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Proteasome Nuclear Import Mediated by Arc3 Can Influence Efficient DNA Damage Repair and Mitosis in Schizosaccharomyces Pombe

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Proteasomes must remove regulatory molecules and abnormal proteins throughout the cell, but how proteasomes can do so efficiently remains unclear. We have isolated a subunit of the Arp2/3 complex, Arc3, which binds proteasomes. When overexpressed, Arc3 rescues phenotypes associated with proteasome deficiencies; when its expression is repressed, proteasome deficiencies intensify. Arp2/3 is best known for regulating membrane dynamics and vesicular transport; thus, we performed photobleaching experiments and showed that proteasomes are readily imported into the nucleus but exit the nucleus slowly. Proteasome nuclear import is reduced when Arc3 is inactivated, leading to hypersensitivity to DNA damage and inefficient cyclin-B degradation, two events occurring in the nucleus. These data suggest that proteasomes display Arc3-dependent mobility in the cell, and mobile proteasomes can efficiently access substrates throughout the cell, allowing them to effectively regulate cell-compartment-specific activities.

INTRODUCTION
The 26S proteasome functions to degrade proteins that are marked by polyubiquitylation (Voges et al., 1999). Ubiquitylated targets for proteasome degradation include proteins that are mis-folded due to abnormal protein synthesis or environmental assaults, as well as regulatory proteins whose timely removal is critical for proper transcription, signaling, and mitosis. As such, the 26S proteasome provides an indispensable machinery to remove damaged (and frequently cytotoxic) proteins and to maintain homeostasis of regulatory molecules.

Proteasome substrates may be produced throughout the cell. Moreover, some events that require proteasomal degradation, such as DNA damage repair, are restricted to a given cell compartment. What mechanisms must be in place so that proteasomes will always be available when a molecule anywhere in the cell needs to be promptly degraded? Is it possible that efficient proteolysis requires proteasomes to be highly mobile? If so, how is this mobility controlled?

Our lab has previously isolated the yin6 gene in the fission yeast Schizosaccharomyces pombe during the study of Ras GTPase (Yen and Chang, 2000). S. pombe yin6 is a homolog of the mammalian INT6, which was first discovered in a genetic screen using the mouse mammary tumor virus as an insertional mutagen to seek the INT genes that are important for breast tumor formation (Marchetti et al., 1995; Sap and Chang, 2005). Int6/Yin6 can bind both proteasome and eIF3 subunits (thus it is also known as eIF3e/eIF3-p48; Asano et al., 1997). In S. pombe, we have uncovered a key evolutionarily conserved role of Yin6, which is to interact with a Ras pathway to regulate the proteasome (Yen and Chang, 2003; Yen et al., 2003b). In wild-type S. pombe cells, proteasomes concentrate at the nuclear membrane (Wilkinson et al., 1998); in contrast, in yin6 null (yin6Δ) cells, some proteasome subunits are improperly assembled or mislocalized in the cytoplasm or both.

To better understand how Yin6 influences proteasome functioning, we have conducted a high-copy suppressor screen to seek S. pombe cDNAs that when overexpressed rescue the growth defects in yin6Δ cells (Sha et al., 2007). In the present study, we focus on one of the isolated cDNA clones, which encodes Arc3, a subunit of the Arp2/3 complex of actin regulatory proteins. Arc3 was chosen for further study for two key reasons: First, although there are two Ras pathways in S. pombe—the Byr2 MAPKK and the Cdc42 GTPase pathways, Yin6 selectively interacts with...
the latter (Yen and Chang, 2000), which in many other organisms has been shown to regulate Arp2/3 (reviewed in Higgs and Pollard, 2001). Second, a ubiquitous function of the Arp2/3 complex is to promote actin polymerization to provide the force required for the budding and motility of COP1/COPII vesicles (Stamnes, 2002). We thus believe that this apparent link between Arc3 and proteasomes may shed light on the issues of whether proteasomes are mobile and whether mobile proteasomes are important for efficient proteolysis.

We report herein that Arc3, as well as other Arp2/3 subunits, are required for proper proteasome functioning in the cell. That is, overexpressing Arc3 rescues phenotypes of arc3 mutants, whereas repressing Arc3 expression intensifies proteasome mutant phenotypes and decreases the efficiency with which proteasomes function in the cell. Although Arc3 is required for efficient proteasome assembly and catalysis can be found when proteasomes are purified from Arc3-repressed cells. However, when Arc3 expression is silenced, proteasome nuclear import decreases, and cells become hypersensitive to DNA damage and inefficient in degrading cyclin-B, two events that occur in the S. pombe nucleus. These data suggest that high mobility is a critical property of the proteasome. Highly mobile proteasomes can rapidly reach every part of the cell to degrade proteins, and this property can reduce the need to transport potentially toxic molecules. Mobile proteasomes are also critical for efficiently regulating cellular events, e.g., DNA damage repair and mitosis that are cell-compartment restricted.

MATERIALS AND METHODS

Growth Conditions and Reagents
Cells were grown in either yeast extract (rich) medium (YEAU) or synthetic minimal medium (MM) with appropriate supplements (Chen et al., 1999). To test for canavanine sensitivity, a stock solution of canavanine sulfate (50 mg/ml, Sigma, St. Louis, MO), was prepared in DMSO. Plates spotted with cells were irradiated with UV light to facilitate the loss of LEU2, and we named the resulting cells ARC3::URA4/arc3::ura4. On thiamine addition, these three arc3::ura4 strains showed completely identical to those with other arc3 mutations (Yen et al., 1996; Morrell et al., 1999). arc3::ura4 rpn1::gfp cells were generated by crossing strain ARCCN3 with strain rpm1-116-gfp (Wilkinson et al., 1998) followed by random spore selection. To analyze interaction between Arc3 and the proteasome by genetics, an arc3::ura4 mutant (strain ARCCN3::URA4) was crossed with the mts1::URA4 mutant (Wilkinson et al., 1997), followed by random spore selection. Multiple sets of wild-type, single, and double mutants were isolated to confirm that they display the same phenotypes. Similarly, the arc3::URA4 mutant (Shaw et al., 2007) was crossed with Arc3::URA4. To disrupt the ura4 marker gene in this strain, cells were transformed with HindIII-cut fragments of JP0DU, and ura4- cells were selected in MM supplemented with 1% 5-FOA. The resulting strain was named ARCCN3::URA4. To further remove the LEU2 marker, the strain ARCCN3::URA4 was transformed with pREP24ARCC and grown for several generations in MM supplemented with leucine to facilitate the loss of LEU2, and we named the resulting cell ARC3::URA4/ARC3::URA4.

Fluorescence Microscopy
To perform live cell imaging, cells were mounted on agar-embedding medium as described (Tran et al., 2004). Samples were excited via an epiFl 100x/1.30 NA oil microscope (Melville, NY), and the images were captured by either an ORCA ER (Hamamatsu, Bridge- water, NJ) or a Retiga 1300 (Q-Imaging, Austin, TX) camera. GFP levels were quantified using the Openlab software (Improvision, Lexington, MA). To measure the lifetime of Cdc13, stacks of three or eight images (at 0.5- or 1-μm intervals) were collected every minute for 1 h, during which focusing was readjusted every 15 min.

Photobleaching Data Acquisition

These studies were carried out by a Zeiss LSM510 confocal microscope with a Plan-Apochromat 63x/1.30 NA oil objective equipped with an objective heater to maintain sample temperature at 30°C. A 25-mW argon laser was turned on at 75% of power. Images were all collected with the pinhole set at 10.42 airy units (AU) to maximize the signal intensity. Yeast cells are small and the GFP signal dim. To measure the fluorescence intensity before photobleaching, the sample was scanned three times (4× zoom at 1% of laser power) and then 10 iterative laser pulses at full power were applied to the
nucleus to photobleach. For local nuclear fluorescence recovery after photobleaching (FRAP) experiments, four iterative laser pulses at full power were applied to photobleach, and this was followed by collecting 20 images at 500-ms intervals. Alternatively, local nuclear FRAP was also measured using a Leica TCS SPS confocal microscope with a 63×/1.4 NA oil objective (Deerfield, IL). We did not find any appreciable difference in the mobility as measured by these two different instruments.

**Photobleaching Data Analysis**

All fluorescence intensity values were divided by the size of the area of interest (in pixels), and the final data were exported from the LSM software (Carl Zeiss) to Excel (Microsoft, Redmond, WA). The relative intensity (RI) in a region of interest was defined as: \( RI = (I_i - I_b)/I_b \), where \( I_b \) is the fluorescence intensity measured before photobleaching and \( I_i \) is the intensity measured at a given time point \( t \). Both \( I_b \) and \( I_i \) values are corrected for background noise by subtracting the intensity in a region that does not have any cell. In addition to the RI values of the photobleached samples, the RI in the nucleus of an unbleached cell in the same field was also measured to control for spontaneous photobleaching. For the calculation of recovery \( \tau_{1/2} \) in local nuclear FRAP, the raw RI values were corrected for spontaneous photobleaching by dividing \( R_{IB} \) with \( R_{UB} \) to yield \( R_{IB}/R_{UB} \). The “Exponential Rise” model in FRAP, the raw RI values were corrected for spontaneous photobleaching by dividing \( R_{IB} \) with \( R_{UB} \) to yield \( R_{IB}/R_{UB} \). The “Exponential Rise” model in FRAP, the raw RI values were corrected for spontaneous photobleaching by dividing \( R_{IB} \) with \( R_{UB} \) to yield \( R_{IB}/R_{UB} \). The “Exponential Rise” model in FRAP, the raw RI values were corrected for spontaneous photobleaching by dividing \( R_{IB} \) with \( R_{UB} \) to yield \( R_{IB}/R_{UB} \). The “Exponential Rise” model in FRAP, the raw RI values were corrected for spontaneous photobleaching by dividing \( R_{IB} \) with \( R_{UB} \) to yield \( R_{IB}/R_{UB} \).

**Protein Half-Life Measurement**

Cells were pregrown to early log phase at 30°C in the minimal medium to a cell concentration such that at the end of the experiment, the OD would be \( \leq 0.5 \). After addition of 250 mM CHX, 20–50 ml of cells was harvested at each time point, and protein levels were measured by immunoblots.

**Cell Fractionation**

Microsomes were isolated as described (Movischoff et al., 2005) from strain Y6HA3MGGPI. The lysates and the microsome and soluble fractions were analyzed by Western blots.

**Immunoblotting**

To detect ubiquitylated proteins and GFP, cells were lysed by glass beads in PBS (pH 7.4). N-ethylmaleimide (10 mM) was added in the lysate buffer to block cleavage of polyubiquitylated chains. Proteins were separated by standard SDS-PAGE and transferred to nitrocellulose membrane. To detect Ub and GFP, antibodies from Sigma (1:200) and Fitzgerald (1:1000; Concord, MA) were used, and the signals were measured by the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

**Chemical Cross-Linking**

arc3-MYC rpn-5HA cells (strain A3MRSHA) were precultured in 1 L YEAC medium and then collected and washed before being resuspended in 5 ml of PBS containing 2 mM dithiobis(succinimidyl propionate) (Thermo Scientific, Waltham, MA) for 2 h at 4°C. Cross-linking was terminated by adding 5 ml 20 mM Tris–Cl (pH 8.0) at room temperature for 15 min with rocking. Cells were then lysed by glass beads in lysis buffer (10 mM HEPES, pH 7.55, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% IGEPAL CA-630, 1 mM PMSF, 10% glycerol, Complete Protease Inhibitor [Roche, Indianapolis, IN]). To quantify protein levels, fluorescently conjugated secondary antibodies were used, and the signals were measured by the Odyssey infrared imaging system (Li-Cor Biosciences, Lincoln, NE).

**RESULTS**

**Arc3 Overexpression Rescues Proteasome Defects in yin6Δ Cells**

To identify genes that regulate the proteasome, we screened an S. pombe cDNA library for genes that when overexpressed rescued the cold-dependent growth defects in yin6Δ cells. From this screen we isolated a full-length cDNA clone encoding a component of the Arp2/3 complex of actin regulatory proteins called Arc3. Arc3 carries a predicted molecular weight of 19.8 kDa and shares 51% identity with the human ARPC3 (actin-related protein 2/3 complex subunit 3; Welch et al., 1997). We have shown previously that proteasome deficiencies cause yin6Δ cells to be hypersensitive to canavanine, an arginine analog, the incorporation of which results in the synthesis of abnormal proteins. Our results here show that Arc3 overexpression also rescued canavanine hypersensitivity in these cells (Figure 1A). Finally, we have shown that proteasomes are improperly mislocalized in cytoplasm in yin6Δ cells. To determine whether Arc3 overexpression can restore proteasome nuclear localization, we tagged one of the endogenous proteasome catalytic subunits α4 with GFP (Sha et al., 2007) and then measured the ratio of α4-GFP signal in the cytoplasm versus the nucleus. As shown in Figure 1B, Arc3 overexpression modestly restored proteasome nuclear accumulation in yin6Δ cells (Figure 1B), such that relatively more α4-GFP could be detected in the nucleus of yin6Δ cells. These results indicate that the pro-
Arc3, as well as Other Arp2/3 Components, Is Required for Proper Proteasome Functioning in the Cell

Because Arc3 overexpression rescues proteasome defects in yin6Δ cells, we investigated using proteasome mutants whether Arc3 is required for proper proteasome functioning. First, our data in Figure 2A showed that Arc3 overexpression rescued the growth defect of the temperature-sensitive (ts) proteasome mutant carrying the rpt2ts/mts2-1 mutation (Gordon et al., 1993). Next, we sought to inactivate Arc3. Like mutants lacking either arp2 or arp3 (Balasubramanian et al., 1996; McCollum et al., 1996; Morrell et al., 1999), arc3Δ cells are inviable (Materials and Methods). We thus generated a strain carrying a conditional arc3 mutation, which we call arc3nmt. In this strain, the endogenous arc3 has been replaced by an nmt-arc3 cassette such that arc3 expression is now under the control of the thiamine-repressible nmt promoter. We confirmed that when arc3 expression was repressed, although there is no global disruption of F-actin structures, F-actin patches and cables become disorganized, as have been previously reported for other arp2/3 mutants. This arc3nmt cells were crossed into a ts proteasome mutant carrying mts4–288/rpn1 ts (Wilkinson, 1997), and the resulting double mutants (Figure 2B) displayed severe growth defects under conditions that did not substantially impair the growth of either single mu-
Figure 3. The Arp2/3 complex and F-actin are required for proper proteasome function. (A) Cells with the indicated genotypes were serially diluted and spotted on YEAU medium and grown at indicated temperatures. (B) Wild-type and arp3p<sup>-1</sup>-ts cells expressing the M-GFP and R-GFP fusion proteins were incubated at 22°C, and the cell lysates were analyzed by Western blots. Tubulins were the loading control. (C) Wild-type cells were treated with Lat A (+, 10 μM) or DMSO alone (−) for 1 or 2 h, and Lat A was then washed out. Cell lysates were analyzed by Western blots using the indicated antibodies. The levels of polyubiquitylated proteins (marked by the bracket) relative to the tubulin loading control were quantified, and this value was set to 1 at each time point for cells incubated in DMSO. (D) Wild-type cells expressing Ub-M-GFP or Ub-R-GFP were treated with cycloheximide (CHX, 250 μM, 1.5 h), with or without Lat A (10 μM, 1.5 h), and the cell lysates were analyzed by Western blots. (E) The act1<sup>-48</sup> mutant and the wild-type strain were pregrown at 30°C. Samples were taken before or after being shifted to 20°C for 7 h. Cell lysates were analyzed by Western blots using the indicated antibodies. Polyubiquitylated proteins (marked by the bracket) relative to the tubulin loading control were quantified, and this value was set to 1 for wild-type samples at either temperature. (F) Cells with indicated genotypes were streaked on the same YEAP plate and grown at 30°C. Synthetic growth defects of <i>rpn7</i><sup>ts</sup> act1<sup>-48</sup> cells are shown by the inability to form colonies.

Another ts proteasome mutation <i>rpn7</i><sup>ts</sup> (Sha et al., 2007) was similarly tested with the same results (data not shown).

Inactivation of proteasomes can lead to accumulation of polyubiquitylated proteins. As shown in Figure 2C, levels of polyubiquitylated proteins increased in the <i>arc3</i><sup>Δ</sup> mutant cells. To more directly measure reduction in cellular proteasome activity, we constructed three Ub fusion proteins as reporters: Ub-M-GFP, Ub-R-GFP, and Ub<sup>Δ</sup>G76V-GFP (Dantuma et al., 2000). Both Ub-M-GFP and Ub-R-GFP are cleaved after the Ub by endogenous Ub C-terminal hydrolases, and the resulting M-GFP is stable, with a half-life >6 h and thus can accumulate in wild-type cells (Supplemental Figure S1). In contrast, R-GFP and Ub<sup>Δ</sup>G76V-GFP are readily degraded by the proteasome via the N-end rule and Ub fusion degradation pathways, respectively. R-GFP and Ub<sup>Δ</sup>G76V-GFP have half-lives of 30 min and can only accumulate in cells with proteasome deficiency (Supplemental Figure S1 and data not shown). As shown in Figure 2D, both Ub<sup>Δ</sup>G76V-GFP and R-GFP, but not M-GFP, also accumulated in the <i>arc3</i><sup>Δ</sup> mutant cells, with a concurrent doubling in half-life (Figure 2E), indicating that Arc3 is required for efficient proteolysis in the cell.

Because Arc3 is an Arp2/3 complex subunit, we have similarly examined mutations in <i>arp2</i> and <i>arp3</i>. Our data showed that the ts <i>arp2</i><sup>-1</sup> and the cold sensitive <i>arp3</i><sup>-c1</sup> mutations (McCollum et al., 1996) also intensified the phenotype of the proteasome <i>rpn7</i><sup>ts</sup> mutants (Figure 3A and data not shown). The <i>arp3</i><sup>-c1</sup> mutant was further examined, and as shown in Figure 3B, R-GFP, but not M-GFP, accumulated to high levels in these cells. By contrast, two myosin mutants (<i>myo1</i> and <i>myo2</i>-Δ2; Bezanilla et al., 1997; Lee et al., 2000) show no detectable accumulation of polyubiquitylated proteins (data not shown). Because Arp2/3 regulates the actin cytoskeleton, we treated wild-type cells with Lat A, which globally depolymerizes F-actin, and found that this induced accumulation of polyubiquitylated proteins (Figure 3C) and R-GFP (Figure 3D). Moreover, we examined the actin mutation <i>act1</i><sup>-48</sup> (McCollum et al., 1999); as shown in Figure 3E, it too caused accumulation of polyubiquitylated proteins and worsened the phenotype of the <i>rpn7</i><sup>ts</sup> mutant (Figure 3F). We conclude from these results that a functional Arp2/3 complex is required for proper proteasome activity in the cell.

Physical Interaction between Arc3 and Proteasomes

Arc3 and other Arp2/3 components appear as dots near the cell ends and cell equator; however, as much as 85% of the Arp2/3 components are not associated with these dots, but rather are diffused in the cytoplasm (Wu and Pollard, 2005). Conversely, although proteasomes are evidently concentrated at the nucleus, a substantial portion of them are also readily detectable in the cytoplasm. We performed laser confocal microscopy to analyze subcellular localization of a proteasome catalytic subunit, α4, tagged with YFP, and Arc3, tagged with CFP, and the data showed that they primarily colocalized in the cytoplasm (Supplemental Figure S2).

The Arp2/3 complex in mammalian cells is associated with the Golgi and cortical actin (Dubois et al., 2005) and is best known for its role in mediating intracellular pro-
tein trafficking between Golgi and ER (e.g., by controlling vesicle formation and maintenance of structural integrity in these membrane organelles). Furthermore, proteasomes have been shown to concentrate in the microsomal fractions, which are enriched with ER and Golgi, as well as other internal membrane structures, of budding yeast (Elkabetz et al., 2004). We thus analyzed whether in S. pombe Arc3 and proteasomes cofractionate in the microsomal fractions. Our data showed that indeed Arc3, as well as at least one other Arp2/3 subunit, Arp3, cofractionated with Yin6 and many proteasome subunits in the microsome fractions, and they evidently cofractionated with the ER-membrane protein Gpi7 (Figure 2F). In contrast, another actin regulatory protein, Cdc8 (tropomyosin), was enriched in the soluble fraction. Finally, we tested whether the proteasome and Arp2/3 proteins form a complex by immunoprecipitation using cells in which endogenous Arc3 and Rpn5 were both epitope-tagged by homologous recombination. Our data show that when Rpn5-HA was immunoprecipitated, in addition to several proteasome subunits (Rpn1 and the catalytic proteasome subunit α4), Arc3-Myc, as well as Arp3, was also found brought down by Rpn5-HA (Figure 2G). Conversely, when Arc3-Myc was immunoprecipitated, Rpn5-HA and other proteasome subunits coprecipitated with it. In contrast, Cdc8 was not detected in the pull-downs. These data support the idea that the Arp2/3 complex interacts with and regulates proteasomes mostly in regions rich with internal membranes.

Normal Levels and Catalytic Activity of Proteasomes Purified from the Arc3 Mutant

Because proteasomes inefficiently degrade their substrates in the arc3 mutant cells, we first examined proteasome subunit levels in crude cell lysate and found that they were not decreased in the arc3"mutant ("input", Supplemental Figure S3A). We then purified proteasomes by pulling down the proteasome subunit Pus1/Rpn10 and found that there was no detectable change in the levels of copurified proteasome subunits (Supplemental Figure S3A). Furthermore, when the purified proteasomes were measured in vitro for the ability to cleave the model substrate Suc-LLVY-AMC, they appeared to be as active as those from wild-type cells (Supplemental Figure S3B). In agreement with this notion, we found that proteasomes from the arc3 mutant cells were able to degrade a recombinant polyubiquitylated protein Sic1 in vitro as efficiently as those from wild-type cells did (Supplemental Figure S3C). These data suggest that when arc3 levels are down-regulated, proteasomes are still properly assembled and retain their full catalytic activity when measured in vitro.

Efficient Nuclear Import of Proteasomes

One of the best-documented functions of the Arp2/3 complex is to mediate cellular transport and maintain proper membrane structures. Therefore, we investigated whether proteasomes are mobile and whether Arc3 and the actin cytoskeleton play any role in controlling their mobility.

Proteasomes can be found in both the cytoplasm and nucleus in many different cell types. In S. pombe, proteasomes accumulate to high levels at the nuclear membrane. How this pattern is established is not entirely clear. We thus performed photobleaching experiments to examine Rpn11, a proteasome regulatory subunit, which when tagged with GFP via homologous recombination is incorporated into proteasomes and fully functional (Wilkinson et al., 1998). To measure nuclear import, we photobleached the entire nuclei of cells carrying Rpn11-GFP and then measured over time the reappearance of the GFP signal in the nucleus. Conversely, to measure nuclear export, the entire cytoplasm was photobleached, and the fluorescence recovery in the cytoplasm was then measured. Control experiments were performed to photobleach the whole cell to reveal that during the course of these experiments, no new proteasome synthesis was detected (Supplemental Figure S4A). Our data showed that proteasome nuclear entry was at least threefold more efficient than proteasome nuclear exit (Figure 4A). We examined another proteasome regulatory subunit, Rpn7, and obtained the same result (Figure 4B and Sha et al., 2009). One leading model suggests that proteasomes are anchored in the nucleus by Cut8 (Tatebe and Yanagida, 2000). Cut8 is thus expected to affect the nuclear export of proteasomes. Indeed, in cut8Δ cells, proteasome nuclear export was nearly as efficient as import (Figure 4B). We conclude that these photobleaching experiments are effective in analyzing proteasome trafficking in and out of the nucleus, demonstrating that proteasomes are efficiently imported into the nucleus and are held back from exiting by Cut8.

Proteasome Nuclear Import Mediated by Arc3

We then investigated whether functional Arc3 and the actin cytoskeleton are required for efficient proteasome nuclear import as measured above. As shown in Figure 4C, although Rpn11-GFP accumulation at the nuclear membrane was observed within 15 min in wild-type cells, it was not detectable until 45 min when arc3 expression was silenced. At 45 min, Rpn11-GFP already accumulated to high levels inside the nucleus in control cells, whereas none was detectable in the arc3-repressed cells.

The actin cytoskeleton may be necessary for maintaining the integrity of the nuclear pore complex because the Arp2/3 complex has been shown to regulate the function of the nuclear pore (Yan et al., 1997). To investigate whether disrupting the actin cytoskeleton may globally disrupt nuclear pore functions, we examined nuclear import of a model cargo, GLFE (Yoshida and Sazer, 2004), in cells treated with Lat A but detected no difference in its import into the nucleus (Supplemental Figure S5A). This suggests that the nuclear pore is functional in the absence of F-actin. This result also demonstrates that F-actin depolymerization does not globally block transport of all proteins in the cell.

Intracellular Proteasome Mobility Modulated by Arc3

During the course of photobleaching nuclear proteasomes, we noted that when a small area of the nucleus of cells expressing Rpn11-GFP was photobleached, Rpn11-GFP from the surrounding area in the nucleus quickly moved to the bleached area, yielding a recovery \( \tau_{1/2} \) of 1.31 ± 0.03 s (Figure 5A). Rpn7 and a catalytic subunit, α4, were similarly measured, and the same results were obtained (\( \tau_{1/2} = 1.46 \pm 0.05 \) and 1.35 ± 0.05 s, respectively). Furthermore, this mobility also requires functional Arc3: it was reduced when Arc3 expression was reduced (Figure 5D), and it was increased when Arc3 was overexpressed (Figure 5E). Consistent with the fact that Arc3 is part of the actin cytoskeleton, Lat A (Figure 5B) and the presence of the act1-48 mutation (Figure 5C) both reduced proteasome mobility without affecting those of Cut11-GFP, R-GFP, and M-GFP (data not shown). These results demonstrate that inactivating Arc3, as well as F-actin, selectively inhibits proteasome nuclear mobility. It seems that Arc3 and the actin cytoskeleton are not only needed
to efficiently deliver proteasomes into the nucleus, they also play a role maintaining proteasomes in a highly mobile state in the nucleus.

Mobile Proteasomes Are Required for DNA Damage Repair and Normal Anaphase in the Nucleus

In budding yeast, proteasomes are critical for repairing DNA double-strand breaks induced by UV and bleomycin (Krogan et al., 2004). Intriguingly, S. pombe cut8 mutant cells, whose proteasomes do not efficiently accumulate in the nucleus, are also defective in DNA damage repair (Kearsey et al., 2007). Furthermore, cut8 mutants are inefficient in degrading cyclin-B (encoded by cdc13) during anaphase (Tatebe and Yanagida, 2000), which like DNA damage control, occurs in the nucleus. These observations support the idea that proteasomes must move and accumulate in the nucleus in order to regulate DNA damage repair and anaphase progression. We confirmed that proteasome and yin6 mutants in S. pombe are also hypersensitive to a compound related to bleomycin, phleomycin (Figure 6A). Intriguingly, this abnormality was more pronounced in the cold, a condition that retards protein mobility. In addition to phleomycin, we also used UV to induce DNA damage and obtained the same results (Figure 6B). Next, we ascertained whether the Arc3-mediated proteasome mobility and/or nuclear import are important for DNA damage repair. As shown in Figures 6, the arc3mut mutant cells were also hypersensitive to both phleomycin and UV at cold temperatures. Consistent with the fact that Arp2/3 regulates actin polymerization, the act1-48 mutant is similarly sensitive to phleomycin and UV (Figures 6, A and B). These data support the hypothesis that Arp2/3 complex–mediated actin polymerization is required to import proteasomes in sufficient quantities to carry out DNA damage repair.
In *S. pombe*, cyclin-B/Cdc13 associates with the spindle at the onset of prometaphase in the nucleus and must later be degraded by the proteasome to facilitate anaphase progression. We performed live-cell time-lapse microscopy to measure the lifespan of cyclin-B/Cdc13 on the spindle in a strain whose endogenous Cdc13 is tagged by GFP (Tatebe and Yanagida, 2000). We defined the onset of prometaphase as the time point at which two Cdc13-GFP dots (attached to the just separated spindle pole bodies) were first detected (Figure 6E), and our results showed that repressing *arc3* expression markedly prolonged Cdc13-GFP life span on the spindle. This delay in cyclin-B degradation does not seem to involve activation of the spindle checkpoint because it also occurs in cells deleted for the checkpoint kinase *bud1* (Figure 6E). Note that the effect of the mutation on Cdc13 degradation, two events occurring in the nucleus. Furthermore, their anaphase B (elongation of nuclear membrane, Figure 6E) started with the same delay after Cdc13 degradation, suggesting that the silencing of *arc3* expression did not substantially impair the onset of anaphase B.

We conclude that highly mobile proteasomes make it possible to efficiently degrade proteins throughout the cell and that their entry into the nucleus is critical for controlling DNA damage repair and anaphase progression.

**DISCUSSION**

Through the cloning of Arc3 as a high copy suppressor for yin6Δ cells, we have been able to address an intriguing and important issue: are proteasomes stationary or mobile in order to efficiently degrade their substrates throughout the cell? Our data show that increasing Arc3 levels rescues the phenotype of proteasome mutants; conversely, decreasing Arc3 levels reduces proteolysis efficiency and intensifies the phenotype of proteasome mutants. Despite the fact that proteasomes are not efficient in the cell when *arc3* expression is repressed, when these proteasomes are examined in vitro as active as those from normal cells. Because components of the Arp2/3 complex are frequently implicated in mediating protein and organelle trafficking and maintaining internal membrane integrity, and our data show that Arp2/3 and proteasome subunits interact with the microsomal membrane fraction, we investigated whether proteasomes are mobile and whether this mobility is regulated by Arc3. We demonstrated by photobleaching that proteasomes are efficiently imported into the nucleus. When Arc3 is inactivated proteasome entry into the nucleus is substantially blocked, leading to inefficient DNA damage repair and delayed cyclin-B degradation, two events occurring in the nucleus.

*Arc3* is a subunit of the Arp2/3 complex of actin regulatory proteins. Consistent with this, we show that other subunits in the Arp2/3 complex are also required for proper proteasome...
functions; in contrast, the two examined myosins are not. Because Arp2/3 regulates the actin cytoskeleton, disruption of F-actin by Lat A or actin mutations also impair proteasome function. In contrast, disruption of microtubule functions by the presence of the mal3A mutation (Beinhauer et al., 1997) or by adding MBC (methyl benzimidazolecarbamate) does not impede proteasome mobility (data not shown). Our data also show that abnormal actin quite selectively affects the mobility of proteasomes but not that of Cut11 and the model substrates M-GFP and R-GFP. These results argue against the possibility that the observed inhibition in proteasome mobility is due to nonspecific depolymerization of cytoskeletal components. Therefore, although we cannot rule out that Arc3 and actin can influence the movement of other molecules necessary for proteolysis, we strongly argue that the mobility of the proteasome itself is a critical factor for efficient proteolysis. Collectively our data support the model that proteasome mobility and activity in the cell are selectively regulated at least in part by the Arp2/3 complex.

Why do proteasomes need to be so mobile? Because many proteasome substrates may be toxic to the cell, highly mobile proteasomes can reduce the need to transport their substrates over long distances, thus reducing exposure to these toxic molecules. Furthermore, because the coordinated interactions between an E3 Ub ligase, a proteasome, and a substrate might render that particular proteasome temporarily unavailable for processing another substrate, high proteasome mobility could serve to assure efficient delivery of proteasomes to that area. Finally, many biological processes are cell-compartment restricted. Beside DNA damage repair and Cdc13/cyclin-B degradation, we have shown previously that proteasome mutants contain abnormal V- or star-shape spindles (Yen et al., 2003a), suggesting that nuclear proteasomes are also needed for proper spindle formation. In addition, the roles of proteasomes in transcription are well recognized (Collins and Tansey, 2006). In short, mobile proteasomes can thus move quickly to the appropriate compartment such as the nucleus in order to properly control these events.

The Arp2/3 complexes serve as nuclei for promoting actin polymerization to form branched meshwork structures. This mode of actin polymerization is frequently catalyzed by members of the WASP (Wiskott-Aldrich syndrome protein) family, which can bind cell membranes and recruit the Arp2/3 complex to promote F-actin polymerization, providing the force and/or structural rigidity needed in remodeling the membrane for motility (e.g., the formation of lamellipodia) and transport (e.g., vesicle fission and movement and motility of organelles, such as mitochondria). Consistent with the idea that Arp2/3 is expected to associate with membrane fractions, our data indicate that the majority of Arc3 and Arp3, as well as proteasome subunits, are found in the microsomal fractions and that they form a complex with proteasomes. These results suggest that Arp2/3 interacts with proteasomes in endomembrane compartments. Mass spectrometry analyses have shown that in budding yeast Arp2 can bind two proteasome subunits (Ho et al., 2002), and with cross-linking, three more Arp2/3 subunits, including Arc18, the budding yeast Arc3 homolog, have been found to copurify with proteasomes (Guerrero et al., 2008). We note that cross-linking is also needed to detect the binding between Arc3 and proteasomes in S. pombe, suggesting that the
interaction between Arp2/3 and proteasomes is transient in many eukaryotes. We note that additional actin-dependent mechanisms may be operative to enhance the efficiency of compartmentalized proteolysis in S. pombe. For example, a recent study shows that the class V myosin Myo52 in S. pombe cooperates with the Ub receptor Dph1 to regulate Tip1 degradation at the cell tips (Martin-Garcia and Mulvihill, 2009), suggesting that Ub receptors and/or E3s may be transported in a myosin–actin-dependent manner to a given cell compartment.

Although the underlying mechanism regulating proteasome mobility is likely to be very complex, one possibility is that proteasomes can associate with vesicles to be transported to different cell compartments. In support of this idea, proteasomes in mammalian cells have been shown to associate with endosomes, ER, and the ER-Golgi intermediate compartment via ECM29 (Gorbea et al., 2004). In budding yeast, Guerrero et al. (2008) showed that Sec26, Sar1, several other vesicle coat components, and many Arp2/3 subunits copurified with budding yeast proteasomes. In a recent article of ours, using LC-MS/MS, we found that proteasomes associate with translation factors and other proteins in a supercomplex, called translasome (Sha et al., 2009). In that study, we also found Sar1 and Sec26 in the translasome. In agreement with the idea that proteasomes may be imported into the nucleus via the cellular trafficking system, our ongoing study shows that expression of a dominant-negative Sar1 similarly impaired proteasome nuclear import (unpublished results).

Subunits in a large protein complex are expected to interact in a stoichiometric manner. It is thus quite intriguing that overexpressing Arc3 can rescue mutant phenotypes. However, we and others have observed similar phenomena when we studied the proteasomes whose subunits are well known to interact stoichiometrically. For example, we have shown that overexpression of S. pombe proteasome subunits Rpt2 and Rpn12 rescues the phenotypes of rpn5Δ cells (Yen et al., 2003a). In addition, S. pombe yin6Δ cells contain mis-assembled proteasomes, but the mutant phenotype can be rescued by overexpressing many proteasome subunits, Rpn5, Rpt2, Rpn11, Rpn1, and Rpn7 (Yen et al., 2003b; Sha et al., 2009). Similar results were obtained in budding yeast and human cells (Kominami et al., 1998; Chondrogianni et al., 2005). One possible explanation that is increased abundance of individual subunits may drive more efficient assembly of the complex. Alternatively, certain subunits may not be present in stoichiometric amounts and may act as limiting factors for the complex.

Activities proposed to require nuclear actin have intrigued many researchers because they include functions critical for cell physiology, such as transcription, chromatin remodeling, and the trafficking of RNA and ribosomes. Arp2/3, in particular, has been shown to mediate nuclear actin polymerization required for the replication of a baculovirus in insect cells (Goley et al., 2006). Moreover, the activator for Arp2/3, WASP, has been implicated in RNA polymerase I and II-dependent transcription (Fomproix and Pericpalle, 2004; Philimonenko et al., 2004). The observation that proteasomes are also mobile inside the nucleus in a partially Arc3-dependent manner suggests that proteasome mobility may be a new activity controlled by the nuclear actin cytoskeleton. How the nuclear actin cytoskeleton can regulate these activities is unclear primarily because no F-actin structure has ever been fully resolved in the nucleus.

We suggest that actin-dependent proteasome mobility may be evolutionarily conserved in mammalian cells. The mammalian dendritic spines in neurons are enriched with the Arp2/3 complex, which is also required for the formation of these dendritic spines (Wegner et al., 2008). It has been shown recently that 26S proteasomes rapidly translocate from the dendritic shafts to the spines within a few minutes after neuron depolarization (Bingol and Schuman, 2006). Although the Arp2/3–actin cytoskeleton was not directly demonstrated in that study to regulate the movement of the proteasomes, the neuronal proteasomes do associate with F-actin after depolarization. It would be interesting to determine whether such proteasome translocation is also similarly mediated by Arp2/3. Reits et al. (1997) examined the mobility of the immuno-proteasome by fluorescence loss in photobleaching (FLIP) using a GFP-tagged subunit β1/MPF2 that was ectopically expressed in HT1080 fibrosarcoma cells. Immuno-proteasomes are structurally unique and function specifically for antigen presentation. Despite this, they too observed that the rate of proteasome nuclear import is greater than that of nuclear export. However using sodium azide, Reits et al. concluded that the immuno-proteasomes move within the nucleus and within the cytoplasm in an ATP-independent manner, implying that they move entirely by passive diffusion in HT1080 cells. It is possible that different proteasomes in different cells can move by different mechanisms. Moreover, Reits et al. measured proteasome mobility by detecting the loss of GFP levels after photobleaching at a single time point. This approach might not have detected the change in the rate of fluorescence loss. Finally, ATP depletion may affect the assembly of these proteasomes, a possibility that can complicate the interpretation of mobility data. We conclude that actin-dependent 26S proteasome movement is evolutionarily conserved; however, different proteasomes may move by different mechanisms, which may be further influenced by cell type.

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