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14. ABSTRACT INT6 is a target of MMTV, whose insertion generates truncated proteins that induce tumor formation. Our lab demonstrate an evolutionarily conserved function of Int6 in the model system *Schizosaccharomyces pombe*: regulation of proteasome transport and assembly. In this project, I propose to test the hypothesis that Int6 acts as a breast tumor suppressor, which functions to regulate the 26S proteasome, in human mammary epithelial cells. Using siRNAs to reduce Int6 expression level, I found that Int6 is important for genomic stability, as proposed. Furthermore, cells in which Int6 expression is knocked down are hypersensitive to a proteasome inhibitor. This result supports our idea that Int6 can regulate the proteasome in human mammary epithelial cells. Finally, I showed that the dosage of Int6 is important for the development of acini in 3D culture system. In parallel to these studies, I further examined how Int6 can regulate the proteasome. My data show that mutating a conserved leucine in the PCI domain of Int6 leads to its mislocalization in human mammary epithelial cells, and overexpressing this mutant protein in MCF10A cells causes malignant growth in 3D culture. Also, by measuring the protein dynamics in *S. pombe* using Fluorescent loss in photo bleaching (FLIP), I showed that the proteasome subunit Pad1-GFP is actively entering the nucleus, and this process is affected by *int6* deletion.

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Introduction

This project centers on the Int6 protein, which has been implicated in the development of breast cancer because it was first identified from an insertional mutagenesis screen using MMTV (Mouse Mammary Tumor Virus) to search for components in mice that can influence breast tumorigenesis. Reduction in *int6* expression has been observed in human breast tumors, which further underscores the importance of Int6 and human breast cancer formation. Based on our study with a genetic model system, the fission yeast *Schizosaccharomyces pombe*, we have proposed a model in which the *int6* gene in humans acts as a tumor suppressor gene whose normal function is to mediate the functioning of the 26S proteasome. In this project we have tested this hypothesis directly by using human mammary epithelial cells. Our specific aims are to investigate whether altering Int6 functions can influence (1) proteasome functioning and (2) chromosome segregation in human mammary epithelial cells.

Report Body

Task 1: Generate cell lines with reduced Int6 levels

a. Int6 antibody production:

I have prepared several polyclonal antibodies (see Annual Report of 2004). These antibodies allow us to evaluate *int6* gene expression and gene knock down, and carry out co-immunoprecipitation experiments.

b. and c. Test candidate DNA sequences for Int6 knockdown:

As reported in the Progress Report of last year, I have successfully identified several siRNA sequences that can effectively knock down *int6* expression in both HeLa and MCF10A cells. I have since then moved on to build lentivirus expression vector pLL3.7 (Rubinson et al., 2003) to express these siRNA sequences as shRNAs. These vectors will be used to establish cell lines in which *int6* expression is stably knocked-down in order to perform long-term experiments in cells (e.g., MCF10A) that are otherwise hard to chemically transfect. My preliminary data show that Int6 is necessary for viability (see last year's report and Figure 1A). Therefore, to prevent losing the cells in which *int6* expression is most efficiently knocked down, I will use the tet-ON and tet-OFF inducible system (e.g., pLenti4/BLOCKIT, Invitrogen) to allow the expression of the shRNA to be regulated. Since HeLa cells can be chemically transfected at high frequencies, I will use these cells first to optimize conditions before moving on to MCF10A cells. I do not yet know whether these shRNA vectors can successfully knock down *int6* expression.

Task 2: Analyze proteasome function in engineered cells

I reasoned that if Int6 is important for proper proteasome functions in mammalian cells, knocking down Int6 expression should lead to proteasome deficiency. To test this idea, I treated *int6* knocked-down ($int6^{kd}$) HeLa cells with a proteasome inhibitor, MG132, and found that indeed $int6^{kd}$ cells are hypersensitive to MG132 (Figure 1E). My next step is to determine whether there is accumulation of polyubiquitinated proteins in these $int6^{kd}$ cells and then move on to examine MCF10A cells. In parallel, I am testing Int6 binding to the proteasome by determining whether Int6 can co-immunoprecipitate proteasome subunits.

Task 3: Analyze genetic stability in engineered cells

In our previous annual report, I have shown that *int6*^{kd} cells display mitotic abnormalities in that they become multinucleated and contain abnormal spindles (see also Figure 1A-D). In this project period, I have used a 3-D culture system to examine whether alteration in Int6 function can lead to transformation of the mammary epithelial cells. This 3D system has been developed to allow human mammary epithelial cells to grow in an *in vitro* environment that at least partly mimics their native environment, and the cells appear to undertake steps that are critical for normal mammary gland development or tumor formation. After seeding, these cells first proliferate and then cells in the outer layer in contact with the matrix polarize into one layer, which encircles the rest of the cells and blocks their contact with the matrix. These encircled cells eventually undergo *apoptosis* to generate a hollow luminal chamber, which resembles the mammary duct.

In human breast tumors *int6* expression is frequently diminished, and I have shown that in *S. pombe* deleting just one copy of their *int6* creates a haploinsufficiency (Yen et al., 2003). These data suggest that maintaining proper protein levels of Int6 is very important for the cell. To test the importance of Int6 protein levels in normal mammary gland development and in tumorigenesis, I overexpressed Int6 or Int6ΔC (a truncated form induced by MMTV insertion) in MCF10A cells and then monitored formation of acini *in vitro*. Data in Figure 2 show that while the majority of spheres generated by cells carrying the vector control are either hollow or only half-filled with cells, the reverse was observed with cells overexpressing full-length Int6 or Int6ΔC—most of their spheres were filled with cells. These results suggest that overexpressing either Int6 or Int6ΔC can block apoptosis during formation of acini.

While exploring the roles of Int6 in tumorigenesis, I also engaged in mechanistic studies to better understand the regulation of the proteasome by Int6. The studies are carried out in both human mammary epithelial cells and *S. pombe* cells.

Additional Progress:

1. Structure-Function analyses of Int6:

MMTV insertion into mouse *int6* generates C-terminal truncated proteins called Int6ΔC, and Int6ΔC can transform human mammary cells. Int6ΔC lack the PCI domain, which is also found in many proteasome subunits, including Rpn5 and Rpn7, thus may play a role in the regulation of proteasome by Int6. Therefore, I carried out structure-function analyses of the PCI domain to define its molecular functions. In last year's annual report, I reported identification of a conserved leucine in the PCI domain, which is essential for proper localization, assembly, and functioning of both Int6 and proteasome subunit Rpn7 in *S. pombe*. I then followed up on this study by testing whether mutating this residue in human Int6 will also affect its localization and function in human cells. To test whether mInt6 mis-localize in human cells, I tagged wild type or mutant Int6 (mInt6) with YFP, and expressed them in HeLa cells. Wild-type Int6-YFP is concentrated at nuclear periphery in HeLa cells. By contrast, mInt6-YFP diffuses throughout the cell (Figure 3A). To test whether mInt6 will affect mammary gland development and cause tumorigenesis, I overexpressed mInt6 in MCF10A cells and performed similar *in vitro* acini formation assay as described in Task 3. My data show that over-expressing mInt6 and Int6ΔC, but not wild type Int6, will lead to formation of larger acini (Figure 3B), suggesting that mInt6, like Int6ΔC, might cause abnormal cell proliferation when overexpressed in human mammary epithelial cells. These results support the hypothesis that the PCI domain plays an important role in proper localization

and functioning of Int6, and that Int6 protein mutated in the PCI domain might cause malignant cell growth and tumorigenesis.

2. Examine whether the regulatory subunits in the proteasome are dynamic by FRAP/FLIP and whether *S. pombe* Int6 can influence this behavior of the proteasome

Our preliminary evidence suggests that Int6 regulates proper nuclear localization and assembly of proteasome subunits. We and others have data suggesting that regulatory subunits of the yeast proteasome are not functionally identical, suggesting that they each may function in a distinct manner, and in mammalian cells FRAP analyses indicate that proteasome subunits are dynamic (Reits et al., 1997). Thus it is possible that proteasome subunits may be assembled in a highly dynamic fashion to coordinate with the need of the cell, and int6 may play a role in regulating this process. Therefore, I performed FRAP/FLIP to determine whether the yeast proteasome subunits are dynamic and whether *S. pombe* Int6 can regulate proteasome dynamics.

I have tagged several proteasome regulatory subunits with GFP chromosomally, and I started with a component of the lid, Rpn11(also known as Pad1)-GFP, since previous studies have shown it to be well incorporated into the 26S proteasome (Wilkinson et al., 1998), and its GFP signal is reasonably strong. First, I used Fluorescent Recovery after Photobleaching (FRAP) to measure dynamics of Pad1-GFP at nuclear periphery, where proteasome is most highly enriched, finding it to be highly dynamic. Next, I used FLIP (Fluorescent Loss in Photobleaching) to test whether photobleaching Pad1-GFP inside the nucleus will lead to loss of fluorescence in the cytoplasm, and vice versa. My data shows that photobleaching Pad1-GFP inside the nucleus lead to a considerable loss of fluorescence in the cytoplasm, but photobleaching Pad1-GFP in the cytoplasm hardly affects fluorescence in the nucleus. (Figure 4A-C) These data suggests that Pad1-GFP is imported into the nucleus, and once it gets in, it does not exit readily. Next, I investigated whether deletion of *int6* in *S. pombe* will affect this process. For this purpose, I used FLIP to compare loss of cytoplasmic Pad1-GFP fluorescence when photobleaching inside the nuclei of wild-type or *int6null* cells. My data showed that in *int6null* cells, loss of fluorescence in the cytoplasm occurred at a much slower rate (Figure 4D. These data are consistent with our hypothesis that Int6 is required for proteasome localization and assembly.

3. Determining the specific importin(s) responsible for proteasome import:

To better understand how Int6 regulates proteasome import and assembly, I have examined the cellular import machinery responsible for the import of proteasome subunits, and whether Int6 plays a role in regulating this process. Although the proteasome concentrates inside the nucleus, many proteasome subunits do not have a classic nuclear localization sequence (NLS). And how proteasome subunits enter the nucleus is poorly understood.

In the last annual report, I reported mis-localization of GFP-Rpn7 in *S. pombe* cells carrying deletion of an importin β *kap123*. I then followed up on this study by investigating whether there is any genetic interaction between *kap123* and *int6*. My data showed that *kap123* is synthetically lethal with *int6* (Figure 5A). To determine whether proteasome subunits are bound and imported by Kap123, I tested the physical interaction between Kap123 and a number of proteasome subunits. My data shows that Kap123 preferentially interacts with a proteasome regulatory base subunit Rpt2 (Figure 5B and C). These data suggests that Kap123 might be involved in the nuclear transport of proteasome, and Int6 might regulate this process. To determine the whether deletion of these importins will affect proteasome functioning, I also tested whether these importin mutants are sensitive to canavanine, an arginine analog which can be incorporated into newly synthesized proteins and

cause proteasome stress. However, my data showed that *kap123null* cells are not as sensitive to canavanine as several other importin deleted cells, such as *sal3null*, suggesting that there are other importins also playing a role in proper functioning of the proteasome. These initial studies suggest that proteasome localization and functioning are preferentially affected by deletion of *kap123* and *sal3* (Figure 5D). Interestingly, both importins show significant genetic interaction with *int6*. Therefore, I believe that further studies on these importins will help clarify the mechanism by which *int6* regulates the proteasome. To provide direct evidence for the importance of these importins for proteasome nuclear transport, I will next test whether proteasome FLIP rate will be affected in these importin null mutant cells.

Key Research Accomplishments

1. I have obtained polyclonal anti-Int6 antibody and tested its efficacy in human cells (as reported in 2004).

2. I have created generated several siRNA sequences that can transiently knockdown Int6 expression in both HeLa and MCF 10A cells (as reported in 2004).

3. I have begun examining cell functions in Int6-reduced HeLa cells and found evidence of abnormal mitosis due to the presence of tri-polar spindles (as reported in 2004).

4. I found that *int6*^{kd} cells are hypersensitive to a proteasome inhibitor, suggesting that reducing int6 expression leads to proteasome abnormalities (Figure 1).

5. I established the use of the 3D culture system to investigate the role of Int6 in tumor formation (Figure 2).

6. I have evidence suggesting that Int6 proteins may be C-terminally truncated in human breast cancer cell lines (as reported in 2004).

7. I have identified a conserved leucine residue in the PCI domain, which is essential for proper localization, assembly and functioning of both Int6 and proteasome subunit Rpn7 in fission yeast. (as reported in 2004)

8. I found that human Int6 with mutation in the same leucine mislocalizes in human cells, and overexpression of this protein in MCF10A cells causes abnormal proliferation and interferes with acini formation. (Figure 3)

9. I found that the Pad1-GFP is dynamically entering nuclei *in S. pombe* cells, and that the rate is decreased when *int6* is deleted (Figure 4)

10. I have screened all the importin α and β null mutant cells, finding that Kap123 and Sal3 are the two importin βs showing most severe proteasome-related defects and genetic interaction with *int6*. (Figure 5)

Reportable Outcomes

1. One polyclonal anti-Int6 antibody.

2. Two siRNA sequences for down regulating Int6 expression in human mammary epithelial cells.

3. Meeting Abstracts:

Sha Z., Cabrera R., and Chang EC. 2004. Regulation of proteasome nuclear import and assembly by Int6. The 26th Annual Department of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. Houston, TX.

Sha Z., Cabrera R., and Chang EC. 2004. Regulation of proteasome nuclear import and assembly by Int6. The 2004 ASBMB/IUBMB Annual Conference. Boston, MA.

Sha Z. and Chang EC. 2004. Regulation of proteasome nuclear import and assembly by Int6.16th Annual Graduate Student Research Symposium, Baylor College of Medicine. Houston, TX.

Sha Z., Cabrera R., and Chang EC. 2005. Regulation of proteasome nuclear transport and assembly by Int6. The 27th Annual Department of Molecular and Cellular Biology Graduate Student Symposium. Baylor College of Medicine. Houston, TX.

Sha Z., and Chang EC., Regulation of proteasome nuclear import and assembly by Int6. The 2004 ASBMB/IUBMB Annual Conference. Boston, MA. June, 2004.

Sha, Z., and Chang, E. C. 2005. Int6 may influence breast cancer formation by regulating the 26S proteasome. DOD Era of Hope meeting.

Sha Z. and Chang EC. 2005. Regulation of proteasome nuclear import and assembly by Int6.17th Annual Graduate Student Research Symposium, Baylor College of Medicine. Houston, TX.

Sha, Z. Suo, J. Yen, S., and Chang, E. 2005. Investigation of the proteasome nuclear import and assembly by Int6. East Coast Fission Yeast meeting.

Sha, Z., and Chang, E. C. 2005. Regulation of the proteasome by yin6/int6 and an actin regulatory protein, Arc21. (Presented at the ASCB Annual Meeting).

Conclusion

I set out to test the hypothesis that Int6 acts as a breast tumor suppressor, which functions to regulate the 26S proteasome, by directly using human mammary epithelial cells. In the first year of my funding, I obtained reagents, anti-Int6 antibodies and siRNAs, which are critical for studying the molecular functions of Int6. I then went on to use these tools and found that Int6 is important for genomic stability, as proposed. Furthermore, cells in which Int6 expression is knocked down are hypersensitive to proteasome inhibitor, and this result supports our idea that Int6 can regulate the proteasome. Finally, with the support of this grant, I have established the 3D culture system and showed that the dosage of Int6 is important for the development of acini. These results demonstrate that our hypothesis is correct and that I can move on to more deeply investigate how Int6 regulates genomic stability and the proteasome.

In parallel, I am also investigating the mechanism by which proteasome import and functioning are regulated by Int6. First, I followed up on the structure-functional analyses on PCI domain. I found that Int6 with PCI mutation mislocalizes in human cells, and overexpressing this protein in MCF10A cells causes abnormal acini formation. These results suggest that the PCI domain plays an important and conserved role in proper functioning of Int6. Next, I have established the protocol of using FRAP/FLIP to study proteasome dynamics in *S. pombe* to more directly visualize and analyze proteasome nuclear transport. I have shown that the proteasome subunit Pad1-GFP is actively entering the nucleus, and this process is affected by deletion of *int6*. Finally, I screened all the importin α and β mutants for defects in proteasome localization, proteasome defects and genetic interaction with *int6*, showing that Kap123 and Sal3 appears to be preferentially involved in proteasome localization and functioning, and both are showing genetic interaction with *int6*. These data further support our hypothesis that *int6* regulates the proteasome transport and functioning, and can provide mechanistic evidence for us to further test in mammalian cells.

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Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Ihrig, M. M., McManus, M. T., Gertler, F. B., *et al*. (2003). A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nat Genet *33*, 401-406.

Wilkinson, C. R., Wallace, M., Morphew, M., Perry, P., Allshire, R., Javerzat, J. P., McIntosh, J. R., and Gordon, C. (1998). Localization of the 26S proteasome during mitosis and meiosis in fission yeast. Embo J *17*, 6465-6476.

Yen, H. C., Gordon, C., and Chang, E. C. (2003). Schizosaccharomyces pombe Int6 and Ras homologs regulate cell division and mitotic fidelity via the proteasome. Cell *112*, 207-217.

Appendices:

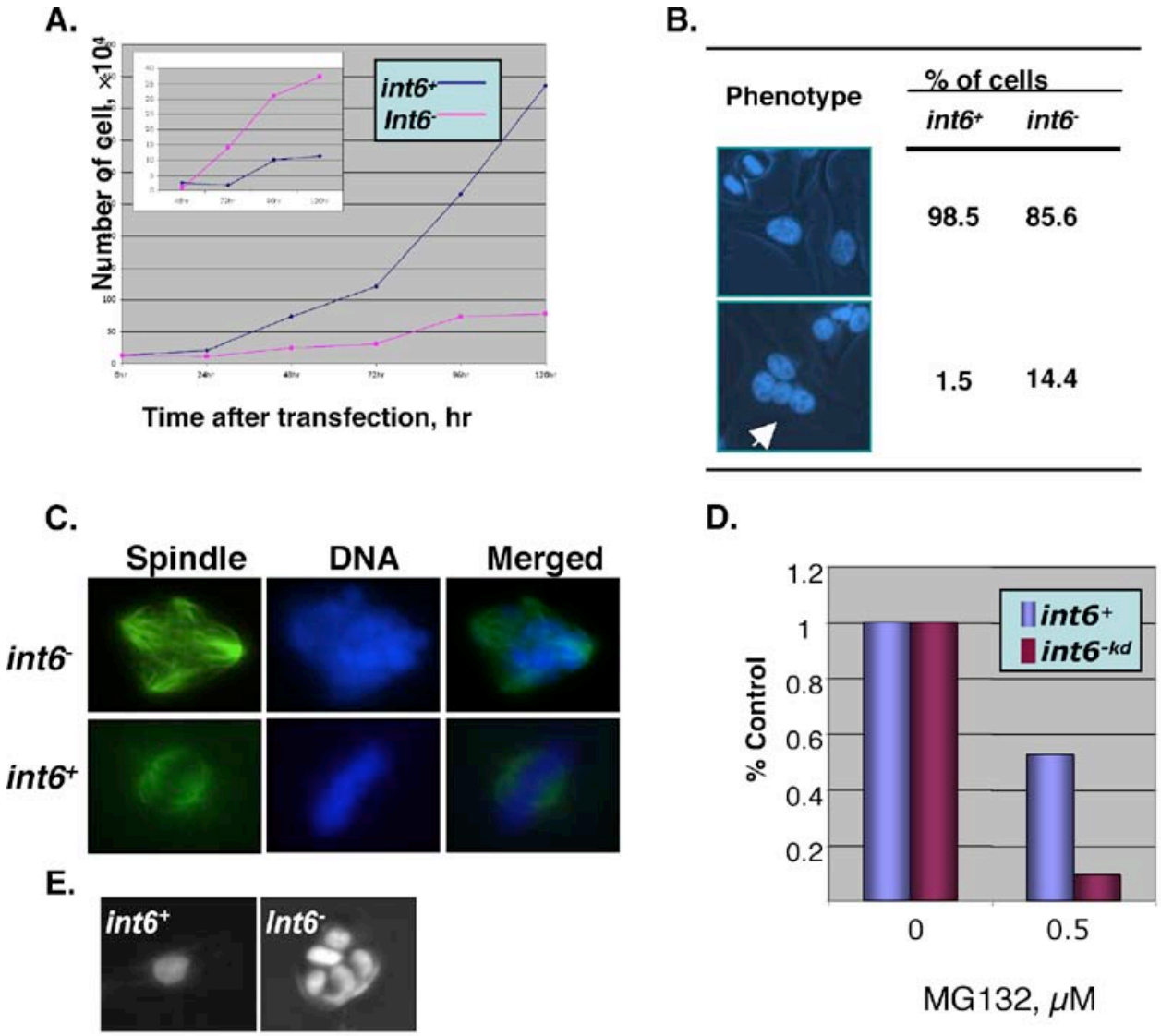


Fig.1. Phenotypes of Int6 knockdown human cells.
HeLa cells were transiently transfected by siRNA against int6 (*int6*$^-$) or control siRNA (*int6*$^+$) and the number of viable cells (A) and dead cells (insert) were counted over time. Correlated with this growth defect is the presence of multinucleated cells (arrow, B), which frequently show multiple spindle poles (C). To test proteasome drug sensitivity, HeLa cells were transfected with control or *int6* siRNA and the cells were seeded with indicated amount of MG132. Over time the cell numbers were counted. The numbers of surviving cells after 4 days of incubation in MG132 were normalized to the controls, which were treated with DMSO, and shown in (D). (E). MCF10A cells show similar mitotic defects with multinucleated cells (as revealed after DAPI staining).

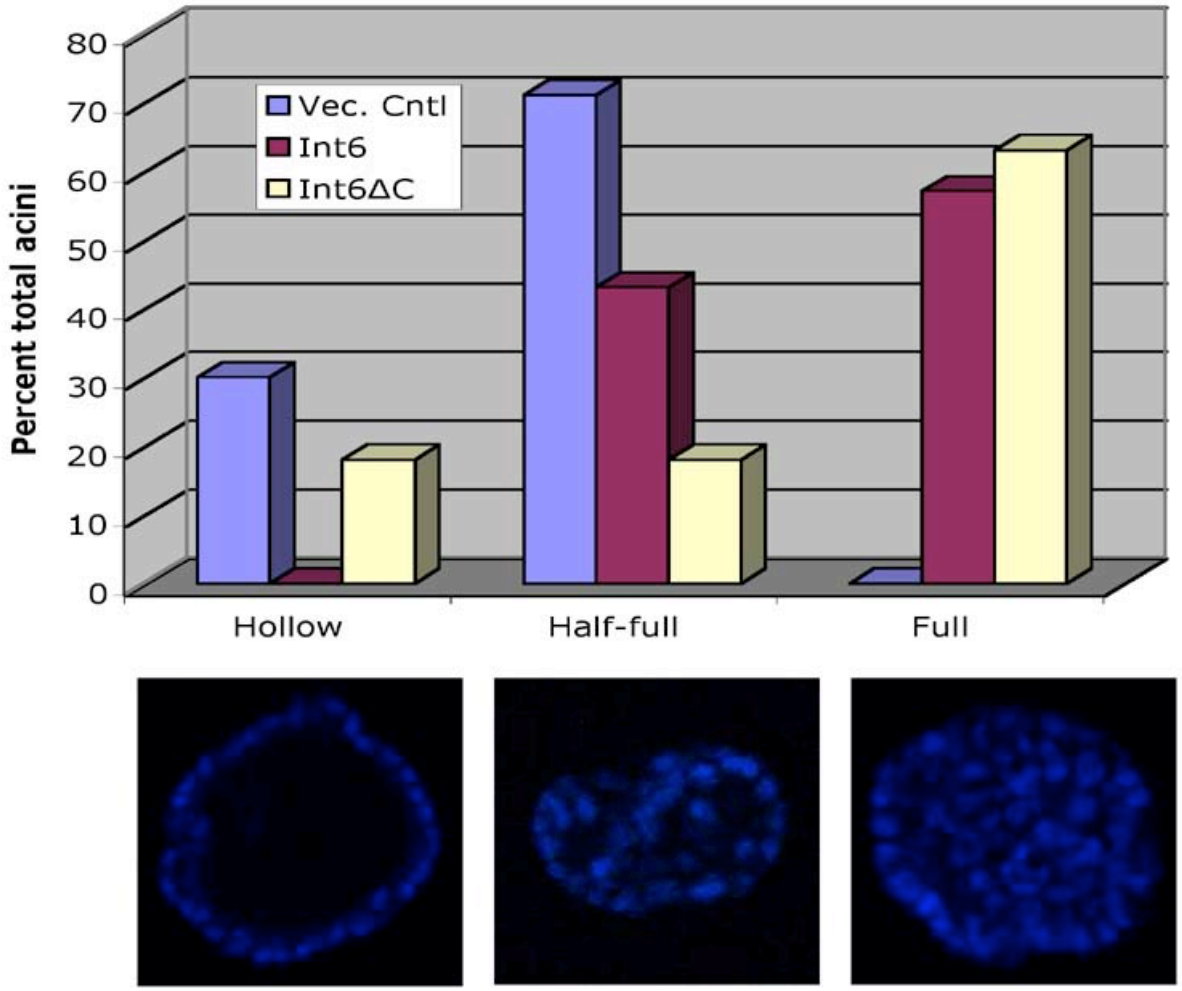


Fig.2. Over expression of Int6 and Int6ΔC interferes with mammary acini formation.
MCF10A cell lines stably expressing indicated Int6 proteins or carrying the vector control were cultured in 3D for 16 days. Acini were fixed and stained by TO-PRO-3 iodide and examined by confocal microscopy to reveal the nuclei of cells in the acini. The frequencies of the sphere that are either hollow, half-full or full of cells in the luminal space were counted.

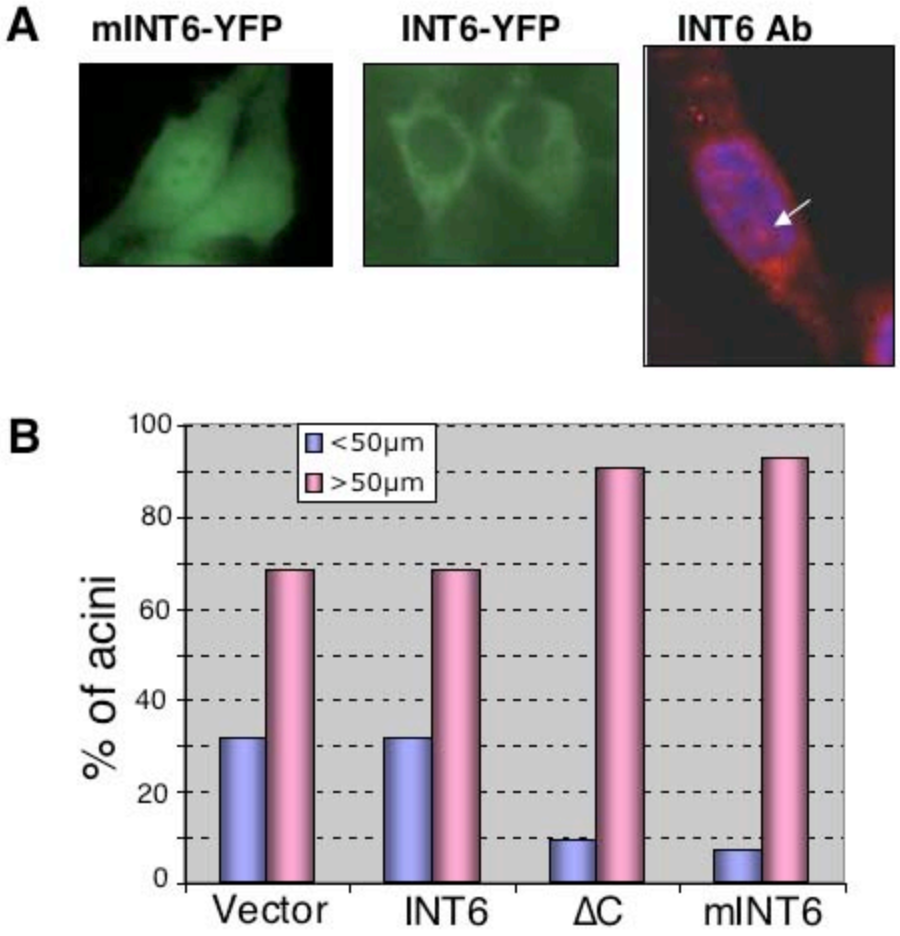


Figure 3: Int6 carrying mutatant PCI domain is mislocalized in human cells, and can interfere with mammary acini formation.
(A) In the left two panels, HeLa cells were transiently transfected by vectors expressing YFP tagged proteins and their localization was examined directly by microscopy. On the right, endogenous Int6 was examined by immunostaining (red) countered stained by DAPI and the image was deconvolved and merged. (B) Stable MCF10A cells expressing indicated proteins were cultured in 3D for 20 days. Percentage of large (>50μm) and small (<50μm) acini was counted under microscope.

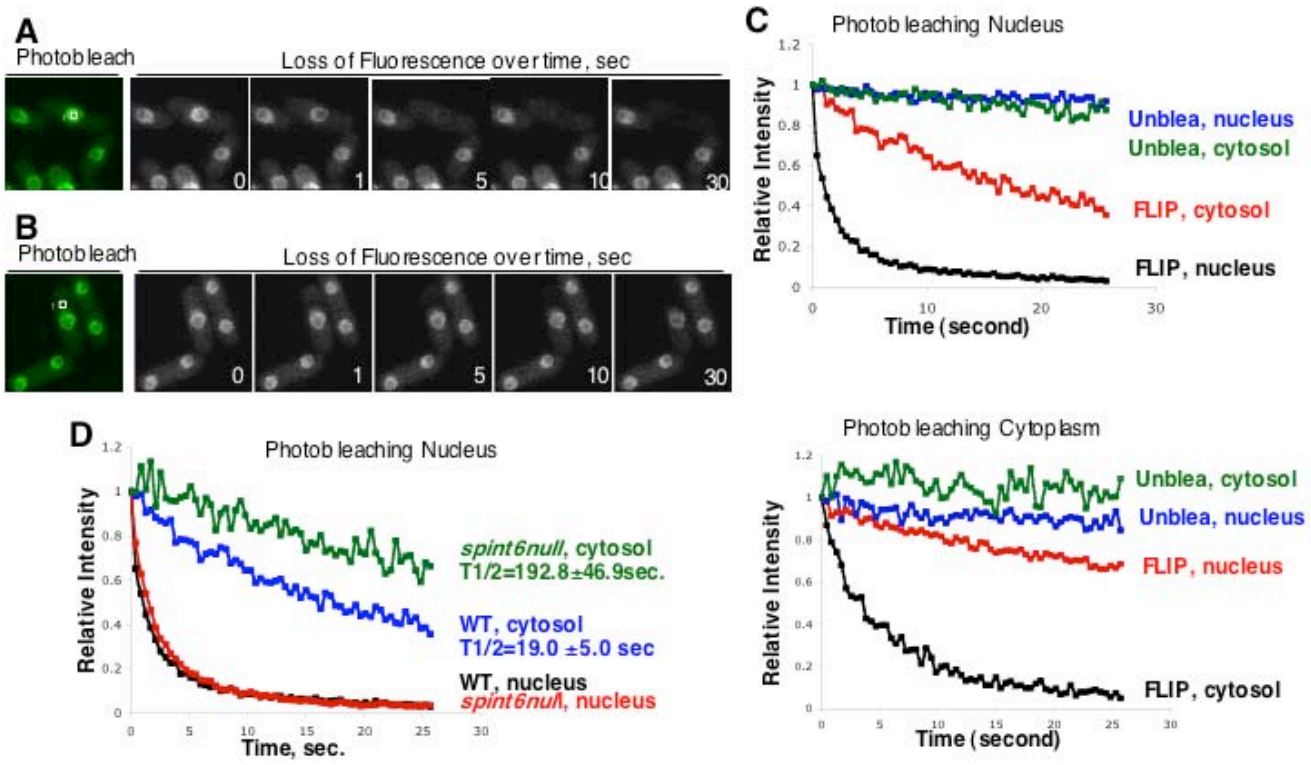
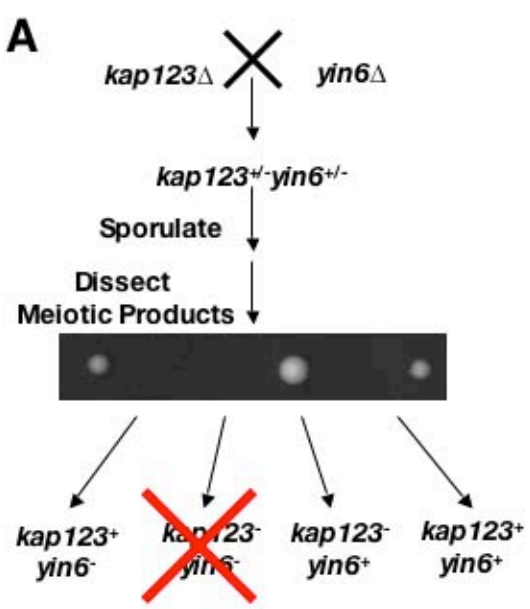


Fig.4. *S. pombe* proteasome is dynamic as measured by FLIP.
Cells carrying Pad1-GFP were photobleached in the marked area, and then the loss of fluorescence was captured over time. (A) Photobleaching in the nucleus leads to loss of Pad1-GFP fluorescence in the cytosol. Conversely, if cytosolic Pad1-GFP was photobleached, the nuclear GFP signal loss very slowly (B). Relative GFP intensities in the nucleus and the cytosol in the photobleached cell, as well as that in a control unbleached cell, in (A) and (B) are plotted over time in panel C. Panel D shows the time course of the loss of cytosolic Pad1-GFP signal after nuclear photobleaching in wild type vs. *yin6Δ* cells. These photobleached cells remained viable as they continued to divide, and the described dynamics of proteasome is highly consistent from cell to cell and from day to day.



B

GAD	LBD	Activation of *lacZ* Reporter
Rpt2	Kap123	
Rpt2	-	
-	Kap123	
-	-	

C

Proteasome Subunits Tested	Two-Hybrid binding with Kap123
Rpn1	-
Rpn5	-
Rpn9	-
Rpn10	-
Rpt2	+
Rpt6	-
Yin6	-

D

		GFP-Rpn7 Loacalition	GFP-NLS Localization	Genetic Interaction with spint6	Canavanine Sensitivity	Binding with proteasome subunits
Kap120	-	+	+	ND	ND	ND
Sal3	Importin 5	+	+	+	+	ND
Kap111	Transportin SR1	+	+	-	±	ND
Kap123	Importin 4	±	+	+	±	Rpt2
Kap114	Importin 9	+	+	-	ND	ND
Kap104	Karyopherin-beta2	+	+	-	+	ND
Imp1	-	+	-	±	+	ND

Fig. 5. *kap123* is synthetically lethal with *int6*, and Kap123 binds Rpt2 in Yeast two-hybrid binding assay. *kap123*$^{+/-}$*int6*$^{+/-}$ heterozygous diploid cells were sporulated, individual asci dissected, and viability of meiotic products was examined. (A) shows the viability of spores from a tetra-type ascus. Only 3 out of 4 spores can germinate, and the unviable spore is *kap123*$^{-}$*int6*$^{-}$ as proved by testing markers. (B) shows yeast two-hybrid binding between Kap123 and Rpt2, indicated by activation of *lacZ* reporter. Results of yeast two-hybrid binding between Kap123 and Yin6 as well as several proteasome subunits are summarized in (C). (D) summarizes the function tested for all the importin mutants: their sensitivity to canavanine, binding with proteasome subunits, genetic interaction with int6, and localization of GFP-Rpn7 in these cells. ND stands for "not determined".