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Schizosaccharomyces pombe Int6 and Ras Homologs Regulate Cell Division and Mitotic Fidelity via the Proteasome

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Summary

Yin6 is a yeast homolog of Int6, which is implicated in tumorigenesis. We show that Yin6 binds to and regulates proteasome activity. Overexpression of Yin6 strengthens proteasome function while inactivation weakens and causes the accumulation of polyubiquitinated proteins including securin/Cut2 and cyclin/Cdc13. Yin6 regulates the proteasome by preferentially interacting with Rpn5, a conserved proteasome subunit, and affecting its localization/assembly. We showed previously that Yin6 cooperates with Ras1 to mediate chromosome segregation; here, we demonstrate that Ras1 similarly regulates the proteasome via Rpn5. In yeast, human Int6 binds Rpn5 and regulates its localization. We propose that human Int6, either alone or cooperatively with Ras, influences proteasome activities via Rpn5. Inactivating Int6 can lead to accumulation of mitotic regulators affecting cell division and mitotic fidelity.

Introduction

The mouse mammary tumor virus (MMTV) has been used as an insertional mutagen to identify genes (*INT* genes) that are important for breast cancer formation (reviewed by Tekmal and Keshava, 1997). So far, a total of nine *INT* genes have been found. Seven of these encode three families of signaling molecules: Wnt, Notch, and fibroblast growth factor (FGF); detailed studies suggest that these molecules are critical for normal regulation of growth and differentiation. Another *INT* gene, *INT5*, encodes aromatase (CYP19), the rate-limiting enzyme for estrogen synthesis. The level of estrogen can profoundly and specifically influence the development of breast tumors. The final *INT* gene, *INT6*, encodes a highly conserved protein whose functions are largely unknown.

Several lines of evidence suggest that normal Int6 function is critical for preventing breast tumorigenesis in mice and humans. In mice, MMTV insertion into *INT6* causes the production of C-terminally truncated proteins (Int6ΔC; Marchetti et al., 1995), which appear to act in a dominant-negative fashion to induce breast cancer. Human and mouse Int6 proteins are identical in amino acid sequence (Miyazaki et al., 1997), and overexpression of Int6ΔC can transform human mammary cells, which then form mammary tumors when injected into nude mice (Rasmussen et al., 2001). Moreover, loss of heterozygosity (LOH) or a reduction in human *INT6* gene (*h-INT6*) expression is detected in 30%–40% of human breast cancers examined so far (Marchetti et al., 2001; Miyazaki et al., 1997; van't Veer et al., 2002).

We have previously isolated an *INT6* homolog, *yin6*, from the fission yeast *Schizosaccharomyces pombe*. Yin6 binds directly to Moe1 (Chen et al., 1999; Yen and Chang, 2000), and thus forms a complex with Scd1 (also known as Ral1; Chang et al., 1994; Fukui and Yamamoto, 1988), a nucleotide exchange factor for Cdc42 and effector of Ras1, the *S. pombe* homolog of the mammalian protooncogenic H-ras G protein (Fukui et al., 1986). Deleting *yin6*, *moe1*, or both (*moe1*Δ *yin6*Δ) produces essentially the same phenotypes: slow growth in the cold and inefficient separation of sister chromatids (Yen and Chang, 2000). These abnormalities are exacerbated by *ras1*Δ, causing severe chromosome missegregation and cell death; by contrast, a constitutively active form of Ras1 rescues these defects. Yin6 and h-Int6 are 43% identical in amino acid sequence; full-length h-Int6, but not h-Int6ΔC, rescues the phenotype of *yin6*Δ cells. These data indicate that yeast and human Int6 are functional homologs and that one of the key functions of Int6 is to maintain chromosome stability, which can be further modulated by Ras activity. However, how Yin6 and Ras1 regulate chromosome segregation has not yet been established.

The C-terminal region that is critical for Int6 function contains a highly conserved PCI domain, which is frequently found in components of three protein complexes: the *P*roteasome, *C*OP9, and e*I*F3 (Hofmann and Bucher, 1998). This raises the possibility that control of chromosome stability by Yin6 may involve an interaction with one or more of these complexes. The eukaryotic proteasome, a multisubunit machinery consisting of regulatory and catalytic complexes, functions to degrade polyubiquitinated proteins, many of which are mitotic regulatory molecules (Ferrell et al., 2000). Two of the best-studied proteasome substrates are securin and cyclin B (called Cut2 and Cdc13, respectively, in *S. pombe*), which are central for proper chromosome segregation and cell cycle progression. Securin/Cut2 accumulation in proteasome mutants blocks cohesin degradation, thus inhibiting sister-chromatid disjunction, while cyclin B/Cdc13 accumulation delays the exit from mitosis.

Other evidence supports the hypothesis that Yin6 can interact with the proteasome. In addition to the fact that Yin6 and numerous proteasome components contain PCI domains, *yin6*Δ cells display phenotypes that are very similar to those in proteasome mutants: they are both defective in sister-chromatid segregation (Gordon et al., 1993; Yen and Chang, 2000), are resistant to microtubule depolymerization drugs such as MBC (methylbenzyl carbamylate) and TBZ (thiabendazole) (Gordon et al., 1993; Yen and Chang, 2000; our unpublished data),

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and are defective in sensing nutrient levels and thus mate even in rich medium (our unpublished results).

In this study, we show definitively that Yin6 binds to and is a positive regulator of the proteasome in *S. pombe*. Proper proteasome functions require Yin6, and in *yin6*Δ cells, in which the proteasome is defective, polyubiquitinated proteins such as securin/Cut2 and cyclin/Cdc13 accumulate abnormally. In addition, we have discovered how Yin6 regulates the proteasome: loss of Yin6 leads to mislocalization of a proteasome subunit, Rpn5 (Glickman et al., 1998; Saito et al., 1997), and improper assembly of the proteasome. Consistent with the hypothesis that Yin6 interacts with Ras1, we demonstrate that Ras1 also mediates proteasome functions and Rpn5 localization. Finally, we show that h-Int6 binds h-Rpn5, and that h-Int6, but not h-Int6ΔC, can substitute for Yin6 to restore Rpn5 localization in yeast. These data support a model in which regulation of the proteasome via Rpn5 is a highly conserved function of the Int6 proteins. We propose that inactivation of mammalian Int6, either alone or together with *RAS* mutations, causes tumorigenesis by inhibiting proteasome functions, which leads to inefficient degradation of mitotic regulators and a disruption of growth control and genetic stability.

Results

Yin6 Positively Regulates the Proteasome

To determine whether Yin6 is important for proteasome activities, we first examined whether Yin6 inactivation causes hypersensitivity to TPCK (tosylsulfonyl phenylalanyl chloromethyl ketone) and canavanine, which are known to inhibit the growth of mutants defective in ubiquitin (Ub)-dependent proteolysis. TPCK and its related compounds are protease inhibitors and have been shown to inhibit cyclin B degradation in clam embryo extracts (Luca and Ruderman, 1989) and to retard growth of *S. pombe* Ub ligase mutants (Grishchuk et al., 1998). Canavanine is an arginine analog that can cause incomplete protein synthesis. The resulting abnormal proteins must be removed by the proteasome; hence, *S. pombe* proteasome mutants are hypersensitive to canavanine (Wilkinson et al., 2000). As shown in Figures 1A and 1B, *yin6*Δ cells are highly sensitive to both drugs.

If proper proteasome function requires Yin6, *yin6*Δ would intensify the growth defect of the proteasome mutants. To test this idea, *yin6* was deleted from mutants carrying temperature-sensitive (ts) mutations in genes encoding regulatory subunits of the proteasome. We found that *yin6*Δ exacerbates the growth defect of all available proteasome mutants except one, Rpn5, which will be discussed later (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/2/207/DC1). Some of these double mutants can barely form colonies, while the growth of other double mutants is severely retarded at semipermissive temperatures. A representative study involving Mts3/Rpn12 and Mts2/Rpt2 is illustrated in Figure 1C. Moreover, if Yin6 is crucial for the proteasome function, overexpression of proteasome components could rescue phenotypes of *yin6*Δ cells and overexpression of Yin6 could rescue the growth defect of the proteasome mutants. Indeed, we found that overexpression of proteasome components weakly rescues the TPCK hypersensitivity of *yin6*Δ cells to various degrees (Figure 1D), as well as the TBZ resistance and cold-sensitive growth defects (data not shown). Conversely, Yin6 overexpression rescues the growth defect of at least two proteasome mutants (see Figure 1E for the *mts3/rpn12* mutant; the data for the *pad1/rpn11* mutant are not shown).

Consistent with the hypothesis that the Yin6-proteasome interaction is important for chromosome segregation, we determined that *yin6*Δ proteasome double mutants die of chromosome missegregation at the semipermissive temperature (an example involving the mts2/rpt2 mutant is illustrated in Figure 2A). A substantial number of these double-mutant cells appear to be blocked in metaphase with a single highly condensed nucleus and a short spindle ($\leq 3\ \mu m$), indicative of inefficient separation of sister chromatids (Figure 2B). Together, these data support the hypothesis that Yin6 positively regulates proteasome activity: Yin6 overexpression apparently strengthens it, while deletion of *yin6* weakens it.

Yin6 Associates with the Proteasome Complex

The proteasome is composed of catalytic and regulatory complexes, and the latter can be further separated into the lid and base subcomplexes (Ferrell et al., 2000). Since Yin6 regulates the proteasome, we investigated whether Yin6 associates with the whole proteasome. Affinity pull-down of proteasome subunits has been demonstrated to be an effective method to isolate the proteasome (Glickman et al., 1998). To determine whether Yin6 can associate with the proteasome, we constructed a strain in which the endogenous Yin6 is tagged by the c-myc epitope, and endogenous Pus1/Rpn10, a base subunit, is tagged with protein A (ProA). As expected, components from the lid (Mts3/Rpn12), the base (Mts2/Rpt2 and Mts4/Rpn1), and the catalytic core (the α subunits) can be efficiently pulled down along with Pus1/Rpn10 by the IgG beads (Figure 3A). Yin6, but not the negative control Cdc8, was also detected in the pull-down sample, demonstrating that Yin6 associates with the proteasome in *S. pombe*.

*yin6*Δ Leads to Accumulation of Polyubiquitinated Proteins, Including Cut2 and Cdc13

If *yin6*Δ weakens proteolysis without substantially affecting the earlier step of polyubiquitination, polyubiquitinated proteins would be expected to accumulate in *yin6*Δ cells. As shown in Figure 4A, *yin6*Δ cells, like the proteasome mutant, clearly contain more polyubiquitinated proteins as detected by immunoblotting. The level of polyubiquitinated proteins can be further increased by canavanine treatment. To determine that the accumulation of these proteins is indeed caused by inefficient protein degradation, we examined total protein degradation in cells by a pulse-chase method. Our data confirm that *yin6*Δ cells are inefficient in protein degradation (Figure 4B).

The degradation of polyubiquitinated Cut2/securin and Cdc13/cyclin B by the proteasome is critical for proper separation of sister chromatids and anaphase exit, both of which are defective in *yin6*Δ cells (Yen and

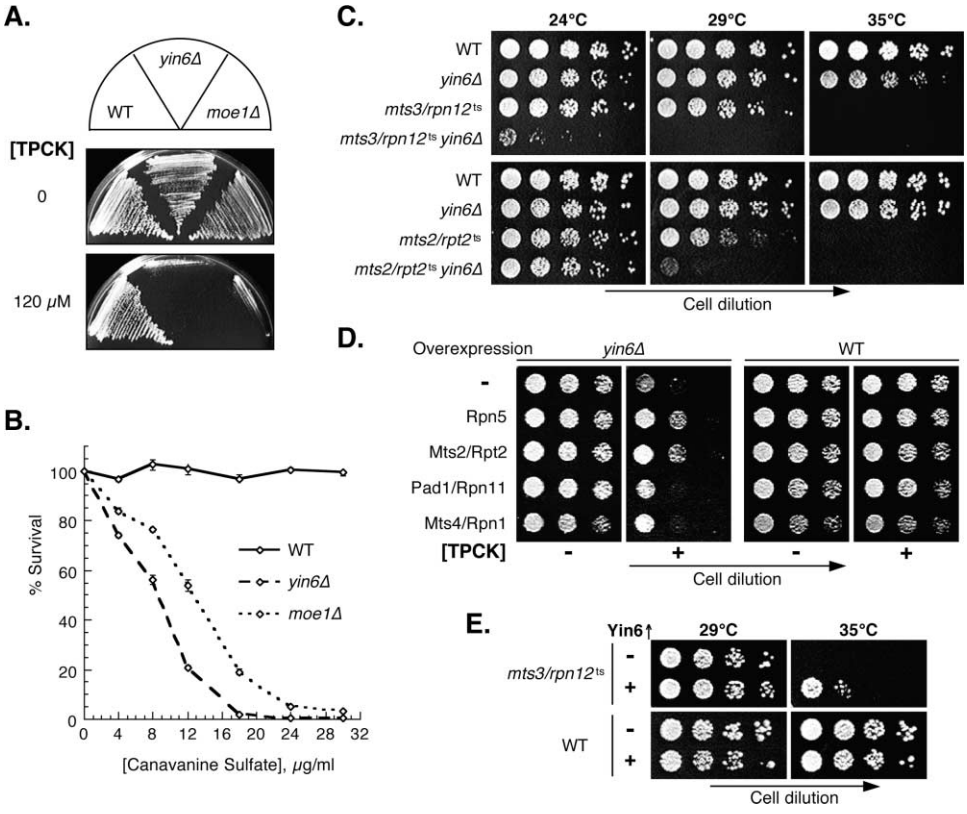

Figure 1. Yin6 Is Important for Proteasome Functions

(A) Cells were streaked on YEAU plates and incubated at 30°C for 3 days.

(B) Cells were pregrown in YEAU at 30°C. Equal numbers of cells were then spread on MM plates with the indicated amount of canavanine and incubated at 30°C for 14 days. The number of colonies that emerged without canavanine was taken as 100% survival. The cells were seeded in triplicate and the numbers shown are mean ± SE (n = 3). The strains tested in (A) and (B) are SP870 (WT, wild-type), YIN6K (*yin6*Δ), and MOE1L (*moe1*Δ).

(C) Serially diluted cells were spotted on YEAU plates and incubated at indicated temperatures. The strain of cells shown on top was derived from a fusion between strain *mts3/rpn12-1* (*mts3/rpn12*^{ts}) and YIN6U (*yin6*Δ), while the bottom was from a fusion between *mts2/rpt2-1* (*mts2/rpt2*^{ts}) and YIN6U (*yin6*Δ).

(D) Wild-type (SP870) and *yin6*Δ (YIN6K) cells transformed with a vector control (−, pREP1) or the same vector carrying various genes were serially diluted and spotted on MM plates with (+) or without (−) 100 μM TPCK. Plates were incubated at 30°C for 5 days.

(E) Wild-type (SP870) and *mts3/rpn12*^{ts} (*mts3/rpn12-1*) strains were transformed with either a vector control (−, pAL1) or a plasmid carrying the genomic version of *yin6* (+, pAL1YIN6g). Serially diluted cells were spotted on MM plates and incubated at indicated temperatures.

Chang, 2000; data not shown). It is possible that the growth defect of *yin6*Δ cells is at least partly caused by inefficient degradation of Cut2 and Cdc13. We carried out two lines of study to investigate this possibility.

First, we overexpressed a polyhistidine-tagged Ub in cells and then examined whether more Cut2 and Cdc13 can be pulled down by Ni^{2+}-beads in *yin6*Δ cells. Our data demonstrated that substantially more Cut2 and Cdc13 were pulled down by the Ni^{2+}-beads in *yin6*Δ cells (Figure 4C), suggesting that these cells are defective in Cut2 and Cdc13 degradation. To exclude the possibility that Ub-Cut2 accumulation in *yin6*Δ cells is caused by irregularities in the cell cycle, not in the proteasome, we examined cells that were synchronized in S phase. Our data show that the S phase-arrested *yin6*Δ cells still contain high levels of Ub-Cut2 (Figure 4D). Second, we postulated that if inefficient degradation of Cut2 and Cdc13 was responsible for the growth defect in *yin6*Δ cells, the growth of *yin6*Δ cells would be more sensitive to Cut2 and Cdc13 overexpression. We therefore overexpressed Cut2 and Cdc13 using the thiamine-repressible *nmt1* promoter in both wild-type and *yin6*Δ cells and examined the ability of the cells to form colonies with and without thiamine. As shown in Figure 4E, overexpression of Cut2 and Cdc13 is more toxic to *yin6*Δ cells. In conclusion, these results support the hypothesis that the abnormalities in *yin6*Δ cells are caused by inefficient degradation of polyubiquitinated proteins. Accumulation of securin and cyclin is at least partly responsible for the mitotic defects observed in *yin6*Δ cells.

Isolation of Rpn5, a Conserved Proteasome Subunit, as a Yin6 Binding Protein

To further understand how Yin6 regulates the proteasome, yeast two-hybrid screens were conducted using both an N- and a C-terminally truncated Yin6, Yin6ΔN, and Yin6ΔC (amino acid residues 220–501 and 1–327, respectively), as baits. From both screens (see Experimental Procedures), we isolated a highly conserved protein, Rpn5, which is a PCI protein in the lid of the protea-

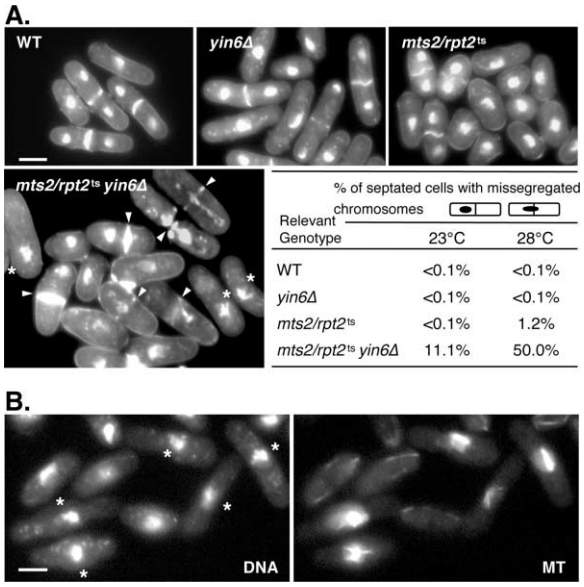

Figure 2. The *yin6* and Proteasome Double Mutants Are Defective in Chromosome Segregation

(A) Cells were pregrown in YEAU at 23°C to early log phase, resuspended in fresh YEAU medium, and then shifted to 28°C to grow for another 17 hr. Cells were stained to reveal DNA and the septum. Shown here are cells after the shift to 28°C. The percentages of septated cells with missegregated chromosomes before and after the shift to 28°C were tabulated.

(B) The same *mts2/rpt2*^{ts} *yin6*Δ cells as in (A) were stained to reveal DNA and microtubules (MT). Arrowheads mark the septa of cells with missegregated chromosomes; asterisks mark those cells with supercondensed chromosomes. Some of these cells have a star-shaped spindle, indicative of defects in spindle formation.

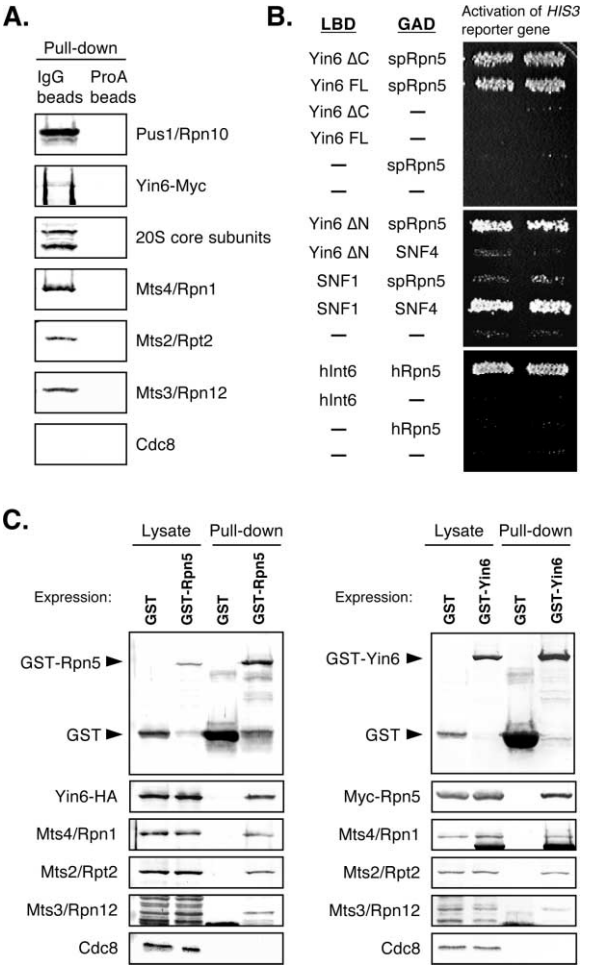

Figure 3. Yin6/Int6 Binds the Proteasome and Rpn5

(A) Lysates prepared from strain *pus1-ProA yin6-MYC* were pulled down with IgG sepharose beads. The protein A (ProA) beads were used as the pull-down control. The pulled-down proteins were analyzed by Western blotting.

(B) Protein-protein interactions as tested by the yeast two-hybrid system: proteins fused with the LexA DNA binding domain (LBD) and Gal4 activation domain (GAD) are as indicated. Note that the *S. pombe* and human Rpn5 are labeled as spRpn5 and hRpn5, respectively. Yin6 FL, Yin6ΔN, and Yin6ΔC contain full-length and C- and N-terminally truncated Yin6. Plasmids used were pLBDYIN6ΔC, pLBDYIN6, pLBDYIN6ΔN, pLBDSNF1, pLBDHINT6, pVJL11, pGADspRPN5, pGADSNF4, pGADhRPN5, and pGADgh. Snf1 and Snf4 were the positive controls (Chen et al., 2000).

(C) On the left, lysates were prepared from strain *yin6-HA* transformed with either a vector control (pAAUGST), which expressed GST, or the same vector carrying *S. pombe rpn5* (pGSTRPN5), which expresses GST-Rpn5. On the right, lysates were made from *yin6*Δ (YIN6K) strain transformed with pMycRpn5 and pAAUGST or pGSTYIN6 (expressing Myc-Rpn5, GST, or GST-Yin6, respectively). The glutathione beads were used for the pull-down. Both the lysate and the pull-down samples were analyzed by Western blots.

some. Rpn5 also binds full-length Yin6 (Figure 3B), and this binding is specific. For example, Rpn5 was isolated multiple times and is the only proteasome component isolated from these screens. Furthermore, Yin6 does not interact with other known proteasome subunits available for the two-hybrid system: Mts1/Rpn9, Mts2/Rpt2, Mts4/Rpn1, Pus1/Rpn10, Sug1/Rpt6, Sug2/Rpt5, and Mss1/Rpt1 (data not shown). To determine whether Yin6 forms a complex with Rpn5 in fission yeast, GST pull-down experiments were performed. As shown in Figure 3C, GST-Yin6 can pull down Rpn5, along with other proteasome components in the lid and the base, but not the control Cdc8 protein; reciprocally, GST-Rpn5 can pull down Yin6, but not Cdc8. Based on these results, we conclude that Yin6 binds Rpn5 with great specificity in *S. pombe*.

Rpn5 Acts Downstream of Yin6

To define the genetic relationship between Yin6 and Rpn5, an *rpn5*Δ strain was constructed (Experimental Procedures). *rpn5*Δ cells show a cold-dependent growth defect (Figure 5A), canavanine hypersensitivity (Figure 5B), and an exacerbation of the growth defect of proteasome mutants (data not shown); all of which are similar to *yin6*Δ cells. We then created a *yin6*Δ *rpn5*Δ strain. Unlike the double mutants carrying *yin6*Δ and other proteasome mutations (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/2/207/DC1), *yin6*Δ *rpn5*Δ cells do not display any synthetic lethality and their phenotypes are very similar to those of the single-null mutants (Figures 5A and 5B). These observations are consistent with the hypothesis that Yin6 and

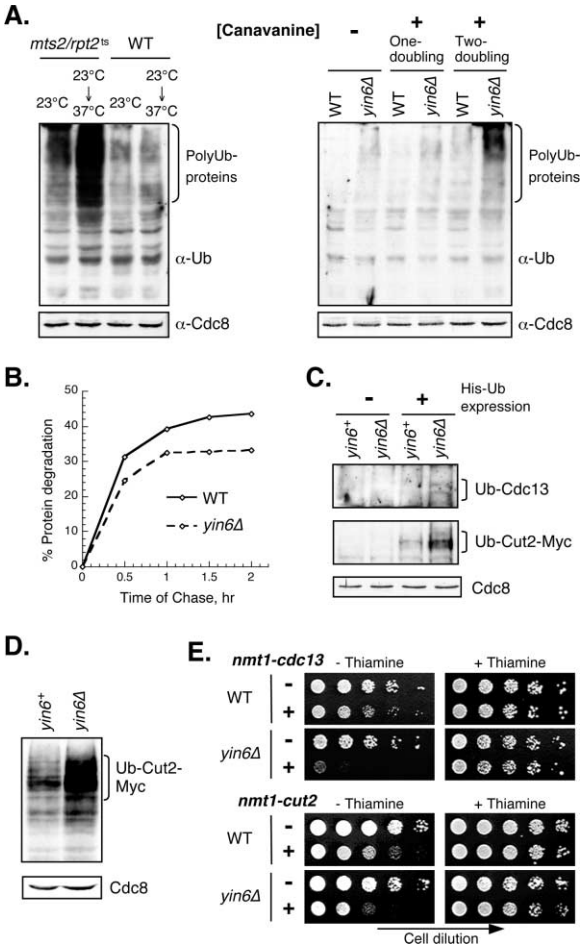

Figure 4. *yin6*Δ Leads to Inefficient Degradation of Polyubiquitinated Proteins

(A) On the left, lysates were prepared from cells grown either at 23°C or pregrown at 23°C then shifted to 37°C for 4.5 hr. On the right, lysates were prepared from cells treated with (+) or without (−) 10 μg/ml canavanine sulfate for one or two doubling times. The levels of ubiquitinated proteins were analyzed by Western blots.

(B) Cells were pulsed with [^{35}S]-Met, and TCA precipitable ^{35}S-signals were measured over time after chase. The "rate" of protein degradation was calculated from the slope of the curves from two experiments, and it is 1.5 ± 0.2 times faster in wild-type cells.

(C) Cell extracts from indicated strains with (+) or without (−) His-Ub expression were pulled down by Ni^{2+}-resin and polyubiquitinated Cut2-Myc and Cdc13 were detected by immunoblotting. Cdc8 is the loading control.

(D) The same as in (C) except that cells were synchronized in S phase (Chen et al., 1999).

(E) Wild-type (WT) and *yin6*Δ cells transformed with either a vector control (−, pREP41) or the same vector carrying the *cdc13* or *cut2* gene (+) were pregrown in MM media with 0.2 μM thiamine. Cells were washed with thiamine-free MM media and spotted on plates containing either no thiamine or 20 μM thiamine. Strains used in (A), (B), and (E) were SP870 (WT), *mts2/rpt2-1* (*mts2/rpt2*^{ts}), and YIN6K (*yin6*Δ). Strains used in (C) and (D) were *cut2-MYC* (*yin6*$^{+}$) and *cut2-MYC*YIN6K (*yin6*Δ).

Rpn5 interact with one another in a linear pathway. To further determine the order in which they act, we examined the effects of gene overexpression on null mutant phenotypes. Rpn5 overexpression partially rescues the sensitivities to canavanine (Figure 5C) and TPCK (Figure 1D) of *yin6*Δ cells, while Yin6 overexpression neither rescues nor worsens the growth defect of *rpn5*Δ cells (Figure 5D). These genetic data collectively show that Yin6 is upstream of Rpn5 in proteasome regulation and raise the possibility that Yin6 can regulate Rpn5.

Yin6 Regulates Rpn5 Localization and Assembly in the Nucleus

In *S. pombe*, the proteasome is assembled in the inner nuclear membrane (Wilkinson et al., 1998). Rpn5 was tagged with GFP to examine its localization. The resulting fusion protein fully rescued the phenotype of *rpn5*Δ cells (data not shown), suggesting that GFP-Rpn5 is biologically functional. In further support of this, like other proteasome components, GFP-Rpn5 is localized in the nucleus and concentrated around the nuclear membrane in *yin6*$^{+}$ cells (Figure 6A); in striking contrast, GFP-Rpn5 is largely cytoplasmic in *yin6*Δ cells (Figure 6A). The apparent reduction of Rpn5 in the nucleus is not caused by global protein degradation since the total Rpn5 protein levels in wild-type and *yin6*Δ cells are essentially the same (Figure 6C). By contrast, Yin6 localization and protein levels are not altered by *rpn5*Δ (data not shown). As shown in Figures 1D and 5C, Rpn5 overexpression partially rescues the phenotypes of *yin6*Δ cells. This result can be partly explained by the fact that nuclear Rpn5 levels in *yin6*Δ cells are increased as Rpn5 expression is elevated (Figure 6B). To quantify how much Rpn5 is lost from the proteasome in *yin6*Δ cells, we examined the contents of the proteasome in *yin6*Δ cells by affinity pull-down. As shown in Figure 6C, more than 50% of the pulled-down proteasomes lack Rpn5. More important, nearly 50% of the isolated proteasomes also lack the catalytic α subunits. These results demonstrate that Yin6 regulates Rpn5 nuclear localization and proper proteasome assembly.

Like *yin6*Δ cells, *S. pombe cut8* mutant cells contain proteasome subunits that are mislocalized in the cytosol and display chromosome instability (Tatebe and Yanagida, 2000). Thus, we investigated whether Yin6 interacts with Cut8. Our data show that *yin6*Δ combined with the *cut8* mutation creates a severe synthetic growth defect (Figure 6D).

Ras1 and Moe1 Are Also Required for Proper Proteasome Function

Our previous studies indicated that Moe1 binds directly to Yin6 and affects the same functions as those controlled by Yin6. Hence, we tested whether Moe1 also interacts with the proteasome. Our genetic data indicate that *moe1*Δ, like *yin6*Δ, similarly augments the growth defect of numerous proteasome mutants (Supplemental Table S1 available at http://www.cell.com/cgi/content/full/112/2/207/DC1). TPCK and canavanine toxicity is greater in *moe1*Δ cells (Figures 1A and 1B), and Rpn5 is mislocalized in *moe1*Δ cells (Figure 6A). Additionally, Moe1 associates with Rpn5 in a Yin6-dependent manner. Moe1 cannot be pulled down by GST-Rpn5 without Yin6 (Figure 7A) and does not bind Rpn5 in the two-hybrid system (data not shown).

We have shown that the Yin6-Moe1 complex interacts with the Ras1 pathway to affect chromosome segregation. *ras1*Δ intensifies while the constitutively active

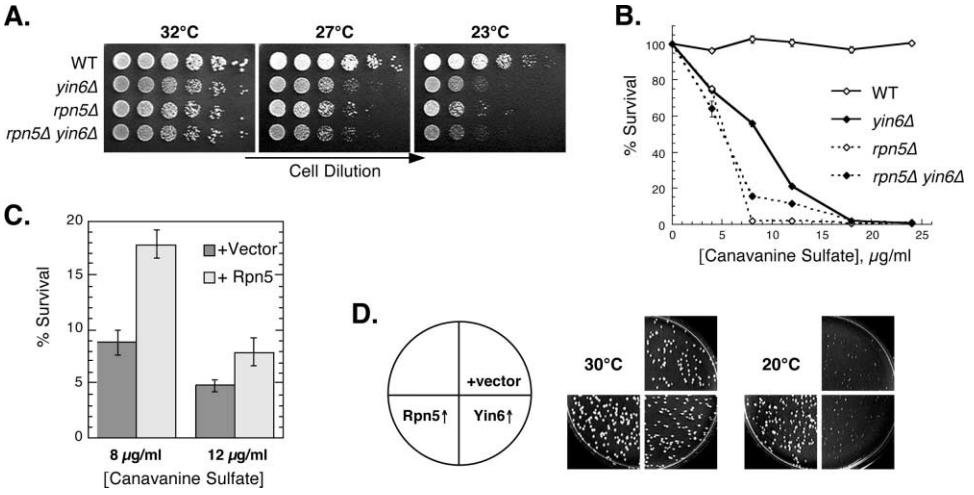

Figure 5. Genetic Interactions between Yin6 and Rpn5

(A) Serially diluted cells were spotted on YEAU plates and grown at indicated temperatures.

(B) Cells were pregrown in YEAU medium and an equal number of cells from each strain were spread on MM plates containing canavanine sulfate. Plates were incubated at 30°C.

(C) *yin6*Δ cells, transformed with either a vector control (pAL1) or the same vector carrying the genomic version of *rpn5* (pAL1RPN5), were pregrown in MM medium and then spread on canavanine plates (0, 8, and 12 μg/ml). The percent survival was assayed as described in Figure 1B. The numbers shown are mean ± SE (n = 3).

(D) *rpn5*Δ cells were transformed with either a vector control (pAL1), or a plasmid carrying the genomic version of either *rpn5* (pAL1RPN5ag) or *yin6* (pAL1YIN6g). Cells were spread on MM plates and incubated at 30°C or 20°C. The strains used were SP870 (WT), RPN5AA (*rpn5*Δ), YIN6K (*yin6*Δ), and R5AAY6K (*rpn5*Δ *yin6*Δ).

Ras1G17V rescues this defect of *yin6*Δ cells. We asked whether Ras1 can modulate Yin6 interaction with the proteasome. Indeed, *ras1*Δ worsens the canavanine (Figure 7B) and TPCK (Figure 7C) toxicity in *yin6*Δ cells. Ras1G17V, in contrast, rescues these abnormalities (Figures 7B and 7C), and most remarkably, it also restores Rpn5 nuclear localization (Figure 6A). Moreover, *ras1*Δ worsens the growth defect of proteasome mutants while Ras1G17V weakly rescues it (Supplemental Table S1 available at above website; Figures 7D and

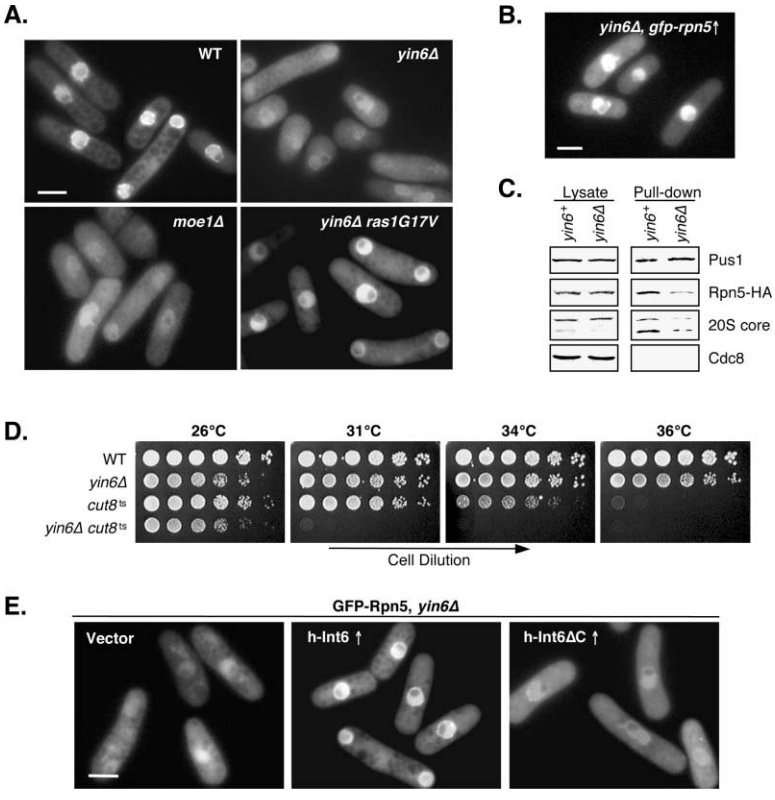

Figure 6. The Yin6-Moe1 Complex and Ras1 Are Required for Proper Rpn5 Localization and Assembly

(A) pREP41GFPRPN5 was integrated into the chromosome to visualize Rpn5 localization. Cells were grown at 30°C. All images collected under identical conditions.

(B) *yin6*Δ cells were transformed with pALGRPN5, which expresses GFP-Rpn5 from the strong *adh1* promoter.

(C) Proteasome pull-down was performed using lysates that were prepared from strains *pus1-ProA rpn5b-HA* (*yin6*$^+$) and *pus1-ProA rpn5b-HA yin6*Δ (*yin6*Δ) grown at 20°C.

(D) The indicated strains were derived from a fusion between *yin6*Δ and the *cut8-563* mutants and were serially diluted and spotted on YEAU plates.

(E) *yin6*Δ cells, transformed with pREP41 GFPRPN5 and either the vector control (pSLF173), pHAHINT6 (expressing full-length h-Int6) or pHAT22 (expressing h-Int6ΔC, containing amino acid residues 1–317), were grown in MM medium to log phase. The strains tested in (A), (B), and (E) were SP870 (WT), YIN6K (*yin6*Δ), MOE1U (*moe1*Δ), and R1VUYIN6K (*yin6*Δ *ras1G17V*).

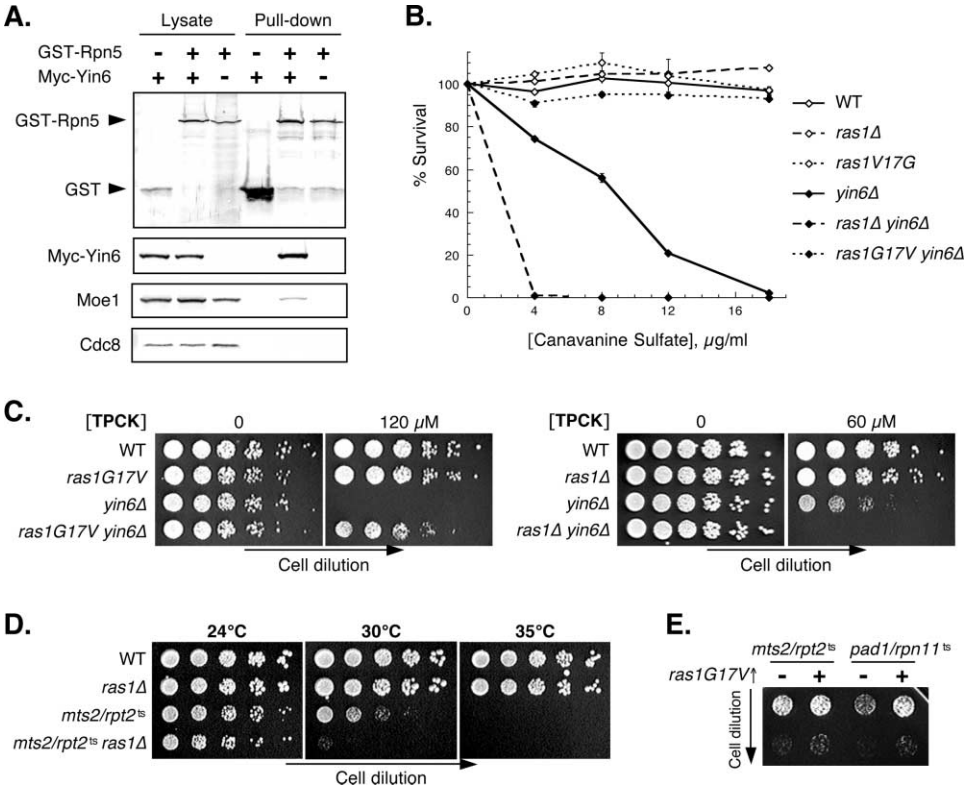

Figure 7. Ras1 and Moe1 Also Mediate the Proteasome Functioning

(A) Lysates used for the proteasome pull-down were from *rpn5*Δ *yin6*Δ cells (R5AAY6K) transformed with (+) or without (−) pGSTRPN5 (expressing GST-Rpn5), and with (+) or without (−) pMYCYIN6 (expressing Myc-Yin6). Lysates and the pull-down samples were analyzed by Western blotting.

(B) The percent survival was measured as described in Figure 1B.

(C) Serially diluted cells were spotted on YEAU plates with or without TPCK and incubated at 30°C.

(D) Cells were spotted on YEAU plates and incubated at indicated temperatures.

(E) *mts2/rpt2*^{ts} and *pad1/rpn11*^{ts} strains, transformed with either a vector control (−, pAL) or a plasmid carrying the genomic version of Ras1G17V (+, pALR1Vg, Nadin-Davis et al., 1986), were spotted on MM plates and incubated at 25°C. The growth of wild-type cells was unaffected by pALR1Vg transformation (data not shown). The strains tested were SP870 (WT), YIN6K (*yin6*Δ), RAS1U (*ras1*Δ), RAS1VU (*ras1G17V*), RAS1UYIN6K (*ras1*Δ *yin6*Δ), R1VUYIN6K (*ras1G17V yin6*Δ), *mts2/rpt2-1* (*mts2/rpt2*^{ts}), *mts2/rpt2-1*RAS1U (*mts2/rpt2*^{ts} *ras1*Δ), and *pad1/rpn11-1* (*pad1/rpn11*^{ts}).

7E). These results show that regulation of Rpn5 is a common molecular mechanism by which both Ras1 and the Yin6-Moe1 complex influence proteasome functions.

The Interaction between Int6 and Rpn5 Is Conserved Evolutionarily

We have shown previously that Yin6 is functionally homologous to h-Int6. We then investigated whether h-Int6 also interacts with h-Rpn5. Our data show that h-Rpn5 and h-Int6 form a protein complex (Figure 3B); more important, full-length h-Int6, but not the Int6ΔC found in tumors, rescues the abnormal distribution of yeast Rpn5 in *yin6*Δ cells (Figure 6E).

Discussion

Int6 has been implicated in tumor formation, but very little is known about its biological functions. In this paper, we studied the functions of the *S. pombe* homolog, Yin6. Our data suggest that one of the key conserved functions of Yin6 is to regulate the proteasome through its interaction with Rpn5, a component of the lid (Supplemental Figure S1, available at http://www.cell.com/cgi/content/full/112/2/207/DC1). In *yin6*Δ cells, Rpn5 is mislocalized in the cytosol, and the proteasome is therefore improperly assembled and impaired in its activity. This leads to the accumulation of mitotic regulators, such as securin/Cut2 and cyclin/Cdc13, causing chromosome instability and abnormal mitosis. Since h-Int6 also binds h-Rpn5 and influences Rpn5 localization in yeast, it is highly probable that h-Int6 also regulates the proteasome in humans. Inactivation of h-Int6 (as a result of LOH or a reduction in *h-INT6* expression) in mammary epithelial cells may lead to inefficient degradation of a subset of mitotic regulators, thus disrupting cell cycle control and/or promoting genetic instability.

Yin6 Regulates Rpn5 Cellular Localization and Proteasome Assembly

The proteasome nuclear localization is critical for proper proteasome functions for both budding yeast and fission yeast. In the former, proteasome components are imported as precursor complexes into the nucleus where they are further assembled to be active (Lehmann et al., 2002). Our data suggest that mislocalization of protea-

some subunits in the cytosol also leads to proteasome inactivation in fission yeast; thus, it is highly probable that fission yeast proteasome similarly reaches maturity in the nucleus. The nuclear import of the proteasome in budding yeast requires an importin-α, encoded by *SRP1* (Lehmann et al., 2002). Interestingly, *S. pombe cut8* encodes a homolog of the budding yeast Sts1, which interacts with Srp1. Hence, it is possible that nuclear import of proteasome subunits in fission yeast also involves importin-α functions. Mammalian proteasomes appear to be both nuclear and cytoplasmic, with the cytoplasmic proteasome being transported into the nucleus (Reits et al., 1997). In some cells, the ratio of cytoplasmic versus nuclear proteasomes varies in a cell cycle dependent manner (Amsterdam et al., 1993; Lafarga et al., 2002; Palmer et al., 1994). Evidently, proteasome nuclear trafficking is conserved among eukaryotes and is pivotal for proper progression of the cell cycle.

Rpn5 lacks an obvious nuclear localization signal (NLS), so it most likely enters the nucleus as a complex with other molecules, such as Yin6. This is supported by the fact that Int6 proteins contain a nuclear export signal and a presumptive bipartite NLS (Guo and Sen, 2000), and that they shuttle in and out of the nucleus in both humans and yeast (Guo and Sen, 2000; Yen and Chang, 2000). Alternatively, because *yin6*Δ severely intensifies the growth defect of the *cut8* mutant, it is possible that Yin6 regulates the function of importin-α, which may in turn control the nuclear import of Rpn5. Intriguingly, even though Yin6 binds Rpn5 with substantial specificity, the whole proteasome appears misassembled in *yin6*Δ cells. It is possible that proteasome assembly requires the cooperative action of all the subunits, such that the absence of any one component could globally affect this process. Alternatively, Rpn5 itself may play a unique role in regulating proteasome assembly. Consistent with this, our ongoing study shows that the proteasome is similarly misassembled in *rpn5*Δ cells (our unpublished data).

Evidence that Int6 in Higher Eukaryotes Can Affect Proteasome Functions

In *Arabidopsis*, Int6 can be linked to the proteasome through its binding to the COP9 complex (Yahalom et al., 2001). One major function of the COP9 is to regulate proteolysis; furthermore, it binds proteasome subunits and regulates their assembly (Kim et al., 2001; Schwechheimer and Deng, 2001). h-Int6 binding to a proteasome component has been reported in a recent two-hybrid study (Hoareau Alves et al., 2002). h-Int6 can also be linked to proteasome regulation through its binding to Tax (Desbois et al., 1996), a viral oncoprotein carried by human T cell leukemia virus type 1. Tax binds and activates the proteasome to facilitate the degradation of IκBα, a key transcription inhibitor of the NFκB pathway (Hemelaar et al., 2001).

Does Yin6/Int6 have functions other than proteasome regulation? Int6 proteins have been presumed to engage in translation initiation because they copurify with the eIF3 complex in both *S. pombe* and mammals (Akiyoshi et al., 2001; Asano et al., 1997; Bandyopadhyay et al., 2000). Paradoxically, cells lacking *yin6* are viable with polysome-ribosome profiles that are nearly indistinguishable from those of wild-type cells (Akiyoshi et al., 2001; Bandyopadhyay et al., 2000). These observations indicate that Yin6 is not essential for global translation initiation in yeast. So why, then, do Int6 proteins bind the eIF3 complex? The proteasome has been shown to tightly associate with the translation machinery, presumably to remove proteins that are improperly folded during translation (Verma et al., 2000). Hence, the association of Int6 with both the proteasome and eIF3 may simply be the result of an interaction between these two complexes during translation.

The Roles of the Proteasome and Genetic Instability in Tumorigenesis

The levels of numerous oncoproteins and tumor suppressors are regulated by the proteasome, thus deregulation of the proteasome can influence a wide range of pathways acting collectively to promote tumorigenesis (Pajonk and McBride, 2001). In support of this, compounds affecting proteasome functions (e.g., MLN341 and PS-341) are emerging as powerful drugs for cancer treatment (Adams, 2002). One of the key functions mediated by the proteasome is genetic stability, which requires the timely and efficient degradation of securin. Human securin is in fact encoded by an oncogene, *PTTG* (pituitary tumor transforming gene; Zou et al., 1999), and is highly expressed in numerous cancers, including breast cancer (Sáez et al., 1999). Overexpression of *PTTG* can transform cells in vitro and injection of these transformed cells induces tumor formation in nude mice (Zhang et al., 1999). Since securin accumulates in *yin6*Δ cells, it is tempting to speculate that Int6 inactivation in humans can cause a similar accumulation, leading to improper chromosome separation.

Genetic instability itself can profoundly influence multiple cell functions because it allows mutations favorable for tumorigenesis to occur efficiently (Lengauer et al., 1998), and breast tumors are among the most genetically unstable. Furthermore, aging is one of the most critical risk factors for breast cancer (Baselga and Norton, 2000). These features argue that genetic instability, occurring over a long period of time, may be particularly crucial in the development of breast cancer. In accordance with this, three of the best-known genes involved in breast cancer, *BRCA1*, *BRCA2*, and *ErbB2/NEU*, mediate genomic stability (Venkitaraman, 2002; Liu et al., 2002); moreover, both *BRCA1* and *BRCA2*, like *yin6,* also participate in Ub-dependent proteolysis (Venkitaraman, 2002).

Breast Tumorigenesis Results from Deregulation of *INT* Genes

We now have a model for the molecular functions of all the *INT* genes. Extracellular peptide signals for cell proliferation are clearly important for breast cancer because three *INT* genes encode FGFs (Hanahan and Weinberg, 2000). The involvement of *WNT1/INT1* and *NOTCH/INT3* underscores the role of cell-cell or cell-matrix communication in the mammary glands (Artavanis-Tsakonas et al., 1999; Wiseman and Werb, 2002). *INT5* encodes an aromatase, thus highlighting the critical role of estrogen levels in breast tumorigenesis (Lon-

ning, 2002). By targeting the proteasome, Int6 can globally affect mitotic regulation and genetic stability.

Additionally, numerous components of other Int pathways, such as NF-κB (from the FGF pathway), β-catenin (from the Wnt pathway), and estrogen receptors, are directly or indirectly regulated by the proteasome (Laney and Hochstrasser, 1999; Leclercq, 2002). As noted earlier, Int6 may regulate NF-κB function by controlling the level of IκBα via its binding to Tax. Hence, it is possible that Int6 may broadly modulate the function of other Int pathways by controlling levels of key components in these pathways. By affecting other Int pathways and genetic stability, *INT6* mutations alone may promote tumorigenesis even when other *INT* genes are normal. In keeping with this, *INT6*, unlike other *INT* genes, was first identified from a feral strain, CZECH II, which has not been inbred for a high propensity of tumor formation and does not have any endogenous MMTV (Marchetti et al., 1995).

The Role of Ras and Int6 Interaction in Breast Tumorigenesis

We have previously reported that the growth defect in *yin6*Δ cells is worsened by *ras1*Δ and rescued by activated Ras1. In this study, we show that both Yin6 and Ras1 can operate through a common molecular mechanism: regulation of proteasome functions and Rpn5 localization (Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/112/2/207/DC1). In keeping with the idea that Ras regulates the proteasome, mammalian Ras has been shown to stabilize the MYC protooncoprotein in a proteasome-dependent manner (Sears et al., 1999).

Our data raise the interesting possibility that activated Ras1 may in fact rescue tumorigenesis induced by Int6 inactivation. We note that although Ras activation is a key driving force for tumorigenesis in most epithelial cell cancers, the role of Ras in breast cancer is uncertain. In contrast to the high frequency of Ras activation mutations in other epithelial cell cancers (e.g., 90% in pancreatic tumors; Rodenhuis, 1992), they are rare in mammary glands (Kiaris and Spandidos, 1995) with a frequency as low as 5% (Schondorf et al., 1999). Moreover, upregulation of Ras expression has been shown to correlate with a more favorable prognosis (Gohring et al., 1999; Schondorf et al., 1999). Clearly, future studies with human mammary epithelial cell lines will be required to fully understand the role of Ras in breast carcinogenesis and to characterize its relationship with Int6.

Experimental Procedures

Strains, Plasmid Constructions, and Growth Conditions

Details of the strains used, the construction of mutant strains and plasmids, and the conditions under which strains were grown are included in the Supplemental Data (available at http://www.cell.com/cgi/content/full/112/2/207/DC1).

The Yeast Two-Hybrid Assay and Cloning of *rpn5*

The reporter strains were L40 and HF7C (Criekinge and Beyaert, 1999). The assays for the activation of the *lacZ* and *HIS3* are as described (Chang et al., 1994). Yin6 autoactivates the reporter genes when it is fused with the DNA binding domain; thus, 3A-T (3-amino-1, 2, 4-traizole) was added to reduce background. For the two-hybrid screen, pGBDYIN6ΔN and pGBDYIN6ΔC were used as baits. The cDNA library used was as described (Chang et al., 1994). Approximately 10^7 cDNA clones were screened with both baits, and full-length *rpn5* was isolated 7 times using pGBD-YIN6ΔN and 168 times using pGBD-Yin6ΔC. We note that there are two *rpn5* genes in *S. pombe*, *rpn5a* and *rpn5b* (accession numbers AL121732 and AL590902) (Wood et al., 2002). They code for identical proteins with 443 amino acid residues, but contain different 5′- flanking sequences. *S. pombe* Rpn5 protein is approximately 40% identical in primary sequence to the human and budding yeast versions (Saito et al., 1997). One of these cDNA clones was named pGADspRPN5 and used for detailed studies.

26S Proteasome Pull-Down Assay

Approximately 30 OD units of cells were lysed in the binding buffer (25 mM Tris, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 0.1% Triton X-100, 20% glycerol, [pH 7.0–7.4]). Total crude lysates were centrifuged at 20,800 × g for 20 min. Supernatants with the same amount of protein were incubated with either 10 μl IgG Sepharose or protein A Sepharose overnight at 4°C. After wash in the binding buffer, beads were incubated with the TEV protease (Invitrogen) to release the proteasome. Eluted proteins were analyzed by SDS-PAGE and Western blots. Moe1, Mts2, Mts3, Mts4, and Pus1 antisera were as described and used at 1:1000 dilution (Chen et al., 2000; Wilkinson et al., 1997). Antibodies against the α-subunits in the catalytic complex (MCP231, 1:1000) and Cdc8 (1:400) were from Affiniti and M. Balasubramanian (University of Singapore; Balasubramanian et al., 1992), respectively. 9E10 (1:1000) was used to detect Myc-tagged proteins.

Detection of Polyubiquitinated Proteins

To detect polyubiquitinated proteins, cells were lysed in PEM buffer (Yen and Chang, 2000). Total crude extracts containing equal amount of proteins were analyzed by immunoblotting with an ubiquitin antibody (1:3500, a kind gift from C. Pickart). The assay of polyubiquitinated Cut2 and Cdc13 levels was essentially as described (Berry et al., 1999). Ub-Cut2-Myc and Ub-Cdc13 were detected by immunoblotting with 9E10 (1:1000) and a Cdc13 antibody (1:2000; Gachet et al., 2001), respectively.

Measurement of Total Protein Degradation

Cells were pregrown in MM medium to log phase at 22°C. Five OD units of cells were washed with sulfate-free MM medium and then resuspended in 1 ml sulfate-free MM medium with shaking for 20 min at 22°C. These cells were pulsed with 50 μCi ^{35}S-Met (Amersham Pharmacia) for 7 min, and then filtered and resuspended in the chase MM medium containing 5 mM Met, 2 mM Cys, and 100 μg/ml cycloheximide (Ayscough and Warren, 1994). The TCA precipitable ^{35}S signal was measured as described (Bandyopadhyay et al., 2000).

GST Pull-Down Assay

Yeast lysates were prepared in PEM buffer plus 0.5% IGEPAL CA-630 (Sigma). Crude lysates were centrifuged twice at 20,800 × g for 20 min. Supernatants were incubated with 10 μl glutathione sepharose (Amersham Pharmacia Biotech) overnight at 4°C. Beads were washed with the lysis buffer, and the proteins were analyzed by the Western analysis. The amount of lysate run as control in our figures was approximately 15% of that used for the pull-down experiment. The anti-GST antibody (1:2000) was from Amersham Pharmacia Biotech. The 12CA5 antibody (1:1000) was used to detect HA-tagged proteins.

Fluorescence Microscopy

The general procedures for calcofluor and 4′-6-diaminodino-2-phenylindole (DAPI) staining and microtubule visualization were essentially as described (Chen et al., 1999), except that for the latter, cells were fixed in 3% formaldehyde and 0.05% glutaraldehyde. All scale bars in the figures equal 5 μm.

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References

Adams, J. (2002). Proteasome inhibition: a novel approach to cancer therapy. Trend. Mol. Med. *8*, 49–54.

Akiyoshi, Y., Clayton, J., Phan, L., Yamamoto, M., Hinnebusch, A.G., Watanabe, Y., and Asano, K. (2001). Fission yeast homolog of murine Int-6 protein, encoded by mouse mammary tumor virus integration site, is associated with the conserved core subunits of eukaryotic translation initiation factor 3. J. Biol. Chem. *276*, 10056–10062.

Amsterdam, A., Pitzer, F., and Baumeister, W. (1993). Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cell cycle control. Proc. Natl. Acad. Sci. USA *90*, 99–103.

Asano, K., Merrick, W.C., and Hershey, J.W. (1997). The translational initiation factor eIF3-p48 subunit is encoded by *int6*, a site of frequent integration by the mouse mammary tumor virus genome. J. Biol. Chem. *272*, 23477–23480.

Artavanis-Tsakonas, S., Rand, M.D., and Lake, R.J. (1999). Notch signaling: cell fate control and signal integration in development. Science *284*, 770–776.

Ayscough, K., and Warren, G. (1994). Inhibition of protein synthesis disrupts the Golgi apparatus in the fission yeast, *Schizosaccharomyces pombe*. Yeast *10*, 1–11.

Balasubramanian, M.K., Helfman, D.M., and Hemmingsen, S.M. (1992). A new tropomyosin essential for cytokinesis in the fission yeast *S. pombe*. Nature *360*, 84–87.

Bandyopadhyay, A., Matsumoto, T., and Maitra, U. (2000). Fission yeast Int6 is not essential for global translation initiation, but deletion of *int6*+ causes hypersensitivity to caffeine and affects spore formation. Mol. Biol. Cell *11*, 4005–4018.

Baselga, J., and Norton, L. (2000). Focus on breast cancer. Cancer Cell *1*, 319–322.

Berry, L.D., Feoktistova, A., Wright, M.D., and Gould, K.L. (1999). The *Schizosaccharomyces pombe* dim1(+) gene interacts with the anaphase-promoting complex or cyclosome (APC/C) component lid1(+) and is required for APC/C function. Mol. Cell. Biol. *19*, 2535–2546.

Chang, E.C., Barr, M., Wang, Y., Jung, V., Xu, H., and Wigler, H.M. (1994). Cooperative interaction of *S. pombe* proteins required for mating and morphogenesis. Cell *79*, 131–141.

Chen, C.-R., Li, Y.-C., Chen, J., Hou, M.C., Papadaki, P., and Chang, E.C. (1999). Moe1, a novel conserved protein in *Schizosaccharomyces pombe*, interacts with a ras effector, Scd1, to affect proper spindle formation. Proc. Natl. Acad. Sci. USA *96*, 517–522.

Chen, C.-R., Chen, J., and Chang, E.C. (2000). A conserved interaction between Moe1 and Mal3 is important for proper spindle formation in *Schizosaccharomyces pombe*. Mol. Biol. Cell *11*, 4067–4077.

Criekinge, W.V., and Beyaert, R. (1999). Yeast two-hybrid: state of the art. Biol. Proc. Online *2*, 1–28.

Desbois, C., Rousset, R., Bantignies, F., and Jalinot, P. (1996). Exclusion of Int-6 from PML nuclear bodies by binding to the HTLV-I Tax oncoprotein. Science *273*, 951–953.

Ferrell, K., Wilkinson, C.R., Dubiel, W., and Gordon, C. (2000). Regulatory subunit interactions of the 26S proteasome, a complex problem. Trends Biochem. Sci. *25*, 83–88.

Fukui, Y., and Yamamoto, M. (1988). Isolation and characterization of *Schizosaccharomyces pombe* mutants phenotypically similar to *ras1*$^-$. Mol. Gen. Genet. *215*, 26–31.

Fukui, Y., Kozasa, T., Kaziro, Y., Takeda, T., and Yamamoto, M. (1986). Role of a *ras* homolog in the life cycle of *Schizosaccharomyces pombe*. Cell *44*, 329–336.

Gachet, Y., Tournier, S., Millar, J.B., and Hyams, J.S. (2001). A MAP kinase-dependent actin checkpoint ensures proper spindle orientation in fission yeast. Nature *412*, 352–355.

Glickman, M.H., Rubin, D.M., Fried, V.A., and Finley, D. (1998). The regulatory particle of the *Saccharomyces cerevisiae* proteasome. Mol. Cell. Biol. *18*, 3146–3162.

Gohring, U.J., Schondorf, T., Kiecker, V.R., Becker, M., Kurbacher, C., and Scharl, A. (1999). Immunohistochemical detection of H-ras protooncoprotein p21 indicates favorable prognosis in node-negative breast cancer patients. Tumour Biol. *20*, 173–183.

Gordon, C., McGurk, G., Dillon, P., Rosen, C., and Hastie, N.D. (1993). Defective mitosis due to a mutation in the gene for a fission yeast 26S protease subunit. Nature *366*, 355–357.

Grishchuk, E.L., Howe, J.L., and McIntosh, J.R. (1998). A screen for genes involved in the anaphase proteolytic pathway identifies *tsm1*(+), a novel *Schizosaccharomyces pombe* gene important for microtubule integrity. Genetics *149*, 1251–1264.

Guo, J., and Sen, G. (2000). Characterization of the interaction between the interferon-induced protein P56 and the Int6 protein encoded by the locus of insertion of the mouse mammary tumor virus. J. Virol. *74*, 1892–1899.

Hanahan, D., and Weinberg, R.A. (2000). The hallmarks of cancer. Cell *100*, 57–70.

Hemelaar, J., Bex, F., Booth, B., Cerundolo, V., McMichael, A., and Daenke, S. (2001). Human T-cell leukemia virus type 1 Tax protein binds to assembled nuclear proteasomes and enhances their proteolytic activity. J. Virol. *75*, 11106–11115.

Hoareau Alves, K., Bochard, V., Rety, S., and Jalinot, P. (2002). Association of the mammalian proto-oncoprotein Int-6 with the three protein complexes eIF3, COP9 signalosome and 26S proteasome. FEBS Lett. *527*, 15–21.

Hofmann, K., and Bucher, P. (1998). The PCI domain: a common theme in three multiprotein complexes. Trends Biochem. Sci. *23*, 204–205.

Kiaris, H., and Spandidos, D.A. (1995). Mutations of ras genes in human tumours. Int. J. Oncol. *7*, 413–421.

Kim, T., Hofmann, K., von Arnim, A.G., and Chamovitz, D.A. (2001). PCI complexes: pretty complex interactions in diverse signaling pathways. Trends Plant Sci. *6*, 379–386.

Lafarga, M., Fernandez, R., Mayo, I., Berciano, M.T., and Castano, J.G. (2002). Proteasome dynamics during cell cycle in rat Schwann cells. Glia *38*, 313–328.

Laney, J.D., and Hochstrasser, M. (1999). Substrate targeting in the ubiquitin system. Cell *97*, 427–430.

Leclercq, G. (2002). Molecular forms of the estrogen receptor in breast cancer. J. Steroid Biochem. Mol. Biol. *80*, 259–272.

Lehmann, A., Janek, K., Braun, B., Kloetzel, P.M., and Enenkel, C. (2002). 20 S proteasomes are imported as precursor complexes into the nucleus of yeast. J. Mol. Biol. *317*, 401–413.

Lengauer, C., Kinzler, K.W., and Vogelstein, B. (1998). Genetic instabilities in human cancers. Nature *396*, 643–649.

Liu, S., Liu, W., Jakubczak, J.L., Erexson, G.L., Tindall, K.R., Chan, R., Muller, W.J., Adhya, S., Garges, S., and Merlino, G. (2002). Genetic instability favoring transversions associated with ErbB2-induced mammary tumorigenesis. Proc. Natl. Acad. Sci. USA *99*, 3770–3775.

Lonning, P.E. (2002). The role of aromatase inactivators in the treatment of breast cancer. Int. J. Clin. Oncol. *7*, 265–270.

Luca, F.C., and Ruderman, J.V. (1989). Control of programmed cyclin destruction in a cell-free system. J. Cell Biol. *109*, 1895–1909.

Marchetti, A., Buttitta, F., Miyazaki, S., Gallahan, D., Smith, G., and Callahan, R. (1995). Int-6, a highly conserved, widely expressed

gene, is mutated by mouse mammary tumor virus in mammary preneoplasia. J. Virol. *69*, 1932–1938.

Marchetti, A., Buttitta, F., Pellegrini, S., Bertacca, G., and Callahan, R. (2001). Reduced expression of INT-6/eIF3-p48 in human tumors. Int. J. Oncol. *18*, 175–179.

Miyazaki, S., Imatani, A., Ballard, L., Marchetti, A., Buttitta, F., Albertsen, H., Nevanlinna, H.A., Gallahan, D., and Callahan, R. (1997). The chromosome location of the human homolog of the mouse mammary tumor-associated gene INT6 and its status in human breast carcinomas. Genomics *46*, 155–158.

Nadin-Davis, S.A., Nasim, A., and Beach, D. (1986). Involvement of *ras* in sexual differentiation but not in growth control in fission yeast. EMBO J. *5*, 2963–2971.

Pajonk, F., and McBride, W.H. (2001). The proteasome in cancer biology and treatment. Radiat. Res. *156*, 447–459.

Palmer, A., Mason, G.G., Paramio, J.M., Knecht, E., and Rivett, A.J. (1994). Changes in proteasome localization during the cell cycle. Eur. J. Cell Biol. *64*, 163–175.

Rasmussen, S.B., Kordon, E., Callahan, R., and Smith, G.H. (2001). Evidence for the transforming activity of a truncated Int6 gene, in vitro. Oncogene *20*, 5291–5301.

Reits, E.A., Benham, A.M., Plougastel, B., Neefjes, J., and Trowsdale, J. (1997). Dynamics of proteasome distribution in living cells. EMBO J. *16*, 6087–6094.

Rodenhuis, M. (1992). *ras* and human tumors. Semin. Cancer Biol. *3*, 241–247.

Sáez, C., Japon, M.A., Ramos-Morales, F., Romero, F., Segura, D.I., Tortolero, M., and Pintor-Toro, J.A. (1999). hpttg is over-expressed in pituitary adenomas and other primary epithelial neoplasias. Oncogene *18*, 5473–5476.

Saito, A., Watanabe, T.K., Shimada, Y., Fujiwara, T., Slaughter, C.A., DeMartino, G.N., Tanahashi, N., and Tanaka, K. (1997). cDNA cloning and functional analysis of p44.5 and p55, two regulatory subunits of the 26S proteasome. Gene *203*, 241–250.

Schondorf, T., Andrack, A., Niederacher, D., Scharl, A., Becker, M., Engel, H., and Gohring, U.J. (1999). H-ras gene amplification or mutation is not common in human primary breast cancer. Oncol. Rep. *6*, 1029–1033.

Schwechheimer, C., and Deng, X.W. (2001). COP9 signalosome revisited: a novel mediator of protein degradation. Trends Cell Biol. *11*, 420–426.

Sears, R., Leone, G., DeGregori, J., and Nevins, J.R. (1999). Ras enhances Myc protein stability. Mol. Cell *3*, 169–179.

Tatebe, H., and Yanagida, M. (2000). Cut8, essential for anaphase, controls localization of 26S proteasome, facilitating destruction of cyclin and cut2. Curr. Biol. *10*, 1329–1338.

Tekmal, R.R., and Keshava, N. (1997). Role of MMTV integration locus cellular genes in breast cancer. Front. Biosci. *2*, 519–526.

van't Veer, L.J., Dai, H., van de Vijver, M.J., He, Y.D., Hart, A.A., Mao, M., Peterse, H.L., van der Kooy, K., Marton, M.J., Witteveen, A.T., Schreiber, G.J., *et al*. (2002). Gene expression profiling predicts clinical outcome of breast cancer. Nature *415*, 530–536.

Venkitaraman, A.R. (2002). Cancer susceptibility and the functions of BRCA1 and BRCA2. Cell *108*, 171–182.

Verma, R., Chen, S., Feldman, R., Schieltz, D., Yates, J., Dohmen, J., and Deshaies, R.J. (2000). Proteasomal proteomics: identification of nucleotide-sensitive proteasome-interacting proteins by mass spectrometric analysis of affinity-purified proteasomes. Mol. Biol. Cell *11*, 3425–3439.

Wilkinson, C.R., Wallace, M., Seeger, M., Dubiel, W., and Gordon, C. (1997). Mts4, a non-ATPase subunit of the 26 S protease in fission yeast, is essential for mitosis and interacts directly with the ATPase subunit Mts2. J. Biol. Chem. *272*, 25768–25777.

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.

Wilkinson, C.R., Ferrell, K., Penney, M., Wallace, M., Dubiel, W., and Gordon, C. (2000). Analysis of a gene encoding Rpn10 of the fission yeast proteasome reveals that the polyubiquitin-binding site of this subunit is essential when Rpn12/Mts3 activity is compromised. J. Biol. Chem. *275*, 15182–15192.

Wiseman, B.S., and Werb, Z. (2002). Stromal effects on mammary gland development and breast cancer. Science *296*, 1046–1049.

Wood, V., Gwilliam, R., Rajandream, M.A., Lyne, M., Lyne, R., Stewart, A., Sgouros, J., Peat, N., Hayles, J., Baker, S., et al. (2002). The genome sequence of *Schizosaccharomyces pombe*. Nature *415*, 871–880.

Yahalom, A., Kim, T.H., Winter, E., Karniol, B., von Arnim, A.G., and Chamovitz, D.A. (2001). *Arabidopsis* eIF3e (INT-6) associates with both eIF3c and the COP9 signalosome subunit CSN7. J. Biol. Chem. *276*, 334–340.

Yen, H.-C.S., and Chang, E.C. (2000). Yin6, a fission yeast Int6 homolog, complexes with Moe1 and plays a role in chromosome segregation. Proc. Natl. Acad. Sci. USA *97*, 14370–14375.

Zhang, X., Horwitz, G.A., Prezant, T.R., Valentini, A., Nakashima, M., Bronstein, M.D., and Melmed, S. (1999). Structure, expression and function of human pituitary tumor-transforming gene (PTTG). Mol. Endocrinol. *13*, 156–166.

Zou, H., McGarry, T.J., Bernal, T., and Kirschner, M.W. (1999). Identification of a vertebrate sister-chromatid separation inhibitor involved in transformation and tumorigenesis. Science *285*, 418–422.

Accession Numbers

The GenBank accession numbers for sequences *rpn5a* and *rpn5b* are AL121732 and AL590902, respectively.