ABSTRACT

The Integrin Interacts with *cki-1/p27^{kip1}*, a Tumor Suppressor Gene, of the Nematode *Caenorhabditis elegans*

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Mentor: Myeongwoo Lee. Ph.D.

Integrin is a heterodimeric cell surface receptor for extracellular matrix (ECM) and plays essential roles in regulating cell behaviors such as cell migration, adhesion, growth and death. The cell-ECM interaction is particularly important for progression and arrest of cell cycle. However, the mechanism by which the ECM influences the cell cycle *in vivo* is poorly understood. To study the role of integrin in cell cycle, we generated a *pat-3, Caenorhabditis elegans* β integrin, transgenic mutant. The *pat-3 (sp)* carrying a defective splicing of intron 7 displayed growth defective phenotypes such as cold-sensitive larval arrest, shorter body length, reduced lifespan, and increased sterility. To study the role of integrin in cell growth, we assessed for the interaction of integrin signaling to CKI-1 that is a *C. elegans* homologue of the cyclin dependent kinase inhibitor p27^{kip1}, a tumor suppressor. In wild type pat-3 (+), CKI-1::GFP is localized to nucleoli in hypodermis; whereas, CKI-1::GFP appeares clumped and scattered in the nucleoplasm of *pat-3 (sp).* In addition, the level of CKI-1::GFP protein was found elevated in *pat-3 (sp).* In an RNAi screen, we found that integrin signaling and SCF E3

ubiquitin ligase genes appeared to involve in the localization and expression of CKI-1. This result suggests that integrin signaling and SCF E3 ligase work together to regulate the cellular distribution of CKI-1. We continued to study the localization of CKI-1 and investigate suppressors of *pat-3 (sp)* defects. RNAi screen revealed that genes involved in ribosome biogenesis, unfolded protein response, chromatin modification, rRNA processing, and ubiquitin-mediated protein degradation are required for the up regulation of CKI-1::GFP, suggesting that integrin signaling links to the function of many genes acting in nucleus, nucleolus, and protein degradation.

My study demonstrated that integrin plays robust roles in regulating the proper localization and level of CKI-1/p27kip1 in concert with integrin signaling, protein degradation, and many nucleus expressed genes including *dnc-1, hda-1, mig-32,* and *lpd-7.* This provides insight into potential molecular mechanism of the mammalian β1 integrin splice variants, β1B and β1C, in regulating cell behavior.

The Integrin Interacts with *cki-1/p27^{kip1}*, a Tumor Suppressor Gene, of the Nematode *Caenorhabditis elegans*

by

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CHAPTER ONE

Introduction

Specialized molecules on the surface of the cell serve to receive and transmit information to the cell interior. Integrins are a family of heterodimeric receptors consist of α and β subunits that bind to ECM ligands, cell-surface ligands and soluble ligands. Each subunit includes large extracellular domain, which contributes to the ligand-binding site, transmembrane, and a short cytoplasmic domain (tail) with its distinct roles. Upon the ligand binding, integrins transmit signals into the cell interior; they can also receive intracellular signals that regulate their ligand-binding affinity of extracellular domain, also known as inside-outside signaling (Hynes, 1987). Integrins also physically link ECM to the inside of the cell (cytoskeleton) and enable bidirectional signaling between interior and exterior of the cell through plasma membrane to regulate cell behavior such as adhesion, migration, differentiation, and growth (Hynes, 1992).

History of Integrin Study

The discovery of integrin is emanated from fibronectin study in late 1970 (Lahav et al., 1982). Around that time, fibronectin emerged as the major extracellular protein participating in the formation of focal adhesion and leaded to the studies about connection between cell and ECM molecule involved in cell behavior such as adhesion, and migration (Gardner and Hynes, 1985). Additional evidences had shown that there must exist transmembrane protein to physically link between extracellular matrix proteins and the intracellular cytoskeleton. Several candidate molecules were considered, based on

a variety of experimental approaches including monoclonal antibodies to isolate the binding complex. CAST, a monoclonal antibody which interferes with adhesion of myoblasts to matrix-coated surfaces, was used to precipitate a complex of several proteins from which the sequences were identified (Tamkun et al., 1986). Identification revealed a transmembrane protein with novel structural features, which was named 'integrin' from the integral membrane protein complex linking the extracellular matrix to the cytoskeleton (Hynes, 1987). The Arg-Gly-Asp (RGD) sequence is a known integrin-binding motif, originally identified in fibronectin as a cell attachment site. RGD is recognized by a number of integrin receptors, other matrix proteins, and ion channels (Ruoslahti, 1987). When integrin was identified with antibodies to integrin α subunits and Arg-Gly-Asp (RGD) peptide, several proteins were also co-precipitated and it became clear that the functional receptors are heterodimer complex composed of α and β subunit.

Integrin Subunits

Integrin is a heterodimeric transmembrane protein that is totally distinct with no detectable homology between the two subunits and restricted to metazoan. In vertebrates 18 α subunits and 8 β subunits in human have been identified to constitute 24 different integrins in various combination (Clark, 1995).

Giancotti et al. have showed that β subunit serves as a binding site for signaling and cytoskeletons, whereas the α subunit has regulatory roles (Giancotti and Ruoslahti, 1999). The extracellular domains of α subunits form elongated stalks and a globular

ligand-binding head region that consists of seven repeats of 30-40 amino acids that fold into a β propeller structure [Figure 1.1]. I-domain refers to an insertion within the β propeller containing a 'metal ion-dependent adhesion site' which directly participates in ligand binding as cation-dependent manner and founded in about half of the integrin molecules (Giancotti and Ruoslahti, 1999). All integrin β subunits contain an 'I-like domain' in head region, which has been suggested to help binding to α subunit.

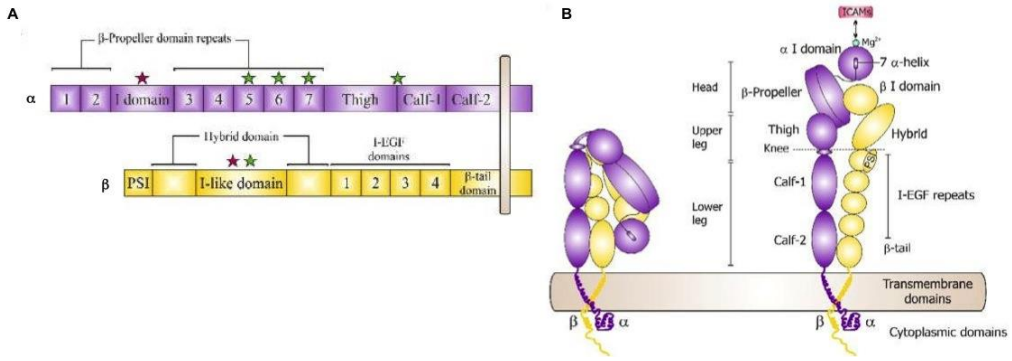

Figure 1.1. Schematic structure of integrin subunits (A) The α and β subunits have distinctive structures in their head region (B) in αβ dimeri (Barczyk et al., 2010).

Integrin cytoplasmic tails are relatively short, less than 75 amino acids [Figure 1.1, right]. β cytoplasmic tails have a homology between the subunits, whereas, α subunit tails are highly divergent except for a conserved motif (GFFKR) for association with the β tail. The NPxY motif in β tail can potentially bind to a PTB-domain of a variety of cytoskeletal or signaling proteins and its tyrosine phosphorylation is essential for the regulation of integrin interactions (O'Toole et al., 1995). Recruited several adaptor proteins which bind actin filaments mediates the cytoplasmic tails integrin-cytoskeleton connection.

The mammalian α subunits are classified into four groups based on I (insert or interaction) domain, also called the A domains, which are inserted into the N-terminus. However, I-domain has not been found in α subunits in invertebrate, suggesting that I-domain is highly conserved only in the vertebrate lineage. Nine α subunits contain an I-domain, which is highly homologous with I-domains of β1 subunit found in collagen receptors (α1, α2, α10 and α11) or, on leukocytes, with β1 and β7 subunit (αD, αE, αL, αM, and αX) [Figure 1.2A].

In mammals, α subunits without I-domain are divided into three groups based on their ligand affinity or structural difference [Figure 1.2A]. One group of α subunits (αIIb, αv, α5, and α8) form complexes with β1 and β3 subunits, and binds to RGD-containing ligands (Giancotti and Ruoslahti, 1999). Second group of α subunit (α3, α6, and α7) drives integrin to bind on laminin mainly with β1 subunit. The third group is consists of two members (α4 and α9), which lack the membrane proximal cleavage site, and pair with β1 or β7 subunit to counter-receptors for the IgG superfamily(Rose et al., 2000), such as VCAM-1(Barczyk et al., 2010) .

All integrin β subunits have a highly conserved region of approximately 240 amino acids near the N-terminus termed the β I-like domain. The integrin β subunit contains a plexin-semaphorin-integrin (PSI) domain (Lee et al., 1995), a hybrid domain, a βI domain, and four cysteine-rich epidermal growth factor (EGF) repeats. Cytoplasmic tail shares homology and contain one or two NPxY motifs as part of recognition sequence for phosphotyrosine-binding (PTB) domains (O'Toole et al., 1995). The β subunits can be classified into three groups two groups for human and one for invertebrates. The first group contains β1, β2 and β7 subunits that associate with α subunits of I-domain. β1

subunit is the most widely expressed in many cell types because of its association with the multiple α subunits (α1-α11 and αv). In contrast, β2 and β7 subunits are restricted to hematopoietic cells (Schneider et al., 2011). The second group includes β3, β4, β5, β6, and β8 subunits are distributed and associated with fewer α chains (Plow et al., 2000). The third group is β4 subunit, which differs from other groups by its unique cytoplasmic domain, and only found in mammals. The β1 and β3 classes predominantly mediate cell-matrix adhesion, while the β2 classes are cell-cell adhesion receptors. The β1 integrins are involved in adhesion to connective tissue macromolecules such as fibronectin, laminin, and collagens, while β3 receptors bind to vascular ligands. β2 integrins are restricted to leukocytes but β1 and β3 integrins have a widespread occurrence and are co-expressed in most cell types (Harris et al., 2000).

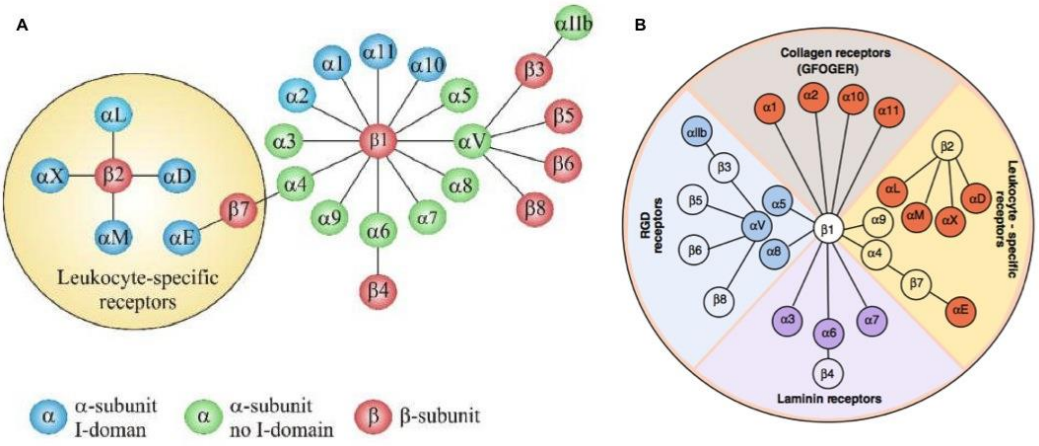

Figure 1.2. Integrin subunits and ligands (A) α and β integrin forms heterodimer with multiple subunits (B) and the heterodimer have preferred ligand (Barczyk et al., 2010).

Integrin and ligands

The ligand-binding site is located in a region at the intersection of the integrin β propeller and the I domain of integrin [Figure 1.1]. The mammalian integrins can be

mainly grouped into laminin-binding, collagen-binding, and RGD- recognizing integrins [Figure 1.2B]. Some integrin receptors display considerable overlap in their ligand-binding specificity, enabling to bind to multiple ligands with dissimilar structures. For example the αvβ3, which mainly binds to vintronectin, shows strong binding affinity to fibronectin, collagen, and fibrinogen. Fibronectin, can bind to more than one integrin (Sonnenberg, 1993).

Integrin and Other Species

Integrin was found in many species including mouse, chicken, *Danio rerio*, *Xenopus laevis*, *Drosophila melanogaster*, and *Caenorhabiditis elegans*. *Drosophila* and *C. elegans* have become preferred experimental model organism for integrin studies due to a simple and tractable properties, such as 1) short life cycle, 2) small number of cells, plenty of progenies, 3) easy to maintain in the laboratory, and 4) conserved genes encoding for integrins, ECM ligands, cytoskeletal proteins with functional overlapping.

Integrins are widely expressed in embryonic tissues of *C. elegans* and *Drosophila* with the minimal set of integrins essential for fundamental process in development. The two integrins are identified in *C. elegans,* including two α subunits (*ina-1* laminin and *pat-2* RGD integrin), and a single β subunit (*pat-3*). The β subunits corresponds to the *Drosophila* PS1 (laminin integrin) and PS2 (RGD integrin). *Drosophila* has five α subunits (PS1- PS5) and two β subunits (βPS and βv) (Gotwals et al., 1994). βPS has high similarity with β subunit of *C. elegans* and in human (Table 1.1) (Wilcox, 1990).

Integrin provides conserved roles such as providing adhesion and maintaining structural integrity during development despite many differences among organisms. For example, RGD-binding integrins are required in embryonic development for muscle

attachment and contraction of body wall muscle cells and vulva tissues in *C. elegans* (Ruoslahti, 1987).

Table 1.1. Expression of integrin in *C. elegans* and *Drosophila (Fornaro and Languino, 1997*

C. elegans Integrin	Ligand	*D. melanogaster* Integrin	Ligand	*H. sapiens* Integrin	Ligand	
αina1βpat3	LN	αPS1βPS	LN	α3β1	LN; TSP	**LAMININ**
				α6β1	LN; *ADAMs*	**RECEPTORS**
				α7β1	LN	
				α6β4	LN	
				α1β1	CO; LN	**FIBRILLAR**
				α2β1	CO; LN; MMP1	**COLLAGEN**
				α10β1	CO	**RECEPTORS**
				α11β1	CO	
αpat2βpat3	?	αPS2βPS	TG	α5β1	FN; *ADAMs*	**RGD**
				α8β1	FN; NN; TN; LAP	**RECEPTORS**
				αvβ1	FN; VN; LAP	
				αvβ3	FN; VN; VWF; OP; TN; BSP; TSP; *CD31*; *ADAMs*; MMP2	
				αIIbβ3	FN; VN; FG; VWF; CD40L; prothrombin	
				αvβ5	VN; BSP; LAP	
				αvβ6	FN; TN; LAP	
				αvβ8	FN; CO; LN; LAP	
		αPS3βPS	?	α4β1	FN; OP; *VCAM*	
		αPS4βPS	?	α9β1	TN; OP; LN; CO; *VCAM*; *ADAMs*	
		αPS5βPS	?			
				α4β7	FN; *VCAM*; *MadCAM*	**LEUKOCYTE-**
				αEβ7	*E-cadherin*	**SPECIFIC**
				αDβ2	*ICAM3*; *VCAM*	**INTEGRINS**
				αLβ2	*ICAM1-5*	
				αMβ2	*ICAM1*; *VCAM*; FG; iC3b	
				αXβ2	FG; iC3b	

Integrin in Animal Development

Cell adhesion mechanisms have been shown important during embryonic development. Especially at early organogenesis, cell-to-cell contacts in dynamic interactions are needed to keep the cells in place during organogenesis. Therefore, integrins in mediated cell adhesion is crucial for studying integrin roles in multiple processes, including embryogenesis and organogenesis.

A role for integrin in development has been confirmed by targeted mutations. Despite widespread expression of integrin in *C. elegans* and *Drosophila*, the genetic deletion of integrin leads to severe abnormalities including defects in muscle, cell migration, gastrulation, adhesion between epithelial cell layers, and lethality (Brown et al., 1989).

Body wall muscle in *C. elegans* and *Drosophila*, integrin-ECM ligation is essential to maintain the muscle in the right place (Brown, 2000). The loss of *pat-3* integrin in *C. elegans*, which is enriched on the basal surfaces of the muscles, leads completely disrupted sarcomere structure and markedly reduced muscle attachment (van Kuppevelt et al., 1989). Similarly, the *Drosophila* muscle without PS integrin has more contractile activity with detachment of the ends of muscles from the ECM (Zusman et al., 1993).

Integrins are also essential for normal cell migration in various tissues. In *C. elegans*, the absence of α *ina-1* integrin causes neuronal cell migration defect (Baum and Garriga, 1997). Failure of cell migration in primordial midgut has been shown to relate to PS integrins (αPS1 and αPS3) deficiency in *Drosophila* (Leptin et al., 1989). Laminin binding integrins promote endodermal cell differentiation, gut morphogenesis (Martin-Bermudo et al., 1999). Deletion of laminin binding integrin leads pharyngeal defects in the *C. elegans ina-1* mutant, and midgut defects in *Drosophila* αPS1 mutant larvae. Morphogenetic defects by the loss of integrin functions, such as notched head phenotype or blistered wing, are observed in both *C. elegans* and *Drosophila* (Brower and Jaffe, 1989).

Integrin and Disease

Mutations in human integrin (α6β4, αIIbβ3, and β2 subunit) have been shown to cause diseases. Ectopic expression of α6β4 leads to the Epidermolysis Bullosa (EB) syndrome that mainly has defects on skin such as blistering and fragile (Jonkman et al., 2002). The blistered skin is caused by the failure of cell adhesion in the dermis, which leads to epidermal and dermal separation (Sterk et al., 2000). Glanzmann's Thrombasthenia is produced by genetic mutations in αIIb or β3, which have a variety of problems including bleeding disorder due to platelet adhesion defects (Jayo et al., 2010). Furthermore, mutations in β3 subunit would have bone cysts or other skeletal defects. Leukocyte adhesion deficiency I (LADI) syndrome is the β2 subunit defects due to mutations in the ligand binding domain (Bunting et al., 2002). LADI syndrome presents a variety of immune deficiencies including variable infection, and it causes wound-healing defects(Etzioni et al., 1999). Since immune cells are unable to attach to the vascular endothelium, reduced leukocytes are recruited and migrated to the sites of infection. Kindlin-3, which binds to a specific domain within the cytoplasmic tail of β subunits, has been mapped from leukocyte adhesion deficiency syndrome patients. Interestingly Kindler's Syndrome, which has mutation in *kindlin-3*, also presents blistering on skin and mucosal due to defects in keratinocyte (Alper et al., 1978). Ectopic expression of integrins, which may lose their connections to ECM or change expression patterns, is very common in many cancer cells (Goel and Languino, 2004). Highly expressed integrins influence the cell invasion and metastasis in many cancers.

Integrin Splice Variants

The important role of integrin cytoplasmic domain in regulating integrin function is well established and emphasized by the existence of unique splice variants, which is product of an alternative splicing mechanism. Alternative splicing occurs in diverse ways including frame shift, premature stop, and skipping of stop codon generates variety of isoforms (Sanchez et al., 2011). Alternative splicing of α and β subunits in the extracellular and cytoplasmic tail regions gives rise to variant forms, which differs in ligand specificity or in cell signaling. Some variants of subunits are expressed in either ubiquitously or a tissue or stage-specific manners, suggesting that each isoform may potentially be involved in unique functions. Differences in the primary sequence of the cytoplasmic domain carry significant effects in a variety of integrin-mediated events.

β1C and α7A-C is typical results of the alternative splicing, which are generated from frame shift in their open reading frame in the adjacent exons. In other case, premature stop codons have been reported either in the alternatively spliced exons or in the translated intron sequences, suggesting further complexity in the integrin system.

In the β1 subunit, the β1B and β1C remain diffuse on the cell surface (Armulik, 2002), while the β1D subunit localizes focal contacts similarly to β1A. In contrast to β1A, the expressions of β1B or β1C subunits negatively affect cellular functions. Negative effects on cell functions might be due to the failure of β1B or β1C recruited to focal contacts where cytoskeletal proteins and other signaling molecules are found. Integrin variants compete with the isoforms present in focal contacts for binding ligands and interfere with signaling events initiated at these sites. Expression of integrin variants, specifically β1C, β1D, α6A and α7A, is induced upon cell and tissue differentiation.

However ectopic expression of integrin variants have inhibitory effects. For example, exogenous expression of β1B inhibited cell adhesion and migration toward fibronectin, in a dominant negative manner (Cali et al., 1999). Also β1C inhibit DNA synthesis and cell proliferation in tumorigenic and normal fibroblasts (Fornaro et al., 2000). The expression of β1C isoform correlates with a non-proliferative phenotype in differentiated prostate epithelial cells and is dramatically downregulated in prostate carcinoma cells (Fornaro et al., 1998).

β1 integrin

β1 integrin forms the biggest integrin subfamily from combining with 12 α subunits, which binds to a wide array of RGD–containing ECM ligands such as collagens, laminin, and fibronectin. β1 integrin is expressed in all mammalian cells and it is required for the development (Danen and Sonnenberg, 2003). Previous studies have shown that β1 activation is also a key regulator in the switch from cancer cell dormancy to metastatic growth *in vitro* and *in vivo*. The complexity of the β1 integrin family is further expanded by the presence of alternative splicing of mRNAs. For human β1 integrin, five different cytoplasmic splice-variants are characterized, β1A, β1B, β1C-1, β1C-2, and β1D with shared common N-terminal part [Figure 1.3] (Armulik, 2002).

Although the ligand specificity for these variants is consistent, receptor affinity toward the ligand varies. The transmembrane and cytoplasmic domain of β1A splice-variant, mostly referred to as β1, is conserved among different species from sponge to human. The last 12 amino acids of β1B are different from β1A, and β1B does not localized to focal contacts on fibronectin in contrast to β1A.

β1B has a dominant negative effect on endogenous β1 integrin, suggesting the competition of β1B with endogenous β1A for available α subunits and the failure of ECM binding. Among the variants, β1C integrin formed from an alternative spicing usually produce 27 amino acids longer cytoplasmic tail than that of β1A splice (Fornaro and Languino, 1997). Like β1B, forced expression of either β1C or its cytoplasmic tail inhibits cell proliferation without affecting cytoskeletal or focal adhesion formation (Fornaro et al., 1998). Because of its inhibitory role of β1C, β1C is expressed in nonproliferative and differentiated epithelium to not become neoplastic, but downregulated in adenocarcinoma inducing rapid cell proliferation. β1D is the only splice-variant that shares significant homology with β1A, only differ by 13 amino acid. Despite the similarity to β1A, ectopic expression of integrin β1D has been shown growth inhibition (Calderwood et al., 2001).

Many studies using β1-null mice and cells have shown the importance of β1 integrin for cell function and development. During differentiation, cells are exposed to a continuously changing environment including alterations in the composition of the ECM, which is accompanied with changes in integrin expression and affinity. β1 integrin is expressed during myogenesis, although some integrins are limited at specific time points during differentiation.

In previous studies, ablation of β1 integrin function decreased the malignant phenotype of cancer cells (Cabodi et al., 2010). Integrin β1 subunits may play an important role in resistance of tumor cells to chemotherapy and ionizing radiation. Low expression or loss of function β1 has been reported in tumor cells that have acquired multidrug resistance. Several lines of evidence showed that β1 signaling plays a

significant role in mediating resistance to cytotoxic chemotherapies by enhancing the cell survival pathway mediated by phosphoinositide 3-kinase (PI3K) and the serine-threonine kinase (Akt) pathway (Jahangiri et al., 2014). Integrin β1 has been implicated in mediating resistance to ionizing radiation through activation of the PI3K/AKT pathway and it is upregulated with radiation (Park et al., 2008). Clinical evidence showed the elevated expression of β1 integrin in invasive breast cancer, which is associated with poor survival rate (dos Santos et al., 2012). Therefore, β1 integrin offers an attractive therapeutic target to inhibit the emergence of tumor.

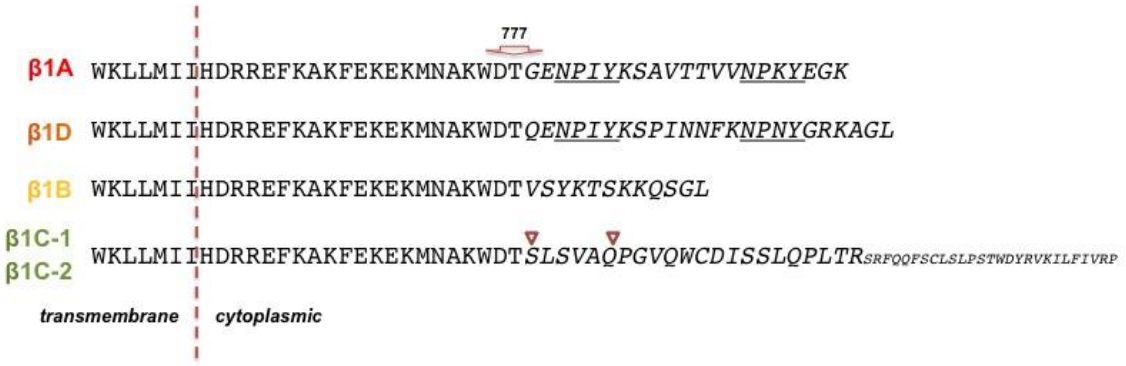

Figure 1.3. The amino acid sequences of the cytoplasmic splice-variants of human integrin β1. The variant specific regions are shown in italic. The NPXY motifs in β1A and β1D are underlined and arrowheads indicate 6 amino acids lacking in β1C-2.

Basic Role of integrin

As their name indicates, integrins create an `integrated' link between the outside and the inside of the cell. Integrin was first defined as a link between ECM and cytoskeleton in cell. For the majority of integrins, the linkage is to actin cytoskeleton. Some proteins in this mechanical linkage such as talin play a dual role and also take part in activating integrins in an ‘inside-out’ signaling mechanisms which control integrin clustering in focal adhesion and increase its affinity to ligands (Chen et al., 1994;

Ginsberg, 1992). This physical link helps the cell signal through plasma membrane and leads to the regulation of cellular behaviors such as adhesion, migration, and differentiation. The cell growth mediated by cell-ECM interaction is an example of cell regulations from the outside of the cell, while intracellular signaling events can also influence the ECM distributions as well as affinity of integrins for ECM ligands.

Integrin ligation to its proper ligand is one of the well-known integrin functions, which controls cell attachment to the ECM being required for cell survival. Complete loss of cell adhesion to ECM, or ectopic attachment to nonspecific ligands, or unligated integrin facilitates apoptosis of the cells such as endothelial cells, epithelial cells, and fibroblasts (Martin-Bermudo and Brown, 2000). In addition, the ectopic expression of dominant negative integrins also inhibits survival by disturbing normal integrin-mediated survival signaling. For example, integrin ligation suppresses caspase 8 activation, but dominant negative integrins induce caspase 8 activation or protein kinase A (PKA), which itself can activate caspase 8. In contrast, tumor cells have abilities to survive as anchorage-independent manner due to its accumulated mutational changes in survival factors, which makes cells independent to integrin-mediated survival signals(Humphries, 1996).

Integrin ligation with ECM regulates cell migration, which is essential for embryonic development, tissue repair, and immune response. The lamellipodium containing an actin cytoskeletal network requires integrin-mediated adhesion to the ECM, at the leading edge of migrating cells, while F-actin stress fibers connect sliding focal contacts for its movement at the trailing ends [Figure 1.4].

Although antagonizing adhesion to the ECM promotes cell migration, recent studies showed that antagonized integrins prevent cell attachment to the ECM. For example, the loss of α5β1 integrin failed to regulate fibroblast migration but α5β1 antagonists such as antibodies, small peptides or small non-peptidic RGD-like molecules activate PKA, which led to inhibit cell migration by disrupting the formation of stress fiber (Russo et al., 2013). Therefore, integrins regulate cell migration by forming focal contacts with the ECM and activating signaling cascades to support migration.

Integrin ligation leads to the intracellular signal cascades, which are referred as "outside-in" signaling, and affect many cell behaviors. Integrins initiate and modulate a number of transduction cascades, such as activation of extracellular signal-regulated protein kinase (ERK), the c-Jun NH_2-terminal kinase (JNK), and activation of phosphatidylinositol-3' kinase/protein kinase B (PKB) (Humphries, 1990). The activation of these signaling pathways also required for cellular responses to growth factors and other receptors.

Integrins, often together with growth factor receptor, upregulate cyclin D1 and E via regulating focal adhesion kinase (FAK) by phosphorylation (Humphries, 1996). Once integrin ligation with ECM is successfully occurred, FAK initiates and stimulates many signaling pathway enhancing the number of growth factor receptors, which contributes to cell cycle progression. Several studies have shown that cell adhesion to the ECM is required for cell cycle progression and integrin signals are important for the down-regulation of cyclin dependent kinase inhibitors (CKIs), including $p21^{cip1}$, $p27^{kip1}$ and $p57^{kip2}$ (Fu et al., 2007). Cell-cycle progression is regulated by cyclin dependent kinases (CDKs), whose activity is controlled by cyclin binding, phosphorylation/dephosphory-

lation, and association with a group of CKIs. Loss of cell anchorage to the ECM recently has been shown to upregulate the expression of $p27^{kip1}$ and $p21^{cip}$.

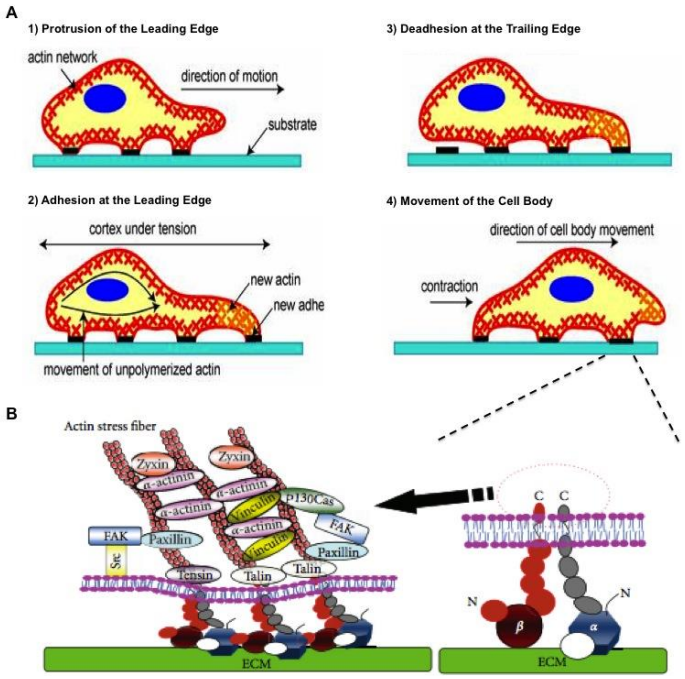

Figure 1.4. Schematic steps of cell migration and integrin activation at focal adhesion (A) At the leading edge, cell extends a protrusion towards the moving direction by actin polymerization. After adhesion of leading edge to the surface, cell deadheres its rear part and pulls the whole cell body forward. (B) The cytoplasmic tail of integrin at focal adhesion recruits multiple proteins, which cross-linked each other to regulate both the actin cytoskeleton and signal transduction (Nagano et al., 2012)

$p27^{kip1}$

$p27^{kip1}$ is a well-studied mammalian CKI protein, abundant in quiescent and G1 phase, and downregulated in proliferating S phase cells. The $p27^{kip1}$ mRNA levels are generally constant throughout the cell cycle but protein levels are mainly controlled by ubiquitin-proteasome dependent proteolysis, as they are highest in cells undergoing senescence and reduced during the G1 to S phases transition (Abukhdeir and Park, 2008). The $p27^{kip1}$ localization is also changed by cell cycle progression and transiently

translocated from nucleus during G0 phase to the cytoplasm at the G1/S transition. In early G1 stage, mitogens induce $p27^{kip1}$ phosphorylation on Ser10 and lead its nuclear export, which incapacitate $p27^{kip1}$, and relieve CDK2 inhibition (Lu and Hunter, 2010). In late G1/S phase, upon activation of cyclin E/CDK2, $p27^{kip1}$ become very unstable and phosphorylation of Thr187 facilitates the interaction of $p27^{kip1}$ with the SCF (Skp1-Cullin-F-box protein)-E3 ligase complex, which ultimately led to its degradation by ubiquitin dependent manner (Sabile et al., 2006). As an important cyclin-dependent kinase inhibitor, $p27^{kip1}$ plays important roles not only in normal cell cycle but also during cell cycle progression of tumor cells [Figure 1.5].

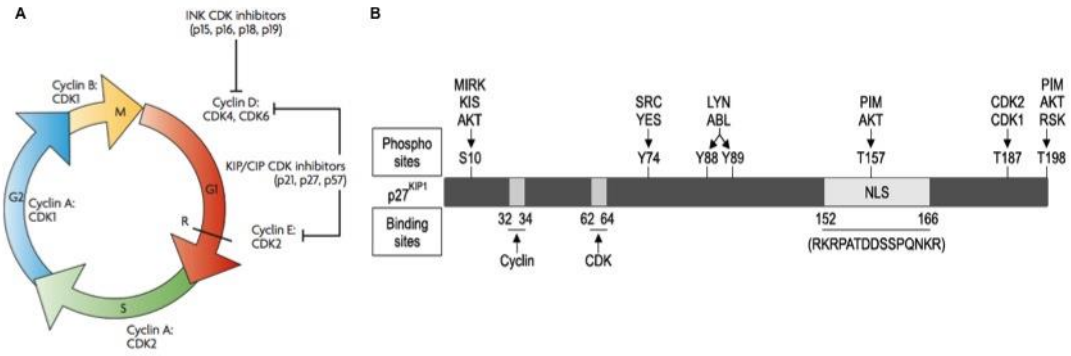

Figure 1.5. Schematic diagram of cell cycle regulation via CKIs and $p27^{kip1}$ phosphorylation (A) and regulation site of $p27^{kip1}$ (B). Nuclear localization sequences (NLS) are also included (Lee and Kim, 2009).

The expression of $p27^{kip1}$ is frequently decreased in human cancers and mislocalized to cytoplasm with low expression level and has been proposed as an adverse prognostic factor that correlates with poor clinical outcome (Steeg and Abrams, 1997). Also ectopic expression of $p27^{kip1}$ inhibits cell proliferation and caused resistance to DNA damaging drugs and increased survival of tumor cells. Accumulating evidence indicated that the expression of $p27^{kip1}$ is mainly regulated post-translationally in many

cancer cells, especially at the protein degradation level (Desdouets and Brechot, 2000). The upregulation of $p27^{kip1}$ protein was mediated by decreased proteasome dependent degradation mechanisms [Figure 1.6].

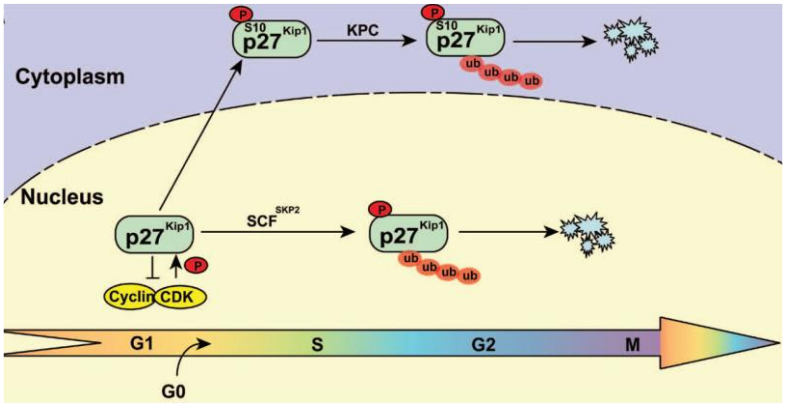

Figure 1.6. *$p27^{kip1}$* expression is regulated by phosphorylation at S10, and ubiquitinated by SCF^{skp2} (Lu and Hunter, 2010) .

Skp2-dependent proteasome degradation of $p27^{kip1}$ protein has been well characterized. Skp2 can specifically recognize Thr187-phosphorylated $p27^{kip1}$ then promote the degradation of $p27^{kip1}$ protein in nucleus (Hengst, 2004). In many cancers, the reduced expression of $p27^{kip1}$ is usually associated with increased expression of Skp2. $p27^{kip1}$ is exported from the nucleus to the cytoplasm at G0-early G1 phase. Moreover, Skp2-dependent proteasome degradation of $p27^{kip1}$ protein occurs during late G1-S phase.

The control of $p27^{kip1}$ activities is regulated by cell adhesion dependent events in different cell types. Integrins are essential for the expression of Skp2 and decrease $p27^{kip1}$ level during cell cycle progression (Hazlehurst et al., 2000). However, recent studies have shown the negative role of integrin in cell growth and upregulation of $p27^{kip1}$ expression as well. For example, integrin β1A upregulated the $p27^{kip1}$ protein amount, both in cytoplasm and nucleus by prolonging the half-life of $p27^{kip1}$ protein and increasing its

stability (Fu et al., 2007). Integrin β1A upregulates the amount of $p27^{kip1}$ protein by suppressing the degradation of $p27^{kip1}$ protein which might be involved in the cell growth inhibition (Fu et al., 2006). The forced expression of β1C specific cytoplasmic domain inhibits cell growth and increases $p27^{kip1}$ protein amount in prostatic adenocarcinoma but the actual mechanism for this and the molecules involved have not been identified.

A number of recent studies have demonstrated the prognostic significance of $p27^{kip1}$ protein in many human cancers. Decreased protein amount of $p27^{kip1}$ is associated with aggressive, high-grade human breast, colorectal, and gastric cancers with poor clinical outcome. Because of the prognostic value, the expression regulation of $p27^{kip1}$, particularly the protein degradation of $p27^{kip1}$, has emerged as a critical area of research in growth control in a wide variety of tumors. Both decreased level and cytoplasmic translocation have been proposed as a negative prognoses sign.

Nucleolus

Information about the direct downstream effector molecules from integrin is lacking, but $p27^{kip1}$ has been identified as one of the nuclear effector molecules of integrin signaling. The nucleolus is the largest subnuclear structure and the number and activity of nucleoli vary during the cell cycle. Many aspects of nucleolar organization and function are conserved within eukaryotic organisms, from yeast to human. The mammalian nucleolus includes several morphologically divided regions—the fibrillar center (FC), the dense fibrillar center (DFC) and the granular component (GC)—that have roles in the various steps of rRNA synthesis [Figure 1.7]. The FC is rich in RNA polymerase I and it includes the transcription factor UBF (Lo et al., 2006). The DFCs are associated with the FCs and contain fibrillarin, an RNA methyltransferase, and nucleolin

that have multiple roles in nucleolar and cellular biology. The GC is the partial maturation and assembly site for pre-ribosomes, and is enriched with ribosomal proteins and assembly factors.

The nucleolus is a dynamic structure that has roles in various processes, from ribosome biogenesis to regulation of the cell cycle and the cellular stress response. The main function of the nucleolus is the rapid production of small and large ribosome subunits by pre-rRNA transcription, processing, and ribosomal RNP assembly at different place in nucleolus. Therefore, the morphology and size of nucleoli are linked to nucleolar activity, which in turn depends on cell growth and metabolism, and is a good marker of ribosome synthesis (Boulon et al., 2010).

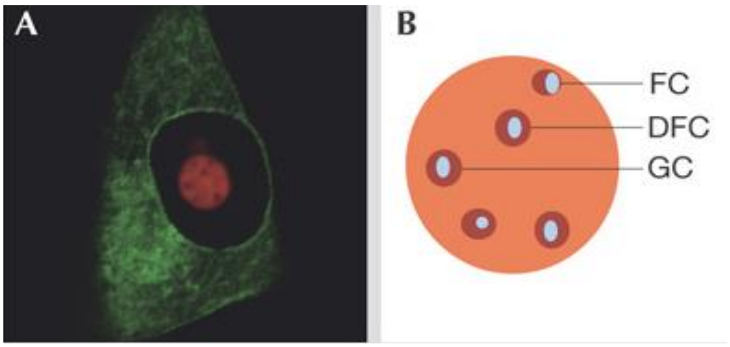

Figure 1.7. Structure of the nucleolus (A, B) Diagrammatic representation of the mammalian nucleolus showing the positions of the FC, DFC, and GC. DFC, dense fibrillar component; FC, fibrillar center; GC, granular components (Emmott and Hiscox, 2009).

In recent studies propose that the nucleolus have multiple functions in health and disease. For example, the nucleolus are involved in the cell cycle regulation and cell growth and in the response to cellular stress (Boulon et al., 2010). The nucleolus can be target for virus infection and disturbance to the nucleolus in a many cellular diseases, from auto-immune disease to cancer (Montanaro et al., 2008). The rapidly dividing cancer cells, exhibit an increase in size and number of nucleolar because of the high rate

of ribosomes synthesis (Emmott and Hiscox, 2009). This hallmark feature provides a marker for pathological diagnosis.

Dissertation Background

C. elegans integrins

C. elegans is a useful model organism for studying integrin function, in part because it has only to three integrin subunits, two α subunit (INA-1 and PAT-2) and one β subunit (PAT-3), form two distinct set of integrins. Mutations in *C. elegans* integrin cause embryonic or larval lethality and developmental defect in muscle and gonad. The INA-1 is the most similar to laminin-binding integrin and ubiquitously expressed at gastrulation (Baum and Garriga, 1997). INA-1 also have been observed in most migrating cells or the cells undergoing morphogenesis such as the uterus, vulva, and the male tail. The failures of axon migration or morphological defects have been observed in multiple organs of *ina-1* mutant including the head, pharynx, vulva, gonad, and uterus. Another α integrin PAT-2 is similar to RGD-binding integrins and highly expressed in body-wall muscle cells, vulva tissues, and DTC immediately after L2 stage (Hsieh et al., 2012).

The PAT-3 is the only β subunit expressed in all tissues. PAT-3 is localized to the dense bodies, M-line, borders between cells of body wall muscle cell, and other tissues such as vulva muscles, spermatheca, neurons, and gonad [Figure 1.8A].

The cytoplasmic tail of PAT-3 have regulatory role via recruiting several molecules such as *talin* and *pat-4.* [Figure 1.8B]. Ectopic expression of the cytoplasmic tail has dominant negative effect on integrin function, for example, the heterologous expression of a HA-βtail showed multiple phenotypes such as larval arrest, uncoordinated movement, and egg laying defects, which have been observed in *pat-3* mutants. These

effects are attributed to the competitive recruitment of signaling molecules to ectopic β tail, which inhibits the endogenous PAT-3 cytoplasmic tails (Lee et al., 2001b).

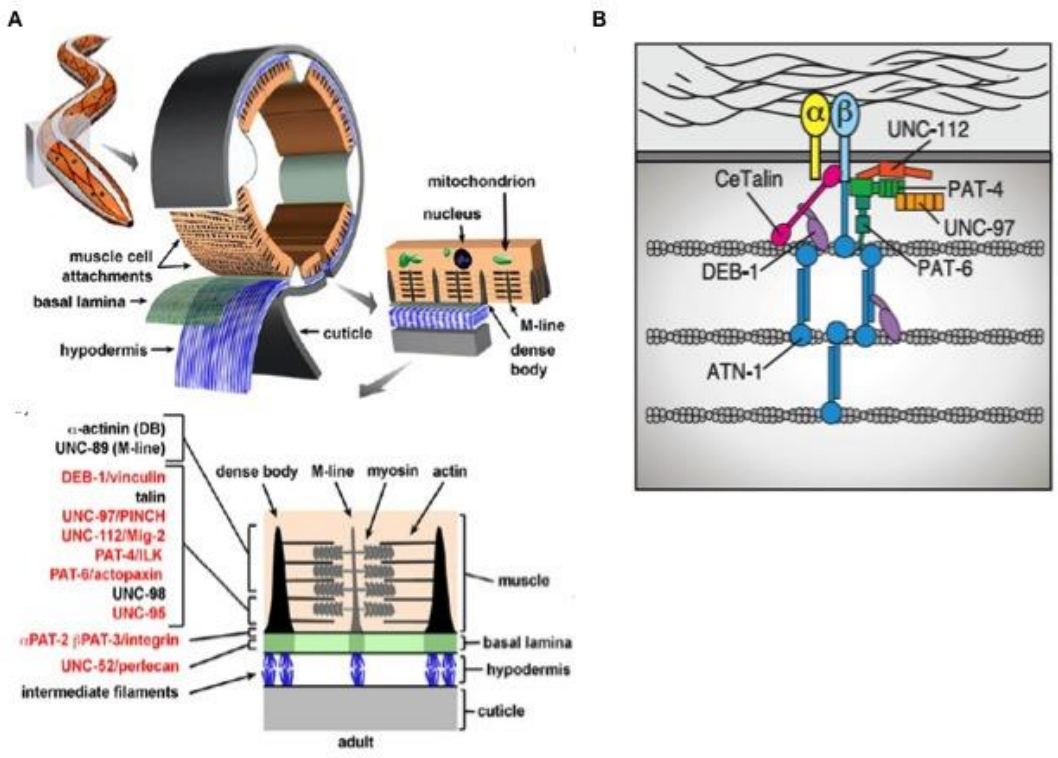

Figure 1.8. Schematic dissection of the *C. elegans* body wall muscle structure: (A) An adult worm with body wall muscle quadrants visible and a body wall cross section. Dense bodies and M-lines attach actin thin filaments and myosin thick filaments, respectively, to the base of sarcomere. (B) Cytoplasmic domain of the PAT-3 integrin subunit making a contact with PAT-4/ILK, talin, alpha-actinin and DEB-1/vinculin at dense body (Moerman and Williams, 2006).

C. elegans Homologue of p27^{kip1}/ CKI-1

The proper formation of a multicellular organism requires the precise coordination of cell division at the correct time and position during development via cell cycle progression. Cell cycle is controlled by cyclins, CDKs, and a group of CKIs, which are the key regulator of cell cycle to maintain exit and entry. The cellular levels of cell cycle regulators are precisely controlled at each stage by activation through phosphorylation or ubiquitin–proteasome dependent degradation as negative regulation.

The *C. elegans* provides an attractive animal model for cell cycle studies. The somatic cell lineage of *C. elegans* has been completely described therefore the timing of development or cell division is described for every cell. Most cell cycle regulators are represented by single gene in *C. elegans* such as cyclin D (*cyd-1*), CDK4/6-related kinase (*cdk-4*) and cyclin E (*cye-1*), to simplify the functional analysis [Figure 1.9].

C. elegans genome encodes two CKIs, *cki-1* and *cki-2*, belonging to the Cip/Kip family which can interact with and inhibit the kinase activity of the Cdk2-cyclin E complex. The CKI-1 and CKI-2 proteins have approximately 30% identity with poorly understood. The *C. elegans* $p27^{kip1}$ homologue *cki-1* has been shown to be one of the downstream effectors of many developmental pathways that confer developmental G1 arrest as negative regulators of the G1/S transition.

Overexpression of *cki-1* causes cell-cycle arrest in G1, while loss of *cki-1* through RNA-mediated interference (RNAi) causes extra larval cell divisions in multiple lineages and induces abnormalities in the organogenesis such as vulva, the somatic gonad, the hypodermis, and intestine (Buck et al., 2009).

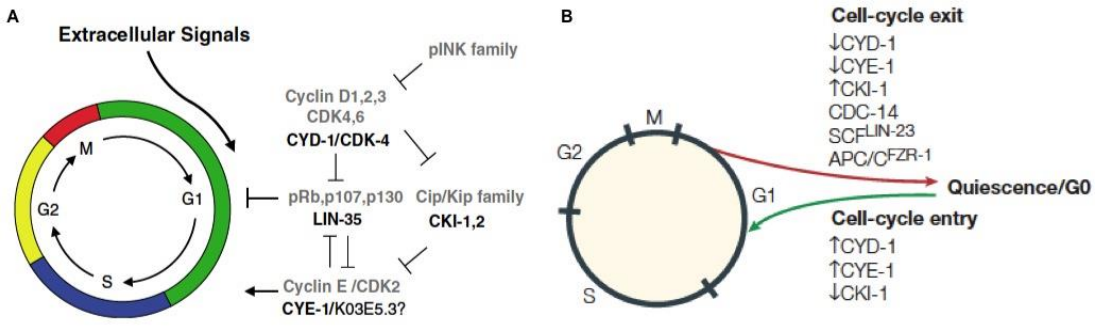

Figure 1.9. General model for regulation of G1 progression. (A) The mammalian genes are indicated in gray above and *C. elegans* orthologs are indicated in bold. (B) Cell cycle regulators are precisely maintained at different stage to progress cell cycle (Kipreos, 2005).

Nucleolus: The Localization of CKI-1

In previous study we found that integrin β1spliced variant, *pat-3 (sp)*, increased the *C. elegans* $p27^{kip1}$/CKI-1 levels with distinct localization in nuclei. In *pat-3* (+), CKI-1::GFP was localized primarily to nucleoli of hypodermal cell as round spots, while CKI-1::GFP have been clumped and disorganized in nucleoplasm of hypodermal cell in *pat-3 (sp)*.

The nucleolus is a recognizable subcompartment within the nucleus of germ cells and hypodermal cells in *C. elegans* (Lee et al., 2012). The main role of *C. elegans* nucleolus is the ribosome production. Therefore, the morphology and size of nucleoli are linked to nucleolar activity, which in turn depends on cell growth and metabolism. Many genes coding for nucleolar proteins found in humans are also conserved in *C. elegans* such as Nopp140 (*dao-5*), a scaffold protein that is required for maintenance of nucleolus integrity. The *C. elegans* "proximal tumor" phenotype in the hermaphrodite gonad was the first phenotype associated with loss of nucleolar integrity and impaired ribosome biogenesis function (Joshi et al., 2010). RNAi screening against other factors involved in ribosome biogenesis revealed a similar gonadogenesis phenotype, suggesting that such phenotypes can result from inefficient ribosome biogenesis. Interestingly, tumor cells not only exhibit rapid cell growth and division, but also often have enlarged nucleoli, which have been observed in the adult cells in *ncl-1* mutant, a higher production rate of rRNA and exhibiting an early appearance of nucleoli in embryos.

Hypothesis and Specific Aims

Integrins are transmembrane αβ heterodimeric receptors that mediate cell adhesion to the extracellular matrix (ECM) and control many cellular functions, such as

proliferation, migration, and differentiation. *C. elegans* is a free-living soil nematode that has been studied as a genetic model organism for molecular analysis of integrin because there are only two integrins, □*pat-2*/□*pat-3* or □*ina-1*/□*pat-3* (Gettner, 1994b). Especially, *C. elegans* □*pat-3* integrin is similar to the human β1 integrin, with the 41% protein sequence identity (Gettner et al., 1995). Mammalian β1 integrin, □1C, an alternatively spliced variant of the β1 subfamily, has been studied for its role in tumor suppression in malignant tissue (Goel and Languino, 2004). The β1C integrin is known to upregulate $p27^{kip1}$. CKI-1 is the $p27^{kip1}$ homolog of *C. elegans*, whose null mutation causes hyperplasia as cell cycle failed in tissues such as the hypodermis, the vulva and the intestine (Kihira et al., 2012). In *C. elegans*, the function of *cki-1* appeared to prevent the cell cycle progression from G1 to S phase. For example, the expression level of *cki-1* reaches a plateau during L4 and young adult stages (Kihira et al., 2012). In the preliminary studies, I have analyzed the role of *pat-3* integrin in regulating CKI-1 expression. Our lab generated nematode *pat-3* rescue lines containing *pat-3 (sp)* integrin or *pat-3 (+)* integrin with CKI-1::GFP fusion protein. The localization pattern of CKI-1::GFP in *pat-3 (sp)* integrin transgenic rescues appeared different in L4 or young adult, comparing to that of *pat-3 (+)* rescues. The subsequent RNA-mediated interference (RNAi) analyses revealed that the distinct localization of CKI-1 depends on the function of cell adhesion molecules and a ubiquitin-mediated protein degradation pathway (Kihira et al., 2012).

Hypothesis

The nuclear CKI-1 localization pattern in pat-3 (sp) animals indicates that CKI-1 nucleolar localization is mediated by cellular stress response in addition to cell adhesion. To test this hypothesis, I proposed to study the following two specific aims.

Aim 1. Demonstrate whether CKI-1 nuclear localization is affected by integrin linked genes and temperature. Cells typically respond quickly to stress and cell cycle checkpoints regulate the right transition throughout the cell cycle in response to cellular stresses including temperature or DNA damage. The increased levels of CKIs are one way to inhibit the cell cycle to keep the genome integrity from stresses response, but the molecular mechanism in the context of integrin signaling is not clear. To link CKI-1 to stress response, I have tested the two □*pat-3* transgenic rescue lines with different temperatures. Temperature changes gave stress to transgenic animals with various levels; larval arrest and lifespan. Expression levels and localization of CKI-1 appeared different at the abnormal temperature.

*Aim 2. The reverse genetic approach to identify genes involved in the CKI-1::GFP localization***.** RNAi screen of *pat-3 (+)* was performed to identify new genes carrying CKI-1 mislocalization phenotypes. We found integrin-linked genes and the Skp2-Cullins-Fbox (SCF) family E3 ligase is important in maintaining the level of CKI-1. After the initial screen, *pat-3 (sp)* was screened for suppressor. The later screen revealed that many nucleus expressed genes, involved in nucleolus functions, unfolded protein response, and RNA splicing, are essential for CKI-1 localization and are linked to integrin.

CHAPTER TWO

β PAT-3 (SP) Integrin, A Splice Mutant, in *Caenorhabditis elegans* Reveals Multiple Function of Integrin in the Cell

Abstract

Integrin is a heterodimeric cell surface receptor for extracellular matrix and plays essential roles in regulating cell behaviors such as cell migration, adhesion, growth, and death. We are interested in studying the role of *Caenorhabditis elegans* β *pat-3* integrin *in vivo*. To obtain a viable allele to characterize the function of *pat-3* integrin, we generated a splice mutation in the cytoplasmic tail of *pat-3* integrin, *pat-3 (sp)*. This mutation provided us with valuable insights on multiple functions of integrin in tissue organization of the nematode. Growth defective phenotypes of *pat-3 (sp)* displayed cold-sensitive larval arrest, shorter body length, and reduced lifespan. To investigate the role of integrin in cell growth, we assessed the interaction of integrin signaling to $p27^{kip1}$/CKI-1. The *pat-3 (sp)* mutant co-expressed with CKI-1::GFP revealed that the majority of integrin linked *pat* genes are the negative regulators of CKI-1 localization while only *pat-12* is the positive regulator of CKI-1, suggesting that integrin signaling plays important roles in regulation of CKI-1. Taken together, our study demonstrated that integrin modulates many different cellular events by modifying its splice pattern.

Introduction

Integrin is an essential molecule for cell-matrix interaction. This heterodimeric cell surface receptor mediates interaction between cell and ECM. In genetics, deletion of integrin largely leads to embryonic arrest or lethality. In contrast to the abundance of α and β integrin molecules in mammals, the nematode *Caenorhabditis elegans* possesses only three integrin genes, β*pat-3*, α*ina-1*, and α*pat-2*. These genes are mainly essential in nature, showing the embryonic or larval arrest in most alleles generated (Cox and Hardin, 2004). Some minor alleles display almost no to mild phenotypes but failed to show distinct and viable phenotypes for further analysis. For example, *ina-1 (gm86) (Baum and Garriga, 1997)*, *pat-3* (*st564) (Williams and Waterston, 1994b)*, *pat-3* (*rh54)(Gettner, 1994a)*, and *pat-3* (*rh151) ((Gettner, 1994a)* display embryonic or larval lethality. In comparison, *ina-1 (gm144) (Huang et al., 2003)* and *pat-3 (ay84)* (M.J. Stern, personal communication) showed mild and leaky cell migration defects. Thus, an allele displaying distinctive and highly penetrant phenotype is essential to analyze genetic or biochemical pathways that *pat-3* gene is involved in.

The *C. elegans* β *pat-3* is the sole β integrin subunit in the nematode. It shows a protein sequence identity to human β1 (42%) integrin and is known to involve in many cellular events (Gettner et al., 1995). The null phenotype of *pat-3* is paralyzed and arrested at two fold (Pat) embryonic stage. This locus was originally isolated in forward genetic screens to look for Pat mutants. Later, additional alleles such *unc-97/PINCH (Mercer et al., 2003)*, *unc-89/obscurin* (Wilson et al., 2012), and *tln-1*/*talin* (Garcia-Alvarez et al., 2003) loci displayed the same Pat phenotypes. Gettner (1994) performed a genetic mosaic analysis and identified that *pat-3* is essential for two major functions,

body wall muscle attachment and gonad and neuronal cell migration (Gettner, 1994b). Although genetic mosaic provided us with valuable information about the role of *pat-3* in development, this technique is usually laborious and unable to measure cellular phenotypes in *C. elegans*. We undertook a molecular approach by creating a mutation in the genomic DNA construct of *pat-3* gene and rescuing a null allele, *pat-3 (st564)*. Transgenic mutants were generated to obtain a viable allele of *pat-3.*

Our interests in characterizing the role of integrin in cell-matrix interaction lead us to create mutations in the cytoplasmic domain of β *pat-3*. Integrin cytoplasmic tail contains conserved motifs such as NPxY (phosphotyrosine binding protein) (O'Toole et al., 1995), WDT (AKT/PKB binding site) (Barczyk et al., 2010), and TT (potential inside-out signaling) (Wennerberg et al., 1998) sequences that are important for binding to the downstream molecules. We have created a mutation that can eliminate such conserved motifs but survive the animals viable so that the phenotype can be studied. In β integrins, there is a small intron before the two NPxY motifs (Hynes, 2002). This β integrin intron is at the same cytoplasmic tail location in many model animals including *C. elegans (Languino and Ruoslahti, 1992)*, *Drosophila (Li et al., 1998)*, and human (Reszka et al., 1992). We created a point mutation at the splice acceptor ag to aa to induce a frame shift mutation that eliminates all functional motifs except WDT (Kihira et al., 2012). This transgenic mutant appears to propagate and provide us with phenotypes that were not described in previous analyses. Briefly, this mutation showed multiple phenotypes such temperature sensitive larval arrests, muscle defects, and other growth defects. Importantly, we addressed the linkage between integrin signaling and CKI-

1/p27^{kip1}, a cell cycle inhibitor for G1, suggesting that integrin plays significant roles in regulating localization and expression of CKI-1 in hypodermis.

Result

Expression of pat-3 (sp) transgene

pat-3 (sp) produced wild-type and mutant mRNA transcripts. To confirm the expression of mutant mRNA species, reverse transcription (RT) PCR analysis was performed. The results showed two mRNA species [Figure 2.1], where upon DNA sequence analysis we confirmed that the larger band, presumably a mutant RNA, included intron sequence, while the smaller band had spliced out and lacked the intron sequence. We expected a few smaller bands in between the two but there were no other minor mRNA's detected. We concluded that this is not a splice variant from an alternative acceptor site in the following exon but it is the mutant transcript including unspliced intron 7 [Figure 2.1A]. Predicted protein sequence indicated that the mutant *pat-3* produces a protein with a longer cytoplasmic tail than the regular splice by adding 19 more amino acids [Figure 2.1B]. As a result, polypeptide chain is terminated at the +2 position of the endogenous TAA stop codon. We expected that this extension may cause instability in 3' UTR of *pat-3* mRNA. Therefore, we analyzed the 3' end of *pat-3* mRNA in *pat-3 (+)* and *pat-3 (sp)* using RACE method [Figure 2.1C]. The sequence analysis confirmed that there was no difference in 3' UTR sequences from both transgenic.

Effects of pat-3 (sp) mutation in C. elegans growth

Unlike the null alleles, *pat-3 (sp)* mutation displayed viable and cold-sensitive growth defects such as larval arrests [Table 2.1]. Eggs were collected from *pat-3 (sp)*

hermaphrodites and placed in the 15ºC incubator. Cold-sensitive larval arrests were observed from the eggs that were incubated at least 10 hours. About 48 % (n= 69) of hatched worms were arrested in early larval stages at 15ºC, compared to *pat-3 (+)* (0%, n=100). Its body length was, however, a little shorter than the *pat-3 (+)* at room temperature. Our analysis showed that the length of *pat-3 (sp)* worms was about 17% (n=34) smaller than that of *pat-3 (+)* grown at room temperature.

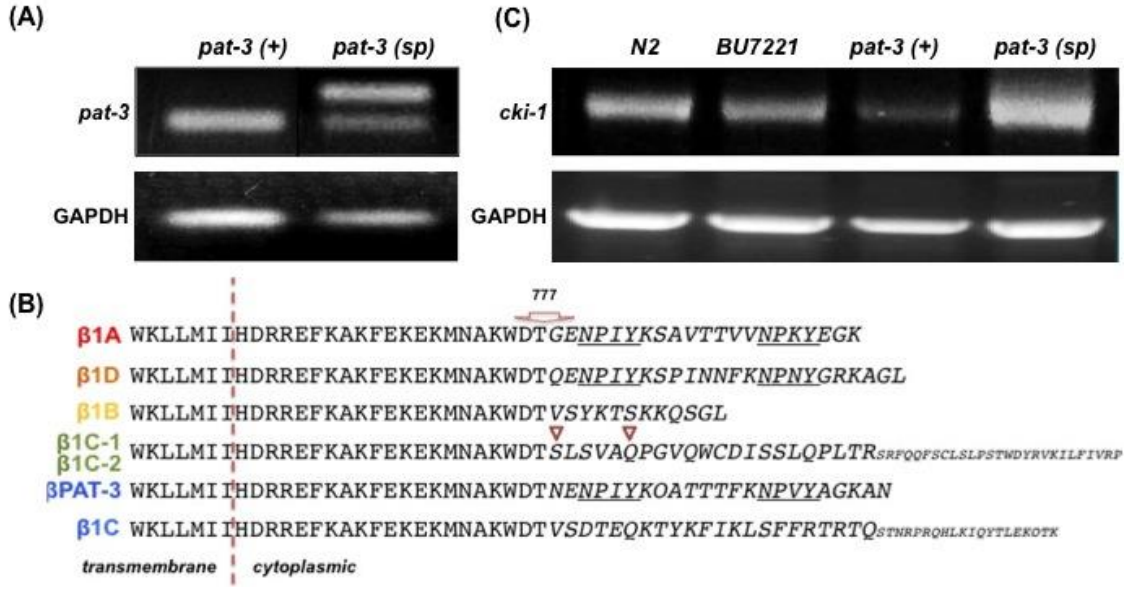

Figure 2.1. RT-PCR analysis of *pat-3* and *cki-1* mRNA in *cki-1*::GFP transgenic and β*pat-3* rescued line. (A) *pat-3* mRNA was measured *pat-3 (+)* and *pat-3 (sp)* animals, respectively. The GAPDH gene, *gpd-1*, was used as an mRNA loading control. (B) Comparison of *C. elegans* PAT-3 and *Homo sapiens* β1 integrin cytoplasmic tails. Protein sequences of *C. elegans pat-3* and human β1A, β1D, and β1C cytoplasmic tails were aligned. (C) The bands represents the 5' RACE product after nested PCR re-amplification using *pat-3* primers in *cki-1*::GFP transgenic and β*pat-3* rescued line.

The growth defects of *pat-3 (sp)* worms led us to investigate the lifespan of this transgenic animal. The lifespan assays were performed at room temperature. The *pat-3 (sp)* mutation clearly displayed a reduced lifespan (Average=16 days, Median=15 days, n=200), compared to N2 (Average=22 days, Median=17 days, n=210) or *pat-3 (+)*

(Average=20.2 days, Median=17 days, n=220) as control. As indicated, *pat-3 (sp)* animals displayed a rapid mortality after 15 days, suggesting that the cumulative effect of the mutation might be emerging later during survival.

Table 2.1. Multiple defects of *pat-3 (sp)* animals

	N2	JE443	*pat-3(+)*	*pat-3(sp)*
Aging (days)	21.7 (17) ± 2.7 (n=200)	21.1 (17) ± 2.6 (n=200)	20.2 (17) ± 1.6 (n=200)	15.9 (15) ± 2.4 (n=200)
Body length (mm)	N/A	N/A	1.4 ± 0.1 (n=37)	1.1 ± 0.8 (n=34)
Cold sensitive arrest (%)	N/A	N/A	0 (n=108)	48 (n=69)

pat-3 (sp) animals exhibit multiple abnormalities. The body length was measured in adult stage animals. The percent of growth defects was determined by counting of unhatched egg and larval arrest animals. N= number of animals. Each data entry represents the average (median) of at least 2 to 3 times test. The errors indicated are the standard deviation. Comparisons between groups were done by student's t-Test and significant was determined at the level of p value 0.05.

The Delocalization of CKI-1 in pat-3 (sp) Suppressed by Low Temperature

This led to an analysis of the transgenic *C. elegans* in growth control. Thus, another *pat-3 (sp)* line with co-expressing *cki-1*::GFP was generated because human β1C variant is linked to $p27^{kip1}$ (Buck et al., 2009), a human *cki-1* homolog, and produces a similar size of cytoplasmic tail of *pat-3 (sp)*. Our study has shown that CKI-1::GFP is localized to nucleoli of hypodermal cells in *pat-3 (+)* while it is delocalized from nucleoli and distributed to the extranucleolar nucleoplasm in *pat-3 (sp)* animals. We measured the *cki-1::GFP* localization at room temperature or 15ºC. The CKI-1::GFP delocalization in *pat-3 (sp)* is greatly reduced when the *pat-3 (sp)* worms were grown at 15ºC. The level of CKI-1::GFP was measured in *pat-3 (sp)* grown at 15ºC using fluorescence microscopy and immunoblot analysis [Figure 2.2A and 2.2B]. The CKI-1::GFP was localized back to nucleoli in 24 hours after the incubation while the amount of CKI-1::GFP, *cki-1* mRNA,

was not decreased [Figure 2.2C] grown at 15ºC compared to the level of grown at room temperature or to the level of *pat-3 (+).* We suggest that this cold sensitivity could restrict the active or preferred growth temperature of the transgenic worms.

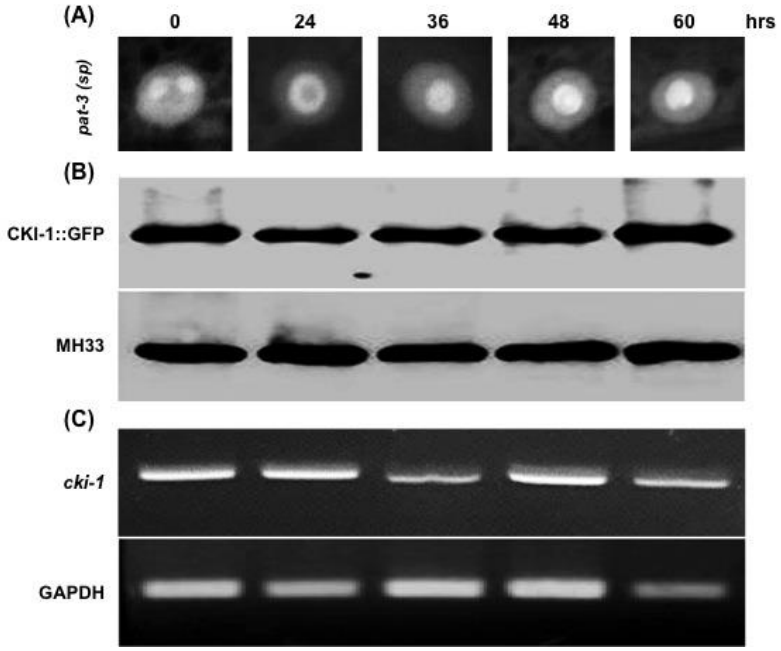

Figure 2.2. Effect of cold stress on CKI-1::GFP localization, protein and mRNA level in *pat-3 (sp)* animals. (A) Thermal stimuli were applied by incubating at 15 °C. Cold stress suppressed the mislocalization of CKI-1::GFP in *pat-3 (sp)*. (B) Western blotting revealed equivalent expression of CKI-1::GFP in *pat-3 (sp)* at each time. An equal loading of proteins was verified by anti-MH33 antibody. (C) The mRNA levels at each time point of the *cki-1*::GFP transgenes in *pat-3 (sp)* were quantified by RT-qPCR, normalized to GAPDH mRNA levels.

The Localization CKI-1::GFP is Linked to Some Pat Genes

The *pat-3* mutations were identified from several forward genetic screens for embryonic lethal mutations (Gettner, 1994b). Such forward genetic analyses defined about twelve Pat genes that can be placed in a linear genetic pathway. To investigate the role of Pat genes to CKI-1 localization, we have performed an RNAi screen of Pat genes [Table 2.2]. We selected RNAi bacterial clones for the Pat genes along with *unc-97*, *unc-52*, *let-2,* and *epi-1,* whose null phenotype is embryonic lethal (Williams and Waterston,

1994b). These genes were knocked down on *pat-3 (+)* or *pat-3 (sp)* background. From the *pat-3 (+)* background, we meant to identify the genes that are linked to *pat-3* or negative regulators of CKI-1 localization. We expected that the removal of the gene would result in the delocalization of CKI-1::GFP in *pat-3 (+)*. In the *pat-3 (sp)* background, on the other hand, we looked for the genes that would suppress CKI-1::GFP delocalization. Presumably the wild type function of the gene is a positive regulator of CKI-1 expression. Among the tested, *unc-52*, *ina-1*, *pat-2, pat-4*, or *unc-97* RNAi displayed delocalization of *cki-1*::GFP in *pat-3 (+)* background. In contrast, only *pat-12* RNAi was able to rescue the delocalization of CKI-1::GFP in *pat-3 (sp),* suggesting that *pat-12* can be placed downstream of *pat-3* and plays an important role in increasing CKI-1 delocalization. In *pat-3 (+)*, integrin signaling might negatively regulate *pat-12* functions [Figure 2.3A]. Removal of negative regulators phenocopied the CKI-1::GFP delocalization in *pat-3 (sp)* [Table 2.2]. Immunoblot analysis to measure CKI-1::GFP protein level in RNAi animals was performed. RNAi of the negative CKI-1 regulators, *unc-52*, *pat-4*, *unc-97*, and *ina-1*, increased the amount of CKI-1::GFP but *pat-12* RNAi, a positive regulator, did not change the level of CKI-1::GFP in *pat-3 (sp)* [Figure 2.3B]. Therefore, we concluded that RNAi of negative regulators not only caused the delocalization but also increased the amount to CKI-1::GFP protein. However, the removal of the positive regulator, *pat-12*, failed to reduce the level of CKI-1::GFP in *pat-3 (+)* or *(sp)*.

Table 2.2. RNAi analysis of genes involved in CKI-1::GFP localization

Sequence name	Gene name	*pat-3 (+)*	*pat-3 (sp)*
C29F9.7	*pat-4*	++ (35)	+++ (35)
C47E8.7	*unc-112*	0 (40)	+++(40)
C48A7.1	*pat-5*	0 (30)	+++ (30)
F01G12.5	*let-2*	0 (20)	+++ (20)
F14D12.2	*unc-97*	+++ (80)	+++ (80)
F54C1.7	*pat-10*	0 (30)	+++ (30)
F54F2.1	*pat-2*	++ (50)	+++ (50)
F54G8.3	*ina-1*	++ (80)	+++ (50)
K08C7.3	*epi-1*	0 (20)	+++ (20)
T17H7.4	*pat-12*	0 (30)	+ (30)
T21D12.4	*pat-6*	0 (60)	+++ (60)
T27B1.2	*pat-9*	0 (30)	+++ (30)
ZC101.2	*unc-52*	++ (50)	+++ (45)
ZC477.9	*pat-8*	0 (30)	+++ (30)
ZK1058.2	*pat-3*	++ (80)	+++ (80)

(N)= the number of animals. Each data entry represents the sum of at least 2 to 3 times test. 0 = 0% mislocalization, + = 1-25% mislocalization, ++ = 26-50% mislocalization, +++ = 51-100% mislocalization.

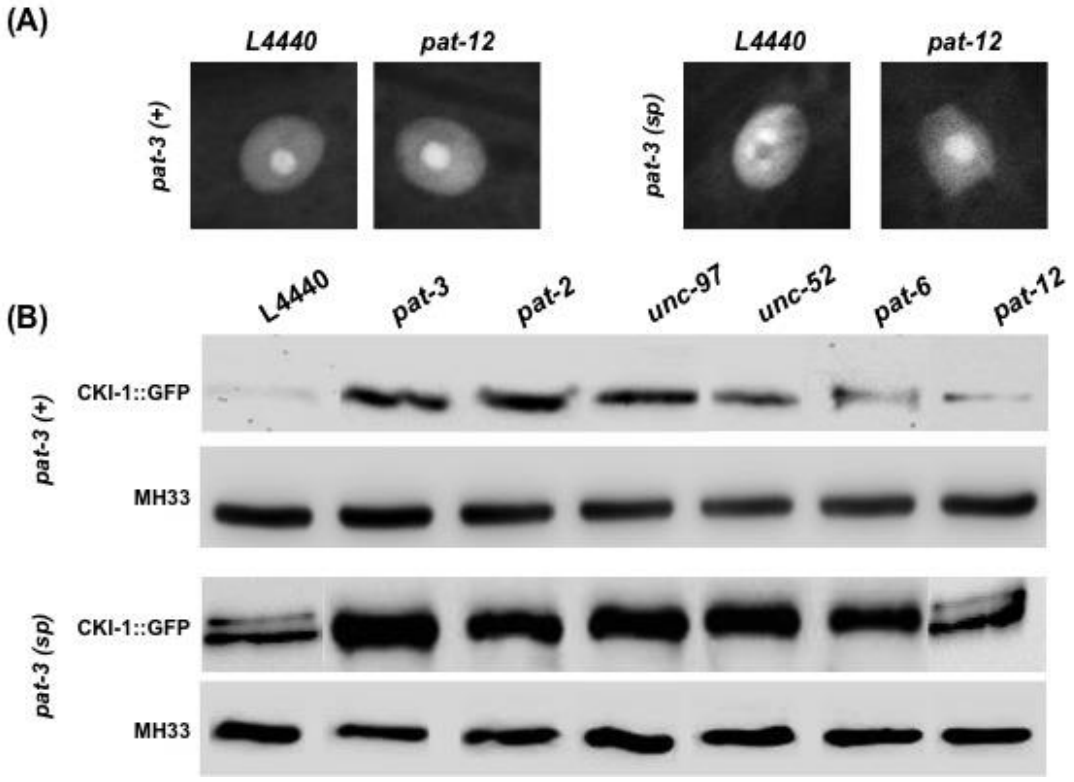

Figure 2.3. Loss of Pat genes induces CKI-1::GFP expression in *pat-3* rescue lines and *pat-12* acts as negative regulator of *cki-1*. (A) Localization of the CKI-1::GFP in *pat-3 (+)* and *pat-3 (sp)* animals with *pat-12* RNAi. *pat-3 (sp)* animals were treated with *pat-12* RNAi and displayed the nucleolar localization compared to control RNAi. However, the same RNAi failed to affect the GFP localization in *pat-3 (+)*. (B) CKI-1::GFP expression affected by Pat gene RNAi. Western blotting showing CKI-1::GFP expression in *pat-3 (+)*, upper two panels, and *pat-3 (sp)*, lower two panel treated with RNAi. *L4440* is a negative RNAi control and loading normalized to MH33.

Discussion

Our study demonstrated that a *pat-3* slice mutation causes multiple defects related to integrin functions. *pat-3 (sp)* appeared to produce two mRNA species, one contains intron sequence but the other does not. Along with the previously reported muscle defects, *pat-3 (sp)* displayed multiple growth defects such as cold-sensitive larval arrests, small body size, and reduced lifespan. The temperature sensitive growth defects prompted us to measure the link between integrin and CKI-1/$p27^{kip1}$ because the similar variant of human β1 integrin is linked to $p27^{kip1}$. In *pat-3 (sp)*, CKI-1::GFP shows delocalized from

nucleolus and accumulated in nucleoplasm. However, incubation at 15ºC was enough to suppress the delocalization. To investigate the interaction between integrin and CKI-1, we depleted pat genes in *pat-3 (+)* or *pat-3 (sp)* background. Results indicated that *unc-52*, *pat-2*, *ina-1*, *pat-4*, and *unc-97* appeared to negatively regulate CKI-1. However, *pat-12* appeared positively regulate CKI-1.

Our results address important molecular functions of integrin in the cell. First, the mutation produced a regular and a mutant splice form, which are resultants of mis-splicing. The similar mRNA splicing mutants, *let-23* and *dpy-10*, were reported in *C. elegans* (Aroian et al., 1993). These two alleles showed similar expression pattern as well as cold-sensitive defects, suggesting that many genes in *C. elegans* share the similar splicing machinery.

Second, *pat-3 (sp)* showed multiple growth defects such as cold-sensitive larval arrest, smaller body length, and shorter lifespan. The reason for the cold sensitivity is largely unknown. We suspect that low temperature grants a detrimental structure formation, unfolded or misfolded, to the mutant protein, which can be toxic during larval development. The shorter lifespan is not surprising but it should be noted that the median lifespan of *pat-3 (sp)* was comparable to that of *pat-3 (+)* but *sp* mutant died rapidly after the median point, suggesting that the mutant protein might have cumulative effects later in the life. As an alternative interpretation, we would also like to suggest that this *sp* mutation provides an alternative integrin function. Perhaps alternative splicing of integrin may have caused temperature adjustment behavior. The regular splice form makes worms fit to a better growing temperature.

Third, temperature affected the cellular event in which integrin is involved; *pat-3 (sp)* incubated at 15ºC for over 24 hours suppresses the delocalization of CKI-1::GFP. However, the amount of CKI-1 expression remains steady at 15ºC. The exact cause of the suppression is unknown. However, in 15ºC, *C. elegans* reduces the growth rate, which may decrease the activity of nucleolus presumably by reducing ribosomal gene activity. Perhaps the reduced nucleolar activity rescues the localization of CKI-1::GFP. Fourth, we intended to study the interaction between integrin and CKI-1. Therefore, we surveyed the interaction of *pat* genes using RNAi in *pat-3 (sp)*. Genes showing interaction are mostly negative regulator of CKI-1. We also found *pat-12* as a positive regulator of CKI-1. It is accepted that integrin cytoplasmic tail forms a complex with *pat-4/ILK*, *pat-6/parvin*, and *unc-97/PINCH* and regulate cell contractility. RNAi analysis revealed that RNAi knock down of *pat-4, unc-97, ina-1,* or *pat-2*, the components of the IPP complex, appeared to delocalize CKI-1::GFP, suggesting that IPP complex is crucial for the nucleolar localization of CKI-1. Our RNAi results indicate that IPP complex might negatively regulate *pat-12,* which is a positive factor for CKI-1. However, the mode of CKI-1 upregulation via *pat-12* remains unanswered.

Materials and Methods

Nematode Strains and Maintenance

Three strains were maintained under standard conditions. Bristol N2, and *pat-3* transgenic line JE443, BU7221, BU444 *kqEx75 [pat-3(+) rab-3::RFP, cki-1::GFP]* and BU7222 *kqEx73 [pat-3(sp) rab-3::RFP, cki-1::GFP]* strains were used in this study. Some are obtained from the Caenorhabditis Genetics Center (CGC, St. Paul, MN).

Fluorescence Microscopy

To visualize CKI-1::GFP localization in hypodermis, L4 to young adult worms were mounted in a drop of M9 buffer containing 0.5mM levamisole on a 24x60 mm coverslip coated with 3% agarose. Images were examined and analyzed using Nikon TE2000-U Diaphot epifluorescence microscope with Nomarski optics, CoolSnap CE monochrome camera (Photometrics, Tucson, AZ), and Metamorph (v8.0, Molecular Devices, Sunnyvale, CA) and ImageJ64 (National Institute of Health, Bethesda, MD) software.

Lifespan Assay

Lifespan assays were conducted at room temperature on NGM plates using standard protocol and were repeated in at least two independent experiments. *C. elegans* were synchronized by performing an egg preparation in 10% sodium hypochlorite solution. One hundred L4 larvae were transferred onto ten NGM plates with ten larvae per the plate. Adult nematodes were regularly transferred to new NGM plates to separate from F1 generations. Surviving and dead animals were counted daily (starting at the first day of adulthood) until all individuals had died. To assess the survival of the worms, the animals were tapped with a platinum wire every two days, nematodes that failed to respond to contact stimuli or stopped pharynx movement were considered as dead. Statistical significance was calculated using the Student's *t* test.

Body length Measurement

Body length by imaging of the animals was done with more than forty L4 animals selected from each line and incubated at room temperature. The same stage animals were

placed on 3 % agar pads on glass slides and were imaged using Metamorph (v8.0, Molecular Devices, Sunnyvale, CA) and a CoolSnap CE monochrome camera (Photometrics, Tucson, AZ) mounted on a Nikon TE2000-U Diaphot epifluorescence microscope (Nikon, Melville, NY, USA). All animals were imaged at 100x magnification. Lengths of animals were determined using the length measurement image tool in ImageJ64 (National Institute of Health, Bethesda, MD) software.

Thermo-sensitive Assays

For thermo-sensitive assays, synchronized L3 animals were incubated at 15 °C then transferred to 20 °C for recovery and the animals were scored after 12 hours. After the cold shock, worms were mounted on a slide and monitored for CKI-1::GFP localization. The distribution of CKI-1::GFP was monitored accordingly. More than 30 animals per trial were tested for each experiment and at least three independent replicates were performed for each assay.

Cold-sensitive Growth Defects of pat-3 Animals

To observed temperature dependent growth defects, embryos were collected by treatment of 10% sodium hypochlorite solution and allowed to hatch at 15 °C. After 10 hours incubation, we counted the total number of eggs and arrested larvae of *pat-3* rescue animals every two hours.

RNA Isolation and Reverse Transcription PCR

Total RNA extraction was performed from fifty L4 to young adult animals using TRI reagent (Sigma-Aldrich, St. Louis, MO), chloroform for RNA precipitation. cDNA were synthesized with approximately 500ng of total RNA used in PCR amplification with

cki-1 primers, *pat-3* primers, and *gpd-1*/GAPDH primers as an internal control. The samples were then resolved in agarose gels and subjected to stain using GelRed (Biotinum, Hayward, CA) for analysis. The following sets of primers were used for amplification.

CKI1 Forward GTCTTCTGCTCGTCGTTGC
CKI1 Reverse CACCGGAGACAGCTTGAT
PAT3PT Forward CTCAACGAAACTACACCCTG
PAT3PT Reverse TTAGTTGGCTTTTCCAGCGT

RNA Interference

C. elegans RNA interference analysis was performed using the bacterial feeding method. Young adult animals were fed with RNAi bacteria containing plasmids directing the expression of dsRNAs on NGM plates with 1 mM lactose and 0.05 mg/ml Ampicillin. F1 progeny of those animals were observed under microscope at L4 to young adult stages for analysis the localization of CKI-1::GFP. The plasmid DNA of all RNAi bacteria used for analysis were isolated and sequenced at Marcrogen (Rockvill, MD) for confirming presence of coding sequence.

Immunoblot Analysis

For immunoblotting, 20 young adults worms were picked and lysed in lysis buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, 1 mM PMSF) with the additional protease inhibitors (Roche Diagnostics, Indianapolis, IN). Samples in lysis buffer were boiled for 10 minutes with 4X Laemmli sample buffer at 100°C then subjected to SDS-PAGE on polyacrylamide gel and transferred onto PVDF membrane. Blots were probed with primary rabbit polyclonal antibody against GFP (Abcam, Cambridge, MA) at 1:2000 and

the respectable secondary antibody. GAPDH (Ambion, AM4300) was used as a loading control.

CHAPTER THREE

A Novel Mutation in β Integrin Reveals an Integrin-mediated Interaction between the Extracellular Matrix and *cki-1/p27^{KIP1}*

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Abstract

The cell-extracellular matrix (ECM) interaction plays an essential role in maintaining tissue shapes and regulates cell behaviors such as cell adhesion, differentiation and proliferation. The mechanism by which the ECM influences the cell cycle *in vivo* is poorly understood. Here we demonstrate that the β integrin PAT-3 regulates the localization and expression of CKI-1, a *C. elegans* homologue of the cyclin dependent kinase inhibitor p27^{KIP1}. In nematodes expressing wild type PAT-3, CKI-1::GFP localizes primarily to nucleoli in hypodermal cells, whereas in animals expressing mutant *pat-3* with a defective splice junction, CKI-1::GFP appears clumped and disorganized in nucleoplasm. RNAi analysis links cell adhesion genes to the regulation of CKI-1. RNAi of *unc-52*/perlecan, *ina-1/α integrin*, *pat-4*/ILK, and *unc-97*/PINCH resulted in abnormal CKI-1::GFP localization. Additional RNAi experiments revealed that the SCF E3 ubiquitin-ligase complex genes, *skpt-1*/SKP2, *cul-1*/CUL1 and *lin-23*/F-box, are required for the proper localization and expression of CKI-1, suggesting that integrin signaling and SCF E3 ligase work together to regulate the cellular distribution of CKI-1. These data also suggest that integrin plays a major role in maintaining proper -1/p27^{KIP1} levels in the cell. Perturbed integrin signaling may lead to the inhibition of SCF

ligase activity, mislocalization and elevation of CKI-1/p27^{KIP1}. These results suggest that adhesion signaling is crucial for cell cycle regulation *in vivo*.

Introduction

Integrins are αβ heterodimeric receptors that mediate bi-directional interactions between cells and extracelluar matrix (ECM)(Arnaout et al., 2005). In mammals, 13α and 8β chains comprise more than twenty heterodimers and play important roles in controlling cell behaviors such as cell adhesion, migration and proliferation (Hynes, 2002). Among the b subunits, b1 integrin is broadly expressed and has multiple splice variants. For example, four β1 splice variants, β1A, β1B, β1C and β1D differing in their cytoplasmic tails, are expressed in many tissues (Fornaro and Languino, 1997). β1A is the dominant splice form and is expressed ubiquitously (Languino and Ruoslahti, 1992). β1D is produced by alternative splicing and is found in striated muscle cells only. Both β1A and β1D forms are localized to focal adhesions and retain the conserved NPxY phosphorylation motif. However, the β1B variant, expressed in keratinocytes and hepatocytes, fails to localize to focal adhesions and exhibits dominant negative activity to β1A-paired integrins. β1B is the result of mis-splicing of intron 7, and retains intronic sequence in its mRNA. β1C is expressed in normal tissues, such as the prostatic epithelium, and is downregulated in cancer cells (Languino and Ruoslahti, 1992). β1C integrin is produced from an alternative splicing event in the cytoplasmic tail of β1 integrin, usually includes exon C and results in a protein 27 amino acids longer than the regular β1A splice form(Meredith et al., 1995).

In many cancerous conditions, integrin lose their connection to the ECM or change their expression patterns (Fornaro and Languino, 1997; Fornaro et al., 1998;

Fornaro et al., 1996). The ECM also undergoes remodeling, resulting in abnormal deposition of proteins or increased ECM stiffness. A change in ECM composition or mechanical properties may upregulate integrin signaling, which promotes cell survival, adhesion and proliferation (Walker and Assoian, 2005). For example, cell detachment from the ECM increases the level of cyclin dependent kinase (CDK) inhibitors thereby preventing advancement to S phase of the cell cycle (Mettouchi et al., 2001).

In some cases, integrin signaling can promote cell cycle arrest (Walker and Assoian, 2005). For example, the expression of integrin β1C in mammalian cells increases the level of $p27^{KIP1}$, a CDK inhibitor(Fornaro et al., 1999). In contrast, lowering the level of $p27^{KIP1}$ allows activation of the CDK/cyclin complex and promotes the cell cycle transition from G1 to S (Guo et al., 1997). In these cells, adhesion to the ECM activates an E3 ubiquitin ligase that is essential for the degradation of $p27^{KIP1}$. The expression of SKP2, an important component of the SCF ubiquitin ligase (E3) complex, is also dependent on cell adhesion at the G1 to S transition(Carrano and Pagano, 2001). However, there is little information available about how integrin signaling regulates the level of cell cycle inhibitors like $p27^{kip1}$ *in vivo*.

The nematode *Caenorhabditis elegans* expresses only two integrins, PAT-3/INA-1 (Baum and Garriga, 1997) and PAT-3/PAT-2 (Gettner et al., 1995), which simplifies the analysis of genetic interactions between integrin and cell cycle control genes. Overexpression of *C. elegans* $p27^{kip1}$/CKI-1 has been found to induce growth arrest and the *cki-1* null mutation results in hyperplasia of tissues such as the hypodermis, the vulva and the intestine (Ambros, 1999; Feng et al., 1999). The disruption of *cki-1* also results in the production of extra distal tip cells from the somatic gonad lineage (Kostic et al., 2003;

Kostic and Roy, 2002). The activity of *cki-1* is regulated by the coordination of stage-specific cellular events such as binding to the CDK/cyclin complex and a sharp increase in expression at the late larval and young adult stages (Buck et al., 2009) (Boxem and van den Heuvel, 2001; Hong et al., 2008)

This study the role of *pat-3 (sp)* (previously known as pat-3(β1C)) builds on our previous work on the function of PAT-3 signaling in *C. elegans* (Xu et al., 2010). *pat-3 (sp) i*s a frameshift mutation in the splice acceptor region (ag to aa) that abolishes conserved interaction domains such as the NPxY motifs and creates a splice variant with an extra 19 amino acids. The *pat-3 (sp)* animals not only produce mutant pat-3, but also express the regular splice form due to utilization of an unusual splice acceptor (Xu et al., 2010). *pat-3(sp)* has similarities to the human β1B and β1C integrins. The *pat-3 (sp)* mutant is similar to β1B because it retains intron sequence in the mRNA and the transgenic line expresses mutant and normal splice forms simultaneously. The mutant is similar to the β1C variant in that it is expected to produce a longer PAT-3 lacking the NPxY motifs (Xu et al., 2010).

In this study, we assessed the role of *pat-3 (sp)* in cell cycle regulation. Briefly, in transgenic pat-3 rescued lines carrying *pat-3 (sp)* (Xu et al., 2010) *cki-1*::GFP (Hong et al., 1998) was upregulated and exhibited a distinct sub-nuclear localization compared to wild type animals. RNA interference analyses revealed that the localization and level of CKI-1 are mediated by focal adhesion molecules and the SCF E3 ubiquitin ligase complex (Deshaies and Joazeiro, 2009). Taken together, our findings suggest that integrin signaling, in conjunction with SCF E3 ligase complex activity, plays a crucial role in the localization and level of CKI-1 *in vivo*.

Result

β pat-3 (sp) increases CKI-1 levels and exhibits a distinct localization in nuclei

PAT-3 β integrin is expressed in virtually all tissues in the nematode *C. elegans* and is required for muscle development and function. Null mutations in *pat-3* cause a fully penetrant embryonic arrest due to defective muscle elongation (Gettner et al., 1995; Williams and Waterston, 1994a). Previously, we created a mutation at the intron 7 splice junction in the cytoplasmic tail of PAT-3 integrin [Figure 3.1] (Fornaro and Languino, 1997; Xu et al., 2010). The transgenic rescued line, *pat-3 (sp)*, is viable but exhibits cold-sensitive larval arrest with gonad and muscle defects. We found that *pat-3 (sp)* expresses the non-spliced as well as the spliced *pat-3* mRNA, suggesting that mutant *pat-3* might inhibit the function of wild type *pat-3*. In this study, we have expanded our analysis to investigate the molecular function of *pat-3 (sp)* (Jannuzi et al., 2002; Jannuzi et al., 2004).

Studies of the mammalian β1B and β1C integrins in mammalian cells revealed that expression of these integrins suppresses cell adhesion and proliferation and upregulates the expression of $p27^{KIP1}$ (Giancotti and Ruoslahti, 1999). To investigate a potential linkage between PAT-3 and CKI-1, the *C. elegans* homolog of mammalian $p27^{KIP1}$ (Hong et al., 1998; Kostic and Roy, 2002; Philipp-Staheli et al., 2001), we created rescued transgenic lines containing *pat-3 (+)* or *pat-3 (sp)* genomic DNA by co-injecting with DNA encoding a CKI-1::GFP fusion protein (Hong et al., 1998; Xu et al., 2010).

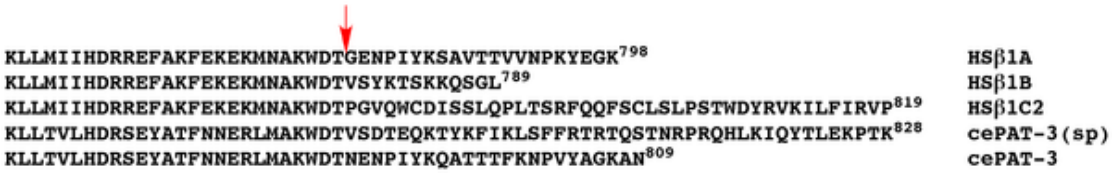

Figure 3.1. Sequences of the PAT-3 cytoplasmic tails. Wild type and mutant PAT-3 tails are compared to human β1A, β1B, and β1C cytoplasmic tails. Location of intron 7 is indicated by the red arrow.

As previously described (Hong et al., 1998; Kostic and Roy, 2002), CKI-1::GFP is expressed in hypodermal cells of late L4 and young adult animals. Nuclear expression is observed within an ER meshwork, typical of the hypodermal syncytium (hyp 7) [Figure 3.2A and 3.2B] (Hedgecock and White, 1985). In *pat-3 (+)* rescued animals, the appearance of CKI-1::GFP is a distinct fluorescent spot within a round green nucleus, suggesting nuclear and nucleolar localization [Figures 3.2A and 3.2C]. To substantiate our interpretation that CKI-1::GFP localizes to the nucleolus, the *ncl-1* gene, disruption of which results in enlarged nucleoli, was depleted using RNAi (Frank and Roth, 1998; Hedgecock and Herman, 1995). In the *pat-3 (+)* background, *ncl-1* (RNAi) significantly increased the size of the CKI-1::GFP spot. ImageJ analysis showed that the size of the green spot was increased by 2.4 fold when compared to that of control RNAi animal [Figure S3.1]. Therefore, we conclude that the observed spots on the nuclei are likely to represent nucleolar localization.

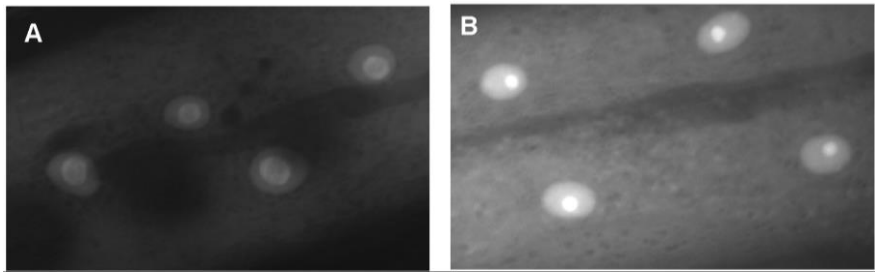

Figure S3.1. *ncl-1(RNAi)* increases the size of the nucleolus in CKI-1::GFP in *pat-3* transgenic animals. Panel A: *ncl-1(RNAi); pat-3(+).* The area of CKI-1::GFP is 2.4 times (P<.001) the size of the area seen in the no RNAi control in panel B: *CKI-1::GFP* in *pat-3(+)* background.

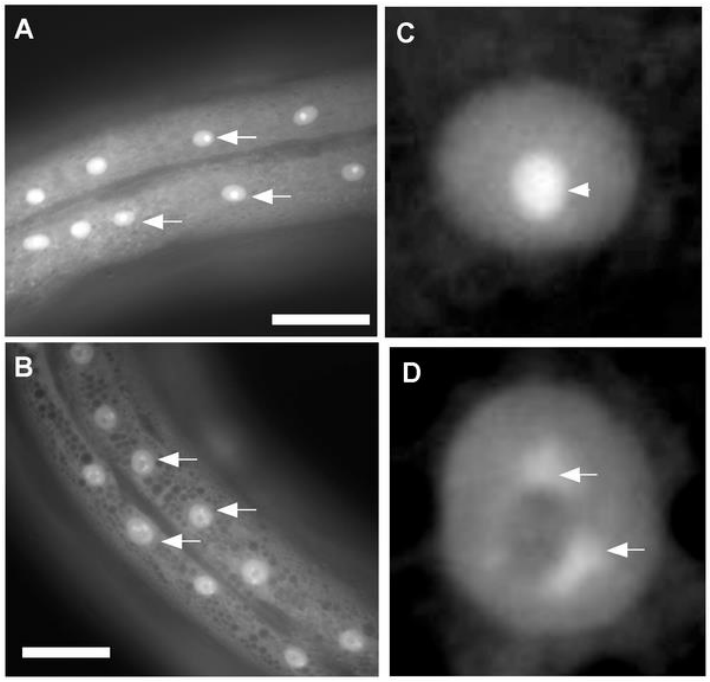

Figure 3.2. CKI-1::GFP is localized to the nucleus and nucleolus in pat-3 transgenic rescued animals. CKI-1::GFP transgenic animals were examined using fluorescence microscopy. Panels A and B show mid-body regions of *pat-3 (+)* and *pat-3 (sp)* rescued animals, respectively. Arrows indicate the nuclei of hypodermal cells at early adult stages. Panels C and D depict a hypodermal nucleus in *pat-3 (+)* and *pat-3 (sp)* worms, respectively. CKI-1::GFP appeared to be nucleolar (arrow heads) in *pat-3 (+),* while the CKI-1::GFP appeared clumped in *pat-3 (sp)* nuclei (arrows). Scale bar = 50 μm.

In the *pat-3 (sp)* rescued animals, CKI-1::GFP localization was visibly different from that seen in *pat-3(+)* animals. In contrast to the compact, nucleolar staining seen in *pat-3(+)* animals, CKI-1::GFP in *pat-3(sp)* was clumped and accumulated in a ring

around a dark center in the nucleus [Figures 3.2B and 3.2D], suggesting mislocalization and possible exclusion from the nucleolus. In addition, the intensity of green fluorescence in *pat-3 (sp)* was increased compared to *pat-3(+)*. In order to test for a possible correlation between the level of CKI-1::GFP and the integrin (*pat-3(+)* or *pat-3(sp)*) expressed, we first analyzed the amount of CKI-1::GFP in the *pat-3* rescued lines. Protein lysates were prepared from an equal number of L4/young adult transgenic animals and tested for CKI-1::GFP protein levels. CKI-1::GFP level in *pat-3(sp)* was ten fold more intense than that seen in *pat-3(+)* lysates [Figure 3.3A], suggesting that PAT-3 signaling may control CKI-1 levels.

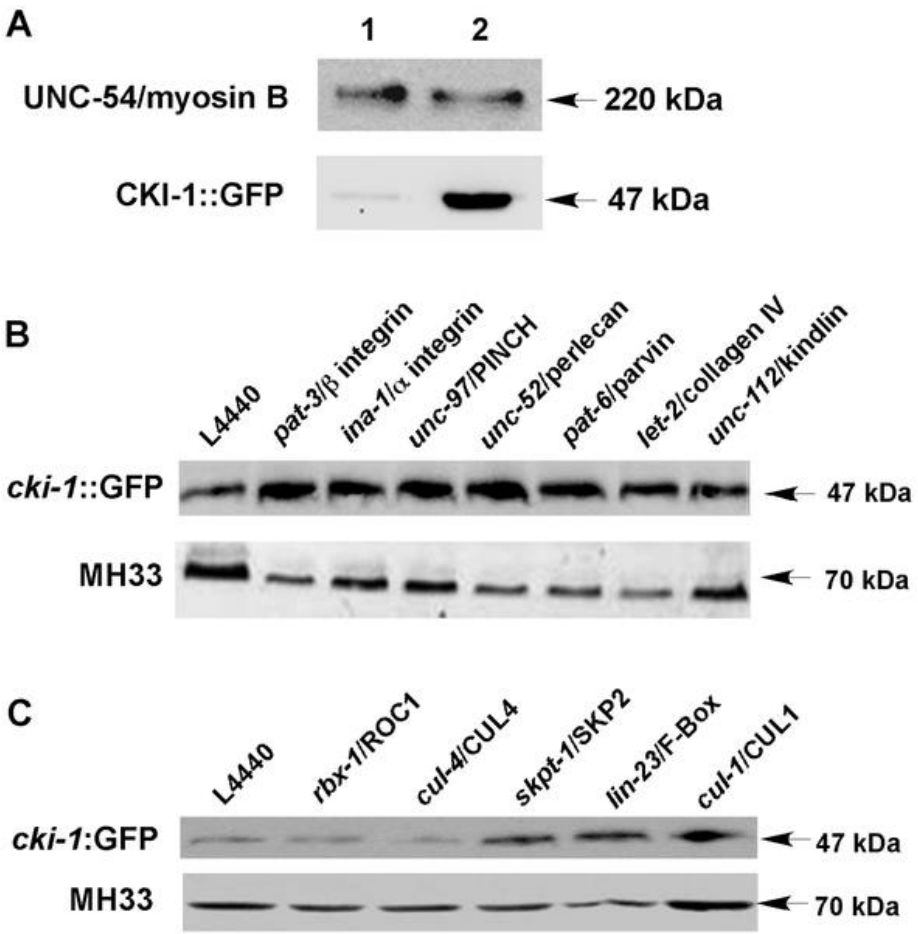

Figure 3.3. Immunoblot analysis of CKI-1::GFP protein in transgenic animals. Panel A: CKI-1::GFP expression levels were assessed in the transgenic rescued lines. Top bands in lanes 1 and 2 show the relative level of UNC-54/myosin B in each sample. Bottom bands indicate the level of CKI-1::GFP. Lanes 1 and 2 represent *pat-3 (+)* and *pat-3 (sp),* respectively. Quantification (Table S1) using ImageJ software revealed that CKI-1::GFP level was 10-fold increased in *pat-3(sp)* animals compared to *pat-3(+)* animals. Panel B: CKI-1::GFP expression levels were assessed in *pat-3(+)* animals treated with RNAi directed against focal adhesion genes. Top bands represent the amount of CKI-1::GFP in extracts prepared from each RNAi condition. L4440 is a negative RNAi control. The *pat-3, ina-1, unc-97, unc-52, pat-6* and *let-2* RNAi caused upregulation of CKI-1::GFP, while unc-112 RNAi had no effect. Bottom bands indicate MH33 [90] levels in each lane as a loading control. Quantification (Table S1) using ImageJ software revealed that CKI-1::GFP level was increased by RNAi of *pat-3, ina-1, unc-97, unc-52, pat-6, and let-2.* Panel C: CKI-1::GFP expression levels were also measured in *pat-3(+)* animals treated with E3 ligase gene RNAi. Top bands represent the amount of CKI-1::GFP in the extracts prepared from each RNAi condition. L4440 is a negative RNAi control. The *skpt-1, lin-23* and *cul-1* RNAi depletions caused upregulation of CKI-1::GFP, while rbx-1 and cul-4 RNAi had no effect. Bottom bands indicate MH33 levels in each lane as a loading control. Quantification (Table S1) using ImageJ software revealed that the CKI-1::GFP level was increased by RNAi of *skpt-1, lin-23* and *cul-1*.

Because the immunoblot results revealed that *pat-3 (sp)* animals produced more CKI-1::GFP protein than *pat-3(+)*, we next assessed the effect on *cki-1* transcription. RNA from each rescued line was isolated and analyzed for the amount of *pat-3* or *cki-1* mRNA using RT-PCR (Figure 3.4A). We also measured the *cki-1* mRNA level in BU7221, a *pat-3 (sp)* rescued line without *cki-1*::GFP (Lee et al., 2001b; Xu et al., 2010; Xu et al., 2005). No significant differences were seen in any of the experiments, suggesting that *pat-3 (sp)* does not significantly increase the level of *cki-1* mRNA compared to controls [Figure 3.4B].

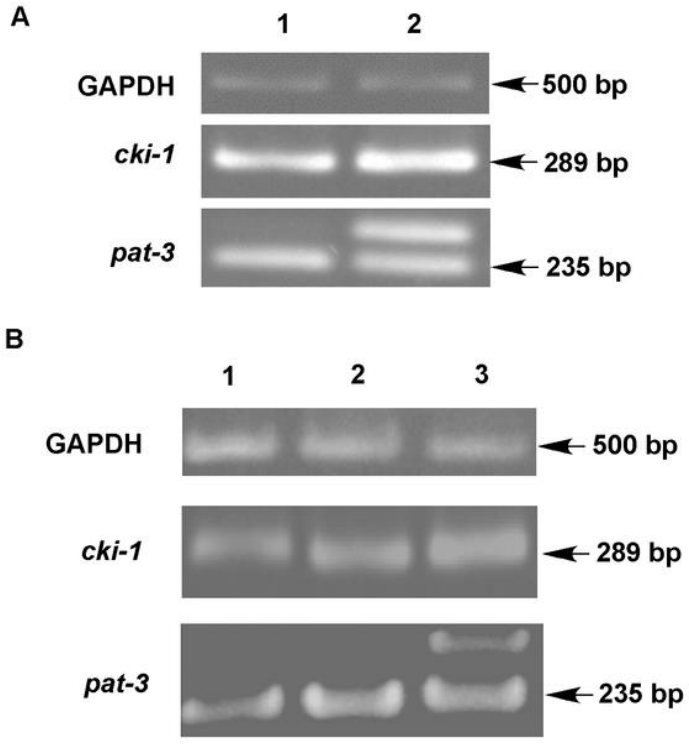

Figure 3.4. RT-PCR analysis of cki-1 mRNA in cki-1::GFP transgenic and βpat-3 rescued lines lacking cki-1::GFP. Panel A: Lanes 1 and 2 display the level of mRNA in *pat-3 (+)* and *pat-3 (sp)* animals, respectively. The GAPDH (glyceraldehyde-3-phosphate dehydrogenase) gene, *gpd-1*, was used as an mRNA loading control. The level of each transcript was measured using ImageJ software. Panel B: *cki-1* mRNA was measured in βpat-3 rescued lines lacking the CKI-1::GFP construct. Lanes 1–3 are N2, JE443 *pat-3 (+),* and BU7221 *pat-3 (sp).* The GAPDH gene, *gpd-1*, was used as an mRNA loading control.

Integrin Signaling Regulates CKI-1 Localization

In order to define genetic pathways that link PAT-3 integrin to CKI-1, a series of RNAi experiments were performed. We hypothesized that the integrin effect on the localization of CKI-1::GFP in *pat-3(sp)* would be mediated by genes that interact with the cytoplasmic domain of β integrin. Thus, candidate genes were selected from known focal adhesion components (Moerman and Williams, 2006). Previous analysis of embryonic muscle development identified 20 essential genes, mostly encoding components of dense bodies and M-lines, which are analogous to focal adhesions (Moerman and Williams, 2006). Integrins are located in the base of these structures and anchor the sarcomeres to the basement membrane. Data from the SAGE database indicated ten of the integrin signaling genes were expressed in the hypodermis. To screen for the genes involved in CKI-1 localization, we tested if RNAi depletion of these integrin signaling components

In *pat-3 (+)* animals, *pat-3 (RNAi)* resulted in CKI-1::GFP accumulation in the nucleoplasm similar to that seen in *pat-3(sp)* [Figures 3.5B and 3.2D]. Next, integrin α subunits were depleted. Depletion of *ina-1* in the *pat-3 (+)* animals also resulted in abnormally clumped CKI-1::GFP [Figures 3.5C], suggesting that the CKI-1 localization is integrin dependent. Among the focal adhesion genes, *pat-4*/ILK, *pat-4*/ILK (Mackinnon et al., 2002), *unc-97*/PINCH (Hobert et al., 1999), and *pat-6*/parvin (Lin et al., 2003a) together form an IPP complex, which is implicated in the control of signaling pathways by the phosphorylation of downstream targets (Wickstrom et al., 2010). RNAi of *pat-4*/ILK or *unc-97*/PINCH in *pat-3 (+)* resulted in the expected uncoordinated phenotypes [Figure S 3.2] and CKI-1 mislocalization in hypodermal nuclei [Figure 3.5D

and 3.5E]. However, in *pat-6*/parvin RNAi animals, CKI-1 maintained its wild-type localization, possibly suggesting that the CKI-1 localization is independent of parvin [Figure 3.5F]. Because *pat-6* RNAi did not result in a strong uncoordinated phenotype [Figure S3.2], it is possible that the *pat-6* RNAi is not as effective as the RNAi to *pat-4* and *unc-97*. However, our data is consistent with the interpretation that ILK and PINCH are mediating integrin signals to control CKI-1 localization in the nucleus.

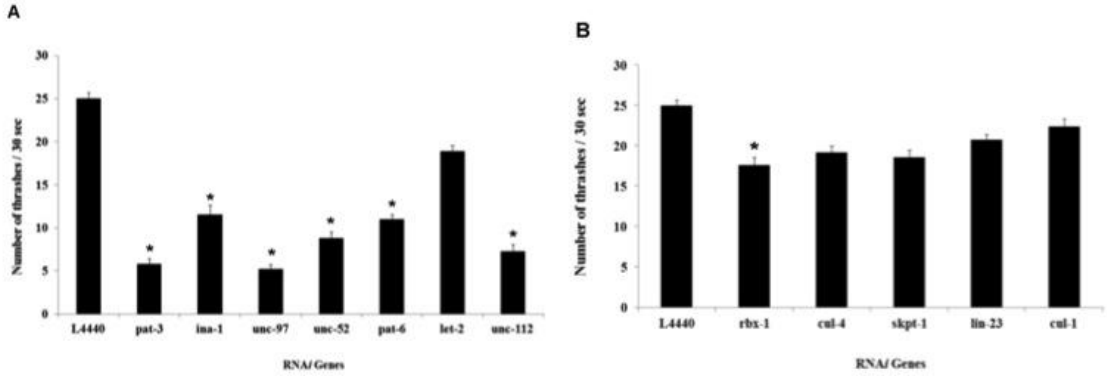

Figure S3.2. Locomotion defects of RNAi animal. Panel A: Number of body bends in 30 seconds was measured in *pat-3(+)* animals treated with RNAi of *pat-3, ina-1, unc-97, unc-52, pat-6, let-2*, and *unc-112* genes. The number of body bends was compared to that of L4440, the negative control RNAi. Black bars indicate the average number of body bends for each RNAi tested. Horizontal bars indicate the standard error of each test. N = 10. *indicates P<.0001 (compared to L4440). Panel B: Number of body bends in 30 seconds was measured in *pat-3 (+)* animals treated with RNAi of *rbx-1, cul-4, skpt-1, lin-23, and cul-1* genes. The number of body bends was compared to that of L4440, the negative control RNAi. Bars indicate the average number of body bends for each RNAi tested. Horizontal bars indicate the standard error of each test. N = 10. *indicates P<.0001 (compared to L4440).

Our RNAi screen also found that *unc-52*/perlecan, a basement membrane component and presumptive integrin ligand (Merz et al., 2003; Rogalski et al., 1995; Rogalski et al., 1993), is required for the proper localization of CKI-1. RNAi of *unc-52* in *pat-3 (+)* affected the CKI-1 localization pattern [Figure 3.5G]. In contrast, depletion of other basement membrane components, such as *let-2*/collagen IV (Sibley et al., 1994),

failed to affect the localization [Figure 3.5H], suggesting that a subset of ECM components is required for CKI-1 localization.

Table 3.1. RNAi analysis of genes involved in localization pattern

Tested Gene	*pat-3(+)*	*pat-3(sp)*	*SAGE search in hypodermis**
pat-3/ β integrin	++ (80)	+++ (80)	Positive
ina-1/ α integrin	++ (80)	+++ (50)	Positive
pat-4/ ILK	++ (35)	+++ (35)	Positive
unc-97/ PINCH	+++ (80)	+++ (80)	Positive
pat-6/ parvin	0 (60)	+++ (60)	Positive
unc-52/ perlecan	++ (50)	+++ (45)	Positive
epi-1/ laminin α	0 (15)	+++ (15)	Positive
let-2/ collagen IV	0 (20)	+++ (20)	Positive
unc-112/ kindlin	0 (40)	+++ (40)	Positive
Y71G12B.11*/talin*	0 (35)	+++ (35)	Positive
lin-23/ F-Box	++ (60)	+++ (60)	Positive
cul-1/ CUL1	++ (80)	+++ (70)	Positive
skpt-1/ SKP2	++ (70)	+++ (70)	Positive
rbx-1/ ROC1	0 (20)	+++ (20)	Positive
cul-4/ CUL4	0 (30)	+++ (30)	Positive
L4440 (vector)	0 (200)	+++ (200)	N/A

% mislocalization refers to animals with mislocalization out of total animals observed. (n) = the number of animals examined.

Ubiquitin-Mediated Protein Degradation Regulates Localization of CKI-1: GFP

Next, we investigated the mechanism by which integrin regulates CKI-1 protein levels without affecting RNA levels. One plausible explanation is that integrin signaling leads to the degradation of CKI-1(Carrano and Pagano, 2001). Integrin-triggered $p27^{KIP1}$ degradation has been observed in mammalian cells. For example, integrin crosstalk with receptor tyrosine kinase (RTK) induces the production of SCF^{SKP2} (Jonason et al., 2007), a member of the SCF E3 ubiquitin ligase complex, which binds to the SKP1 (Bai et al., 1996), CUL1 (Lyapina et al., 1998), and FBX-1 (Carrano and Pagano, 2001) E3 ligase

complex. This SCF complex targets CDK/cyclin inhibitors such as $p27^{kip1}$ and $p21^{cip1}$ (Carrano et al., 1999).

We hypothesized that the SCF complex might play a similar role in the localization and level of CKI-1 in response to integrin signals. To test this hypothesis, we performed RNAi analysis of *skpt-1*/SKP2 (Kim and Kipreos, 2007), *cul-1*/CUL1 (Kipreos et al., 1996), and *lin-23*/F-Box (Hebeisen and Roy, 2008) and monitored CKI-1 localization [Figure 3.5I, 3.5J, and 3.5K]. We first examined *skpt-1/*SKP2 (RNAi) in the *pat-3 (+)* background. CKI-1::GFP accumulation in the *pat-3 (+)*; *skpt-1* (RNAi) nucleoplasm was almost identical to that seen in *pat-3 (sp)* animals [Figure 3.5I]. Similar results were obtained in *cul-1* and *lin-23* RNAi in the *pat-3 (+)* strain [Figure 3.5J and 3.5K], suggesting ubiquitin-mediated protein degradation is responsible for the proper localization of CKI-1. RNAi of another E3 ligase complex gene, *rbx-1/ROC1(Carrano and Pagano, 2001),* did not alter the localization pattern of CKI-1 [Table 3.1]. Studies have also suggested that the DDB-1/CUL-4 associated factors (DCAF) complex is responsible for p27 degradation in mammals and *Drosophila* (Higa et al., 2006). Our analysis of *cul-4 (RNAi)* treated *pat-3(+)* animals suggests that this complex is unlikely to be involved in CKI-1 localization in *C. elegans* [Table 3.1]. Taken together, our RNAi analyses suggested that the members of SCF complex play a role in the CKI-1 localization.

Immunoblot Analysis Demonstrates a Correlation between CKI-1 Overexpression and Mislocalization

We next investigated whether depletion of the focal adhesion and SCF complex genes would affect expression levels of CKI-1/$p27^{KIP1}$ in addition to affecting nuclear

localization patterns (Figures 3.5; Table 3.1). Immunoblot analyses of CKI-1::GFP were performed using protein extracts from RNAi-treated adult animals [Figure 3.5A and 3.5B]. The amount of CKI-1::GFP generally increased in protein extracts of focal adhesion and SCF complex RNAi treated animals. Depletion of focal adhesion genes such as *pat-3, ina-1, unc-97, unc-52, pat-6* and *let-2* resulted in an up to 3-fold increase in CKI-1::GFP levels [Figure 3.3B]. RNAi of SCF E3 ligase genes such as *skpt-1, lin-23,* and *cul-1* also produced significant increases in CKI-1::GFP levels. [Figure 3.3C]. Interestingly, even though nucleolar localization was not affected by *pat-6* and *let-2* RNAi, protein levels were significantly increased [Figure 3.3B]. Our analysis suggested that disruption of integrin signaling or SCF–mediated protein degradation can result in the mislocalization as well as increased expression of CKI-1::GFP. However, the *let-2* and *pat-6* RNAi results suggest there may not be a direct relationship between protein levels and localization.

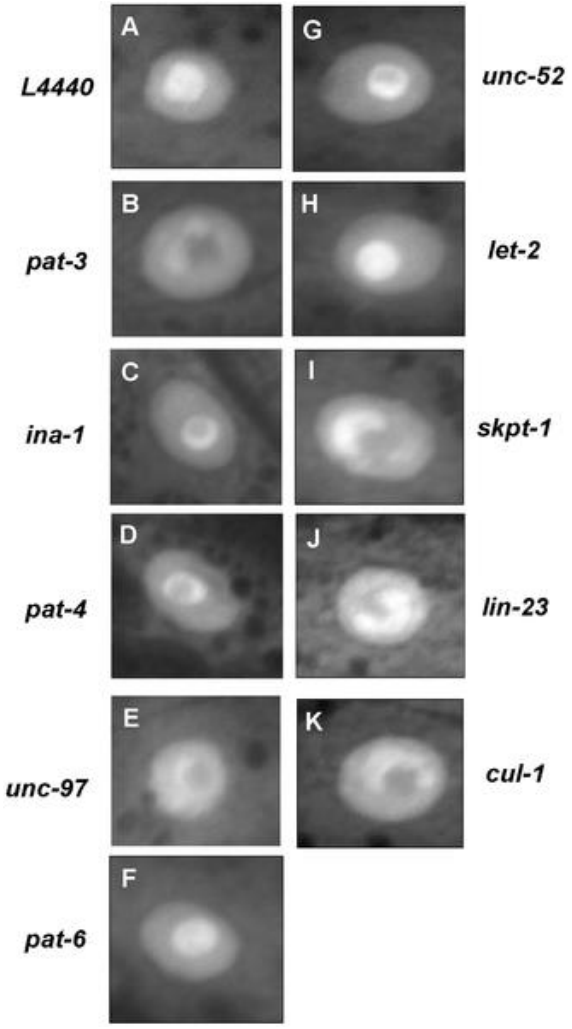

Figure 3.5. RNAi analysis showed that some focal adhesion and E3 ligase genes are required for CKI-1::GFP localization. Panels depict the results of RNAi of focal adhesion genes, dense body or M-line components, on the localization of CKI-1::GFP.

Discussion

In this study, integrin regulation of CKI-1 was assessed *in vivo*. Our analysis revealed that CKI-1/p27^{kip1} had an abnormal localization pattern in the nucleoplasm of animals expressing a mutant integrin, *pat-3 (sp)*. In the mutant animals, CKI-1::GFP was overexpressed and clumped in the nucleoplasm, while in animals expressing wild type integrin, CKI-1::GFP was localized predominantly to the nucleolus [Figure 3.2]. Further studies revealed that the amount of CKI-1::GFP protein was increased in the *pat-3* mutant.

To delineate the genetic pathway responsible for the upregulation of CKI-1/p27^{KIP1}, we depleted focal adhesion and SCF E3 ubiquitin ligase genes in *pat- 3(+)* animals and found that these genes are essential for the proper localization and expression of CKI-1::GFP. We conclude that the inhibition of integrin signaling and protein degradation significantly affects CKI-1 protein localization and expression level *in vivo*.

Previous studies have suggested a link between the mammalian integrin splice variant β1C and p27^{kip1}. For example, increased expression of β1C integrin elevated p27^{KIP1} in prostate cancer cell lines (Fornaro et al., 2001; Fornaro et al., 1998; Moro et al., 2004). Our *pat-3 (sp)* mutant splice form is an artificial variant (Xu et al., 2010). However, our study found that *pat-3(sp)* behaves similarly to the β1C variant of mammalian β1. *pat-3(sp)* increases CKI-1/p27^{kip1} expression, possibly leading to a cell cycle arrest.

CKI-1 is Localized to the Nucleolus

Our observations of nuclear morphology and experiments using *ncl-1* (RNAi) strongly suggest a nucleolar localization for CKI-1::GFP in *pat-3(+)*. In contrast, CKI-1::GFP appeared clumped and was irregularly distributed in the nucleoplasm of *pat-3(sp)* animals, suggesting ectopic accumulation of CKI-1. This nucleolar to nuclear transition in CKI-1::GFP localization suggests a possible link between CKI-1 and nucleolar function.

The nucleolus is the main site of ribosome biogenesis and ribosomal RNA (rRNA) synthesis (Lo et al., 2006). The rRNAs are synthesized and assembled into a ribosomal complex in the nucleolus. In mammals, stressed ribosomal synthesis leads to cell cycle arrest via increased p27^{kip1} levels (Juan and Cordon-Cardo, 2001). In addition,

mutations in the cytoplasmic tail of β4 integrin or p27 β4 binding protein ($p27^{BBP/\ eIF6}$) result in an inability of β4 to localize to hemidesmosomes and a defect in assembly of the 80S ribosomal subunit, suggesting a connection between integrin signaling and ribosome biogenesis (Carmo-Fonseca et al., 2000). Although further studies are required, we speculate that the splice defective *pat-3 (sp)* integrin may cause a decrease in ribosome biosynthesis.

Proper CKI-1 localization is Linked to integrin and integrin-Associated Molecules

Integrins are αβ heterodimers and both subunits are necessary for integrin function. Therefore, depletion of the subunit would have the same effect as disruption of the β subunit, PAT-3. Indeed, CKI-1::GFP mislocalization and increased expression were observed when the α integrin gene *ina-1* was depleted by RNAi, suggesting that CKI-1 upregulation is a result of disrupted integrin function and that integrin activity is normally required for proper CKI-1 localization and protein levels. In addition, RNAi analysis of the IPP complex showed that *pat-4*/ILK (Mackinnon et al., 2002; Xu et al., 2006) and *unc-97*/PINCH (Hobert et al., 1999), not *pat-6*/parvin (Lin et al., 2003b), are also required for correct expression and/or localization of CKI-1. Some studies have suggested that these components act together and are degraded if *pat-4*/ILK or *unc-97*/PINCH is not present (Fukuda et al., 2003) but other studies, including ours, suggest these molecules may have independent roles (Clark et al., 2003; Zhang et al., 2002). In addition, PINCH or parvin binding to ILK is mutually exclusive, suggesting that PINCH might provide a different mode of signaling than parvin (Montanez et al., 2009; Wang et al., 2008). ILK has been shown to play an important role in cell proliferation in tissue culture (Grashoff et al., 2003) and PINCH is frequently upregulated in human cancers

(Wang-Rodriguez et al., 2002). Disruption of ILK or PINCH inhibits cell proliferation and increases the expression level of $p27^{KIP1}$ and pRb (Cruet-Hennequart et al., 2003), suggesting that cells might generally respond to perturbed cell adhesion by inducing $p27^{kip1}$ expression and halting the cell cycle. In addition, our analysis links the ECM ligand, *unc-52*/perlecan, to CKI-1 localization, suggesting that perlecan-bound integrin might regulate CKI-1 by activating *pat-4*/ILK and *unc-97*/PINCH.

SCF E3 ligase complex is Required for the Proper Localization of CKI-1

This study revealed that ubiquitin-mediated protein degradation plays a crucial role in regulating CKI-1/$p27^{kip1}$. RNAi of SCF E3 ligase genes resulted in mislocalization of CKI-1::GFP. The E3 ligase includes a scaffold protein (CUL-1/CUL1) that assembles the ubiquitin ligation complex along with ubiquitin transferase (RBX-1/ROC1), an adaptor (SKPT-1/SKP2), and a substrate-binding protein (LIN-23/F-Box). The SKP2/Cullins/F-box (SCF) E3 ligase complex is involved in the degradation of cellular proteins such as cell cycle inhibitors, transcription factors and other signaling effectors (Skaar and Pagano, 2009). It has been reported that phosphorylated $p27^{kip1}$ is degraded in an SCF-dependent manner and that the loss of SCF is associated with pathological conditions such as cancer (Chu et al., 2007; Skaar and Pagano, 2009; Tsvetkov et al., 1999). Our RNAi analyses showed that SKPT-1/SKP2 (Bai et al., 1996; Kim and Kipreos, 2007) and CUL-1/CUL1 (Kipreos et al., 1996; Lyapina et al., 1998; Tsvetkov et al., 1999) are required for the proper localization of CKI-1, while RBX-1/ROC1 showed no effect. This might indicate that SCF ligase forms a complex with a protein other than RBX-1 for the degradation of CKI-1.

Integrin Signaling may Influence the Cell Cycle by Regulating CKI-1 Level via SCF ubiquitin ligases

We propose a potential model for the role of integrin signaling in CKI-1 regulation. Our preferred model assumes the presence of functional integrin in the hypodermal cells. Integrin is activated by binding to ECM ligand, and signaling is initiated and propagated by molecules such as *pat-4*/ILK and *unc-97*/PINCH to SCF ligase which degrades CKI-1 [Figure 3.6]. *pat-3(sp)* may interfere with the formation of the SCF complex and the degradation of CKI-1 by inhibiting the function of wild-type PAT-3 integrins or by acting as a non-functional β subunit that significantly dilutes integrin signaling. SAGE analysis indicates that *pat-3* and *ina-1* are expressed in hypodermal cells (Griffith et al., 2005; Pleasance et al., 2003) consistent with a previously identified role for *ina-1/* α integrin function in hypodermis (Baum and Garriga, 1997). Although we have identified a role for the SCF complex in CKI-1 degradation, our work does not specifically address whether SCF activation is directly linked to integrin signaling in a linear manner, as displayed in the model [Figure 3.6]. Future genetic studies should determine the cell autonomy and epistatic relationships of the genes in the pathway from integrin to cell cycle control.

In addition to muscle and gonad morphogenesis, our work identifies another important function of integrin in *C. elegans*, the regulation of CKI-1/$p27^{kip1}$. This finding brings new insight onto cell cycle control. Integrins appear to regulate the level of $p27^{KIP1}$ via signaling mediators such as *pat-4*/ILK and *unc-97*/PINCH and to maintain SCF ubiquitin ligase activity. Importantly, *unc-52* RNAi produces a similar phenotype, suggesting that the cell-matrix interaction balances the amount of CKI-1 in the cell. This

information will be useful in understanding the mechanism on how the cell-ECM interaction regulates cell cycle progression.

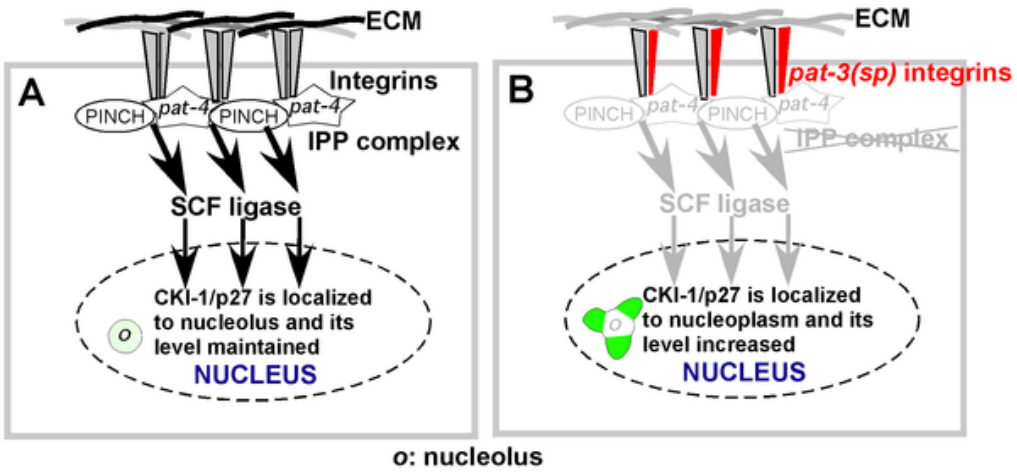

Figure 3.6. Model for the integrin regulation of CKI-1. When ECM ligand binds to integrin on the cell surface, the IPP complex delivers signals to the SCF E3 ligase complex resulting in sequestration of CKI-1/p27 to the nucleolus. In *pat-3 (sp),* the mutant β integrin interferes with the function of wild-type *pat-3(+).* Consequently, integrin does not signal to the IPP complex. We suggest the reduced integrin signals may leave the SCF complex inactive. This allows an increase in the amount of CKI-1/p27 and alters the location of the protein in the nucleus.

Materials and Methods

Mutant pat-3 Constructs and Germline Transformation

pPAT3 (+)-PB12K and mutant constructs for pPAT3-sp were created using overlap extension PCR and have been described previously (Xu et al., 2010). Germline transformation was performed using the standard protocol for microinjections (Mello et al., 1991). Briefly, pPAT3 constructs were mixed with *cki-1::GFP* (Hong et al., 1998) and *rab-3::RFP* or *myo-3::RFP* (Frokjaer-Jensen et al., 2008). All rescued lines were made at a mixture of 5 µg/ml of pPAT3, 2 µg/ml of pVT352G (*cki-1*::GFP), and 100 µg/ml of pGH8 (*rab-3::RFP)* or 100 µg/ml of pCFJ104 (*myo-3*::RFP) in TE buffer (pH 7.5). This mixture was injected into a distal gonad of the RW3600 qC1 *dpy-19(e1259)*

glp-1(q339)/pat-3(st564) III animal. F2 generation animals with 100% red progeny were isolated and more than 10 generations elapsed before the characterization of phenotypes and multiple lines were used for confirmation (Lee et al., 2001a).

Animals and Culture

Caenorhabditis elegans were cultured on nematode growth medium (NGM) agar plates seeded with OP50 *E. coli* under standard conditions (Brenner, 1974). RW3600 qC1 *dpy-19 (e1259) glp-1 (q339)/pat-3 (st564)* III (Williams and Waterston, 1994a) were acquired from the *Caenorhabditis* Genetics Center (St. Paul, MN). The *pat-3* transgenic rescued lines used in this study are listed in Table 3.2. All transgenic lines were cultured under standard conditions (Brenner, 1974).

Table 3.2. Transgenic *C. elegans* used in this study

Mutant designation	Constructs injected	Reference
*pat-3(+) rab-3::RFP, cki-1::GFP (*BU444 *kqEx75)*	*pat-3(st564)*, pPAT3(+), pGH8 *rab-3*::RFP, pVT352G *cki-1*::GFP	This study
*pat-3(+) myo-3::RFP, cki-1::GFP (*BU445 *kqEx76)*	*pat-3(st564)*, pPAT3(+), pCFJ104 *myo-3*::RFP, pVT352G *cki-1*::GFP	This study
pat-3(+) rab-3::RFP (BU446 kqEx77)	*pat-3(st564)*, pPAT3(+), pGH8 *rab-3*::RFP	This study
pat-3(+) sur-5::GFP (JE443)	*pat-3(st564)*, pPAT3(+), TG96 *sur-5*::GFP	[Lee, 2001 #1095]
*pat-3(sp rab-3::RFP, cki-1::GFP (*BU7222 *kqEx73)*	*pat-3(st564)*, pPAT3-sp, pGH8 *rab-3::*RFP, pVT352G *cki-1::*GFP	This study
*pat-3(sp) myo-3::RFP, cki-1::GFP (*BU7223 *kqEx74)*	*pat-3(st564)*, pPAT3-sp, pCFJ104 *myo-3::*RFP, pVT352G *cki-1::*GFP	This study
*pat-3(sp) sur-5::GFP (*BU7221 *kqEx21)*	*pat-3(st564)*, pPAT3-sp, TG96 *sur-5*::GFP	[Xu, 2010 #9713]

Phenotype Characterization and Nuclei Expression Pattern Identification

To characterize rescued lines, young adult worms were mounted in a drop of M9 buffer containing 1% NaN_3 (Sigma Chemical Co., St. Louis, MO) or 0.5 mM levamisole

on a 24 X 60 mm coverslip coated with 4% agarose and examined on a Nikon TE2000-U Diaphot epifluorescence microscope. Images were captured using a CoolSnap *cf* monochrome camera (Roper Scientific, Tucson, AZ) and analyzed with Metavue imaging software (version 7.5, Molecular Devices Co., Downingtown, PA). Typically, CKI-1::GFP became visible at the late L4 stage. In the wild-type rescue *pat-3*(+) animals, GFP was apparently nucleolar: a strong green spot on a larger green nucleus. To photograph the image, the camera was set at the exposure time of 2000 milliseconds. However, in *pat-3(sp)* animals, the CKI-1::GFP expression was much brighter than in the *pat-3*(+). For *pat-3(sp),* images were taken at the exposure time of 300 milliseconds. About 20 hypodermal nuclei in the midbody, an area including vulva at the ventral midline, of each animal was observed for nuclear morphology characterization. Displayed images are patterns seen most commonly under the described conditions.

To analyze the size of nucleoli, the area was measured using ImageJ software (version 1.33, National Institute of Health, Rockville, MD) (Dello et al., 2007). To determine the nucleolar to nuclear ratio, the area value of nucleolus, a brighter spot on a nucleus, was divided by that of nucleus. Five measurements for each rescued line were averaged for comparison.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To analyze *cki-1*, *pat-3*, and *gpd-1*/GAPDH mRNA levels, animals were partially synchronized by isolating embryos using 20% alkaline hypochlorite solution. After 48 hours, forty L4 to young adult animals were picked into 10 µL of M9 buffer. RNA was extracted using 250 µL Tri-Reagent (Sigma-Aldrich, St. Louis, MO) and 50 µL chloroform (1/5 volume of Tri-Reagent) and RNA was precipitated from the extract with

isopropanol. Approximately 1 μg of total RNA was used to synthesize cDNA with Transcriptor Reverse Transcription Kit (Roche, Carlsbad, CA) primed with random hexamers in a total 20 μL reaction volume. Total of 1 μL cDNA was used in PCR amplification with *cki-1* primers, *pat-3* primers, and with control *gpd-1*/GAPDH primers previously described (Xu et al., 2010). Primer sequences listed below were used for amplification:

CKI1 Forward 2:	5'-GGAGTTCTACAGAACC-3'
CKI1 Reverse 2:	5'-CACCGGAGACAGCTTG-3'
PAT3PT Forward1:	5'-CTCAACGAAACTACACCCTGCC-3'
PAT3PT Reverse 1:	5'-TTAGTTGGCTTTTCCAGCGTATACTGG-3'

Immunoblot Analysis

For quantitative analysis of CKI-1 protein levels in each strain, we first picked 30 young adults into 10 μL of M9 buffer and 10 μL of 2X Laemmli Sample Buffer (Bio-Rad Laboratories, Hercules, CA) premixed with 1:1000 β-mercaptoethanol. Sample solutions were then boiled for 10 minutes at 100°C and electrophoresed through a 10% SDS-polyacrylamide gel at 180V for 1 hour. Isolated proteins bands were electrotransferred onto a nitrocellulose membrane (Whatman Ltd., Dassel, Germany) using wet transfer at 100V for 75 minutes in BSN transfer buffer with no methanol or SDS. This nitrocellulose membrane was then blocked for 1 hour in 5% milk solution at room temperature. Rabbit polyclonal IgG anti-GFP antibody (ab290, Abcam Inc., Cambridge, MA) at 1:2000 was applied overnight at 4^{o}C as primary antibody and goat anti-rabbit IgG HRP conjugated (ab6721, Abcam Inc., Cambridge, MA) at 1:5000 was applied for 1 hour at RT. For control blots, LS25, a monoclonal antibody against UNC-54, or MH33, a monoclonal antibody against a gut specific intermediate filament protein, were diluted at 1:1000 and 1:2000. The primary antibody solutions were applied and detected by the goat anti-mouse

IgG HRP conjugated (Sigma Chemical, Mo) secondary antibody. ECL chemiluminescence reagents (Thermo Fisher Scientific, Rockford, IL) were added to the membrane for 1 minute before exposure to the ULTRA-LUM gel imager (Ultra-Lum Inc., Claremont, CA) and analyzed with UltraQuant software (Ultra-Lum Inc., Claremont, CA). Individual band intensity was quantified using ImageJ software (version 1.66, National Institute of Health, Rockville, MD) that measured the integrated density of each band to analyze the intensity of bands.

RNA-Mediated Interference of Gene Expression (RNAi) Analysis

C. elegans RNA interference analysis was performed using the bacterial feeding method (Fire et al., 1998; Timmons et al., 2003). In addition to the standard RNAi protocol, we synchronized the stage of animals; embryos were collected using the standard 20% alkaline hypochlorite solution method. After washes, collected embryos were placed onto RNAi plates, which were incubated in 20°C for 3 to 4 days until young adulthood before characterization. About 20 hypodermal nuclei in the midbody of transgenic worms were observed for the characterization of nuclear morphology using Metavue software. To verify the efficiency, all RNAi animals were examined for behavioral phenotypes linked to the RNAi gene. For example, focal adhesion gene RNAi was subjected to thrashing assays because uncoordinated (Unc) phenotypes were previously reported for RNAi of these genes (Figure S2). Each RNAi-inducing plasmid (Geneservice, Hinxton, UK) used in this study was isolated and sequenced in order to verify the gene targeted by the construct.

Acknowledgements

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CHAPTER FOUR

The Nucleolar Localization of $p27^{kip1}$/CKI-1 reveals Nuclear Genes interacting with Integrin

Abstract

The mechanism by which cell-matrix interaction regulates the cell cycle remains incompletely understood. We have shown previously that the location and levels of *C. elegans* CKI-1/$p27^{kip1}$ depend on proper integrin signaling to SCF E3 ligase. In wild-type hypodermal cells, CKI-1::GFP localizes to nucleoli. However, when *pat-3* integrin signaling is disrupted by a expression of an incorrectly spliced variant, β*pat-3 (sp)* CKI-1 localizes to the nucleoplasm. To identify additional factors regulating the nucleolar localization of CKI-1, an RNAi screen was performed and 33 suppressor or enhancer genes of *pat-3 (sp)* were identified in the functional categories of RNA splicing and processing, chromatin binding and histone modification, nucleolus, cell signaling, heterochronic genes, unfolded protein response and degradation. In addition, RNAi of *dnc-1*, *mfap-1*, *hda-1*, or *mig-32* decreased overall CKI-1::GFP levels. Subsequent immunoblot analysis revealed that *lpd-7/pescadillo* RNAi decreased the protein level of PAT-3, and resulted in abnormal CKI-1::GFP nucleolar localization even in wild type animals, suggesting that LPD-1 normally balances the role of integrin in CKI-1 localization. These identified genes are new mediators of CKI-1 localization and can be placed in downstream or parallel of integrin signaling, therefore, these results provide new insights into the role of integrin in CKI-1/$p27^{kip1}$ regulation.

Introduction

Tissues maintain integrity by forming cell-cell interactions or adhering to the extracellular matrix (ECM). The cell-ECM interaction is mediated by integrins, αβ heterodimeric transmembrane receptors for ECM. In addition to providing attachment to the ECM, integrin has many other functions, including a cell cycle regulatory function. For example, cell attachment from the ECM increases the G1 to S transition in fibroblast (Assoian, 1997). An alternative splice form of integrin β1 subunits, β1C, is downregulated in prostate adenocarcinoma. Its overexpression has been shown to inhibit cancer cell proliferation, suggesting that β1C integrin downregulation might promote the proliferation of cancer cells (Manzotti et al., 2000). In prostatic adenocarcinoma, forced expression of integrin β1C inhibits cell growth and increases $p27^{kip1}$ protein amount, while β1A, a regular β1 splice form, maintains the level of $p27^{kip1}$ protein (Perlino et al., 2000).

$p27^{kip1}$ is a well-characterized mammalian CDK inhibitor abundant in G1/G0 and degraded as the cell entered S phase. Upon activation of cyclin E/CDK2 in late G1/S phase, $p27^{kip1}$ becomes unstable and is degraded by the SCF (Skp1-Cullin-F-box protein)-SKP2-dependent E3 ubiquitin ligase complex (Carrano and Pagano, 2001; Hershko et al., 2001). The $p27^{kip1}$ localization is also changed by cell cycle progression and transiently translocated from nucleus during G0 to the cytoplasm at the G1/S transition. In early G1, mitogens induce $p27^{kip1}$ phosphorylation and lead its nuclear export, which incapacitates $p27^{kip1}$ and releases CDK2 inhibition. In addition, $p27^{kip1}$ plays an important role as a transcriptional repressor forming a complex with histone deacetylase (HDAC1) and E2F4 to regulate the expression of target genes that are

involved in a wide range of cellular functions (Pippa et al., 2012). It also controls cell migration, independently of its role as a CDK inhibitor, by binding to RhoA to inhibit its activation (Assoian, 2004; Besson et al., 2004).

Our previous study found that the *pat-3* β integrin splice mutant, *pat-3 (sp)*, increased *C. elegans* CKI-1 /$p27^{kip1}$ levels and resulted in a distinct re-localization of p27 within the nucleus. *pat-3 (sp)* is a frameshift mutation in the splice acceptor of the seventh exon and creates a longer cytoplasmic tail with an extra 19 amino acids as well as producing a small percentage of normally spliced transcript (Xu et al., 2010). *pat-3 (sp)* resembles the β1C splice form in that it creates a longer cytoplasmic tail than β1A and increases CKI-1/$p27^{kip1}$ levels. In animals expressing the wild type *pat-3 (+)*, CKI-1::GFP localizes primarily to nucleoli of hypodermal cells, while CKI-1::GFP is more highly expressed and appears clumped and disorganized in the nucleoplasm of the hypodermal cells expressing the *pat-3 (sp)* mutant (Kihira et al., 2012). Our RNAi analysis also indicated that *pat-3* is linked to *pat-4*/ILK (Mackinnon et al., 2002), *pat-6*/parvin (Lin et al., 2003b), and *unc-97*/PINCH (Hobert et al., 1999) complex as well as SCF family E3 ubiquitin ligase complex (Tsvetkov et al., 1999) in terms of the CKI-1 nucleolus localization.

The nucleolus of the eukaryotic cell is densely organized with pre-ribosomal RNAs for ribosome biogenesis. The main function of the nucleolus is the rapid production of ribosome subunits by pre-rRNA transcription, processing, and ribosomal RNP assembly (Lo et al., 2006). In *C. elegans*, the nucleolus is easily identified in the cell as a subcompartment within the nucleus.

Disrupted nucleolar organization or inhibiting rRNA synthesis may lead to "nucleolar stress" (Marquez-Lona et al., 2012). These conditions are pathogenic in humans. In fact, several human genetic disorders such as Bowen-Conradi syndrome (CHH), dyskeratosis congenital (DC), Diamond-Blackfan anemia (DBA), Shwachman-Diamond syndrome (SDS), and Treacher-Collin syndrome are originated from the defective human ribosomal proteins genes and their homolog are found in *C. elegans* (Lee et al., 2012). The defects of nucleoli in *C. elegans* have also been observed in *ncl-1* mutants, which have higher production rate of rRNA and exhibits an early appearance of nucleoli in embryos (Frank and Roth, 1998; Hedgecock and Herman, 1995).

Nucleolar localization of CKI-1::GFP in *pat-3 (+)* and the mislocalization in *pat-3 (sp)* prompted us to further analyze the nucleolus in *C. elegans*. To determine additional elements that contribute to CKI-1 localization, we performed an RNAi screen of nucleolar or nuclear genes and identified suppressors and enhancers of the *pat-3 (sp)* mislocalization phenotype. Our analysis revealed that integrin signaling is linked to the many genes, which act in nucleus, nucleolus, and in protein degradation.

Result

In order to investigate the role of integrin in cell cycle, the interaction of integrin to a cell cycle inhibitor, *cki-1*/p27^{kip1}, was studied. Our previous analysis revealed that *cki-1*/p27^{kip1} is localized to the nucleolus in hypodermis, while *cki-1*/p27^{kip1} was shifted to nucleoplasm and its protein level was increased in the *pat-3 (sp)* splice mutant (Figure 4.1). RNAi analyses found that the SCF E3 ubiquitin ligase is linked to *pat-3* integrin, in that the integrin splice mutation caused the increased level of *cki-1*/p27^{kip1} protein due to

the impaired protein degradation (Kihira et al., 2012). However, the mechanism of CKI-1 localization was remained elusive.

Localization of CKI-1::GFP Depends on pat-3 mRNA Species

CKI-1::GFP localizes to hypodermal nucleoli in L4 and young adult *pat-3 (+)* animals (Figure 1D). In the mutant *pat-3 (sp),* CKI-1::GFP accumulates significantly before the L4 stage as patchy clumps in the nucleus [Figure 4.1 A and 4.D]. RT-PCR analysis showed that *pat-3 (sp)* produces a mutant as well as a wild type mRNA species [Figure 4.1C]. In eukaryotes, mutant mRNA carrying premature termination codons or intron sequences usually undergoes rapid mRNA decay, known as nonsense mediated decay (NMD)(Lykke-Andersen et al., 2000). NMD in *C. elegans* is mediated by *smg* genes, which work together to remove unwanted RNA species (Pulak and Anderson, 1993). To determine the responsible integrin mRNA for the localization of CKI-1::GFP, *smg-1* gene (Grimson et al., 2004), which encodes a protein kinase required for NMD, was knocked down by RNAi in *pat-3 (sp)* animals. The *smg-1* RNAi animals showed disrupted CKI-1::GFP expression, with clumps localized to the nucleoplasm of hypodermal nuclei [Figure 4.1B; Table S1]. However, *smg-1* RNAi in *pat-3 (+)* failed to disrupt the nucleolar CKI-1::GFP localization. This result suggests that the mutant *pat-3 (sp)* mRNA might be a target of NMD and that the mutant mRNA from *pat-3 (sp)* is the cause of CKI-1::GFP mislocalization. To verify that the mutant message is a target of NMD, mRNA from *pat-3 (sp)*; *smg-1* RNAi animals was isolated and the amount of mutant *pat-3* mRNA was measured. The mutant RNA increased more than 4 times compared to that of the control [Figure S4.1], confirming that the incorrectly spliced message produced by *pat-3 (sp)* mRNA is degraded by NMD.

PAT-12 is Linked to CKI-1::GFP Localization

The localization patterns of CKI-1::GFP in *pat-3 (+)* or *(sp)* animals [Figure 4.1] prompted us to search for genes involved in CKI-1 localization. Our previous analysis showed that RNAi of SCF E3 ubiquitin ligase or cell adhesion proteins such as *pat-4*/ILK, *pat-6*/parvin, or *unc-97*/PINCH, phenocopied *pat-3 (sp)(Kihira et al., 2012)*, suggesting that the patchy appearance of CKI-1::GFP in the *pat-3 (sp)* mutant could be linked to the specific signaling pathways involved in cell adhesion or protein degradation.

To address this, we attempted to suppress the clumpy nuclear GFP phenotype in hypodermal nuclei. PAT-3 integrin was isolated in a genetic screen of genes required for embryonic muscle elongation (Williams and Waterston, 1994a) because *pat-3* integrin was isolated from a genetic screen to isolate genes required for embryonic muscle elongation (Lee et al., 2001b). Our initial approach was to test all *pat* (paralyzed arrested at two-fold) genes because many of them interact genetically and are organized into a linear pathway. It is reasonable to assume that there might be a downstream *pat* gene linked to CKI-1.

We tested the role of the *pat* genes, *pat-5(Laine et al., 2011)*, *pat-8* (Barstead and Waterston, 1991), *pat-9 (Liu et al., 2012), pat-10* (Terami et al., 1999), and *pat-11(Williams and Waterston, 1994a) ,* in the CKI-1::GFP localization. RNAi of *pat* genes revealed that only *pat-12* RNAi suppressed the nuclear localization in *pat-3 (sp)* [Figure 4.2D]. PAT-12, a hemidesmosome component, is expressed in the hypodermis. However, *pat-12* RNAi in *pat-3 (+)* background displayed no changes in the location of the GFP [Figure 4.2B], suggesting that there are potentially integrin signaling genes linked to the localization of CKI-1::GFP in hypodermis.

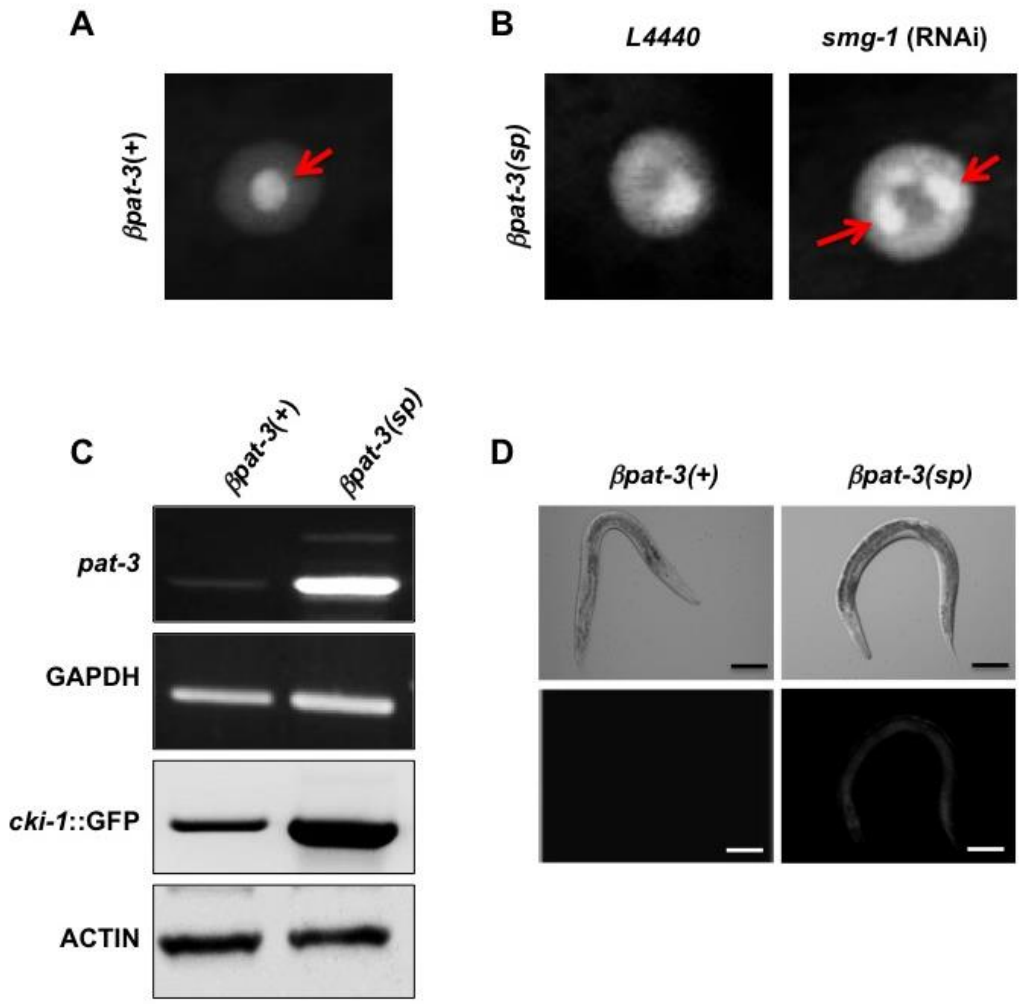

Figure 4.1. CKI-1::GFP expression and localization. A. CKI-1::GFP is localized to nucleolus in *pat-3 (+)* background (arrow). B. The GFP is scattered and clumped in the nucleus (extranucleolar nucleus) shown in *pat-3 (sp)* mutant. The *smg-1* RNAi enhanced the distribution pattern of CKI-1::GFP in *pat-3 (sp)*. C. It appears that *pat-3* integrin is elevated in *pat-3 (sp)* mutant (right), comparing to *pat-3 (+)* (left). The top two rows show the amount of *pat-3* mRNA detected in the worm extracts. Note that *pat-3 (sp)* produced mutant mRNA (arrow) larger in size than the regular message. GAPDH is a loading control. The bottom two rows represent immunoblots showing the level of CKI-1::GFP and actin, a loading control, in *pat-3 (sp)*. D. CKI-1::GFP started to appear in the L4 larval stage in *pat-3 (sp)*. Each animal represents *pat-3 (+)* or *pat-3 (sp)* at the same stage, early L4. The animal on the right, *pat-3 (sp)*, started to show the appearance of GFP at this stage. However, the green fluorescence was not detected in *pat-3 (+)* at this stage. Scale bars indicate 100 μm.

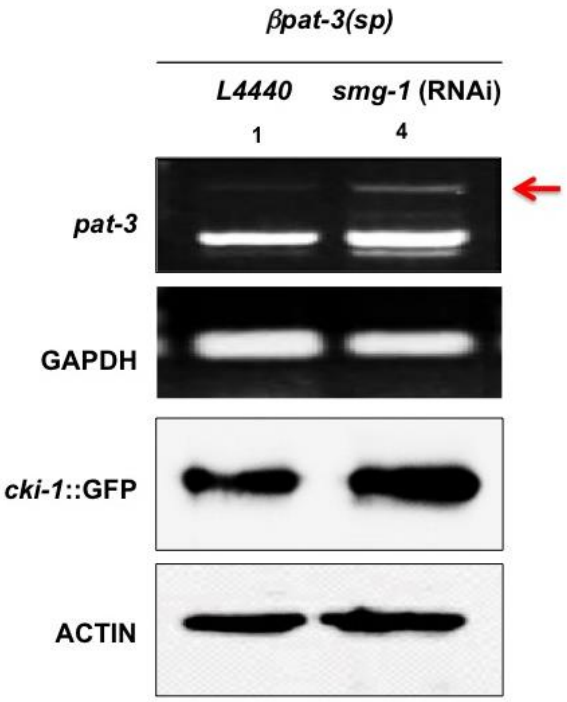

Figure S4.1. *smg-1* RNAi increase the level of CKI-1::GFP. *pat-3 (sp)* animals were treated with *smg-1* RNAi and showed the level of pat-3 mRNA and CKI-1::GFP.

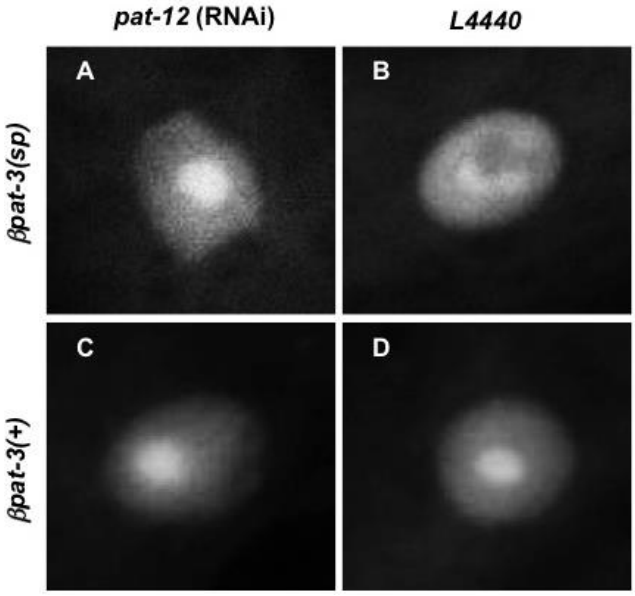

Figure 4.2. pat-12 RNAi suppresses the mislocalization of CKI-1::GFP. pat-3 (sp) animals were treated with pat-12 RNAi and displayed the nucleolar localization (A), compared to control RNAi (B). However, the same RNAi failed to affect the GFP localization in pat-3 (+), panels C and D.

RNAi screen to Identify the Genes Linked to CKI-1 Localization

The obvious nuclear localization of CKI-1::GFP in *pat-3 (+)* and *(sp)* [Figure 4.1 A and B] led us to investigate genes expressed in the nucleus (referred "nuclear genes" throughout this paper). To identify candidate genes involved in the CKI-1::GFP localization, we identified 133 genes tagged with the search term "nucleus" and "nucleolar" in WORMBASE (Chen et al., 2005). Among the selected genes, we were able to obtain 108 RNAi clones from the Ahringer library (Qu et al., 2011). Feeding RNAi was performed in *pat-3 (+)* and *pat-3 (sp)* backgrounds. RNAi clones that reverted the nuclear CKI-1::GFP of *pat-3 (sp)* to the proper nucleolar localization seen in *pat-3 (+),* and that showed no other severe behavioral or developmental defects were further characterized. For each RNAi, more than 10 animals were examined for CKI-1::GFP localization. Clones that provided a reverting phenotype, with normal nucleolar GFP localization, in more than 50% of hypodermal nuclei were regarded as positive.

RNAi of 33 nuclear genes reverted CKI-1::GFP localization in *pat-3 (sp)* to the pattern seen in pat-3 (+) what is the phenotype in N2 [Table 4.1; Figure 4.3]. The suppressor genes can be categorized into the seven functional categories [Table 4.2]. The first group (5 genes) is involved in RNA splicing and processing. The second group (7 genes) functions in chromatin binding and histone modification. The third group (8 genes) is involved in the nucleolus organization genes. The fourth and fifth groups include cell signaling (4 genes) and heterochronic genes (3 genes). In addition, a few unfolded protein response (UPR) and protein degradation genes (6 genes) were involved in the localization. We also performed RNAi of the suppressing genes in *pat-3 (+)* background to find whether these genes may cause the nuclear CKI-1::GFP defect in the

pat-3 (+) background. The RNAi of 32 clones failed to show any change in the CKI-1::GFP localization of *pat-3 (+)* [Figure 4.2 A and B].

RNA Splicing/Processing

This group includes 5 genes such as C12D8.1/single strand binding protein (Yook et al., 2012), *mfap-1/*microfibrillar-associated protein 1 (Ma et al., 2012), *rsp-6*/SRp20 splice factor (Loomis et al., 2009), ZK418.9/single-strand RNA binding protein (Yook et al., 2012) and *szy-20*/RNA binding protein (Song et al., 2008). Four genes appeared to possess a function in common, RNA splicing, including C12D8.1, which database information suggests is a single strand binding and potential splicing factor (Yook et al., 2012). The STRING protein-protein interaction database (Szklarczyk et al., 2011) suggests that C12D8.1 interacts with ZK418.9 and *szy-20*. *mfap-1* is a known RNA splicing factor (Ma et al., 2012). *rsp-6* is involved in RNA splicing and transcription (Huang and Steitz, 2001). These data suggest that *pat-3* signaling may affect RNA splicing in nucleus. Conversely, defective splicing in *pat-3 (sp)* may have increased the activity of C12D8.1, *mfap-1,* or *rsp-6* splicing factor, which led to the increased expression of CKI-1::GFP.

Table 4.1. Genes required for CKI-1::GFP localization

Sequence name	Gene	Functional Class	Number of positive cell (%)	Total number of nucleus
B0547.1	*csn-5*	Protein degradation	47 (33)	143
C12D8.1		RNA splicing/processing	77 (54)	143
C17G10.4	*cdc-14*	Cell signaling	131 (100)	131
C18E9.3	*szy-20*	RNA splicing/processing	49 (40)	123
C33H5.12	*rsp-6*	RNA splicing/processing	128 (100)	128
C48D5.1	*mcm-2*	Chromosome binding/ histone modification	77 (53)	145
C53A5.3	*hda-1*	Chromosome binding/ histone modification	145 (96)	151
C54D1.6	*bar-1*	Cell signaling	205 (100)	205
F11A10.3	*mig-32*	Chromosome binding/ histone modification	63 (50)	126
F19B6.2	*ufd-1*	ERAD and mitochondrial UPR genes	140 (100)	140
F26E4.10	*drsh-1*	Heterochronic genes	63 (46)	136
F28D1.1	*wdr-46*	Nucleolus organization	174 (89)	196
F29B9.2	*jmjd-1.2*	Chromosome binding/ histone modification	145 (99)	147
F43G9.10	*mfap-1*	RNA splicing/processing	92 (100)	92
F45E4.9	*hmg-5*	ERAD and mitochondrial UPR genes	109 (73)	149
F46F11.4	*ubl-5*	ERAD and mitochondrial UPR genes	99 (77)	129
F54C9.8	*ire-1*	Cell signaling	123 (80)	153
F58H12.1	*kin-29*	Cell signaling	90 (58)	155
K10G6.3	*sea-2*	Heterochronic genes	132 (100)	132
R13A5.12	*lpd-7*	Nucleolus organization	67 (51)	131
R186.4	*lin-46*	Heterochronic genes	62 (48)	130
T01C3.7	*fib-1*	Nucleolus organization	155 (100)	155
T03F1.9	*hcp-4*	Chromosome binding/ histone modification	77 (61)	127
T19A6.2	*ngp-1*	Nucleolus organization	81 (61)	133
T23F6.4	*rbd-1*	Nucleolus organization	146 (93)	157
W04B5.5		Cell signaling	72 (62)	117
Y52B11A.9		Nucleolus organization	91 (67)	135
Y56A3A.32	*wah-1*	ERAD and mitochondrial UPR genes	142 (88)	162
ZC8.4	*lfi-1*	Chromosome binding/ histone modification	142 (104)	142
ZK1127.5		Nucleolus organization	150 (89)	168
ZK265.6		Nucleolus organization	158 (85)	185
ZK418.9		RNA splicing/processing	59 (48)	122
ZK593.5	*dnc-1*	Chromosome binding/ histone modification	158 (100)	158

Chromatin binding/histone Modification Group

dnc-1/dynactin (Terasawa et al., 2010), *hcp-4*/mammalian centromere protein (Moore et al., 2005), *hda-1*/histone deacetylase (Ranawade et al., 2013), *jmjd-1.2*/histone demethylase (Kauffman et al., 2011) , *lfi-1*/LIN-5 binding protein (Lorson et al., 2000), *mcm-2*/replication licensing factor (Keyomarsi, 2008), and *mig-32/PRC1* histone ubiquitination (Karakuzu et al., 2009). The RNAi of these genes showed suppression of the *pat-3 (sp)* phenotype. This group can be divided into two subgroups by functions: DNA binding which includes *dnc-1*, *hcp-4*, *mcm-2*, and *lfi-1*; and histone modification which includes *hda-1*, *jmjd-1.2*, and *mig-32*. DNA binding subgroup involved in DNA replication, *hcp-4* and *mcm-2*, or chromosome movement during cell division, *dnc-1* and *lfi-1*, suggested that CKI-1::GFP localization is mediated by the machinery of DNA replication or cell division. The histone modification genes (*hda-1*, *jmjd-1.2*, and *mig-32*) are regulators of chromosome structural changes which modulate gene expression by preventing loosening nucleosome structure. Depletion of these genes may increase the expression of genes involved in the proper localization of CKI-1::GFP.

Nucleolus Organization

This category includes *lpd-7*/pescadillo (Lerch-Gaggl et al., 2002), *ngp-1*/nucleolar GTP binding protein (Chennupati et al., 2011), *rbd-1*/rRNA processing (Bjork et al., 2002), *wdr-46*/18S rRNA processing (Xie et al., 2007) , ZK265.6/nop16-like (Kundel et al., 2012), *fib-1*/methyltransferase for rRNA (Schousboe et al., 2008), ZK1127.5/3'-phosphate cyclase (Chakravarty et al., 2011), *cdc-14*/phosphatase(Saito et al., 2004) , and Y52B11A.9/DOX-1, a zinc finger RNA binding protein (Henis-Korenblit et al., 2010). This major group is expressed in the same compartment, involved in the

proper localization of CKI-1::GFP, and showed two different RNAi phenotypes. The 7 genes showed that the enhanced phenotype (Figure 3 U, V, DD to HH), CKI-1::GFP is clumped in the center of nucleolus as well as distributed over the cortex of nucleus, suggesting that these genes maintain the structure of nucleolus. However, *lpd-7* RNAi showed the typical nucleolar localization, suggesting that this gene could be mediating the nucleolar/nuclear shift of the CKI-1::GFP in the *pat-3 (sp)* background. The *cdc-14* RNAi also caused the nucleolar localization of CKI-1::GFP. CDC-14 is expressed in nucleolus (Saito et al., 2004) and known as a phosphatase promoting stability of CKI-1 levels in the nucleus leading to cell cycle arrest (Buck et al., 2009). Reduction of CDC-14 should cause a reduction of CKI-1 level.

Cell Signaling

The three genes in this category cover many different functions. The *bar-1* gene encodes a β-catenin, a downstream effector of WNT signaling pathway (Eisenmann et al., 1998). It can be explained that BAR-1 might be elevated in *pat-3 (sp)* due to the defective protein degradation and that led to elevation or translocation of CKI-1 in nucleus (Natarajan et al., 2004).

KIN-29, a serine/threonine protein kinase containing AMP-activated kinase (AMPK) domain, regulates body size and is linked to *dbl-1*/TGFβ (Maduzia et al., 2005). W04B5.5 is a phosphoinositide-independent AKT/PKB kinase (Yook et al., 2012). Both genes suppressed CKI-1::GFP localization in *pat-3 (sp).* These data suggested that *pat-3 (sp)* may have increased the capability of these genes to translocate the CKI-1::GFP to nucleus.

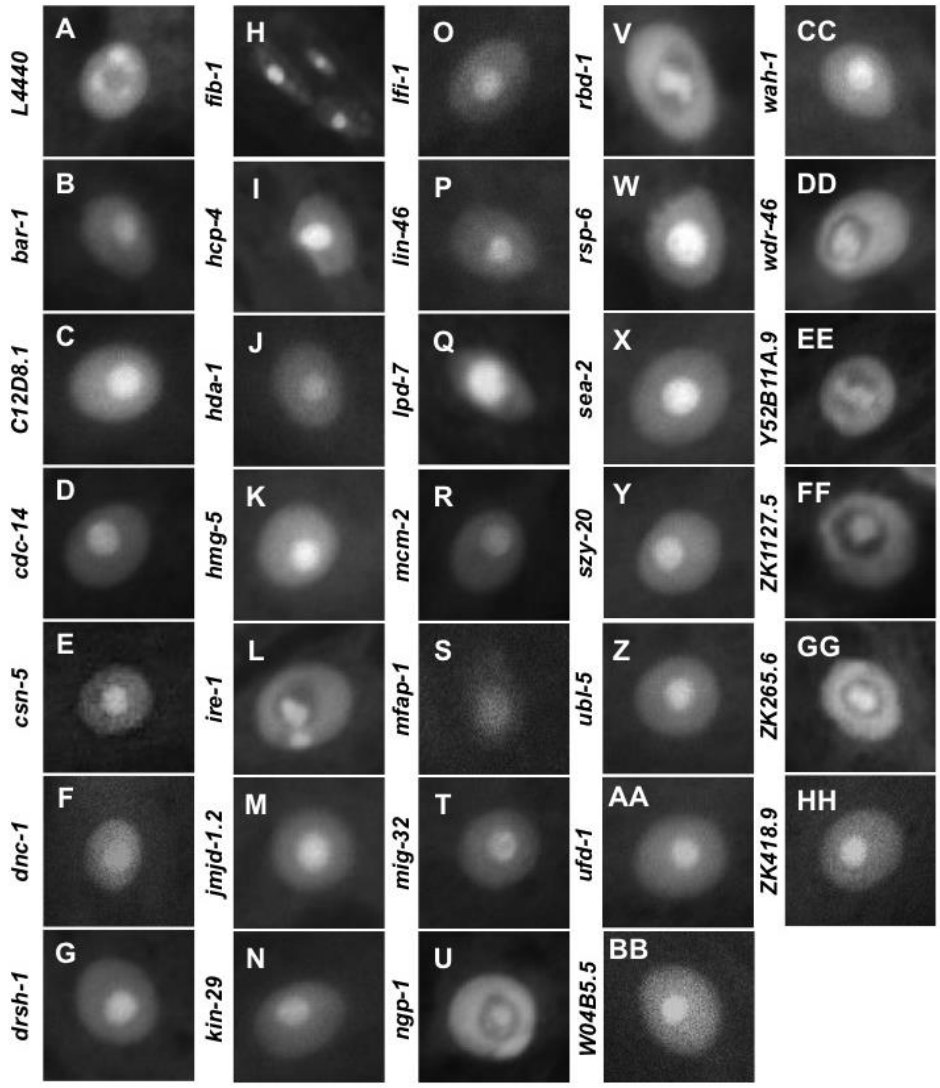

Figure 4.3. The localization of CKI-1::GFP with RNAi of suppressor and enhancer genes in *pat-3 (sp)*. A. L4440, a control RNAi. B to HH. On the left of each panel, the name of the RNAi gene was indicated. Each photograph is taken from representative animals.

Heterochronic Genes

drsh-1 is an endonuclease that cleaves primary miRNA to produce pre miRNA in nucleus (Warf et al., 2011). *sea-2* encodes for a zinc finger protein playing a role as an autosomal signaling element (ASE) during sex determination (Huang et al., 2011) as well as controls developmental timing by regulating *lin-28* (Moss et al., 1997). *lin-46* encodes a novel protein playing important role in developmental timing of hypodermal

development (Pepper et al., 2004). The RNAi of these genes suppressed the phenotype of *pat-3 (sp)* CKI-1::GFP localization. These genes are known to involve in microRNA processing and expressed in hypodermal cells. In addition, the heterochronic genes are altering the developmental timing of larval molts, so the timing of expression at L4 may be altered by their RNAi phenotype.

Protein Degradation and Unfolded Protein Response (UPR) Genes

Signalosome is an enzyme that removes NED-8/NEDD8 from target proteins (Cope et al., 2002). CKI-1 may be a target of NEDD8, an ubiquitin-like molecule that binds to a target protein. This reaction is called neddylation (Yang et al., 2002). In mammalian cells, deneddylation of SCF E3 ligase is essential for the degradation of $p27^{kip1}$ (Saha and Deshaies, 2008). This process is mediated by COP9 signalosome (CSN), a protease complex that regulates the activity of cullin-RING ligase (CRL) family E3 complexes (Lyapina et al., 2001). CSN-5 is a component of the *C. elegans* CSN. The knockdown of *csn-5* may stabilize CKI-1/$p27^{kip1}$ in nucleolus. In *C. elegans*, *ned-8* encodes for NEDD8 homolog and *ubc-12* is an NEDD8 conjugation enzyme. Depletion of *csn-5* or *ubc-12* results in defective neddylation-deneddylation of substrates (Deshaies et al.). Therefore, the *ubc-12* E2 conjugation enzyme was also knocked down in *pat-3 (sp)*. About 93% (N=?) of examined nuclei in *ubc-12* RNAi animals displayed nucleolar CKI-1::GFP, suggesting that protein neddylation is important in the proper localization of CKI-1 (Figure S4.2). We also measured the effect of *ned-8* RNAi but the knockdown animals showed severe behavioral defects unrelated to the localization of CKI-1::GFP

ufd-1/ubiquitin recognition protein is an adapter protein with CDC48/p92/VCP-specific activity in endoplasmic reticulum (ER)-associated degradation (ERAD)

(Dobrynin et al., 2011) . The localization of CKI-1::GFP appears linked to the UPR. *hmg-5/*mitochondrial transcription factor A (TFAM) produces a putative binding protein for mitochondrial DNA (Sumitani et al., 2011). *ubl-5* encodes for a ubiquitin-like protein specific for the degradation of mitochondrial unfolded proteins (mtUPR) (Aldridge et al., 2007). RNAi of *ubl-5* appears to suppress the nuclear localization CKI-1::GFP and mtUPR, suggesting that CKI-1 mislocalization may also cause a stress to mitochondria. *wah-1/*apoptosis-inducing factor (AIF) is a stress protein released from mitochondria to induce apoptosis and also is involved in nuclear DNA fragmentation, suggesting that *pat-3 (sp)* may be linked to apoptosis.

IRE-1 is a transmembrane serine/threonine protein kinase/site-specific endoribonuclease required for the unfolded protein response (UPR) in endoplasmic reticulumn (ER) (Safra et al., 2013). During the UPR, IRE-1 causes mRNA splicing of *xbp-1*, a bZIP transcription factor orthologous to X Box binding protein (Richardson et al., 2011). RNAi of *ire-1* caused the dispersed CKI-1::GFP over nucleolus and nucleoplasm, which resembles the RNAi phenotypes of nucleolus specific genes such as *ngp-1*, *wdr-46,* and Y52B11A.9. We then tested whether *xbp-1* [Table S1] affects the localization of CKI-1::GFP in nucleolus or not. *xbp-1* RNAi was performed in *pat-3 (+)* or *pat-3 (sp)* background [Figure S2]. The RNAi knockdown of *xbp-1* suppressed the nuclear CKI-1::GFP in *pat-3 (sp)*, suggesting that IRE-1/XBP-1 pathway can be linked to nucleolus function.

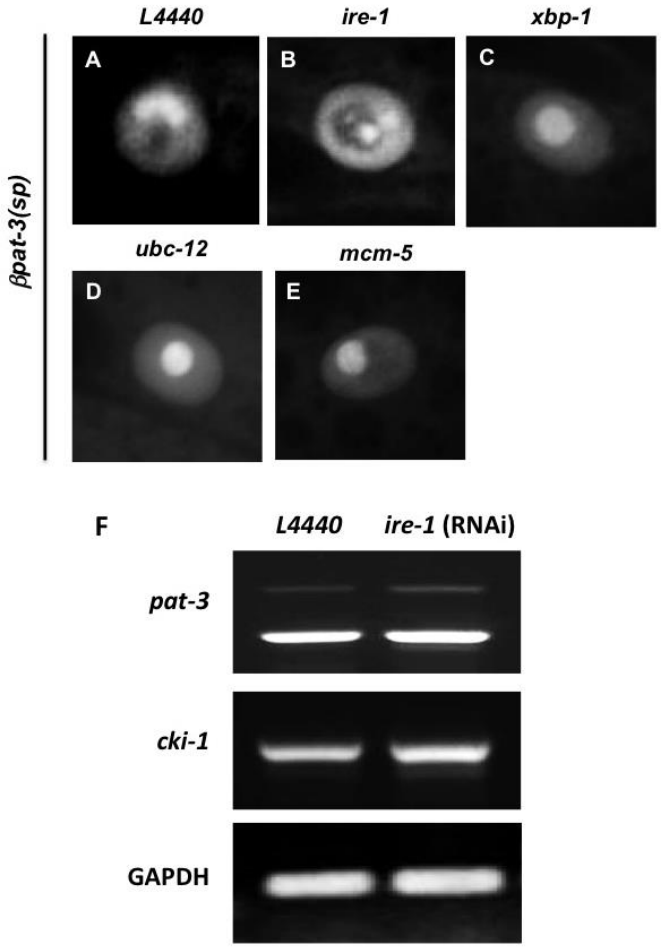

Figure S4.2. Suppressor of the mislocalization of CKI-1::GFP. (A-E) *pat-3 (sp)* animals were treated with *ire-1, xbp-1, ubc-12, and mcm-5* RNAi and displayed the nucleolar localization. L4440 is empty vector RNAi (F). *ire-1* RNAi did not affect the mRNA level of *pat-3 and cki-1*.

Level of CKI-1::GFP Protein in the RNAi Animals

Our RNAi screen revealed that many nuclear genes are required for the localization of CKI-1 and supported the idea that these genes are potentially linked to integrin functions. Defective integrin resulted in CKI-1 mislocalization mediated by these genes, suggesting that the integrin signaling might influence the activities of these genes. The suppression of CKI-1 mislocalization could be due to the reduction in CKI-1::GFP protein level. Therefore, we assessed the protein level of CKI-1::GFP in the RNAi animals to understand whether the suppression is the result of decrease in the protein

level. Young adult stage RNAi animals from *pat-3 (+)* or *pat-3 (sp)* background were collected. Immunoblot analyses were performed using anti-GFP antibodies (Materials and Methods). The triplicate sample analyses revealed that RNAi of four genes, *hda-1*, *mfap-1*, *mig-32*, and *dnc-1* significantly reduced the level of CKI-1::GFP [Figure 4.4]. The RNAi of enhancer nucleolus genes was also tested, but these did not result in an increase in the level of CKI-1::GFP [Figure 4.4].

Because most of the suppressors did not affect overall levels of protein CKI-1::GFP, we reasoned that the suppression might result from an decrease or increase in the level of *pat-3* integrin. In order to address the role of these genes in integrin expression, RNAi was performed in *pat-3::HA* background, a transgenic line in which *pat-3 (st564)* was rescued with pPAT3 (+)::HA [Materials and Methods]. The same set of RNAi experiments were analyzed for the level of PAT-3::HA using immunoblotting. Among the suppressor genes, *lpd-7* RNAi resulted in a significantly reduced PAT-3::HA level, while RNAi of *hda-1, mfap-1, mig-32,* or *dnc-1* did not affect the level of PAT-3::HA integrin expression [Figure 4.5]. To demonstrate the specificity of *lpd-7* on PAT-3, a line carrying a translational GFP fusion of the α integrin INA-1 was treated with *lpd-7* RNAi. INA-1::GFP protein levels were not reduced in *lpd-7* RNAi animals (not shown). Therefore, we concluded that the effect of *lpd-7* is at least somewhat specific to PAT-3 integrin. We next investigated the effect of *lpd-7* RNAi in the *pat-3 (+)* background. In contrast to *pat-3 (sp)* animals, *lpd-7* RNAi caused defective localization of CKI-1::GFP in the *pat-3 (+)* background [Figure 4.6], suggesting that *lpd-7* antagonizes *pat-3* to promote proper *cki-1* localization.

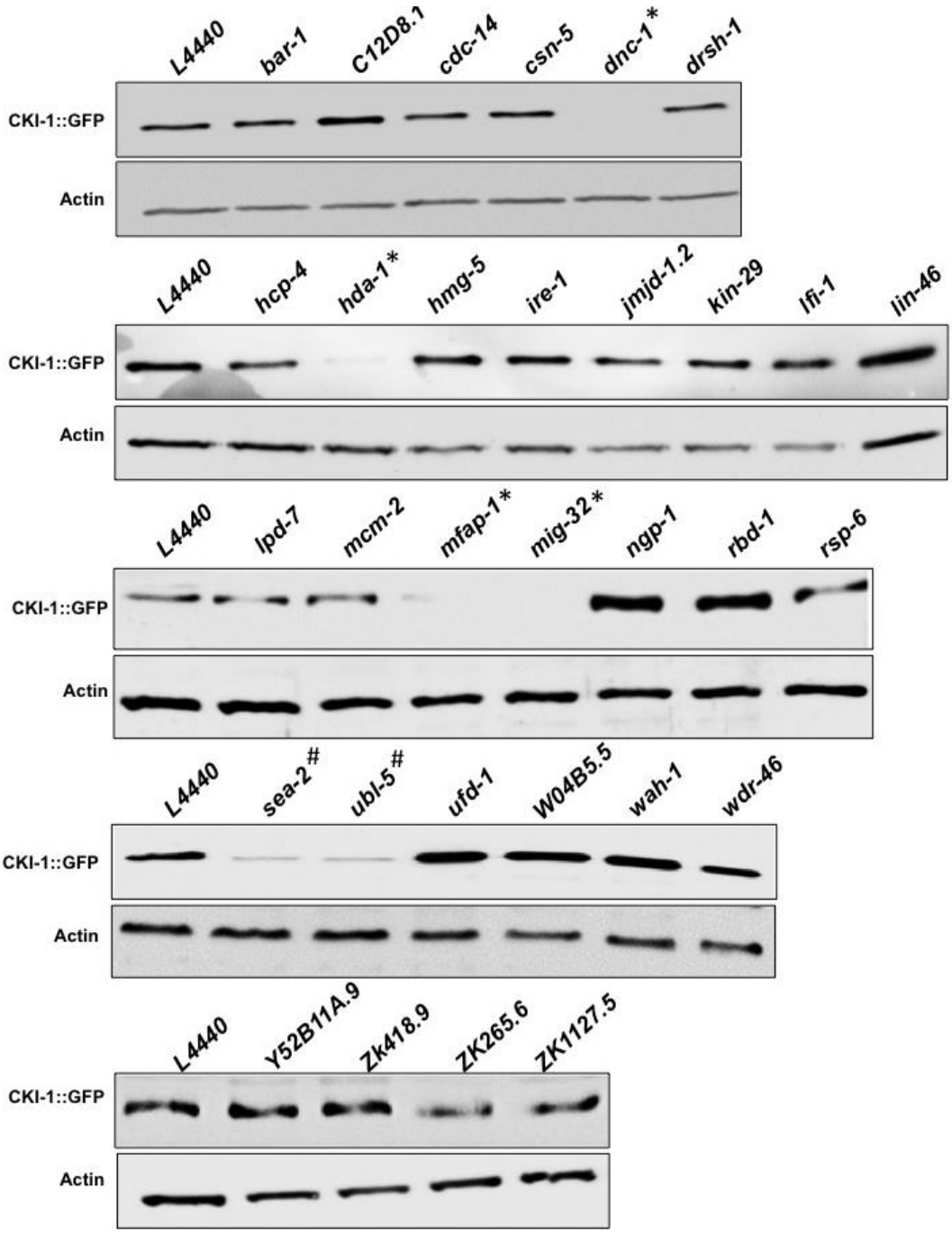

Figure 4.4. Immunoblot analysis to measure the amount of CKI-1::GFP in RNAi animals. CKI-1::GFP expression levels were assessed in *pat-3 (sp)* animals treated with RNAi directed against suppressor and enhancer genes. Top bands represent the amount of CKI-1::GFP in extracts prepared from each RNAi condition (labeled on top of each lane). The *dnc-1, hda-1, mfap-1,* and *mig-32* RNAi caused decreased CKI-1::GFP.

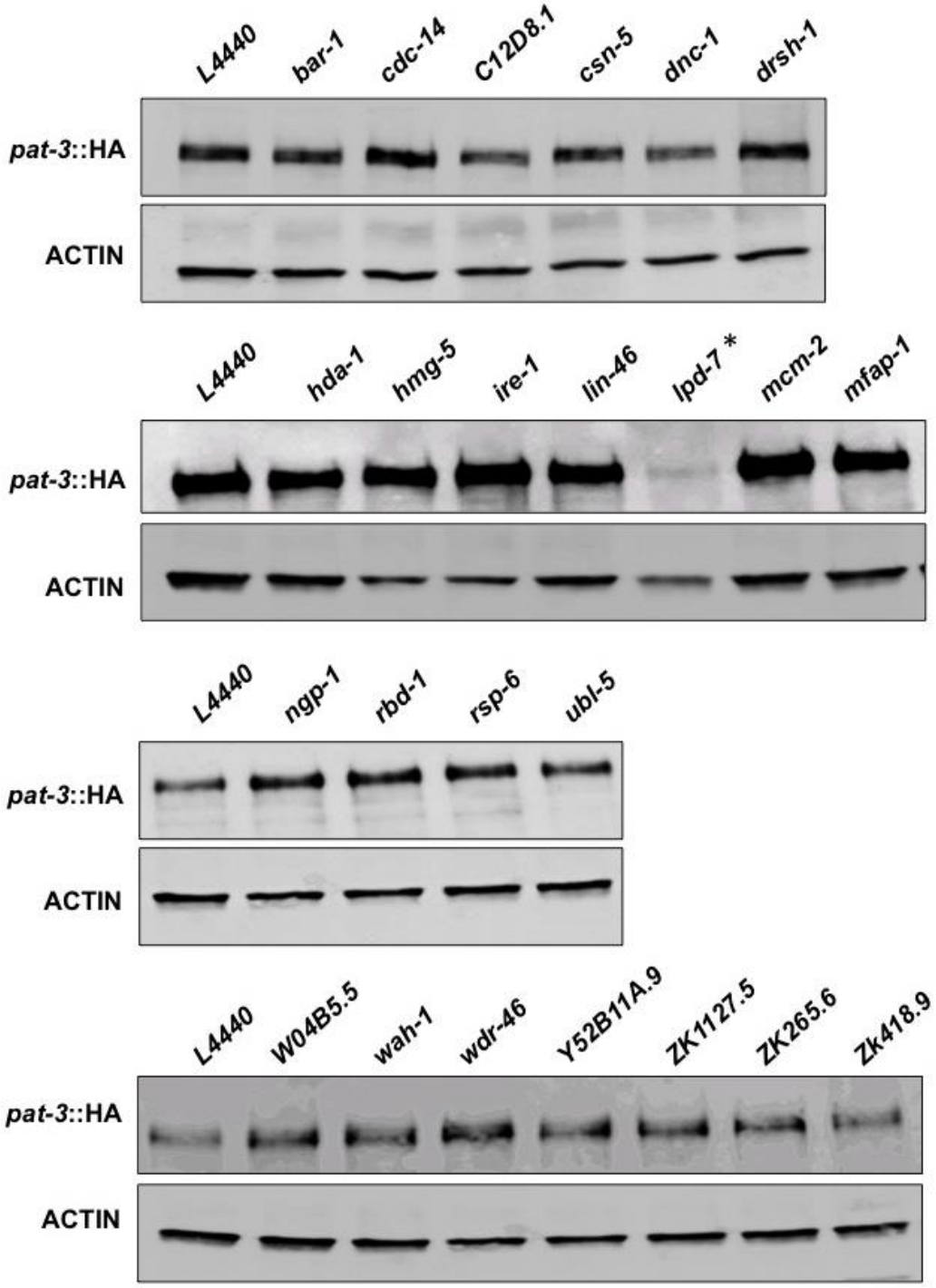

Figure 4.5. Immunoblot analysis to measure the amount of PAT-3::HA in RNAi animals. PAT-3::HA expression levels were assessed in pat-3 (+) animals treated with RNAi directed against suppressor and enhancer genes. Top bands represent the amount of PAT-3::HA in extracts prepared from each RNAi condition (labeled on top of each lane). L4440 is a negative RNAi control. All RNAi failed to cause changes in PAT-3::HA level, except *lpd-7* RNAi. Bottom bands indicate actin levels in each lane as a loading control.

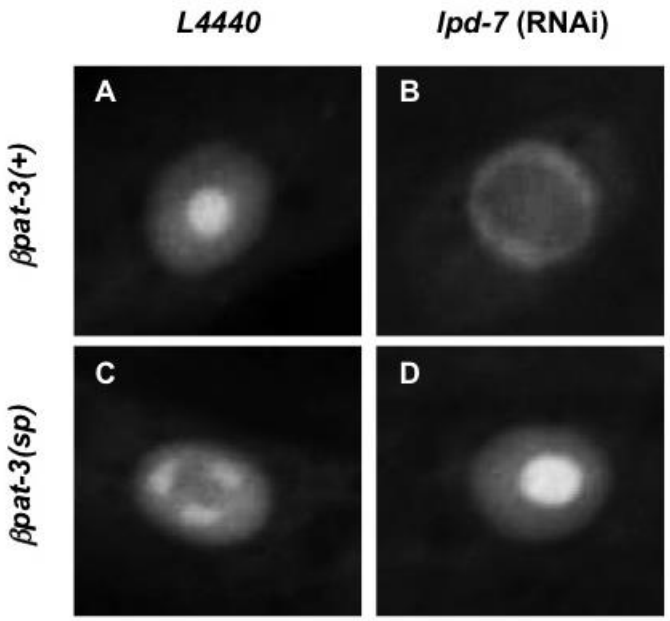

Figure 4.6. *lpd-7* RNAi antagonizes pat-3 integrin in CKI-1::GFP localization. A and B. *lpd-7* RNAi disrupted the nucleolar localization of CKI-1::GFP in *pat-3 (+).* In C and B, *lpd-7* RNAi suppressed the mislocalization of CKI-1::GFP in *pat-3 (sp).*

Discussion

The importance of our findings is in the identification of the nuclear genes linked to integrin signaling and CKI-1::GFP localization. Our results demonstrate that many nuclear genes play important roles in the localization of CKI-1 and that the *pat-3 (sp)* mutant mRNA is the major cause of CKI-1 mislocalization [Figure 4.1]. These genes represent many nuclear functions. The majority are involved in RNA splicing, nucleolus organization, and chromatin binding/histone modification [Figure 4.3]. In addition, genes involved in cell signaling, unfolded protein response (UPR), or protein degradation are also represented. Most of the RNAi treatments failed to reduce the level of CKI-1::GFP in the *pat-3 (sp)* background [Figure 4.4]. However, RNAi of *dnc-1*, *mfap-1*, *hda-1*, and *mig-32* genes did decrease the CKI-1::GFP level in *pat-3 (sp)*. However, none of the RNAi treatments reduced the level of CKI-1::GFP in *pat-3 (+)*. The above results led us

to conclude that these nuclear genes are the mediators of CKI-1::GFP localization and are linked to integrin signaling.

Although our analysis could not rule out the possibility that the suppression is due to a reduction in *pat-3* expression, this does not seem likely. Immunoblots of *pat-3*::HA animals revealed that most suppressor genes failed to affect the level of *pat-3* integrin. In contrast, only *lpd-7* RNAi decreased the protein level of *pat-3::*HA and resulted in anuclear CKI-1::GFP phenotype in *pat-3 (+)*, suggesting that the *lpd-7* potentially negatively regulates integrin-directed CKI-1 localization [Figure 4.5]. The mutations of *pat-3* β integrin were originally isolated in Pat mutant screen. Our previous analysis showed that *pat-4* or *unc-97* RNAi disrupted CKI-1::GFP nucleolus localization. In this study, we screened for all Pat class genes in *pat-3 (sp)* background using RNAi. A Pat gene expressed in hypodermis, *pat-12*, suppressed the nuclear localization of CKI-1::GFP phenotypes, suggesting that *pat-12* is a mediator that links *pat-3* signals to CKI-1 in hypodermis [Figure 4.2].

Our data provide an insight on how a tumor suppressor protein, CKI-1/$p27^{kip1}$, might be localized and regulated in a cell adhesion defective condition, the *pat-3*/β integrin defective background. It appears that CKI-1 localizes to nucleolus in *pat-3 (+)* background. Its activator CDC-14 also localizes to hypodermal nucleoli (Saito et al., 2004). Therefore, we should consider CKI-1 as a nucleolar gene. In response to cell adhesion/integrin, CKI-1 appears to relocate to nuclear matrix. Although further analysis is needed, our current study reveals multiple regulators of CKI-1 localization of CKI-1 as well as the important nuclear function of CKI-1.

RNA Splicing/Processing and Nucleolus Genes may Link to UPR

Figure 4.7 depicts the functional groups and their genes independent or in common. Genes expressed in the nucleolus are also involved in RNA processing such as rRNA processing (*ngp-1*, *rbd-1*, ZK265.6, ZK1127.5, and *wdr-46*), ribosome biogenesis (*wdr-46* and *fib-1*), RNA splicing (ZK418.9), and transcription control (*lpd-7*, Y52B11A.9 and *szy-20*). Role of SZY-20 and ZK418.9 appears common in RNA splicing and nucleolus function categories. Interactions among C12D8.1/splice factor, SZY-20, and ZK418.9 have been established (Yook et al., 2012). Importantly, a novel splicing factor, *mfap-1* RNAi significantly reduced the level of CKI-1::GFP, suggesting the importance of RNA splicing in the CKI-1 localization. The depletion of *mfap-1* may modulate the level of the mutant *pat-3* mRNA in *pat-3 (sp)*. Y52B11A.9/*dox-1* (Henis-Korenblit et al., 2010) is a zinc finger protein and present in nucleolus, whose function is linked to UPR and stress response activities, suggesting that UPR or ER stress signal is delivered to nucleoli via the IRE-1/XBP-1/DOX-1 axis. Nucleolus genes such as rRNA synthesis and ribosome biogenesis, together with IRE-1, displayed the enhancer phenotype of CKI-1::GFP. We speculate that UPR signal is delivered to nucleolus via Y52B11A.9/*dox-1* activity.

LPD-7, a nucleolar protein, is responsive to a starvation stress (Kinoshita et al., 2001) and a homolog of yeast *pescadillo* (*pes1*), a transcription factor with BRCA1 motif. Studies have shown that *pes1* plays important roles in ribosome biogenesis and cell cycle regulation (Lerch-Gaggl et al., 2002).]. *lpd-7* RNAi suppresses nuclear GFP of *pat-3 (sp)*, while it causes the nuclear GFP in *pat-3 (+).* The *lpd-7* RNAi decreased the protein level of PAT-3::HA integrin, while *lpd-7* RNAi failed to reduce the level of CKI-1::GFP. Our

previous analysis demonstrated that *pat-3* or *ina-1* RNAi disrupts the localization of CKI-1::GFP (Kihira et al., 2012). A simple explanation for the antagonism is that *lpd-7* is required for the proper level of *pat-3*. The RNAi of *lpd-7* in *pat-3 (+)* should be the same effect on CKI-1::GFP seen in the previous analysis (Kihira et al., 2012), while *lpd-7* RNAi in *pat-3 (sp)* may decrease PAT-3 amount to a wild-type level. Another possibility could be that the wild-type functions of *lpd-7* and *pat-3* both worked together as CKI-1 repressor for cell cycle advance. Removal of both could return CKI-1 to the default location, nucleolus, to keep the cell cycle.

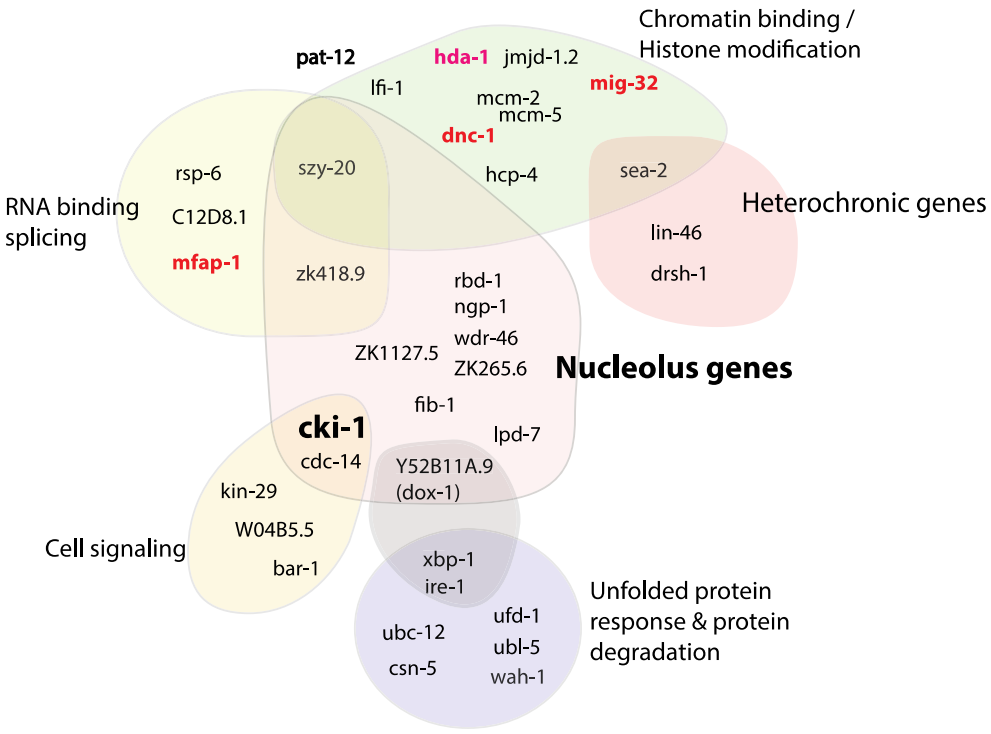

Figure 4.7. How do the nuclear genes regulate the CKI-1/p27^{kip1} localization in nucleolus.

Chromatin binding and histone Modification

The human homolog of *hcp-4*, *mcm-2*, or *dnc-1* has been characterized as CDK targets (Tsutsui et al., 2011). *lfi-1* is a coiled-coil protein interacts to LIN-5, a

chromosome segregation protein, and overlaps its expression with LIN-5 during metaphase. Several proteomics screens found that it is physically interacting with *pat-12* (Hetherington et al., 2011). It is suggested that *lfi-1* may play a role in the localization of CKI-1 in conjunction with *pat-12*.

The RNAi of chromatin binding/DNA replication gene may remove the CDK targets that are also targets of CKI. The RNAi of *hda-1/HDAC1, mig-32/H2 E3 ligase,* or *jmjd-1.2/histone demethylase* significantly reduced the level of CKI-1::GFP. These proteins presumably strengthen the nucleosome structure to prevent gene expression but its RNAi suppresses the CKI-1::GFP mislocalization, suggesting that *pat-3* integrin can be linked to histone modification. The mechanism on how the histone modification regulates to CKI-1::GFP expression is largely unknown. However, it has been reported that *cki-1*/$p27^{kip1}$ may play a role as a gene repressor forming a complex with HDAC1, 4, or 5 (Wilting et al., 2010). Perhaps *cki-1* plays a new role as gene expression repressor acting integrin downstream in hypodermal cell. The heterochronic genes such as *sea-2*, *drsh-1*, and *lin-46* are involved in miRNA processing and their RNAi knockdown suppressed *pat-3 (sp)*. There are few examples of $p27^{kip1}$ being controlled by miRNA. For example, the expression of $p27^{kip1}$ protein is repressed by miR221 (Zhang et al., 2009). Inhibition of miRNA production should affect the level of $p27^{kip1}$ and other proteins. The knockdown of these genes may perturb a proper timing for CKI-1::GFP expression, suggesting that these genes provide a proper stage for the *cki-1* expression in response to *pat-3* signaling.

Cell Signaling Genes

The degradation of *bar-1/β-catenin* might be deregulated in *pat-3 (sp)* background due to defective protein degradation. It is expected that accumulated BAR-1 should translocate to the nucleus and activate target gene expression (Natarajan et al., 2004). There must be a target of *bar-1/ β-catenin* playing an important role in CKI-1 expression. Both KIN-29 and W04B5.5 genes are involved in AKT/PKB pathways and translocate from cytoplasm to nucleus (Maduzia et al., 2005). Currently, the role of these two genes in CKI-1 localization is still speculative. However, it is obvious that RNAi of those genes should reduce the amount of KIN-29 and W04B5.5 in nucleus.

Protein Degradation and UPR Genes

These genes, *ubc-12* and *csn-5*, are involved in neddylation/deneddylation cycle (Hjerpe et al., 2012). There are reports that *ubc-12* and *csn-5* are important in activating $p27^{kip1}$ by neddylation (Morimoto et al., 2000). The RNAi knockdown of either gene reduces the amount of neddylated CKI-1, which may lead to the nucleolar localization. For UPR genes, we suggest that this is due to the accumulation of unfolded *pat-3 (sp)* protein in the mutant. The frame shift mutation in intron 7 should include the irrelevant protein sequence at the C-terminal end (Komaru et al., 2005). The mutant end might be unable to fold properly. It is reasonable to assume that the misfolded protein affects multiple responses in ER and mitochondria. Perhaps *udf-1*, *ubl-5*, *wah-1*, *xbp-1*, and *ire-1* are activated in the *pat-3 (sp)*. However, *ire-1* RNAi enhanced the nuclear localization of CKI-1::GFP in *pat-3 (sp)*. Perhaps *ire-1* may be linked to nucleolus functions such as ribosome biogenesis and there are multiple IRE-1 targets in the nucleolus. However, *xbp-1* RNAi in *pat-3 (sp)* suppressed the mislocalization of CKI-1. There have been reports

that ERAD and UPR impose stresses on rRNA synthesis and nucleolus functions (Boulon et al., 2010). Y52B11A.9/DOX-1 (downstream of *xbp-1*) is a zinc finger protein involved in RNA splicing and processing and its activity dependent on spliced *xbp-1*. Perhaps the UPR stress is delivered to nucleolus via *ire-1/xbp-1/dox-1* axis.

Figure 4.7 summarizes the genes identified in this study. All 33 genes plus several linked genes are added in the picture. It is suggested that all are linked to integrin but the *pat-3* integrin was not indicated in this diagram. Previous analysis revealed that *pat-3 (sp)* caused defects in ubiquitin mediated protein degradation (Kihira et al., 2012). It is reasonable to expect that defective protein degradation would cause UPR. This ER stress might be translated into nucleolar stress mediated by IRE-1 and DOX-1; the CKI-1::GFP mislocalization could be due to a nucleolar stress delivered from UPR. Other gene activities such as chromatin binding/DNA replication, histone modification, cell signaling, and heterochronic genes might be the results of nucleolar stresses that affect transcription and genome stability (Vihervaara et al., 2013). Although the limited number of nuclear genes was tested, we have identified more than 30 nuclear genes involved in the localization of *cki-1*. The genetic interaction to CKI-GFP occurred only in the *pat-3 (sp)* background and failed to display suppression in *pat-3 (+),* suggesting that these genes are linked to integrin functions or signaling.

Why is Understanding of cki-1/p27^{kip1} Nuclear Localization Essential?

This study demonstrates the dynamic role of integrin signaling in controlling the localization of the cell cycle inhibitor CKI-1/p27^{kip1}. The response of CKI-1/p27^{kip1} localization to integrin signaling seems to be evolutionarily conserved. In prostate cancer cell lines, p27^{kip1} protein levels are linked to β1C integrin expression (Fornaro et al.,

1996). Although there is no known linkage of β1B integrin to $p27^{kip1}$, this splice variant showed a different distribution other than in the focal adhesions. It accumulates in the ER and shows significantly reduced adhesion activities (Balzac et al., 1994; Kee et al., 2000). *pat-3 (sp)* is an artificial mutation introduced into *C. elegans* using transgenic rescue. However, *pat-3 (sp)* functions similarly to the B and C splice variants of mammalian β1 integrin (Gettner et al., 1995) with anti-adhesive and anti-proliferative activities, respectively (Lee et al., 2001b). Our study suggests that integrin signaling may impact the many nuclear genes to properly localize CKI-1 in the nucleolus. The similar variants of mammalian β1 integrin may also play *pat-3 (sp)*-like roles in response to the signals from the ECM.

Materials and Methods

Animal Strains

The nematode *Caenorhabditis elegans* were grown on nematode growth medium (NGM) agar plates with OP50 *E. coli* bacteria under standard conditions (Brenner, 1974). The rescue transgenic lines, BU444 *kqEx75 [pat-3(+) rab-3::RFP, cki-1::GFP]* and BU7222 *kqEx73 [pat-3(sp) rab-3::RFP, cki-1::GFP],* are described in Kihira *et al.* (Kihira et al., 2012). BU7231 *kqEx85* [*pat-3::HA, rab-3::GFP*] was generated in this study. The RW3600 qC1 *dpy-19(e1259) glp-1(q339)/pat-3(st564)* III was used for generating *pat-3* transgenic. Some *C. elegans* strains used in this study were obtained from the Caenorhabditis Generics Center (St. Paul, MN).

DNA Constructs and Germline Transformation

The *pat-3* genomic DNA construct, pPAT(+)-PB12K, was excised from the ZK1058 cosmid using restriction enzymes and inserted between PstI and SmaI sites of pSP73 plasmid vector (Promega, Madison, WI) (Lee et al., 2001b). To generate HA epitope tagging of *pat-3* translational fusion, an HA cassette with three HA coding sequences in tandem was PCR amplified from pMR2307 (Kurihara et al., 1996) with an XbaI site added at each end and cloned into the unique NheI site in the pPAT3ENE800 construct, an NheI site replaced stop codon of *pat-3*. The *pat-3*::HA fragment was excised from the ENE800::3XHA using restriction enzymes MscI and EcoRI and inserted into pPAT(+)-PB12K between MscI and KpnI sites of pPAT(+)-PB12K and isolated and purified by ethanol precipitation. Germline transformation was performed using the standard protocol for microinjection (Lee et al., 2001b). pPAT-3::HA constructs was injected into the distal gonads of the RW3600 *qC1 dpy-19(e1259) glp-1(q339)/pat-3(st564)*III animals at 5μg/ml as pool with pGH8 *rab-3::mCherry* plasmid as a coinjection pan-neuronal marker at 100 μg/ml. After the initial generation of F1 red worms, those animals were propagated by selfing a single hermaphrodite growing on a NGM plate. F2 generation animal with 100% red progeny were isolated and more than 10 generations elapsed before the characterization of phenotypes.

Fluorescence Microscopy

To visualize CKI-1::GFP localization in hypodermis, L4 to young adult worms were mounted in a drop of M9 buffer containing 0.5mM levamisole on a 24X60mm coverslip coated with 4% agarose. Images were examined and analyzed using Nikon TE2000-U Diaphot epifluorescence microscope with Nomarski optics, CoolSnap CE

monochrome camera (Photometrics, Tucson, AZ), and Metamorph (v8.0, Molecular Devices, Sunnyvale, CA) and ImageJ64 (National Institute of Health, Bethesda, MD) softwares. About 20 hypodermal nuclei in the midbody, an area including vulva at the ventral midline, of each animal was observed for nuclear morphology characterization.

RNA Isolation and Reverse Transcription PCR

Fifty L4 to young adult animals were collected in TRI reagent (Sigma-Aldrich, St. Louis, MO) for total RNA extraction and followed by chloroform precipitation. Approximately 700 ng of total RNA was used to synthesize cDNA using Transcriptor Reverse Transcription kit (Invitrogen, Grand Island, NY) primed with random hexamer. Subsequently, total of 2 µl cDNA was used in PCR amplification with *cki-1* primers, *pat-3* primers, and *gpd-1*/GAPDH primers as an internal control. The samples were then subjected to analysis on a GelRed (Biotinum, Hayward, CA) stained agarose gel.
The following sets of primer were used for amplification.

CKI1 Forward GTCTTCTGCTCGTCGTTGC
CKI1 Reverse CACCGGAGACAGCTTGAT
PAT3PT Forward CTCAACGAAACTACACCCTG
PAT3PT Reverse TTAGTTGGCTTTTCCAGCGT

Target Gene Selection

To find genes whose knockdown causes the changes in *cki-1/p27^{kip1}* expression or localization, we identified candidate target genes from the WormBase (www.wormbase.org) using various criteria and focused on nucleus or nucleolar-enriched genes to confine the outcomes. To avoid any results from outsources closely related to worms, *C. remanei* or *C. briggase* for instance, target organisms were restricted to *C.*

elegans. Data searching began with one subject, “For A Gene”, using “nucleus” or “nucleolar” as keywords to obtain the maximum number of genes. To exclude the falsely selected genes, we filtered the genes by the expression pattern of genes and RNA library availability as well. This search led to the identification of 133 genes (Tables S1 and S2) and 33 RNAi, the final positive candidates, were classified into 7 groups based on their molecular function, biological process, and partner protein class.

RNA Interference

C. elegans RNA interference analysis was performed using the bacterial feeding method. Young adult animals were fed with bacteria containing plasmids directing the expression of dsRNAs on NGM plates with 1mM lactose and 0.1mg/ml Ampicillin. F1 progeny of those animals were observed under the microscope at L4 to young adult stage to analyze the localization of CKI-1::GFP. To verify the RNAi efficiency, all RNAi animals were examined for behavioral phenotypes linked to the RNAi gene. All RNAi bacteria used in this study were liquid cultured to isolate plasmid DNA and the sequences of plasmids were sent to Macrogen (Rockvill, MD) to confirm the presence of coding sequence for each gene. The sequence data were confirmed by BLASTn search.

Immunoblot Analysis

For immunoblotting, 20 young adult worms were collected and lysed in the lysis buffer (50 mM Tris–HCl (pH 7.4), 1 mM EDTA, and 1 mM PMSF containing protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN). *C. elegans* samples were boiled for 10 minutes in 4X Laemmli sample buffer then resolved on 10% SDS polyacrylamide gel and transferred onto PVDF membrane. Blots were probed with primary rabbit

polyclonal antibody against GFP (Abcam, Cambridge, MA) at 1:2000 or a mouse monoclonal antibody against HA (Covance, Princeton, NJ) and the respective secondary antibodies.

BIBLIOGRAPHY

Abukhdeir, A.M., and B.H. Park. 2008. P21 and p27: roles in carcinogenesis and drug resistance. *Expert Rev Mol Med*. 10:e19.

Aldridge, J.E., T. Horibe, and N.J. Hoogenraad. 2007. Discovery of genes activated by the mitochondrial unfolded protein response (mtUPR) and cognate promoter elements. *PLoS One*. 2:e874.

Alper, J.C., H.P. Baden, and L.A. Goldsmith. 1978. Kindler's syndrome. *Archives of dermatology*. 114:457.

Ambros, V. 1999. Cell cycle-dependent sequencing of cell fate decisions in Caenorhabditis elegans vulva precursor cells. *Development*. 126:1947-1956.

Armulik, A. 2002. Splice variants of human beta 1 integrins: origin, biosynthesis and functions. *Front Biosci*. 7:d219-227.

Arnaout, M.A., B. Mahalingam, and J.P. Xiong. 2005. Integrin structure, allostery, and bidirectional signaling. *Annu Rev Cell Dev Biol*. 21:381-410.

Aroian, R.V., A.D. Levy, M. Koga, Y. Ohshima, J.M. Kramer, and P.W. Sternberg. 1993. Splicing in Caenorhabditis elegans does not require an AG at the 3' splice acceptor site. *Mol Cell Biol*. 13:626-637.

Assoian, R.K. 1997. Anchorage-dependent cell cycle progression. *Journal of Cell Biology*. 136:1-4.

Assoian, R.K. 2004. Stopping and going with p27kip1. *Dev Cell*. 6:458-459.

Bai, C., P. Sen, K. Hofmann, L. Ma, M. Goebl, J.W. Harper, and S.J. Elledge. 1996. SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. *Cell*. 86:263-274.

Balzac, F., S.F. Retta, A. Albini, A. Melchiorri, V.E. Koteliansky, M. Geuna, L. Silengo, and G. Tarone. 1994. Expression of beta 1B integrin isoform in CHO cells results in a dominant negative effect on cell adhesion and motility. *J Cell Biol*. 127:557-565.

Barczyk, M., S. Carracedo, and D. Gullberg. 2010. Integrins. *Cell Tissue Res*. 339:269-280.

Barstead, R.J., and R.H. Waterston. 1991. Vinculin is essential for muscle function in the nematode. *Journal of Cell Biology*. 114:715-724.

Baum, P.D., and G. Garriga. 1997. Neuronal migrations and axon fasciculation are disrupted in *ina-1* integrin mutants. *Neuron*. 19:51-62.

Besson, A., M. Gurian-West, A. Schmidt, A. Hall, and J.M. Roberts. 2004. p27Kip1 modulates cell migration through the regulation of RhoA activation. *Genes Dev*. 18:862-876.

Bjork, P., G. Bauren, S. Jin, Y.G. Tong, T.R. Burglin, U. Hellman, and L. Wieslander. 2002. A novel conserved RNA-binding domain protein, RBD-1, is essential for ribosome biogenesis. *Mol Biol Cell*. 13:3683-3695.

Boulon, S., B.J. Westman, S. Hutten, F.M. Boisvert, and A.I. Lamond. 2010. The nucleolus under stress. *Mol Cell*. 40:216-227.

Boxem, M., and S. van den Heuvel. 2001. lin-35 Rb and cki-1 Cip/Kip cooperate in developmental regulation of G1 progression in C. elegans. *Development*. 128:4349-4359.

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

Brower, D.L., and S.M. Jaffe. 1989. Requirement for integrins during Drosophila wing development. *Nature*. 342:285-287.

Brown, N.H. 2000. Cell-cell adhesion via the ECM: integrin genetics in fly and worm. *Matrix Biol*. 19:191-201.

Brown, N.H., D.L. King, M. Wilcox, and F.C. Kafatos. 1989. Developmentally regulated alternative splicing of Drosophila integrin PS2 alpha transcripts. *Cell*. 59:185-195.

Buck, S.H., D. Chiu, and R.M. Saito. 2009. The cyclin-dependent kinase inhibitors, cki-1 and cki-2, act in overlapping but distinct pathways to control cell cycle quiescence during C. elegans development. *Cell Cycle*. 8:2613-2620.

Bunting, M., E.S. Harris, T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2002. Leukocyte adhesion deficiency syndromes: adhesion and tethering defects involving beta 2 integrins and selectin ligands. *Curr Opin Hematol*. 9:30-35.

Cabodi, S., M. del Pilar Camacho-Leal, P. Di Stefano, and P. Defilippi. 2010. Integrin signalling adaptors: not only figurants in the cancer story. *Nat Rev Cancer*. 10:858-870.

Calderwood, D.A., A. Huttenlocher, W.B. Kiosses, D.M. Rose, D.G. Woodside, M.A. Schwartz, and M.H. Ginsberg. 2001. Increased filamin binding to beta-integrin cytoplasmic domains inhibits cell migration. *Nat Cell Biol*. 3:1060-1068.

Cali, G., C. Mazzarella, M. Chiacchio, R. Negri, S.F. Retta, M. Zannini, F. Gentile, G. Tarone, L. Nitsch, and C. Garbi. 1999. RhoA activity is required for fibronectin assembly and counteracts beta1B integrin inhibitory effect in FRT epithelial cells. *J Cell Sci*. 112 (Pt 6):957-965.

Carmo-Fonseca, M., L. Mendes-Soares, and I. Campos. 2000. To be or not to be in the nucleolus. *Nat Cell Biol*. 2:E107-112.

Carrano, A.C., E. Eytan, A. Hershko, and M. Pagano. 1999. SKP2 is required for ubiquitin-mediated degradation of the CDK inhibitor p27. *Nat Cell Biol*. 1:193-199.

Carrano, A.C., and M. Pagano. 2001. Role of the F-box protein Skp2 in adhesion-dependent cell cycle progression. *J Cell Biol*. 153:1381-1390.

Chakravarty, A.K., P. Smith, and S. Shuman. 2011. Structures of RNA 3'-phosphate cyclase bound to ATP reveal the mechanism of nucleotidyl transfer and metal-assisted catalysis. *Proc Natl Acad Sci U S A*. 108:21034-21039.

Chen, N., T.W. Harris, I. Antoshechkin, C. Bastiani, T. Bieri, D. Blasiar, K. Bradnam, P. Canaran, J. Chan, C.K. Chen, W.J. Chen, F. Cunningham, P. Davis, E. Kenny, R. Kishore, D. Lawson, R. Lee, H.M. Muller, C. Nakamura, S. Pai, P. Ozersky, A. Petcherski, A. Rogers, A. Sabo, E.M. Schwarz, K. Van Auken, Q. Wang, R. Durbin, J. Spieth, P.W. Sternberg, and L.D. Stein. 2005. WormBase: a comprehensive data resource for *Caenorhabditis* biology and genomics. *Nucleic Acids Res*. 33 Database Issue:D383-389.

Chen, Y.-P., T.E. O'Tooke, T. Shipley, J. Forsyth, S.E. LaFlamme, K.M. Yamada, S.J. Shattil, and M.H. Ginsberg. 1994. Inside-out signal transduction inhibited by isolated integrin cytoplasmic domains. *Journal of Biological Chemistry*. 269:18307-18301.

Chennupati, V., D. Datta, M.R. Rao, N. Boddapati, M. Kayasani, R. Sankaranarayanan, M. Mishra, P. Seth, C. Mani, and S. Mahalingam. 2011. Signals and pathways regulating nucleolar retention of novel putative nucleolar GTPase NGP-1(GNL-2). *Biochemistry*. 50:4521-4536.

Chu, I., J. Sun, A. Arnaout, H. Kahn, W. Hanna, S. Narod, P. Sun, C.K. Tan, L. Hengst, and J. Slingerland. 2007. p27 phosphorylation by Src regulates inhibition of cyclin E-Cdk2. *Cell*. 128:281-294.

Clark, E., Brugge, JS. 1995. Integrins and signal transduction pathways: the road taken. *Science*. 268:233-239.

Clark, K.A., M. McGrail, and M.C. Beckerle. 2003. Analysis of PINCH function in Drosophila demonstrates its requirement in integrin-dependent cellular processes. *Development*. 130:2611-2621.

Cope, G.A., G.S. Suh, L. Aravind, S.E. Schwarz, S.L. Zipursky, E.V. Koonin, and R.J. Deshaies. 2002. Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science*. 298:608-611.

Cox, E.A., and J. Hardin. 2004. Sticky worms: adhesion complexes in C. elegans. *J Cell Sci*. 117:1885-1897.

Cruet-Hennequart, S., S. Maubant, J. Luis, P. Gauduchon, C. Staedel, and S. Dedhar. 2003. alpha(v) integrins regulate cell proliferation through integrin-linked kinase (ILK) in ovarian cancer cells. *Oncogene*. 22:1688-1702.

Danen, E.H., and A. Sonnenberg. 2003. Integrins in regulation of tissue development and function. *J Pathol*. 201:632-641.

Dello, S.A., R.M. van Dam, J.J. Slangen, M.C. van de Poll, M.H. Bemelmans, J.W. Greve, R.G. Beets-Tan, S.J. Wigmore, and C.H. Dejong. 2007. Liver volumetry plug and play: do it yourself with ImageJ. *World J Surg*. 31:2215-2221.

Desdouets, C., and C. Brechot. 2000. p27: a pleiotropic regulator of cellular phenotype and a target for cell cycle dysregulation in cancer. *Pathol Biol (Paris)*. 48:203-210.

Deshaies, R.J., E.D. Emberley, and A. Saha. Control of cullin-ring ubiquitin ligase activity by nedd8. *Subcell Biochem*. 54:41-56.

Deshaies, R.J., and C.A. Joazeiro. 2009. RING domain E3 ubiquitin ligases. *Annu Rev Biochem*. 78:399-434.

Dobrynin, G., O. Popp, T. Romer, S. Bremer, M.H. Schmitz, D.W. Gerlich, and H. Meyer. 2011. Cdc48/p97-Ufd1-Npl4 antagonizes Aurora B during chromosome segregation in HeLa cells. *J Cell Sci*. 124:1571-1580.

dos Santos, P.B., J.S. Zanetti, A. Ribeiro-Silva, and E.I. Beltrao. 2012. Beta 1 integrin predicts survival in breast cancer: a clinicopathological and immunohistochemical study. *Diagn Pathol*. 7:104.

Eisenmann, D.M., J.N. Maloof, J.S. Simske, C. Kenyon, and S.K. Kim. 1998. The beta-catenin homolog BAR-1 and LET-60 Ras coordinately regulate the Hox gene lin-39 during Caenorhabditis elegans vulval development. *Development*. 125:3667-3680.

Emmott, E., and J.A. Hiscox. 2009. Nucleolar targeting: the hub of the matter. *EMBO Rep*. 10:231-238.

Etzioni, A., C.M. Doerschuk, and J.M. Harlan. 1999. Of man and mouse: leukocyte and endothelial adhesion molecule deficiencies. *Blood*. 94:3281-3288.

Feng, H., W. Zhong, G. Punkosdy, S. Gu, L. Zhou, E.K. Seabolt, and E.T. Kipreos. 1999. CUL-2 is required for the G1-to-S-phase transition and mitotic chromosome condensation in Caenorhabditis elegans. *Nat Cell Biol*. 1:486-492.

Fire, A., S.-Q. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, and C.C. Mello. 1998. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*. 319:806-811.

Fornaro, M., and L.R. Languino. 1997. Alternatively spliced variants: a new view of the integrin cytoplasmic domain. *Matrix Biol*. 16:185-193.

Fornaro, M., T. Manes, and L.R. Languino. 2001. Integrins and prostate cancer metastases. *Cancer Metastasis Rev*. 20:321-331.

Fornaro, M., M. Manzotti, G. Tallini, A.E. Slear, S. Bosari, E. Ruoslahti, and L.R. Languino. 1998. Beta1C integrin in epithelial cells correlates with a nonproliferative phenotype: forced expression of beta1C inhibits prostate epithelial cell proliferation. *Am J Pathol*. 153:1079-1087.

Fornaro, M., C.A. Steger, A.M. Bennett, J.J. Wu, and L.R. Languino. 2000. Differential role of beta(1C) and beta(1A) integrin cytoplasmic variants in modulating focal adhesion kinase, protein kinase B/AKT, and Ras/Mitogen-activated protein kinase pathways. *Mol Biol Cell*. 11:2235-2249.

Fornaro, M., G. Tallini, C.J. Bofetiado, S. Bosari, and L.R. Languino. 1996. Down-regulation of beta 1C integrin, an inhibitor of cell proliferation, in prostate carcinoma. *Am J Pathol*. 149:765-773.

Fornaro, M., G. Tallini, D.Q. Zheng, W.M. Flanagan, M. Manzotti, and L.R. Languino. 1999. p27(kip1) acts as a downstream effector of and is coexpressed with the beta1C integrin in prostatic adenocarcinoma. *J Clin Invest*. 103:321-329.

Frank, D.J., and M.B. Roth. 1998. ncl-1 is required for the regulation of cell size and ribosomal RNA synthesis in Caenorhabditis elegans. *J Cell Biol*. 140:1321-1329.

Frokjaer-Jensen, C., M.W. Davis, C.E. Hopkins, B.J. Newman, J.M. Thummel, S.P. Olesen, M. Grunnet, and E.M. Jorgensen. 2008. Single-copy insertion of transgenes in Caenorhabditis elegans. *Nat Genet*. 40:1375-1383.

Fu, Y., Z. Fang, Y. Liang, X. Zhu, P. Prins, Z. Li, L. Wang, L. Sun, J. Jin, Y. Yang, and X. Zha. 2007. Overexpression of integrin beta1 inhibits proliferation of hepatocellular carcinoma cell SMMC-7721 through preventing Skp2-dependent degradation of p27 via PI3K pathway. *J Cell Biochem*. 102:704-718.

Fu, Y., L.Y. Wang, Y.L. Liang, J.W. Jin, Z.Y. Fang, and X.L. Zha. 2006. Integrin beta(1A) upregulates p27 protein amount at the post-translational level in human hepatocellular carcinoma cell line SMMC-7721. *Acta Biochim Biophys Sin (Shanghai)*. 38:523-530.

Fukuda, T., K. Chen, X. Shi, and C. Wu. 2003. PINCH-1 is an obligate partner of integrin-linked kinase (ILK) functioning in cell shape modulation, motility, and survival. *J Biol Chem*. 278:51324-51333.

Garcia-Alvarez, B., J.M. de Pereda, D.A. Calderwood, T.S. Ulmer, D. Critchley, I.D. Campbell, M.H. Ginsberg, and R.C. Liddington. 2003. Structural determinants of integrin recognition by talin. *Mol Cell*. 11:49-58.

Gardner, J.M., and R.O. Hynes. 1985. Interaction of fibronectin with its receptor on platelets. *Cell*. 42:439-448.

Gettner, S.N. 1994a. Isolation and functional analysis of Caehorhabditis elegans integrin Bpat-3 : a novel integrin beta subunit. University of California, San Francisco. x, 152 leaves.

Gettner, S.N. 1994b. Isolation and functional analysis of *Caenorhabditis elegans* integrin bpat-3. University of California, San Francisco, CA.

Gettner, S.N., C. Kenyon, and L.F. Reichardt. 1995. Characterization of bpat-3 heterodimers, a family of essential integrin receptors in *C. elegans*. *Journal of Cell Biology*. 129:1127-1141.

Giancotti, F.G., and E. Ruoslahti. 1999. Integrin signaling. *Science*. 285:1028-1032.

Ginsberg, M.H., X. Du, and E.F. Plow. 1992. Inside-out integrin signalling. *Curr. Opin. Cell Biol*. 4:766-771.

Goel, H.L., and L.R. Languino. 2004. Integrin signaling in cancer. *Cancer Treat Res*. 119:15-31.

Gotwals, P.J., S.E. Paine-Saunders, K.A. Stark, and R.O. Hynes. 1994. Drosophila integrins and their ligands. *Curr Opin Cell Biol*. 6:734-739.

Grashoff, C., A. Aszodi, T. Sakai, E.B. Hunziker, and R. Fassler. 2003. Integrin-linked kinase regulates chondrocyte shape and proliferation. *EMBO Rep*. 4:432-438.

Griffith, O.L., E.D. Pleasance, D.L. Fulton, M. Oveisi, M. Ester, A.S. Siddiqui, and S.J. Jones. 2005. Assessment and integration of publicly available SAGE, cDNA microarray, and oligonucleotide microarray expression data for global coexpression analyses. *Genomics*. 86:476-488.

Grimson, A., S. O'Connor, C.L. Newman, and P. Anderson. 2004. SMG-1 is a phosphatidylinositol kinase-related protein kinase required for nonsense-mediated mRNA Decay in Caenorhabditis elegans. *Mol Cell Biol*. 24:7483-7490.

Guo, Y., G.N. Sklar, A. Borkowski, and N. Kyprianou. 1997. Loss of the cyclin-dependent kinase inhibitor p27(Kip1) protein in human prostate cancer correlates with tumor grade. *Clin Cancer Res*. 3:2269-2274.

Harris, E.S., T.M. McIntyre, S.M. Prescott, and G.A. Zimmerman. 2000. The Leukocyte Integrins. *The Journal of Biological Chemistry*. 275:23409-23412.

Hazlehurst, L.A., J.S. Damiano, I. Buyuksal, W.J. Pledger, and W.S. Dalton. 2000. Adhesion to fibronectin via beta1 integrins regulates p27kip1 levels and contributes to cell adhesion mediated drug resistance (CAM-DR). *Oncogene*. 19:4319-4327.

Hebeisen, M., and R. Roy. 2008. CDC-25.1 stability is regulated by distinct domains to restrict cell division during embryogenesis in C. elegans. *Development*. 135:1259-1269.

Hedgecock, E.M., and R.K. Herman. 1995. The ncl-1 gene and genetic mosaics of Caenorhabditis elegans. *Genetics*. 141:989-1006.

Hedgecock, E.M., and J.G. White. 1985. Polyploid tissues in the nematode Caenorhabditis elegans. *Dev Biol*. 107:128-133.

Hengst, L. 2004. A second RING to destroy p27(Kip1). *Nat Cell Biol*. 6:1153-1155.

Henis-Korenblit, S., P. Zhang, M. Hansen, M. McCormick, S.J. Lee, M. Cary, and C. Kenyon. 2010. Insulin/IGF-1 signaling mutants reprogram ER stress response regulators to promote longevity. *Proc Natl Acad Sci U S A*. 107:9730-9735.

Hershko, D., G. Bornstein, O. Ben-Izhak, A. Carrano, M. Pagano, M.M. Krausz, and A. Hershko. 2001. Inverse relation between levels of p27(Kip1) and of its ubiquitin ligase subunit Skp2 in colorectal carcinomas. *Cancer*. 91:1745-1751.

Hetherington, S., C. Gally, J.A. Fritz, J. Polanowska, J. Reboul, Y. Schwab, H. Zahreddine, C. Behm, and M. Labouesse. 2011. PAT-12, a potential anti-nematode target, is a new spectraplakin partner essential for Caenorhabditis elegans hemidesmosome integrity and embryonic morphogenesis. *Dev Biol*. 350:267-278.

Higa, L.A., X. Yang, J. Zheng, D. Banks, M. Wu, P. Ghosh, H. Sun, and H. Zhang. 2006. Involvement of CUL4 ubiquitin E3 ligases in regulating CDK inhibitors Dacapo/p27Kip1 and cyclin E degradation. *Cell Cycle*. 5:71-77.

Hjerpe, R., Y. Thomas, and T. Kurz. 2012. NEDD8 overexpression results in neddylation of ubiquitin substrates by the ubiquitin pathway. *J Mol Biol*. 421:27-29.

Hobert, O., D.G. Moerman, K.A. Clark, M.C. Beckerle, and G. Ruvkun. 1999. A conserved LIM protein that affects muscular adherens junction integrity and mechanosensory function in Caenorhabditis elegans. *J Cell Biol*. 144:45-57.

Hong, F., M.D. Larrea, C. Doughty, D.J. Kwiatkowski, R. Squillace, and J.M. Slingerland. 2008. mTOR-raptor binds and activates SGK1 to regulate p27 phosphorylation. *Mol Cell*. 30:701-711.

Hong, Y., R. Roy, and V. Ambros. 1998. Developmental regulation of a cyclin-dependent kinase inhibitor controls postembryonic cell cycle progression in Caenorhabditis elegans. *Development*. 125:3585-3597.

Hsieh, H.H., T.Y. Hsu, H.S. Jiang, and Y.C. Wu. 2012. Integrin alpha PAT-2/CDC-42 signaling is required for muscle-mediated clearance of apoptotic cells in Caenorhabditis elegans. *PLoS Genet*. 8:e1002663.

Huang, X., P. Huang, M.K. Robinson, M.J. Stern, and Y. Jin. 2003. UNC-71, a disintegrin and metalloprotease (ADAM) protein, regulates motor axon guidance and sex myoblast migration in C. elegans. *Development*. 130:3147-3161.

Huang, X., H. Zhang, and H. Zhang. 2011. The zinc-finger protein SEA-2 regulates larval developmental timing and adult lifespan in C. elegans. *Development*. 138:2059-2068.

Huang, Y., and J.A. Steitz. 2001. Splicing factors SRp20 and 9G8 promote the nucleocytoplasmic export of mRNA. *Mol Cell*. 7:899-905.

Humphries, M.J. 1990. The molecular basis and specificity of integrin-ligand interactions. *J. Cell Sci.* 97:585-592.

Humphries, M.J. 1996. Integrin activation: the link between ligand binding and signal transduction. *Current Opinion in Cell Biology*. 8:632-640.

Hynes, R.O. 1987. Integrins: a family of cell surface receptors. *Cell*. 48:549-554.

Hynes, R.O. 1992. Integrins: Versatility, modulation and signaling in cell adhesion. *Cell*. 69:11-25.

Hynes, R.O. 2002. Integrins: bidirectional, allosteric signaling machines. *Cell*. 110:673-687.

Jahangiri, A., M.K. Aghi, and W.S. Carbonell. 2014. beta1 integrin: Critical path to antiangiogenic therapy resistance and beyond. *Cancer Res*. 74:3-7.

Jannuzi, A.L., T.A. Bunch, M.C. Brabant, S.W. Miller, L. Mukai, M. Zavortink, and D.L. Brower. 2002. Disruption of C-terminal cytoplasmic domain of betaPS integrin subunit has dominant negative properties in developing Drosophila. *Mol Biol Cell*. 13:1352-1365.

Jannuzi, A.L., T.A. Bunch, R.F. West, and D.L. Brower. 2004. Identification of integrin beta subunit mutations that alter heterodimer function in situ. *Mol Biol Cell*. 15:3829-3840.

Jayo, A., I. Conde, P. Lastres, C. Martinez, J. Rivera, V. Vicente, and C. Gonzalez-Manchon. 2010. L718P mutation in the membrane-proximal cytoplasmic tail of beta 3 promotes abnormal alpha IIb beta 3 clustering and lipid microdomain coalescence, and associates with a thrombasthenia-like phenotype. *Haematologica*. 95:1158-1166.

Jonason, J.H., N. Gavrilova, M. Wu, H. Zhang, and H. Sun. 2007. Regulation of SCF(SKP2) ubiquitin E3 ligase assembly and p27(KIP1) proteolysis by the PTEN pathway and cyclin D1. *Cell Cycle*. 6:951-961.

Jonkman, M.F., H.H. Pas, M. Nijenhuis, G. Kloosterhuis, and G. Steege. 2002. Deletion of a cytoplasmic domain of integrin beta4 causes epidermolysis bullosa simplex. *J Invest Dermatol*. 119:1275-1281.

Joshi, P.M., M.R. Riddle, N.J. Djabrayan, and J.H. Rothman. 2010. Caenorhabditis elegans as a model for stem cell biology. *Dev Dyn*. 239:1539-1554.

Juan, G., and C. Cordon-Cardo. 2001. Intranuclear compartmentalization of cyclin E during the cell cycle: disruption of the nucleoplasm-nucleolar shuttling of cyclin E in bladder cancer. *Cancer Res*. 61:1220-1226.

Karakuzu, O., D.P. Wang, and S. Cameron. 2009. MIG-32 and SPAT-3A are PRC1 homologs that control neuronal migration in Caenorhabditis elegans. *Development*. 136:943-953.

Kauffman, E.C., B.D. Robinson, M.J. Downes, L.G. Powell, M.M. Lee, D.S. Scherr, L.J. Gudas, and N.P. Mongan. 2011. Role of androgen receptor and associated lysine-demethylase coregulators, LSD1 and JMJD2A, in localized and advanced human bladder cancer. *Mol Carcinog*.

Kee, W.J., E.R. Li, and F.M. Watt. 2000. beta1B integrin subunit contains a double lysine motif that can cause accumulation within the endoplasmic reticulum. *J Cell Biochem*. 78:97-111.

Keyomarsi, K. 2008. A novel MCM-2 fragment with potential biological function in senescence. *Cell Cycle*. 7:3480.

Kihira, S., E.J. Yu, J. Cunningham, E.J. Cram, and M. Lee. 2012. A novel mutation in beta integrin reveals an integrin-mediated interaction between the extracellular matrix and cki-1/p27KIP1. *PLoS One*. 7:e42425.

Kim, Y., and E.T. Kipreos. 2007. The Caenorhabditis elegans replication licensing factor CDT-1 is targeted for degradation by the CUL-4/DDB-1 complex. *Mol Cell Biol*. 27:1394-1406.

Kinoshita, Y., A.D. Jarell, J.M. Flaman, G. Foltz, J. Schuster, B.L. Sopher, D.K. Irvin, K. Kanning, H.I. Kornblum, P.S. Nelson, P. Hieter, and R.S. Morrison. 2001. Pescadillo, a novel cell cycle regulatory protein abnormally expressed in malignant cells. *J Biol Chem*. 276:6656-6665.

Kipreos, E.T. 2005. C. elegans cell cycles: invariance and stem cell divisions. *Nat Rev Mol Cell Biol*. 6:766-776.

Kipreos, E.T., L.E. Lander, J.P. Wing, W.W. He, and E.M. Hedgecock. 1996. cul-1 is required for cell cycle exit in C. elegans and identifies a novel gene family. *Cell*. 85:829-839.

Komaru, K., Y. Ishida, Y. Amaya, M. Goseki-Sone, H. Orimo, and K. Oda. 2005. Novel aggregate formation of a frame-shift mutant protein of tissue-nonspecific alkaline phosphatase is ascribed to three cysteine residues in the C-terminal extension. Retarded secretion and proteasomal degradation. *FEBS J*. 272:1704-1717.

Kostic, I., S. Li, and R. Roy. 2003. cki-1 links cell division and cell fate acquisition in the C. elegans somatic gonad. *Dev Biol*. 263:242-252.

Kostic, I., and R. Roy. 2002. Organ-specific cell division abnormalities caused by mutation in a general cell cycle regulator in C. elegans. *Development*. 129:2155-2165.

Kundel, D.W., E. Stromquist, A.L. Greene, O. Zhdankin, R.R. Regal, and T.A. Rose-Hellekant. 2012. Molecular characterizations of Nop16 in murine mammary tumors with varying levels of c-Myc. *Transgenic Res*. 21:393-406.

Kurihara, L.J., B.G. Stewart, A.E. Gammie, and M.D. Rose. 1996. Kar4p, a karyogamy-specific component of the yeast pheromone response pathway. *Mol Cell Biol*. 16:3990-4002.

Lahav, J., M.A. Schwartz, and R.O. Hynes. 1982. Analysis of platelet adhesion with a radioactive chemical crosslinking reagent: interaction of thrombospondin with fibronectin and collagen. *Cell*. 31:253-262.

Laine, V., C. Frokjaer-Jensen, H. Couchoux, and M. Jospin. 2011. The alpha1 subunit EGL-19, the alpha2/delta subunit UNC-36, and the beta subunit CCB-1 underlie voltage-dependent calcium currents in Caenorhabditis elegans striated muscle. *J Biol Chem*. 286:36180-36187.

Languino, L.R., and E. Ruoslahti. 1992. An alternative form of the integrin beta 1 subunit with a variant cytoplasmic domain. *J Biol Chem*. 267:7116-7120.

Lee, J., and S.S. Kim. 2009. The function of p27 KIP1 during tumor development. *Exp Mol Med*. 41:765-771.

Lee, J.O., L.A. Bankston, M.A. Arnaout, and R.C. Liddington. 1995. Two conformations of the integrin A-domain (I-domain): a pathway for activation? *Structure*. 3:1333-1340.

Lee, L.W., C.C. Lee, C.R. Huang, and S.J. Lo. 2012. The nucleolus of Caenorhabditis elegans. *Journal of biomedicine & biotechnology*. 2012:601274.

Lee, M., E.J. Cram, B. Shen, and J.E. Schwarzbauer. 2001a. Role of bpat-3 integrins in development and function of *Caenorhabditis elegans* muscles and gonads. *J. Biol. Chem.* 276:36404-36410.

Lee, M., E.J. Cram, B. Shen, and J.E. Schwarzbauer. 2001b. Roles for beta(pat-3) integrins in development and function of Caenorhabditis elegans muscles and gonads. *J Biol Chem*. 276:36404-36410.

Leptin, M., T. Boegart, R. Lehman, and M. Wilcox. 1989. The function of PS integrins during *Drosophila* embryogenesis. *Cell*. 56:401-408.

Lerch-Gaggl, A., J. Haque, J. Li, G. Ning, P. Traktman, and S.A. Duncan. 2002. Pescadillo is essential for nucleolar assembly, ribosome biogenesis, and mammalian cell proliferation. *J Biol Chem*. 277:45347-45355.

Li, X., M.W. Graner, E.L. Williams, C.E. Roote, T.A. Bunch, and S. Zusman. 1998. Requirements for the cytoplasmic domain of the alphaPS1, alphaPS2 and betaPS integrin subunits during Drosophila development. *Development*. 125:701-711.

Lin, X., H. Qadota, D.G. Moerman, and B.D. Williams. 2003a. C. elegans PAT-6/actopaxin plays a critical role in the assembly of integrin adhesion complexes in vivo. *Curr Biol*. 13:922-932.

Lin, X., H. Qadota, D.G. Moerman, and B.D. Williams. 2003b. *C. elegans* PAT-6/Actopaxin plays a critical role in the assembly of integrin adhesion complexes *in vivo*. *Current Biology*. 13:922-932.

Liu, Q., T.I. Jones, R.A. Bachmann, M. Meghpara, L. Rogowski, B.D. Williams, and P.L. Jones. 2012. C. elegans PAT-9 is a nuclear zinc finger protein critical for the assembly of muscle attachments. *Cell & bioscience*. 2:18.

Lo, S.J., C.C. Lee, and H.J. Lai. 2006. The nucleolus: reviewing oldies to have new understandings. *Cell Res*. 16:530-538.

Loomis, R.J., Y. Naoe, J.B. Parker, V. Savic, M.R. Bozovsky, T. Macfarlan, J.L. Manley, and D. Chakravarti. 2009. Chromatin binding of SRp20 and ASF/SF2 and dissociation from mitotic chromosomes is modulated by histone H3 serine 10 phosphorylation. *Mol Cell*. 33:450-461.

Lorson, M.A., H.R. Horvitz, and S. van den Heuvel. 2000. LIN-5 is a novel component of the spindle apparatus required for chromosome segregation and cleavage plane specification in Caenorhabditis elegans. *J Cell Biol*. 148:73-86.

Lu, Z., and T. Hunter. 2010. Ubiquitylation and proteasomal degradation of the p21(Cip1), p27(Kip1) and p57(Kip2) CDK inhibitors. *Cell Cycle*. 9.

Lyapina, S., G. Cope, A. Shevchenko, G. Serino, T. Tsuge, C. Zhou, D.A. Wolf, N. Wei, and R.J. Deshaies. 2001. Promotion of NEDD-CUL1 conjugate cleavage by COP9 signalosome. *Science*. 292:1382-1385.

Lyapina, S.A., C.C. Correll, E.T. Kipreos, and R.J. Deshaies. 1998. Human CUL1 forms an evolutionarily conserved ubiquitin ligase complex (SCF) with SKP1 and an F-box protein. *Proc Natl Acad Sci U S A*. 95:7451-7456.

Lykke-Andersen, J., M.D. Shu, and J.A. Steitz. 2000. Human Upf proteins target an mRNA for nonsense-mediated decay when bound downstream of a termination codon. *Cell*. 103:1121-1131.

Ma, L., X. Gao, J. Luo, L. Huang, Y. Teng, and H.R. Horvitz. 2012. The Caenorhabditis elegans gene mfap-1 encodes a nuclear protein that affects alternative splicing. *PLoS Genet*. 8:e1002827.

Mackinnon, A.C., H. Qadota, K.R. Norman, D.G. Moerman, and B.D. Williams. 2002. C. elegans PAT-4/ILK functions as an adaptor protein within integrin adhesion complexes. *Curr Biol*. 12:787-797.

Maduzia, L.L., A.F. Roberts, H. Wang, X. Lin, L.J. Chin, C.M. Zimmerman, S. Cohen, X.H. Feng, and R.W. Padgett. 2005. C. elegans serine-threonine kinase KIN-29 modulates TGFbeta signaling and regulates body size formation. *BMC Dev Biol*. 5:8.

Manzotti, M., P. Dell'Orto, P. Maisonneuve, M. Fornaro, L.R. Languino, and G. Viale. 2000. Down-regulation of beta(1C) integrin in breast carcinomas correlates with high proliferative fraction, high histological grade, and larger size. *Am J Pathol*. 156:169-174.

Marquez-Lona, E.M., Z. Tan, and S.S. Schreiber. 2012. Nucleolar stress characterized by downregulation of nucleophosmin: a novel cause of neuronal degeneration. *Biochem Biophys Res Commun*. 417:514-520.

Martin-Bermudo, M.D., I. Alvarez-Garcia, and N.H. Brown. 1999. Migration of the Drosophila primordial midgut cells requires coordination of diverse PS integrin functions. *Development*. 126:5161-5169.

Martin-Bermudo, M.D., and N.H. Brown. 2000. The localized assembly of extracellular matrix integrin ligands requires cell-cell contact. *J Cell Sci*. 113 Pt 21:3715-3723.

Mello, C.C., J.M. Kramer, D. Stinchcomb, and V. Ambros. 1991. Efficient gene transfer in C.elegans: extrachromosomal maintenance and integration of transforming sequences. *EMBO J*. 10:3959-3970.

Mercer, K.B., D.B. Flaherty, R.K. Miller, H. Qadota, T.L. Tinley, D.G. Moerman, and G.M. Benian. 2003. Caenorhabditis elegans UNC-98, a C2H2 Zn finger protein, is a novel partner of UNC-97/PINCH in muscle adhesion complexes. *Mol Biol Cell*. 14:2492-2507.

Meredith, J., Jr., Y. Takada, M. Fornaro, L.R. Languino, and M.A. Schwartz. 1995. Inhibition of cell cycle progression by the alternatively spliced integrin beta 1C. *Science*. 269:1570-1572.

Merz, D.C., G. Alves, T. Kawano, H. Zheng, and J.G. Culotti. 2003. UNC-52/perlecan affects gonadal leader cell migrations in *C. elegans* hermaphrodites through alterations in growth factor signaling. *Dev. Biol.* 256:173-186.

Mettouchi, A., S. Klein, W. Guo, M. Lopez-Lago, E. Lemichez, J.K. Westwick, and F.G. Giancotti. 2001. Integrin-specific activation of Rac controls progression through the G(1) phase of the cell cycle. *Mol Cell*. 8:115-127.

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

Montanaro, L., D. Trere, and M. Derenzini. 2008. Nucleolus, ribosomes, and cancer. *Am J Pathol*. 173:301-310.

Montanez, E., S.A. Wickstrom, J. Altstatter, H. Chu, and R. Fassler. 2009. Alpha-parvin controls vascular mural cell recruitment to vessel wall by regulating RhoA/ROCK signalling. *EMBO J*. 28:3132-3144.

Moore, L.L., G. Stanvitch, M.B. Roth, and D. Rosen. 2005. HCP-4/CENP-C promotes the prophase timing of centromere resolution by enabling the centromere association of HCP-6 in Caenorhabditis elegans. *Mol Cell Biol*. 25:2583-2592.

Morimoto, M., T. Nishida, R. Honda, and H. Yasuda. 2000. Modification of cullin-1 by ubiquitin-like protein Nedd8 enhances the activity of SCF(skp2) toward p27(kip1). *Biochem Biophys Res Commun*. 270:1093-1096.

Moro, L., E. Perlino, E. Marra, L.R. Languino, and M. Greco. 2004. Regulation of beta1C and beta1A integrin expression in prostate carcinoma cells. *J Biol Chem*. 279:1692-1702.

Moss, E.G., R.C. Lee, and V. Ambros. 1997. The cold shock domain protein LIN-28 controls developmental timing in C. elegans and is regulated by the lin-4 RNA. *Cell*. 88:637-646.

Nagano, M., D. Hoshino, N. Koshikawa, T. Akizawa, and M. Seiki. 2012. Turnover of focal adhesions and cancer cell migration. *International journal of cell biology*. 2012:310616.

Natarajan, L., B.M. Jackson, E. Szyleyko, and D.M. Eisenmann. 2004. Identification of evolutionarily conserved promoter elements and amino acids required for function of the C. elegans beta-catenin homolog BAR-1. *Developmental Biology*. 272:536-557.

O'Toole, T.E., J. Ylanne, and B.M. Culley. 1995. Regulation of integrin affinity states through an NPXY motif in the beta subunit cytoplasmic domain. *J Biol Chem*. 270:8553-8558.

Park, C.C., H.J. Zhang, E.S. Yao, C.J. Park, and M.J. Bissell. 2008. Beta1 integrin inhibition dramatically enhances radiotherapy efficacy in human breast cancer xenografts. *Cancer Res*. 68:4398-4405.

Pepper, A.S., J.E. McCane, K. Kemper, D.A. Yeung, R.C. Lee, V. Ambros, and E.G. Moss. 2004. The C. elegans heterochronic gene lin-46 affects developmental timing at two larval stages and encodes a relative of the scaffolding protein gephyrin. *Development*. 131:2049-2059.

Perlino, E., M. Lovecchio, R.A. Vacca, M. Fornaro, L. Moro, P. Ditonno, M. Battaglia, F.P. Selvaggi, M.G. Mastropasqua, P. Bufo, and L.R. Languino. 2000. Regulation of mRNA and protein levels of beta1 integrin variants in human prostate carcinoma. *Am J Pathol*. 157:1727-1734.

Philipp-Staheli, J., S.R. Payne, and C.J. Kemp. 2001. p27(Kip1): regulation and function of a haploinsufficient tumor suppressor and its misregulation in cancer. *Exp Cell Res*. 264:148-168.

Pippa, R., L. Espinosa, G. Gundem, R. Garcia-Escudero, A. Dominguez, S. Orlando, E. Gallastegui, C. Saiz, A. Besson, M.J. Pujol, N. Lopez-Bigas, J.M. Paramio, A. Bigas, and O. Bachs. 2012. p27Kip1 represses transcription by direct interaction with p130/E2F4 at the promoters of target genes. *Oncogene*. 31:4207-4220.

Pleasance, E.D., M.A. Marra, and S.J. Jones. 2003. Assessment of SAGE in transcript identification. *Genome Res*. 13:1203-1215.

Plow, E.F., T.A. Haas, L. Zhang, J. Loftus, and J.W. Smith. 2000. Ligand binding of integrins. *Journal of Biological Chemistry*. 275:21785-21788.

Pulak, R., and P. Anderson. 1993. mRNA surveillance by the Caenorhabditis elegans smg genes. *Genes Dev*. 7:1885-1897.

Qu, W., C. Ren, Y. Li, J. Shi, J. Zhang, X. Wang, X. Hang, Y. Lu, D. Zhao, and C. Zhang. 2011. Reliability analysis of the Ahringer Caenorhabditis elegans RNAi feeding library: a guide for genome-wide screens. *BMC Genomics*. 12:170.

Ranawade, A.V., P. Cumbo, and B.P. Gupta. 2013. Caenorhabditis elegans histone deacetylase hda-1 is required for morphogenesis of the vulva and LIN-12/Notch-mediated specification of uterine cell fates. *G3*. 3:1363-1374.

Reszka, A.A., Y. Hayashi, and A.F. Horwitz. 1992. Identification of amino acid sequences in the integrin beta 1 cytoplasmic domain implicated in cytoskeletal association. *J Cell Biol*. 117:1321-1330.

Richardson, C.E., S. Kinkel, and D.H. Kim. 2011. Physiological IRE-1-XBP-1 and PEK-1 signaling in Caenorhabditis elegans larval development and immunity. *PLoS Genet*. 7:e1002391.

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., B.D. Williams, G.P. Mullen, and D.G. Moerman. 1993. Products of the unc-52 gene in Caenorhabditis elegans are homologous to the core protein of the mammalian basement membrane heparan sulfate proteoglycan. *Genes Dev*. 7:1471-1484.

Rose, D.M., P.M. Cardarelli, R.R. Cobb, and M.H. Ginsberg. 2000. Soluble VCAM-1 binding to a4 integrins is cell type-specific, activation-dependent, and disrupted during apoptosis in T cells. *Blood*:in press.

Ruoslahti, E., and M. D. Pierschbacher. 1987. New perspectives in cell adhesion: RGD and integrins. *Science*. 238:491-497.

Russo, M.A., M. Paolillo, Y. Sanchez-Hernandez, D. Curti, E. Ciusani, M. Serra, L. Colombo, and S. Schinelli. 2013. A small-molecule RGD-integrin antagonist inhibits cell adhesion, cell migration and induces anoikis in glioblastoma cells. *Int J Oncol*. 42:83-92.

Sabile, A., A.M. Meyer, C. Wirbelauer, D. Hess, U. Kogel, M. Scheffner, and W. Krek. 2006. Regulation of p27 degradation and S-phase progression by Ro52 RING finger protein. *Mol Cell Biol*. 26:5994-6004.

Safra, M., S. Ben-Hamo, C. Kenyon, and S. Henis-Korenblit. 2013. The ire-1 ER stress-response pathway is required for normal secretory-protein metabolism in C. elegans. *J Cell Sci*. 126:4136-4146.

Saha, A., and R.J. Deshaies. 2008. Multimodal activation of the ubiquitin ligase SCF by Nedd8 conjugation. *Mol Cell*. 32:21-31.

Saito, R.M., A. Perreault, B. Peach, J.S. Satterlee, and S. van den Heuvel. 2004. The CDC-14 phosphatase controls developmental cell-cycle arrest in C. elegans. *Nat Cell Biol*. 6:777-783.

Sanchez, S.E., E. Petrillo, A.R. Kornblihtt, and M.J. Yanovsky. 2011. Alternative splicing at the right time. *RNA Biol*. 8:954-959.

Schneider, J.G., S.R. Amend, and K.N. Weilbaecher. 2011. Integrins and bone metastasis: integrating tumor cell and stromal cell interactions. *Bone*. 48:54-65.

Schousboe, I., B.T. Nystrom, and G.H. Hansen. 2008. Differential binding of factor XII and activated factor XII to soluble and immobilized fibronectin--localization of the Hep-1/Fib-1 binding site for activated factor XII. *FEBS J*. 275:5161-5172.

Sibley, M.H., P.L. Graham, N. von Mende, and J.M. Kramer. 1994. Mutations in the alpha 2(IV) basement membrane collagen gene of Caenorhabditis elegans produce phenotypes of differing severities. *Embo J*. 13:3278-3285.

Skaar, J.R., and M. Pagano. 2009. Control of cell growth by the SCF and APC/C ubiquitin ligases. *Curr Opin Cell Biol*. 21:816-824.

Song, M.H., L. Aravind, T. Muller-Reichert, and K.F. O'Connell. 2008. The conserved protein SZY-20 opposes the Plk4-related kinase ZYG-1 to limit centrosome size. *Dev Cell*. 15:901-912.

Sonnenberg, A. 1993. Integrins and their ligands. *Curr. Top. Micro. Immun.* 184:7-35.

Steeg, P.S., and J.S. Abrams. 1997. Cancer prognostics: past, present and p27. *Nat Med*. 3:152-154.

Sterk, L.M., C.A. Geuijen, L.C. Oomen, J. Calafat, H. Janssen, and A. Sonnenberg. 2000. The tetraspan molecule CD151, a novel constituent of hemidesmosomes, associates with the integrin alpha6beta4 and may regulate the spatial organization of hemidesmosomes. *J Cell Biol*. 149:969-982.

Sumitani, M., K. Kasashima, J. Matsugi, and H. Endo. 2011. Biochemical properties of Caenorhabditis elegans HMG-5, a regulator of mitochondrial DNA. *J Biochem*. 149:581-589.

Szklarczyk, D., A. Franceschini, M. Kuhn, M. Simonovic, A. Roth, P. Minguez, T. Doerks, M. Stark, J. Muller, P. Bork, L.J. Jensen, and C. von Mering. 2011. The STRING database in 2011: functional interaction networks of proteins, globally integrated and scored. *Nucleic Acids Res*. 39:D561-568.

Tamkun, J.W., D.W. DeSimone, D. Fonda, R.S. Patel, C. Buck, A.F. Horwitz, and R.O. Hynes. 1986. Structure of integrin, a glycoprotein involved in the transmembrane linkage between fibronectin and actin. *Cell*. 46:271-282.

Terami, H., B.D. Williams, S. Kitamura, Y. Sakube, S. Matsumoto, S. Doi, T. Obinata, and H. Kagawa. 1999. Genomic organization, expression, and analysis of the troponin C gene pat-10 of Caenorhabditis elegans. *J Cell Biol*. 146:193-202.

Terasawa, M., M. Toya, F. Motegi, M. Mana, K. Nakamura, and A. Sugimoto. 2010. Caenorhabditis elegans ortholog of the p24/p22 subunit, DNC-3, is essential for the formation of the dynactin complex by bridging DNC-1/p150(Glued) and DNC-2/dynamitin. *Genes Cells*. 15:1145-1157.

Timmons, L., H. Tabara, C.C. Mello, and A.Z. Fire. 2003. Inducible systemic RNA silencing in Caenorhabditis elegans. *Mol Biol Cell*. 14:2972-2983.

Tsutsui, T., R. Fukasawa, A. Tanaka, Y. Hirose, and Y. Ohkuma. 2011. Identification of target genes for the CDK subunits of the Mediator complex. *Genes Cells*.

Tsvetkov, L.M., K.H. Yeh, S.J. Lee, H. Sun, and H. Zhang. 1999. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. *Curr Biol*. 9:661-664.

van Kuppevelt, T.H., L.R. Languino, J.O. Gailit, S. Suzuki, and E. Ruoslahti. 1989. An alternative cytoplasmic domain of the integrin beta 3 subunit. *Proc Natl Acad Sci U S A*. 86:5415-5418.

Vihervaara, A., C. Sergelius, J. Vasara, M.A. Blom, A.N. Elsing, P. Roos-Mattjus, and L. Sistonen. 2013. Transcriptional response to stress in the dynamic chromatin environment of cycling and mitotic cells. *Proc Natl Acad Sci U S A*. 110:E3388-3397.

Walker, J.L., and R.K. Assoian. 2005. Integrin-dependent signal transduction regulating cyclin D1 expression and G1 phase cell cycle progression. *Cancer Metastasis Rev*. 24:383-393.

Wang, X., K. Fukuda, I.J. Byeon, A. Velyvis, C. Wu, A. Gronenborn, and J. Qin. 2008. The structure of alpha-parvin CH2-paxillin LD1 complex reveals a novel modular recognition for focal adhesion assembly. *J Biol Chem*. 283:21113-21119.

Wang-Rodriguez, J., A.D. Dreilinger, G.M. Alsharabi, and A. Rearden. 2002. The signaling adapter protein PINCH is up-regulated in the stroma of common cancers, notably at invasive edges. *Cancer*. 95:1387-1395.

Warf, M.B., W.E. Johnson, and B.L. Bass. 2011. Improved annotation of C. elegans microRNAs by deep sequencing reveals structures associated with processing by Drosha and Dicer. *RNA*. 17:563-577.

Wennerberg, A., R. Fassler, B. Warmegard, and S. Johansson. 1998. Mutational analysis of the potential phosphorylation sites in the cytoplasmic domain of integrin b1A. *Journal of Cell Science*. 111:1117-1126.

Wickstrom, S.A., A. Lange, E. Montanez, and R. Fassler. 2010. The ILK/PINCH/parvin complex: the kinase is dead, long live the pseudokinase! *EMBO J*. 29:281-291.

Wilcox, M. 1990. Genetic analysis of the Drosophila PS integrins. *Cell Differ Dev*. 32:391-399.

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

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

Wilson, K.J., H. Qadota, P.E. Mains, and G.M. Benian. 2012. UNC-89 (obscurin) binds to MEL-26, a BTB-domain protein, and affects the function of MEI-1 (katanin) in striated muscle of Caenorhabditis elegans. *Mol Biol Cell*. 23:2623-2634.

Wilting, R.H., E. Yanover, M.R. Heideman, H. Jacobs, J. Horner, J. van der Torre, R.A. DePinho, and J.H. Dannenberg. 2010. Overlapping functions of Hdac1 and Hdac2 in cell cycle regulation and haematopoiesis. *EMBO J*. 29:2586-2597.

Xie, X., Z. Wang, and Y. Chen. 2007. Association of LKB1 with a WD-repeat protein WDR6 is implicated in cell growth arrest and p27(Kip1) induction. *Mol Cell Biochem*. 301:115-122.

Xu, X., J.H. Ahn, P. Tam, E.J. Yu, S. Batra, E.J. Cram, and M. Lee. 2010. Analysis of conserved residues in the betapat-3 cytoplasmic tail reveals important functions of integrin in multiple tissues. *Dev Dyn*. 239:763-772.

Xu, X., D. Lee, H.Y. Shih, S. Seo, J. Ahn, and M. Lee. 2005. Linking integrin to IP(3) signaling is important for ovulation in Caenorhabditis elegans. *FEBS Lett*. 579:549-553.

Xu, X., S.C. Rongali, J.P. Miles, K.D. Lee, and M. Lee. 2006. *pat-4*/ILK and *unc-112*/Mig-2 are required for gonad function in *Caenorhabditis elegans*. *Exp Cell Res*. 312:1475-1483.

Yang, X., S. Menon, K. Lykke-Andersen, T. Tsuge, X. Di, X. Wang, R.J. Rodriguez-Suarez, H. Zhang, and N. Wei. 2002. The COP9 signalosome inhibits p27(kip1) degradation and impedes G1-S phase progression via deneddylation of SCF Cul1. *Curr Biol*. 12:667-672.

Yook, K., T.W. Harris, T. Bieri, A. Cabunoc, J. Chan, W.J. Chen, P. Davis, N. de la Cruz, A. Duong, R. Fang, U. Ganesan, C. Grove, K. Howe, S. Kadam, R. Kishore, R. Lee, Y. Li, H.M. Muller, C. Nakamura, B. Nash, P. Ozersky, M. Paulini, D. Raciti, A. Rangarajan, G. Schindelman, X. Shi, E.M. Schwarz, M. Ann Tuli, K. Van Auken, D. Wang, X. Wang, G. Williams, J. Hodgkin, M. Berriman, R. Durbin, P. Kersey, J. Spieth, L. Stein, and P.W. Sternberg. 2012. WormBase 2012: more genomes, more data, new website. *Nucleic Acids Res*. 40:D735-741.

Zhang, C., G. Wang, C. Kang, Y. Du, and P. Pu. 2009. [Up-regulation of p27(kip1) by miR-221/222 antisense oligonucleotides enhances the radiosensitivity of U251 glioblastoma]. *Zhonghua Yi Xue Yi Chuan Xue Za Zhi*. 26:634-638.

Zhang, Y., K. Chen, Y. Tu, A. Velyvis, Y. Yang, J. Qin, and C. Wu. 2002. Assembly of the PINCH-ILK-CH-ILKBP complex precedes and is essential for localization of each component to cell-matrix adhesion sites. *J Cell Sci*. 115:4777-4786.

Zusman, S., Y. Grinblat, G. Yee, F.C. Kafatos, and R.O. Hynes. 1993. Analyses of PS integrin functions during Drosophila development. *Development*. 118:737-750.