A Novel Mutation in β Integrin Reveals an Integrin-Mediated Interaction between the Extracellular Matrix and cki-1/p27\textsuperscript{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\textsuperscript{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 cytoplasm. RNAi analysis links cell adhesion genes to the regulation of CKI-1. RNAi of unc-52/perlecan, ina-1/ι 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, skp-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 CKI-1/p27\textsuperscript{kip1} levels in the cell. Perturbed integrin signaling may lead to the inhibition of SCF ligase activity, mislocalization and elevation of CKI-1/p27\textsuperscript{kip1}. These results suggest that adhesion signaling is crucial for cell cycle regulation in vivo.

Introduction

Integrins are αβ heterodimeric receptors that mediate bidirectional interactions between cells and extracellular matrix (ECM) [1]. 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 [2]. Among the β subunits, β1 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 [3]. β1A is the dominant splice form and is expressed ubiquitously [3]. β1D is produced by alternative splicing and is found in striated muscle cells only [4]. Both β1A and β1D forms are localized to focal adhesions and retain the conserved NPXY phosphorylation motif [5,6]. However, the β1B variant, expressed in keratinocytes and hepatocytes, fails to localize to focal adhesions and exhibits dominant negative activity to β1A-paired integrins [7,8]. β1B is the result of mis-splicing of intron 7, and retains intronic sequence in its mRNA [9]. β1C is expressed in normal tissues, such as the prostatic epithelium, and is downregulated in cancer cells [10]. 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 [5,11].

In many cancerous conditions, integrins lose their connection to the ECM or change their expression patterns [3,12,13]. 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 [14,15]. 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 [16]. In some cases, integrin signaling can promote cell cycle arrest [17]. For example, the expression of integrin β1C in mammalian cells increases the level of p27\textsuperscript{kip1}, a CDK inhibitor [18]. In contrast, lowering the level of p27\textsuperscript{kip1} allows activation of the CDK/cyclin complex and promotes the cell cycle transition from G1 to S [19–22]. In these cells, adhesion to the ECM activates an E3 ubiquitin ligase that is essential for the degradation of p27\textsuperscript{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 [23]. However, there is little information available about how integrin signaling regulates the level of cell cycle inhibitors like p27\textsuperscript{kip1} in vivo.
The nematode *Caenorhabditis elegans* expresses only two integrins, PAT-3/INA-1 [24] and PAT-3/PAT-2 [25], which simplifies the analysis of genetic interactions between integrin and cell cycle control genes. Overexpression of *C. elegans* p27KIP1/CKI-1 has been found to induce growth arrest and the *dki-1* null mutation results in hyperplasia of tissues such as the hypodermis, the vulva and the intestine [26–28]. The disruption of *dki-1* also results in the production of extra distal tip cells from the somatic gonad lineage and the intestine [26–28]. The disruption of *pat-3(sp)* results in hyperplasia of tissues such as the hypodermis, the vulva and the intestine [26,31–33]. The disruption of *pat-3(sp)* causes simultaneously. The mutant is similar to the human β1B and β1C integrins. The *pat-3(sp)* mutant is similar to β1B because it retains intron sequence in the **p27KIP1** gene, disruption of which results in enlarged nucleoli, was depleted using RNAi [42,43]. In the *pat-3(+)* background, *ncl-1* (RNAi) significantly increased the size of the **p27KIP1** spot. Image analysis showed that the size of the green spot was increased by 2.4 fold when compared to that of control RNAi animals (Figure S1, Table S1). Therefore, we conclude that the observed spots on the nucleoli are likely to represent nucleolar localization.

In the *pat-3(sp)* rescued animals, hCKI-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, hCKI-1::GFP in *pat-3(sp)* was clumped and accumulated in a ring around a dark center in the nucleus (Figures 2B and 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 hCKI-1::GFP and the integrin (*pat-3(+)) or *pat-3(sp)) expressed, we first analyzed the amount of hCKI-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 hCKI-1::GFP protein levels. hCKI-1::GFP level in *pat-3(sp)* was ten fold more intense than that seen in *pat-3(+)* lysates (Figure 3A), suggesting that PAT-3 signaling may control hCKI-1 in vivo.

### Results

#### βpat-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* [37], and is required for muscle development and function. Null mutations in *pat-3* cause a fully penetrant embryonic arrest due to defective muscle elongation [25,30]. Previously, we created a mutation at the intron 7 splice junction in *pat-3* (previously known as *pat-3(1B)*). The disruption of *pat-3(1B)* results in enlarged nucleoli, was depleted using RNAi [42,43]. In the *pat-3(+)* background, *ncl-1* (RNAi) significantly increased the size of the **p27KIP1** spot. Image analysis showed that the size of the green spot was increased by 2.4 fold when compared to that of control RNAi animals (Figure S1, Table S1). Therefore, we conclude that the observed spots on the nucleoli are likely to represent nucleolar localization.

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 2B and 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 3A), suggesting that PAT-3 signaling may control CKI-1 levels.

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 *dki-1* transcription. RNA from each rescued line was isolated and analyzed for the amount of *pat-3* or *dki-1* mRNA using RT-PCR (Figure 4A). We also measured the *dki-1* mRNA level in BU7221, a *pat-3(sp)* rescued line without *dki-1::GFP* [34]. No significant differences were seen in any of the experiments, suggesting that *pat-3(sp)* does not significantly increase the level of *dki-1* mRNA compared to controls (Figure 4B).

**Figure 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.

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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 [44]. 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 [44–45]. Integrins are located in the base of these structures and anchor the sarcomeres to the basement membrane. Data from the SAGE database [37] 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 [44] in pat-3(+) would result in mislocalization of CKI-1::GFP in the nucleoplasm, similar to that seen in pat-3(sp) animals (Results summarized in Table 1).

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 5B and 2D). Next, integrin α subunits were depleted. Depletion of inta-1 in the pat-3(+) animals also resulted in abnormally clumped CKI-1::GFP (Figures 5C), suggesting that the CKI-1 localization is integrin dependent. Among the focal adhesion genes, pat-4/ILK [46], unc-97/PINCH [47] and pat-6/parvin [48] together form an IPP complex, which is implicated in the control of signaling pathways by the phosphorylation of downstream targets [49]. RNAi of pat-4/ILK or unc-97/PINCH in pat-3(+) resulted in the expected uncoordinated phenotypes (Figure S2) and CKI-1 mislocalization in hypodermal nuclei (Figure 5D and 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 5F). Because pat-6 RNAi did not result in a strong uncoordinated phenotype (Figure S2), 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.

Our RNAi screen also found that unc-52/perlecan, a basement membrane component and presumptive integrin ligand [50–52], is required for the proper localization of CKI-1. RNAi of unc-52 in pat-3(+) affected the CKI-1 localization pattern (Figure 5G). In contrast, depletion of other basement membrane components, such as let-27/collagen IV [53], failed to affect the localization (Figure 5H), suggesting that a subset of ECM components is required for CKI-1 localization.

Figure 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.

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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 [23]. Integrin-triggered p27KIP1 degradation has been observed in mammalian cells. For example, integrin crosstalk with receptor tyrosine kinase (RTK) induces the production of SCF^SKP2 [54], a member of the SCF E3 ubiquitin ligase complex, which binds to the SKP1 [55], CUL1 [56] and FBX-1 [23] E3 ligase complex. This SCF complex targets CDK/cyclin inhibitors such as p27KIP1 and p21CIP1 [57].

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 [33], cul-1/CUL1 [58] and lin-23/F-Box [59] and monitored CKI-1 localization (Figure 5I, 5J, and 5K). We first examined skpt-1/SKP2 (RNAi) in the pat-3(sp) background. CKI-1::GFP accumulation in the pat-3(sp) animals compared to pat-3(+). 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 [90] 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.

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Ubiquitin-mediated Protein Degradation Regulates Localization of CKI-1::GFP
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/p27KIP1 in addition to affecting nuclear localization patterns (Figures 5A and 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 let-2, ina-1, unc-97, unc-52, pat-6 RNAi results suggest there may not be a direct relationship between protein levels and localization.

Discussion

In this study, integrin regulation of CKI-1 was assessed in vivo. Our analysis revealed that CKI-1/p27KIP1 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 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/p27KIP1, 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.

Table 1. RNAi analysis of genes involved in localization pattern.

| Tested Gene       | pat-3(+) | pat-3(sp) | SAGE search in hypodermis* |
|-------------------|----------|-----------|---------------------------|
| pat-3/integrin    | ++ (80)  | ++ (80)   | Positive                  |
| ina-1/integrin    | ++ (80)  | ++ (50)   | Positive                  |
| pat-4/ILK         | ++ (35)  | ++ (35)   | Positive                  |
| unc-97/PINCH      | +++ (80) | +++ (80)  | Positive                  |
| pat-6/parvin      | 0 (80)   | +++ (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                  |
| Y71G128.11/talin  | 0 (35)   | +++ (35)  | Positive                  |
| lin-23/F-Box      | ++ (60)  | +++ (60)  | Positive                  |
| cul-1/CUL1        | ++ (80)  | +++ (70)  | Positive                  |
| skp-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. 0 = 0% mislocalization, + = 1–25% mislocalization, ++ = 26–50% mislocalization, +++ = 51–75% mislocalization.

*Name of the gene was queried individually against the SAGE database. doi:10.1371/journal.pone.0042425.t001

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*Name of the gene was queried individually against the SAGE database.
mammals, stressed ribosomal synthesis leads to cell cycle arrest via increased p27KIP1 levels [63]. In addition, mutations in the cytoplasmic tail of β4 integrin or p27 β4 binding protein (p27β4BP) result in 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.

**Figure 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. Panels A: the negative control L4440 plasmid, B: pat-3 RNAi, C: ina-1 RNAi, D: pat-4 RNAi, E: unc-97 RNAi, F: pat-6 RNAi, G: unc-52 RNAi, H: let-2 RNAi, I: skpt-1 RNAi, J: lin-23 RNAi and K: cul-1 RNAi. doi:10.1371/journal.pone.0042425.g005
Although further studies are required, we speculate that the splice defective \textit{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 \( \alpha \beta \) heterodimers and both subunits are necessary for integrin function. Therefore, depletion of the \( \alpha \) subunit would have the same effect as disruption of the \( \beta \) subunit, \textit{PAT-3}. Indeed, CKI-1::GFP mislocalization and increased expression were observed when the \( \alpha \) integrin gene \textit{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 \textit{pat-4/ILK} [46,67], \textit{unc-97/PINCH} [47] and \textit{pat-6/parvin} [48] 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 \textit{pat-4/ILK} or \textit{unc-97/PINCH} is not present [68] but other studies, including ours, suggest these molecules may have independent roles [69,70]. In addition, PINCH or parvin binding to ILK is mutually exclusive, suggesting that PINCH might provide a different mode of signaling than parvin [71,72]. ILK has been shown to play an important role in cell proliferation in tissue culture [73] and PINCH is frequently upregulated in human cancers [74]. Disruption of ILK or PINCH inhibits cell proliferation and increases the expression level of \( \mu_2 \)-\textit{KIP1} and pRb [75], suggesting that cells might generally respond to perturbed SCF ligase forms a complex with a protein other than RBX-1 for while RBX-1/ROC1 showed no effect. This might indicate that \textit{SKP2/Cullins/transferase (RBX-1/ROC1), an adaptor (SKPT-1/SKP2), and SCF E3 ligase genes resulted in mislocalization of CKI-1::GFP. 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 ligands and signaling is initiated and propagated by molecules such as \textit{pat-4/ILK} and \textit{unc-97/PINCH} to SCF ligase which degrades CKI-1 (Figure 6). \textit{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 \textit{PAT-3} integrins or by acting as a non-functional \( \beta \) subunit that significantly dilutes integrin signaling [8]. SAGE analysis indicates that \textit{pat-3} and \textit{ina-1} are expressed in hypodermal cells [79,80] consistent with a previously identified role for \textit{ina-1}/\( \alpha \) integrin function in hypodermis [28]. 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 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 \textit{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 \textit{pat-4/ILK and unc-97/PINCH} and to maintain SCF ubiquitin ligase activity. Importantly, \textit{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.

**Materials and Methods**

**Animals and Culture**

\textit{Caenorhabditis elegans} were cultured on nematode growth medium (NGM) agar plates seeded with OP50 \textit{E. coli} under standard conditions [81]. \textit{RW3600 qC1 \textit{dyf-19} (e1259) \textit{glp-1} (q339)/\textit{pat-3} (ts564) III} [38] were acquired from the \textit{Caenorhabditis Genetics Center (St. Paul, MN)}. The \textit{pat-3} transgenic rescued lines used in this study are listed in Table 2. All transgenic lines were cultured under standard conditions [81].

**Mutant \textit{Pat-3} Constructs and Germline Tranformation**

\textit{pPAT3 (+)-PB12K} and mutant constructs for \textit{pPAT3-sp} were created using overlap extension PCR and have been described previously [34]. Germline transformation was performed using the standard protocol for microinjections [82]. Briefly, \textit{pPAT3} constructs were mixed with \textit{cki-1:GFP} [26] and \textit{rab-3:RFP} or \textit{myo-3:RFP} [83]. All rescued lines were made at a mixture of 5 \( \mu \)g/ml of \textit{pPAT3}, 2 \( \mu \)g/ml of \textit{pVT352G (cki-1:GFP)}, and 100 \( \mu \)g/ml of \textit{pGH8 (rab-3:RFP)} or 100 \( \mu \)g/ml of \textit{pCF104 (myo-3:RFP)} in TE buffer (pH 7.5). This mixture was injected into a distal gonad of the \textit{RW3600 qC1 \textit{dyf-19(e1259) glp-1(q339)/pat-3(ts564)} 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 [84].

**Phenotype Characterization and Nuclei Expression Pattern Identification**

To characterize rescued lines, young adult worms were mounted in a drop of M9 buffer containing 1% Na\textsubscript{2}CO\textsubscript{3} (Sigma Chemical Co., St. Louis, MO) or 0.5 mM levamisole on a 24×60 mm coverslip coated with 4% agarose and examined on a Nikon TE2000-U Diaphot epifluorescence microscope. Images were captured using a CoolSnap f 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 \textit{pat-3(+)} animals, GFP was apparently nuclear: 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) [85]. 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 [86]. 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 [34]. Primer sequences listed below were used for amplification:

| Primer  | Sequence                  |
|---------|---------------------------|
| cki-1 Forward 2 | 5'-GGAGTTCTACAGAACC-3' |
| cki-1 Reverse 2 | 5'-CACCGGAGACAGCCTTG-3' |
| PAT3PT Forward 1 | 5'-CTCAAGGAAACTACACCCTG-3' |
| PAT3PT Reverse 1 | 5'-TTAGTTGGCTTTTCCACCGTA-3' |

Table 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(s1564), pPAT3(+), pGH8 rab-3:RFP, pVT352G cki-1:GFP | This study |
| pat-3(+)+myo-3:RFP, cki-1:GFP (BU445 kqEx76) | pat-3(s1564), pPAT3(+), pCF1104 myo-3:RFP, pVT352G cki-1:GFP | This study |
| pat-3(+)+rab-3:RFP (BU446 kqEx77) | pat-3(s1564), pPAT3(+), pGH8 rab-3:RFP | This study |
| pat-3(+)+sur-5:GFP (BU443) | pat-3(s1564), pPAT3(+), TG96 sur-5:GFP | [84] |
| pat-3(sp)+rab-3:RFP, cki-1:GFP (BU2222 kqEx73) | pat-3(s1564), pPAT3-sp, pGH8 rab-3:RFP, pVT352G cki-1:GFP | This study |
| pat-3(sp)+myo-3:RFP, cki-1:GFP (BU2223 kqEx74) | pat-3(s1564), pPAT3-sp, pCF1104 myo-3:RFP, pVT352G cki-1:GFP | This study |
| pat-3(sp)+sur-5:GFP (BU2221 kqEx21) | pat-3(s1564), pPAT3-sp, TG96 sur-5:GFP | [34] |

Multiple transgenic lines of each pat-3 rescue were analyzed.
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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 180 V for 1 hour. Isolated protein bands were electrotransferred onto a nitrocellulose membrane (Whatman Ltd., Dassel, Germany) using wet transfer at 100 V 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°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 chemiluminesence 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 [87,88]. In addition to the standard RNAi protocol, we synchronized the stage of animals; embryos were collected using the standard 20% alkaline hypochlorite solution method [86]. 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 hypo-morphic L4440 animals were observed onto RNAi plates, which were incubated in 20°C 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°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 chemiluminesence 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.

Supporting Information

Figure S1 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 < 0.001) the size of the area seen in the no RNAi control in panel B: CKI-1::GFP in pat-3(+).background. (TIF)

Figure S2 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, unc-112, and unc-111 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 < 0.0001 (compared to L4440). Panel B: Number of body bends in 30 seconds was measured in pat-3(+), animals treated with RNAi of c79-1, unc-112, and unc-111 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 < 0.0001 (compared to L4440). (TIF)

Table S1 ImageJ analysis data. (XLSX)
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