genetics. 77: 71-94

Brown, Michael C., Leslie A. Cary, Jennifer S. Jamieson, Jonathan A. Cooper, and Christopher E. Turner. 2005. Src and FAK Kinases Cooperate to Phosphorylate Paxillin Kinase Linker, Stimulate Its Focal Adhesion Localization, and Regulate Cell Spreading and Protrusiveness. *Mol. Biol. Cell*. 16(9): 4316-4328.

Burridge, Keith, and Magdalena Chrzanowska-Wodnicka. 1996. Focal adhesions, contractility, and signaling. *Annu. Rev. Cell Dev. Biol.* 12:463–519.

Calderwood, David A., Yosuke Fujioka, Jose M. de Pereda, Begon˜ a Garcı´a-Alvarez, Tetsuya Nakamoto, Ben Margolis, C. Jane McGlade, Robert C. Liddington, and Mark H. Ginsberg. 2003. Integrin β cytoplasmic domain interactions with phosphotyrosine-binding domains: A structural prototype for diversity in integrin signaling. *Proc Natl Acad Sci U S A*. 100(5): 2272-2277.

Calderwood, David A. Integrin activation. 2004. *J. Cell Sci.* 117:657−666.

Campbell, Iain D., Mark H. Ginsberg. 2004. The talin-tail interaction places integrin activation on FERM ground. *Trends Biochem. Sci.* 29(8): 429-435.

Czuchra, Aleksandra, Hannelore Meyer, Kyle R. Legate, Cord Brakebusch, and Reinhard Fassler. 2006. Genetic analysis of β1 integrin "activation motifs" in mice. *J. Cell. Biol.* 174(6): 889–899.

Dallas, Sarah L., Qian Chen, and Pitchumani Sivakumar. 2006. Dynamics of Assembly and Reorganization of Extracellular Matrix Proteins. *Curr. Top. Dev. Biol.* 75: 1-24.

Debelle, L. and A. M. Tamburro. 1999. Elastin: molecular description and function. *Int. J. Biochem. Cell Biol.* 31: 261-72

Dong, Chun, Joaquin M. Muriel, Sarah Ramirez, Harald Hutter, Edward M. Hedgecock, Leonid Breydo, Ilia V. Baskakov, and Bruce E. Vogel. 2006. Hemicentin Assembly in the Extracellular Matrix Is Mediated by Distinct Structural Modules. *J. Biol. Chem*. 281(33): 23606-23610.

Eyre, David R. 1984. Cross-linking in collagen and elastin. *Ann. Rev. Biochem.* 53: 717-48.

Fitzgerald, M. C. and Jean E. Schwarzbauer. 1998. Importance of the basement membrane protein SPARC for viability and fertility in *Caenorhabditis elegans. Curr. Biol*. 8(23): 1285-1288.

Francis, R., and R. H. Waterston. 1991. Muscle cell attachment in *Caenorhabditis elegans*. *J. Cell. Biol.* 114: 465–479.

Geiger, B., A. Bershadsky, R. Pankov, and KM. Yamada. 2001. Transmembrane crosstalk between the extracellular matrix and the cytoskeleton. *Nat. Rev. Mol. Cell Biol.* 2: 793-805.

Gettner, Sonya N., Cynthia Kenyon, and Louis E Reichardt. 1995. Characterization of βpat-3 Heterodimers, a Family of Essential Integrin Receptors in *C. elegans*. *J. Cell Biol.* 129 (4): 1127-1141.

Ginsberg, Mark H., Anthony Partridge, and Sanford J Shattil. 2005. Integrin regulation. *Curr Opin Cell Biol.* 17: 509-516.

Gorski, Jeff P., Bjorn R. Olsen. 1998. Mutations in extracellular matrix molecules. *Curr Opin Cell Biol.* 10: 586-593.

Graham, P. L., Jeffrey J. Johnson, Shaoru Wang, Marion H. Sibley, Malini C. Gupta, and James M. Kramer Characterization of *a*1(IV) Collagen Mutations in *Caenorhabditis elegans* and the Effects of *a*1 and *a*2(IV) Mutations on Type IV Collagen Distribution. *J. Cell Biol.*137(5): 1171–1183

Gupta, M. C., Patricia L. Graham, and James M. Kramer. 1997. Characterization of *a*1(IV) Collagen Mutations in *Caenorhabditis elegans* and the Effects of *a*1 and *a*2(IV) Mutations on Type IV Collagen Distribution. *J. Cell Biol.*137(5): 1185-1196.

Hesselson, Daniel, and Judith Kimble. 2006. Growth control by EGF repeats of the *C. elegans* Fibulin-1C isoform. *J. Cell. Biol.* 175(2): 217-223.

Hocking, Denise C., and Cecilia H. Chang. 2003. Fibronectin matrix polymerization regulates small airway epithelial cell migration. *Am J Physiol Lung Cell Mol Physiol.* 285: L169–L179.

Howe, Alan, Andrew E. Aplin, Suresh K. Alahari, and R. L. Juliano. 1998. Integrin signaling and cell growth control. *Curr. Opin. Cell Biol.* 10(2): 220-231.

Hresko, M. C., B.D. Williams, and R.H. Waterston. 1994. Assembly of body wall muscle and muscle cell attachment structures in *Caenorhabditis elegans*. *J. Cell. Biol.* 124: 491-506.

Huang, Sui, and Donald E. Ingber. 1999. The structural and mechanical complexity of cell-growth control. *Nat. Cell Biol.* 1: E131–E138.

Huang,C. C., David H. Hall, Edward M. Hedgecock, Gautam Kao, Vassiliki Karantza, Bruce E. Vogel, Harald Hutter, Andrew D. Chisholm, Peter D. Yurchenco, and William G. Wadsworth. 2003. Laminin a subunits and their role in *C. elegans development*. *Development* 130: 3343-3358.

Hynes, Richard O. 2002. Integrins: Bidirectional, Allosteric Signaling Machines. *Cell.* 110: 673-687.

Iozzo, Renato V. 1998. Matrix proteoglycans: from molecular design to cellular Function. *Annu. Rev. Biochem.* 67: 609-652.

Isnenghi, E., K. Cassada, C. Smith, K. Denitch, A. Radnia, and G. von Ehrenstein. 1983. Maternal effects and temperature-sensitive period of mutations affecting embryogenesis in *Caenorhabditis elegans. Dev. Biol.* 98:465–480.

Johnson, RP, and S. W. Craig. 1995. F-actin binding site masked by the intramolecular association of vinculin head and tail domains. *Nature.* 373:261–64.

Kang, S.H., and James M. Kramer. 2000. Nidogen is nonessential and not required for normal type IV collagen localization in *Caenorhabditis elegans*. *Mol. Biol. Cell* 11: 3911–3923.

Kao, Gautam, Cheng-chen Huang, Edward M. Hedgecock, David H. Hall, William G. Wadsworth. 2006. The role of the laminin β subunit in laminin heterotrimer assembly and basement membrane function and development in *C. elegans*. *Dev. Biol.* 290: 211 – 219.

Kim, S., and William G. Wadsworth. 2000. Positioning of longitudinal nerves in *elegans* by nidogen. *Science* 288: 150–154.

Law, D.A., et al. 1999. Integrin cytoplasmic tyrosine motif is required for outside-in αIIbβ3 signalling and platelet function. *Nature*. 401: 808-811.

Lebleu, Valerie S., Brian MacDonald, and Raghu Kalluri. 2007. Structure and Function of Basement Membrane. *Exp Biol Med.* 232:1121−1129.

Lee, Myeongwoo, Erin J. Cram, Bing Shen, and Jean E. Schwarzbauer. 2001. Roles for βpat-3 Integrins in Development and Function of *Caenorhabditis elegans* Muscles and Gonads. *J. Biol. Chem.* 276(39): 36404-36410.

Meighan, Christopher M., and Jean E. Schwarzbauer. 2007. Control of C. elegans hermaphrodite gonad size and shape by vab-3/Pax6-mediated regulation of integrin receptors. *Genes & Dev.* 21: 1615-1620.

Montell, D.J. 1999. The genetics of cell migration in Drosophila melanogaster and Caenorhabditis elegans development. *Development*. 126(14): 3035-3046.

Mullen, G. P., Teresa M. Rogalski, Jason A. Bush, Poupak Rahmani Gorji, and Donald G. Moerman. 1998. Complex Patterns of Alternative Splicing Mediate the Spatial and Temporal Distribution of Perlecan/UNC-52 in *Caenorhabditis elegans*. *Mol. Biol. Cell.*10: 3205–3221.

Muriel, J. M., Chun Dong, Harald Hutter, and Bruce E. Vogel. 2005. Fibulin-1C and Fibulin-1D splice variants have distinct functions and assemble in a hemicentin-dependent manner. *Development*. 132: 4223-4234.

Ono, Kanako, Robinson Yu, and Shoichiro Ono. 2007. Structural Components of the Nonstriated Contractile Apparatuses in the *Caenorhabditis elegans* Gonadal Myoepithelial Sheath and Their Essential Roles for Ovulation. *Dev. Dyn.* 236:1093−1105.

Oxley, Camilla L., Nicholas J. Anthis, Edward D. Lowe, Loannis Vakonakis, Iain D. Campbell, and L. Wegener. An Integrin Phosphorylation Switch. *J. Biol. Chem* 283(9): 5420-5426.

Phillips, David R., K. S. Srinivasa Prasad, Jeanne Manganello, Ming Bao, and Lisa Nannizzi-Alaimo, 2001. Integrin tyrosine phosphorylation in platelet signaling. *Curr Opin Cell Biol.* 13(5): 546-554.

Qin, Jun, Olga Vinogradova, and Edward F. Plow. 2004. Integrin Bidirectional Signaling: A Molecular View. *PLoS Biol.* 2(6): 726-729.

Reszka, A.A., Y. Hayashi, and A.F. Horwitz. 1992. Identification of amino acid sequences in the integrin β1 cytoplasmic domain implicated in cytoskeletal association. *J. Cell Biol*. 117: 1321-1330.

Rogalski, T. M., E. J. Gilchrist, G. P. Mullen, and D. G. Moerman. 1995. Mutations in the *unc-52* gene responsible for body wall muscle defects in adult *Caenorhabditis elegans* are located in alternatively spliced exons. *Genetics* 139: 159-169.

Rogalski, T. M., G. P. Mullen, J. A. Bush, E. J. Gilchrist and D. G. Moerman. 2001. UNC-52/perlecan isoform diversity and function in *Caenorhabditis elegans*. *Biochem. Soc. Tran.* 29: 171-176.

Sakai, T., et al. 1998. Modulation of β1A integrin functions by tyrosine residues in the β1 cytoplasmic domain. *J. Cell Biol.* 141: 527-538.

Schwarzbauer, Jean E., and Jan L. Sechler. 1999. Fibronectin fibrillogenesis: a paradigm for extracellular matrix assembly. *Curr Opin Cell Biol.* 11(5): 622-627.

Stroeken, P.J., et al. 2000. Cytoplasmic domain mutants of β1 integrin, expressing β1-knockout lymphoma cells, have distinct effects on adhesion, invasion, and metastasis. *Oncogene.* 19: 1232-1238.

Sulston, J.E., and H. R. Horvitz. 1977. Post-embryonic cell lineages of the nematode, *Caenorhabditis elegans*. *Dev. Biol*. 56: 110–156.

Timpl, R. 1989. Structure and activity of basement membrane proteins. *Eur. J. Biochem.* 180:487–502.

Tucker, Morgan, and Min Han. 2008. Muscle cell migrations of C. elegans are mediated by the α-integrin INA-1, Eph receptor VAB-1, and a novel peptidase homologue MNP-1. *Dev. Biol*. 318: 215−223.

Turner, C. E., J. R. Jr Glenney, K. Burridge. 1990. Paxillin: a new vinculin-binding protein present in focal adhesions. *J. Cell Biol.* 111:1059–68.

Vignoud, Lucile, Corinne Albiges-Rizo, Philippe Frachet, and Marc R. Block. 1997. NPXY motifs control the recruitment of the α5β1 integrin in focal adhesions independently of the association of talin with the β1 chain. *J. Cell Sci.* 110: 1421-1430.

Vogel, B. E., and Edward M. Hedgecock. 2001. Hemicentin, a conserved extracellular member of the immunoglobulin superfamily, organizes epithelial and other cell attachments into oriented lineshaped junctions. *Development* 128: 883-894.

Vu, Thiennu H., and Zena Werb. 2000. Matrix metalloproteinases: effectors of development and normal physiology. *Genes & Dev.* 14(17): 2123-2133.

Williams, B. D., and R. H. Waterston. 1994. Genes critical for muscle development and function in *Caenorhabditis elegans* identified through lethal mutations. *J. Cell. Biol.* 124: 475-490.

Zhang, Zhi-Gang, Ingo Bothe, Frank Hirche, Manon Zweers, Donald Gullberg, Gabriele Pfitzer, Thomas Krieg, Beate Eckes, and Monique Aumailley, 2006. Interactions of primary fibroblasts and keratinocytes with extracellular matrix proteins: contribution of α2β1 integrin. *J. Cell. Sci.* 119: 1886-1895.