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The Fission Yeast *Schizosaccharomyces pombe* Has Two Importin-α Proteins, Imp1p and Cut15p, Which Have Common and Unique Functions in Nucleocytoplasmic Transport and Cell Cycle Progression

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ABSTRACT

The nuclear import of classical nuclear localization signal-containing proteins depends on importin-α transport receptors. In budding yeast there is a single importin-α gene and in higher eukaryotes there are multiple importin-α-like genes, but in fission yeast there are two: the previously characterized *cut15* and the more recently identified *imp1*. Like other importin-α family members, Imp1p supports nuclear protein import *in vitro*. In contrast to *cut15*, *imp1* is not essential for viability, but imp1Δ mutant cells exhibit a telophase delay and mild temperature-sensitive lethality. Differences in the cellular functions that depend on Imp1p and Cut15p indicate that they each have unique physiological roles. They also have common roles because the imp1Δ and the cut15-85 temperature-sensitive mutations are synthetically lethal; overexpression of *cut15* partially suppresses the temperature sensitivity, but not the mitotic delay in imp1Δ cells; and overexpression of *imp1* partially suppresses the mitotic defect in cut15-85 cells but not the loss of viability. Both Imp1p and Cut15p are required for the efficient nuclear import of both an SV40 nuclear localization signal-containing reporter protein and the Pap1p component of the stress response MAP kinase pathway. Imp1p and Cut15p are essential for efficient nuclear protein import in *S. pombe*.

NUCLEOCYTOPLASMIC transport is a process specific to eukaryotes in which the chromosomes are physically separated from the cytoplasm by the nuclear envelope (NE). In all eukaryotes, the receptor-mediated transport of nuclear proteins across the NE from their site of synthesis in the cytoplasm is essential for all nuclear processes. The precise tissue-specific and temporal regulation of nuclear protein import is also critical in the regulation of cell cycle progression and in developmental and signal transduction pathways (reviewed in Kaffman and O'Shea 1999).

Proteins are targeted to the nucleus by an NLS (*n*uclear *l*ocalization *s*ignal). There are two types of classical NLSs, both of which must bind to an importin-α adaptor for transport to the nucleus: the mono-partite NLS that consists of 4 or more basic amino acids preceded by a helix-breaking residue and the bipartite NLS that has two short stretches of basic amino acids separated by a 9- to 12-amino-acid spacer (reviewed in Izaurralde and Adam 1998). Both the mono-partite SV40 and the bipartite nucleoplasmin NLS are competent to direct cargo proteins to the nucleus in fission yeast, budding yeast, and other organisms (reviewed in Yoshida and Sazer 2004).

The importin-β family of transport receptors, also called karyopherins, or more specifically, importins or exportins, carries cargoes both into and out of the nucleus. Some import cargoes bind directly to an importin-β receptor, while others interact with an importin-α adaptor, which associates with an importin-β receptor (Gorlich and Kutay 1999). The importin-β subunit of both of these types of transport complex targets them to the NE by binding to proteins at the nuclear pore complex (reviewed in Gorlich and Kutay 1999).

There are multiple importin-α proteins in metazoan organisms and they have been categorized on the basis of amino-acid sequence comparisons (Malik *et al.* 1997; Mason *et al.* 2002). Three subfamilies, whose members have different expression patterns, different functions, and/or different cargo-binding specificities, have been identified in plants and animals (Kohler *et al.* 1999; Talcott and Moore 2000; Geles and Adam 2001; Geles *et al.* 2002; Mason *et al.* 2002). In Drosophila, importin-α isoforms have nonoverlapping functions: the

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gametogenesis defects of a null mutation in importin-α2 can be rescued by importin-α1 or -α3 in male flies, but only by importin-α2 in females (Mason *et al.* 2002). The *in vivo* cargoes of the importin-α1, -α2, and -α3 families remain largely unknown. In budding yeast there is a single importin-α-like gene, *SRP1*, but in fission yeast there are two, *imp1* and *cut15*, which are in the same importin-α1 subfamily (Malik *et al.* 1997; Mason *et al.* 2002). This makes *Schizosaccharomyces pombe* an excellent experimental system in which to investigate the specialized roles of multiple importin-α proteins in eukaryotic cells.

Nucleocytoplasmic transport is also dependent on the Ran GTPase (Spi1p in fission yeast), an evolutionarily conserved GTPase whose nucleotide-bound state is regulated by a nuclear, chromatin-bound, *g*uanine nucleotide *e*xchange *f*actor (RanGEF) and a cytoplasmic *G*TPase *a*ctivating *p*rotein (RanGAP) (Sazer and Dasso 2000; Salus and Sazer 2001). The compartmentation of these regulatory proteins results in a high concentration of Ran-GTP in the nucleus and a low concentration in the cytoplasm (Kalab *et al.* 2002). This Ran-GTP gradient across the nuclear envelope distinguishes the nuclear and cytoplasmic compartments and thereby imposes directionality on nucleocytoplasmic transport by influencing the stability of cargo-containing complexes (Gorlich *et al.* 1996; Izaurralde *et al.* 1997; Gorlich 1998). Proteins destined for import form stable complexes with their import carriers in the cytoplasm, where the concentration of Ran-GTP is low, but are dissociated from their carriers in the nucleus, where the concentration of Ran-GTP is high.

After nuclear envelope breakdown at mitosis in higher eukaryotes, chromatin-associated RanGEF generates a high local concentration of Ran-GTP surrounding the chromosomes (Kalab *et al.* 2002). This gradient is important for other Ran-dependent functions, including mitotic spindle assembly and nuclear envelope reformation and structure, which are independent of its role in nucleocytoplasmic transport (reviewed in Hetzer *et al.* 2000; Zhang and Clarke 2000, 2001; Dasso 2001; Arnaoutov and Dasso 2003) but are executed using the same mechanism. Cargo-containing import complexes are destabilized by Ran-GTP, both in the nucleus of interphase cells, to disassemble the import complex, and in the immediate vicinity of the chromosomes in mitotic cells, to release proteins necessary for mitotic and postmitotic events from an inhibitory association with their transport carriers. Importin-α has been shown to play an important role in nucleocytoplasmic transport, mitotic spindle assembly, and nuclear envelope structure and assembly (Clarke and Zhang 2001; Dasso 2001; Geles *et al.* 2002; Hatchet *et al.* 2004).

In fission yeast, the Ran GTPase (Spi1p) and its regulators are essential for cell cycle progression. When the Ran GTPase is misregulated, *S. pombe* cells arrest after mitosis with hypercondensed, unreplicated chromosomes, fragmented nuclear envelopes, and a wide medial septum (Sazer and Nurse 1994; Demeter *et al.* 1995; Matynia *et al.* 1996). A nucleocytoplasmic-transport-independent role for Ran in regulating microtubule structure has also been established (Fleig *et al.* 2000; Salus *et al.* 2002). Cells in which the level of active Ran protein is lowered by the spi1-25 mutation or by a decrease in RanGEF function have abnormal microtubules but are competent for nucleocytoplasmic transport (Fleig *et al.* 2000; Salus *et al.* 2002), suggesting that Ran-dependent processes are differentially sensitive to the level of active Ran.

The role of Ran in nucleocytoplasmic transport has been well characterized in higher eukaryotes and in budding yeast (Gorlich 1998). The high degree of structural and functional conservation of components of the Ran GTPase system (Sazer 1996) make it likely that Ran directly participates in transport in *S. pombe* as well. However, aside from a mutation in the nuclear export receptor *crm1* (Fukuda *et al.* 1997), no previously characterized *S. pombe* mutants are defective in nucleocytoplasmic transport, including null mutations of six transport factors and six nucleoporins (Chen *et al.* 2004). Even *cut15*, an essential gene that encodes a nuclear import receptor of the importin-α type, has been reported to be competent for the transport of an SV40 NLS fusion protein *in vivo* (Matsusaka *et al.* 1998).

This article reports the identification of the first two fission yeast genes required for efficient nuclear protein import, both of which encode importin-α proteins. One is the previously characterized *cut15* and the other is *imp1*, which was first identified in the *S. pombe* genome-sequencing project (Wood *et al.* 2002). Imp1p has the signature motifs of an importin-α protein and supports nuclear protein import *in vitro*. The efficient import of both monopartite- and bipartite-containing import substrates depends on Imp1p and Cut15p. Genetic and physiological analyses indicate that these two import adaptors are likely to have both unique and common binding partners.

MATERIALS AND METHODS

Yeast cell culture: Standard methods were used for culture medium [yeast extract, EMM (Edinburgh minimal medium), amino acid supplements, and phloxine B] and genetic techniques (Moreno *et al.* 1991). Strains used in this study are listed in Table 1. Transformations were by either lithium acetate (Moreno *et al.* 1991) or EZ yeast transformation II kit (Zymo Research, Orange, CA). Viability was assayed by growing cells in liquid medium to midlog phase and spotting an equal number of cells in fivefold dilutions onto plates, some of which also contained phloxine B, a vital dye that accumulates in dead cells and turns the colonies dark pink. Hydrogen peroxide (Sigma, St. Louis) was used to activate the stress response pathway (Yoshida and Sazer 2004). DNA was visualized in cells fixed in 70% ethanol using 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) (Sigma-Aldrich) (Moreno *et al.* 1991). GFP and YFP fusion proteins

TABLE 1

Strains used in this study

Strain name	Genotype	Source
SS446	*h⁻ leu1-32 ura4-D18 ade6-M210*	Our stock
SS482	*h⁻ leu1-32 ura4-D18 ade6-M216 int::*pREP4X-SV40NLS-GFP-LacZ	Our stock[a]
SS767	*h⁻ leu1-32 ura4-D18 ade6-M210 int::*pREP42-*pap1::LEU2*	Our stock[b]
SS959	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4*	This study
SS1167	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85*	M. Yanagida[c]
SS1168	*h⁺ leu1-32 ura4-D18 ade6-M216 cut15-85*	M. Yanagida[c]
SS1233	*h⁺ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4 int::*pREP41X-*pap1::leu1*	This study
SS1250	*h⁻ leu1-32 ura4-D18 ade6-M210 cut15-85 int::*pREP41X-*pap1::leu1*	This study
SS1254	*h⁻/h⁺ leu1-32/leu1-32 ura4-D18/ura4-D18 ade6-M210/ade6-M216 imp1Δ::ura4/imp1⁺ cut15⁺/cut15-85*	This study
SS1339	*h⁻ leu1-32 ura4-D18 ade6-M210* + pR1GsvNLSF1(G-NLS-F)	This study
SS1343	*h⁻ leu1-32 ura4-D18 ade6-M210* + pR1GrevNESF1(G-NES-F)	This study
SS1355	*h⁻ leu1-32 ura4-D18 ade6-M210* + pR1GsvNLSrevNES1(G-NLS-F-NES)	This study
SS1359	*h⁺ leu1-32 ura4-D18 ade6-M216 cut15-85* + pR1GsvNLSF1(G-NLS-F)	This study
SS1363	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pR1GsvNLSF1(G-NLS-F)	This study
SS1386	*h⁺ leu1-32 ura4-D18 ade6-M216 cut15-85* + pR1GrevNESF1(G-NES-F)	This study
SS1390	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pR1GrevNESF1(G-NES-F)	This study
SS1392	*h⁺ leu1-32 ura4-D18 ade6-M216 cut15-85* + pR1GsvNLSrevNES1 (G-NLS-F-NES)	This study
SS1396	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pR1GsvNLSrevNES1(G-NLS-F-NES)	This study
SS1429	*h⁻ leu1-32 ura4-D18 ade6-M210 cut15-85 int::nmt1-cut15-YFP::leu1*	This study
SS1430	*h⁺ leu1-32 ura4-D18 ade6-M216 cut15-85 int::nmt1-cut15-YFP::leu1*	This study
SS1517	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85 int::*pREP4X-*SV40NLS-GFP-LacZ*	This study
SS1519	*h⁺ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4 int::*pREP4X-*SV40NLS-GFP-LacZ*	This study
SS1543	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85 int::nmt1-imp1-YFP::leu1*	This study
SS1630	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pREP81X-*cut15*	This study
SS1634	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP3X	This study
SS1635	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP3X-*imp1*	This study
SS1636	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP41X-*imp1*	This study
SS1637	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP81X-*imp1*	This study
SS1638	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP3X-*cut15*	This study
SS1639	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP41X-*cut15*	This study
SS1658	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pREP3X	This study
SS1661	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pREP81X-*imp1*	This study
SS1712	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP3X-*GFP*	Our stock[d]
SS1716	*h⁻ leu1-32 ura4-D18 ade6-M210 int::imp1-TAP::kan^R*	This study
SS1717	*h⁻ leu1-32 ura4-D18 ade6-M210 int::cut15-TAP::kan^R*	This study
SS1741	*h⁻ leu1-32 ura4-D18 ade6-M210 cut15-85 imp1Δ::ura4 int::nmt1-cut15-YFP::leu1*	This study
SS1742	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP81X-*cut15*	This study
SS1743	*h⁻ leu1-32 ura4-D18 ade6-M210* + pREP3X-*GFP-imp1*	This study
SS1744	*h⁻ leu1-32 ura4-D18 ade6-M210 int::cut15-TAP::kan^R* + pREP3X-*GFP*	This study
SS1745	*h⁻ leu1-32 ura4-D18 ade6-M210 int::cut15-TAP::kan^R* + pREP41X-*GFP-pap1*	This study
SS1746	*h⁻ leu1-32 ura4-D18 ade6-M210 int::imp1-TAP::kan^R* + pREP3X-*GFP*	This study
SS1747	*h⁻ leu1-32 ura4-D18 ade6-M210 int::imp1-TAP::kan^R* + pREP41X-*GFP-pap1*	This study
SS1748	*h⁻ leu1-32 ura4-D18 ade6-M210 imp1Δ::ura4* + pREP81X-*cut15*	This study
SS1749	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85* + pREP3X	This study
SS1750	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85* + pREP81X-*cut15*	This study
SS1751	*h⁻ leu1-32 ura4-D18 ade6-M216 cut15-85* + pREP81X-*imp1*	This study
SS1767	*h⁻ leu1-32 ura4-D18 ade6-M210 int::imp1-GFP::kan^R*	This study

[a] Yoshida and Sazer (2004).
[b] Matynia *et al.* (2001).
[c] Matsusaka *et al.* (1998).
[d] Kadura *et al.* (2005).

were visualized in living cells. Transcription from the *nmt1* gene promoter (Maundrell 1990) in plasmids pREP3X, pREP41X, or pREP81X (Forsburg 1993), which carry the high-, medium-, or low-strength versions of the promoter, was repressed by the addition of 5 μg/ml thiamine or was induced by washing away the thiamine and incubating cells in thiamine-free medium.

Animal cell culture: HeLa cell lines were cultured in Dulbecco's modified Eagle's medium (GIBCO, Gaithersburg, MD) supplemented with 10% fetal bovine serum (GIBCO) at 37° in a humidified atmosphere containing 10% CO_2.

Fluorescence microscopy: Cells were observed on a Zeiss Axioskop fluorescence microscope and photographed with a

DVC 1300 black-and-white CCD camera using QED software at equivalent exposure times.

Strain construction: A pDUAL vector (Matsuyama *et al.* 2004) containing *cut15* tagged with YFP, FLAG, and His_6 (a generous gift of Minoru Yoshida) was integrated at the *leu1* locus by linearizing the DNA using *Not*I and transforming it into wild-type haploid cells (SS959). The imp1Δ mutant was constructed by replacing the open reading frame with an *ura4* gene cassette. The upstream region (nucleotides −1050–−59) of the *imp1* ORF was amplified by PCR, digested with *Not*I and *Bam*HI, and subcloned into the *Not*I and *Bam*HI site in pKS-*ura4* (Bahler *et al.* 1998) to create pBSII-*imp1*N-*ura4*. The *imp1* C-terminal noncoding region (nucleotides +1651–+2655) was amplified by PCR, digested with *Eco*RI and *Hinc*II, and subcloned into the *Eco*RI-*Hinc*II sites of pBSII-*imp1*N-*ura4*, creating plasmid pSS389, which was digested with *Not*I and *Hinc*II and the insert transformed into the wild-type diploid strain SS531. PCR and Southern blotting confirmed the disruption. The *imp1* and *cut15* genes were fused to the tandem affinity purification (TAP) epitope at their endogenous loci with the single-step PCR-based method (Bahler *et al.* 1998) using pFA6a-kanMX6-CTAP2 (Tasto *et al.* 2001) to generate strains SS1716 and SS1717, respectively. To visualize Imp1p localization, the *imp1* gene was fused to the GFP epitope at its endogenous locus using the single-step PCR-based method (Bahler *et al.* 1998) and pFA6a-GFP-kanMX6 as a template to generate strain SS1767. To construct the imp1Δ cut15-85 heterozygous diploid (SS1254), cut15-85 (SS1168) (Matsusaka *et al.* 1998) was crossed to imp1Δ (SS959) and diploids were identified. The imp1Δ cut15-85 haploid, kept alive by expression of *cut15* (SS1747), was constructed by crossing SS1430 with SS959.

Plasmid construction: The 1.6-kb *imp1* cDNA was amplified from the *S. pombe* cDNA library λACT (generous gift from S. Elledge) by PCR. The product was digested with *Sal*I and *Sma*I and the insert subcloned into pBluescript II SK (+) to create pBSK-*imp1*. For overexpression studies, the *Sal*I-*Sma*I insert of pBSK-*imp1* was subcloned into the *Sal*I-*Sma*I sites of pREP3X (Forsburg 1993) (pREP3X-*imp1*) and the *Xho*I-*Sma*I sites of pREP41X (pREP41X-*imp1*) and pREP81X (pREP81X-*imp1*). To express a His-Imp1p fusion protein in bacteria, the *imp1 Sal*I-*Sma*I fragment was subcloned into the *Xho*I-*Pvu*II sites of the pRSET B vector (Invitrogen, San Diego) to make pRSET B-*imp1*. The 1.6-kb *cut15* cDNA was amplified from the *S. pombe* cDNA library in λACT by PCR. The PCR product was digested with *Sal*I and *Sma*I and subcloned into pBluescript II SK(+), resulting in pBSK-*cut15*, which was digested with *Sal*I and *Sma*I, and the *cut15* fragment was subcloned into the *Sal*I and *Sma*I sites of pREP3X (pREP3X-*cut15*). The *cut15* cDNA fragment was inserted into an *Xho*I-*Sma*I site in pREP41X (pREP41X-*cut15*), pREP81X (pREP81X-*cut15*), and pRSET B (pRSET B-*cut15*). To monitor the localization of untagged GFP as a control for the localization studies, the GFP gene was amplified by PCR from the template plasmid pFA6a-GFPS65T-kanMX6 and the oligonucleotides were designed to introduce a stop codon following the coding region. The 0.75-kb GFP-containing fragment was digested with *Xho*I and *Sma*I and subcloned into *Xho*I and *Sma*I sites in the pREP3X vector (pREP3X-GFP).

Protein purification from bacteria: *Escherichia coli* BL21 (DE3) strains transformed with pRSET B-*imp1* or pRSET B-*cut15* were lysed and the His-tagged proteins purified on a Ni-NTA agarose column (QIAGEN, Chatsworth, CA), eluted with 300 mm imidazole (Sigma) in PBS, and then dialyzed with cold-TB buffer [20 mm Hepes-KOH, pH 7.3, 110 mm K acetate, 2 mm Mg acetate, 1 mm EGTA, and 2 mm dithiothreitol (DTT)] at 4°.

Yeast nuclear protein import assays: Cells carrying an integrated copy of the plasmid pREP4X-SV40 NLS-GFP (Yoshida and Sazer 2004) (SS482), a plasmid-borne copy of pR1GLFE1 encoding GST-SV40 T NLS-GFP-Rev nuclear export signal (NES), pR1GEF1 with only the NES, pR1GLF2 with only the NLS (Kudo *et al.* 1997), or an integrated copy of *pap1*-GFP (SS791) were grown to midlog phase in supplemented EMM without thiamine. To induce Pap1p-GFP nuclear localization, cells were exposed to hydrogen peroxide (0.003%) (Yoshida and Sazer 2004).

Immunoprecipitation: Cell extracts were prepared by standard methods (Moreno *et al.* 1991) with minor modifications. For immunoprecipitations, cut15-TAP or imp1-TAP cells were transformed with pREP41X-GFP-*pap1* or pREP3X-GFP, grown in supplemented EMM with thiamine, washed, and incubated in supplemented EMM without thiamine. Extracts were prepared in ice-cold HG buffer [25 mm HEPES (pH 7.4), 25 mm NaCl, 5 mm $MgCl_2$, and 1 mm DTT] containing a proteinase inhibitor cocktail (EDTA-free complete, Roche, Indianapolis) and 1 mm phenylmethylsulfonyl fluoride (PMSF) using the glass bead (425–600 μm) disruption method. Two milligrams of soluble protein was incubated with prewashed IgG beads (Amersham, Buckinghamshire, UK) for 2 hr at 4°. The beads were washed five times with ice-cold NP-40 buffer [150 mm NaCl, 50 mm Tris-HCl (pH 8.0), 2 mm EDTA, 1.0% NP-40, and 0.1% SDS] plus 100 mg/ml PMSF and denatured in SDS sample buffer. For Western blotting, one-fourth of the immunoprecipitates was resolved by 12% Tris-HCl SDS-PAGE. Mouse anti-GFP antibody (U.S. Biological, clone GF200) (diluted 1:1000) to detect GFP fusion proteins or peroxidase-antiperoxidase (Sigma) (diluted 1:1000) to detect TAP fusion proteins were used as primary antibodies.

HeLa cell nuclear protein import assay: This import assay was performed essentially as described (Schwoebel *et al.* 1998). HeLa cells were cultured at 37° on cover slips to ~70% confluence, incubated on ice, washed one time with cold TB buffer, treated with TB buffer containing 70 mg/ml digitonin in DMSO for 5 min on ice, and then incubated in reaction mixture containing 5 μg/ml rhodamine-NLS-BSA (rhodamine-labeled BSA coupled to peptides containing the NLS of the SV40 T antigen), 1 mm GTP, 2 mg/ml BSA, purified human import factors (100 μg/ml Ran, 3 μg/ml p10/NTF2, and 25 μg/ml importin-β), plus 20 μg/ml of His-Imp1p, His-Cut15p, or human importin-α in TB buffer at room temperature for 20 min. After washing three times, cells were fixed with 3% paraformaldehyde in TB buffer.

Sequence analysis: The putative NLS sequence of Pap1p was identified by searching the database of *S. pombe* proteins containing consensus NLS sequences (http://cubic.bioc.columbia.edu/cgi/var/nair/predictNLS/Genome.pl) assembled by the Columbia University Bioinformatics Center (Nair *et al.* 2003). Alignments of importin-α family members [*S. pombe* Cut15p (accession no. BAA24518), *S. pombe* Imp1p (accession no. T39506), *Saccharomyces cerevisiae* Srp1p (accession no. AAA35090), *Drosophila melanogaster* (D.m.), importin-α1 (accession no. CAB64597), and *Homo sapiens* karyopherin-α1 (accession no. AAP35605)] were generated using Clustal W and displayed using Box Shade.

RESULTS

Fission yeast has two importin-α genes: Cut15p is a previously characterized *S. pombe* importin-α family member that is essential for viability but, unexpectedly, is not required for the import of a classical SV40 NLS-containing fusion protein *in vivo* (Matsusaka *et al.* 1998). The *S. pombe* genome-sequencing project identified *imp1* (Wood *et al.* 2002), whose predicted product is clearly an importin-α family member (Figure 1).

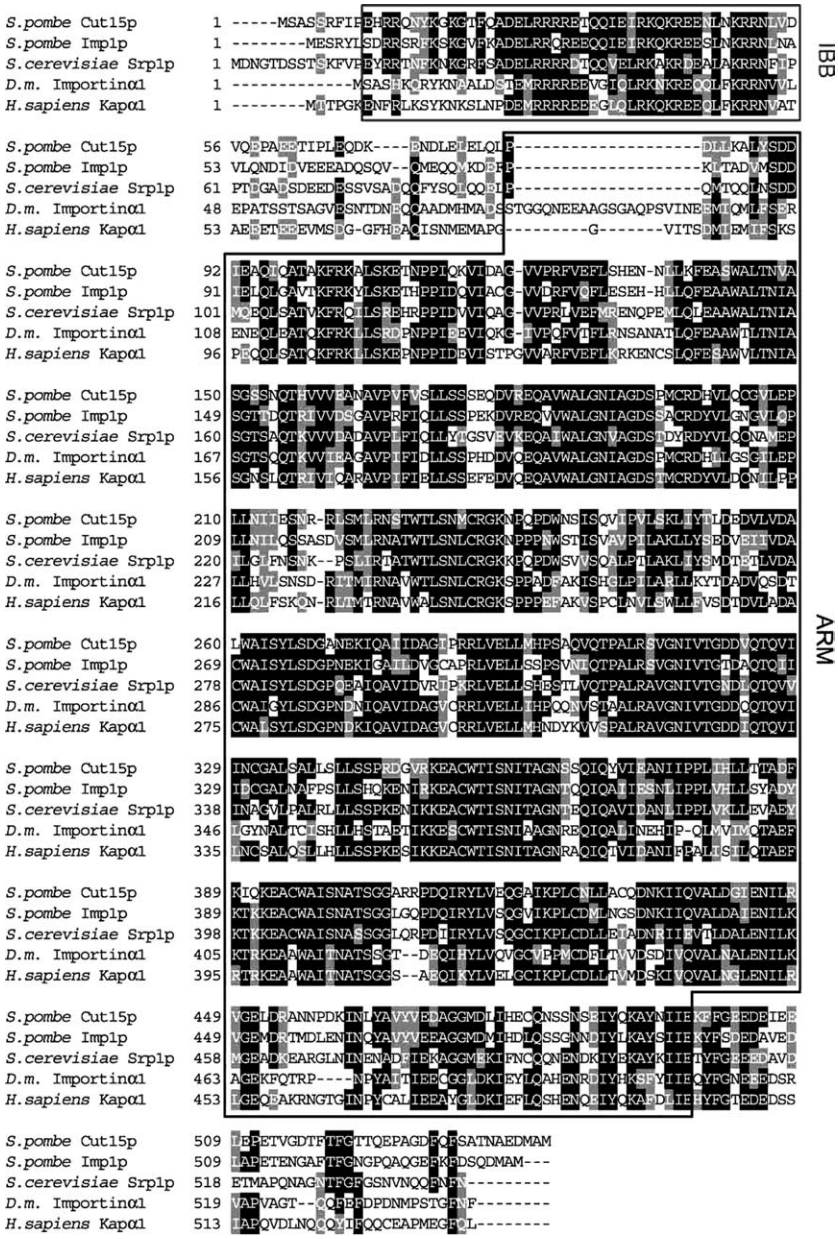


FIGURE 1.—*S. pombe imp1* encodes a putative importin-α family member. Amino-acid sequence alignment of *S. pombe* Cut15p, *S. pombe* Imp1p, *S. cerevisiae* Srp1p, *D. melanogaster* (D.m.) importin-α1, and *H. sapiens* karyopherin-α1 (Kap-α1). Identical amino acids are on a black background and similar amino acids are on a gray background. The IBB and ARM repeats are indicated.

Imp1p and Cut15p are both in the importin-α1 subfamily (MALIK *et al.* 1997; GOLDFARB *et al.* 2004). Imp1p is 62% identical and 79% similar to Cut15p and >50% identical to the next 40 closest BLAST matches, which represent importin-α family members from a wide range of organisms. Sequence alignment shows that the similarity between Imp1p and other importin-α family members extends throughout the protein (Figure 1) and therefore encompasses the highly conserved *i*mportin-*b*eta *b*inding domain (IBB) at the N terminus, by which importin-α binds to importin-β, and the multiple armadillo (ARM) repeats that form the cargo-binding region of the protein (CONTI *et al.* 1998; CONTI and KURIYAN 2000). The IBBs of Imp1p and Cut15p are 55% and 65% similar, respectively, to the IBB consensus sequence in the NCBI Conserved Domain Database, and they are the only two IBB-containing *S. pombe* proteins.

Imp1p has nuclear protein import activity *in vitro*: To ask whether Imp1p is an authentic importin-α, His-Imp1p and His-Cut15p fusion proteins were expressed in and purified from bacteria (Figure 2A). An *in vitro* nuclear protein import assay was performed in which the ability of digitonin-permeabilized HeLa cells to import rhodamine-NLS-BSA into the nucleus was assessed (SCHWOEBEL *et al.* 1998). As previously shown using this assay system (see MATERIALS AND METHODS) import of rhodamine-NLS-BSA into the nucleus is dependent on the addition of human importin-α to the other essential components of the transport system (Figure 2B-1). Similarly, His-Imp1p (Figure 2B-3) and His-Cut15p (Figure 2B-5) had import activity

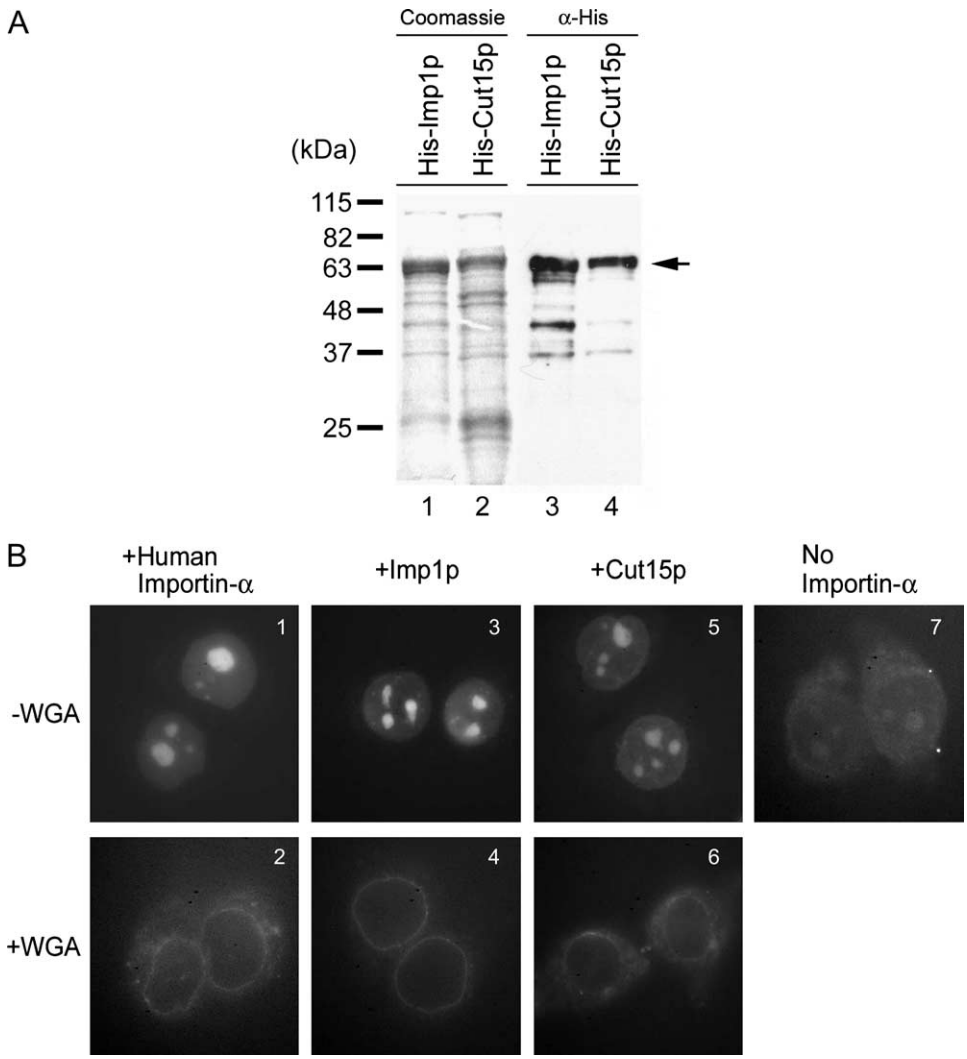


FIGURE 2.—*S. pombe* Imp1p has nuclear protein import activity *in vitro*. (A) His-Imp1p (lanes 1 and 3) and His-Cut15p (lanes 2 and 4) were expressed in and purified from bacteria. The supernatants were passed over a Ni-NTA column and the two His-fusion proteins, both of which migrate at ~67 kDa (arrow), were eluted with imidazole. Eluates of His-Imp1p (lane 1) and His-Cut15p (lane 2) were separated using SDS-PAGE and visualized by Coomassie staining. His-Imp1p (lane 3) and His-Cut15p (lane 4) were visualized using an anti-His antibody on a Western blot. (B) Permeabilized HeLa cells incubated with rhodamine-SV40 NLS-BSA, purified import factors, and other components (see MATERIALS AND METHODS). (1 and 2) Human importin-α, (3 and 4) His-Imp1p, or (5 and 6) His-Cut15p, either with (2, 4, and 6) or without (1, 3, and 5) wheat germ agglutinin (WGA). (7) Control assay with no added importin-α and no WGA.

in vitro. Accumulation of rhodamine-NLS-BSA at the periphery of the nucleus, but not within the nucleoplasm, when nuclear pore function was inhibited with wheat germ agglutinin (Figure 2B-2, B-4, and B-6), indicated that substrate accumulation in the nucleus was the result of passage through the pores. Import was also dependent on the addition of importin-α (Figure 2B-7). Therefore, Imp1p is a functional importin-α protein that has import activity similar to that of human importin-α and the other *S. pombe* importin-α protein, Cut15p.

The imp1Δ mutant has a cell cycle defect different from that of a cut15 mutant: A heterozygous imp1Δ diploid strain was constructed by replacing one copy of the open reading frame with the selectable *ura4* gene. Tetrad analysis revealed that, in contrast to *cut15*, the imp1Δ mutant was viable at 25°, but was slightly temperature sensitive at 36° (Figure 3, A and D). In a normal cell cycle after mitosis, the two nuclei move to the tips of the cells and then relocalize to what will be the middle of each daughter cell after cytokinesis (Figure 3B-1). In the imp1Δ strain (Figure 3B-2), binucleated cells in which the DNA remains at the cell tips accounted for 10.4%, compared to 1.1% of wild-type cells. Mono-nucleated cells in which the nucleus is not in the center of the cell, a phenotype never seen in wild-type cells, represented 2.1% of the imp1Δ population. This phenotype differed from that of the temperature-sensitive cut15-85 mutant (Figure 3B-3) in which cells fail to condense their chromosomes at mitosis and then to undergo cytokinesis before the completion of mitosis (MATSUSAKA *et al.* 1998). The medial septum (cell wall) then cuts through the unsegregated chromosomes, resulting in cell lethality after 2 hr at the restrictive temperature (Figure 3C). In contrast, the viability of imp1Δ cells was comparable to that of wild-type cells after 0–4 hr at 36° (Figure 3C) and similar results were seen when cells were incubated for 24 or 48 hr at 36° before shifting them back to 25° (data not shown).

***imp1* and *cut15* mutations were synthetically lethal:** Imp1p and Cut15p are the only importin-α-like proteins in the fission yeast genome. If they both function as importin-α receptors *in vivo*, then cells lacking both

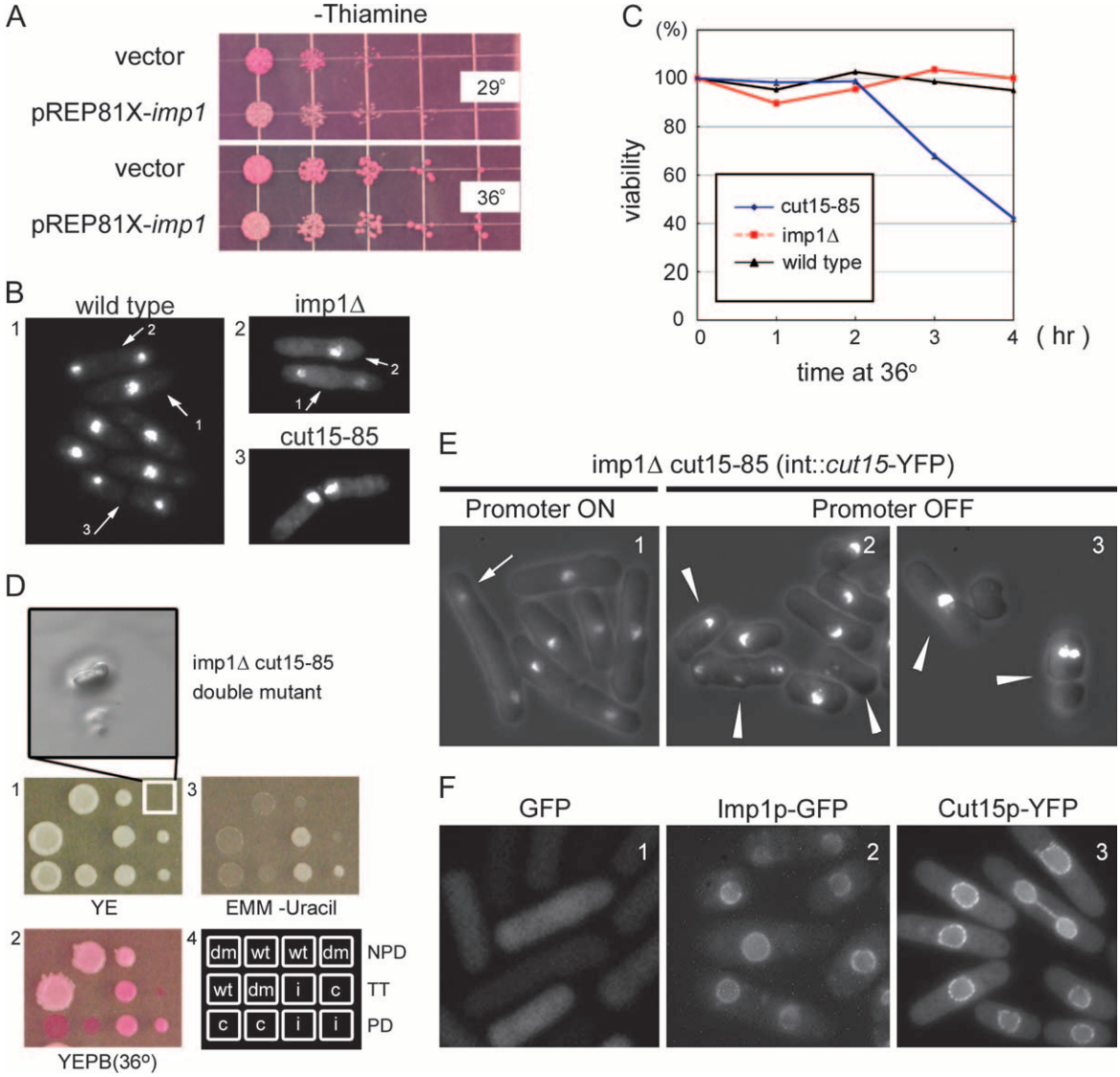


FIGURE 3.—*imp1* and *cut15* mutations cause different cellular defects and are synthetically lethal, but the Imp1p and Cut15p proteins have similar intracellular localizations. (A) imp1Δ cells with pREP81X-*imp1* or empty vector were spotted in fivefold serial dilutions onto plates containing the vital dye phloxine B at 29° or 36° for 2 days. (B) Wild-type (B-1), imp1 null (B-2), or cut15-85 (B-3) strains were incubated at 36° for 4 hr, fixed in ethanol, and the DNA was visualized with DAPI. Wild-type interphase (B-1, cell 1) and postmitotic cells without (B-1, cell 2) or with (B-1, cell 3) a medial septum. imp1Δ cells (B-2) with nuclei at the tips of both unseptated (B-2, cell 1) and septated (B-2, cell 2) cells. cut15-85 cells septated before the completion of chromosome separation (B-3). (C) Equal numbers of cut15-85, imp1Δ, or wild-type cells grown in liquid YE at 25° were spread in triplicate onto YE plates incubated at 36° for 1–4 hr, and then at 25° for 3 days, and average colony number per plate was determined. (D) Tetrad analysis of the heterozygous imp1Δ cut15-85 diploid. Thirty-seven complete tetrads were dissected on YE plates (D-1) and replica plated to YE with phloxine B (YEPB) at 36° to monitor temperature sensitivity (D-2) and to EMM lacking uracil to monitor uracil auxotrophy (D-3). Representative parental ditype (PD), nonparental ditype (NPD), and tetratype (TT) tetrads are shown. The four possible cell types are ura4$^-$ cut15-85^{ts} (c), ura4$^+$ imp1Δ (i), ura4$^-$ wild type (wt), and inviable imp1Δ cut15-85^{ts} double mutants (dm) (D-4). (E) The imp1Δ cut15-85^{ts} haploid double mutant with cut15-YFP was grown without (E-1) or with thiamine for 20 hr (E-2 and E-3), fixed in ethanol, and the DNA was visualized by DAPI. Arrow indicates cell with DNA at the tips. Arrowheads indicate abnormal mitosis. (F) Imp1p-GFP and Cut15p-YFP accumulate at the nuclear periphery and to a lesser extent in the nucleus (F-2 and F-3) compared to untagged GFP (F-1). Imp1p-GFP was expressed from its own promoter at the endogenous locus. An integrated copy of Cut15-YFP was expressed from the *nmt1* gene promoter.

genes might be inviable, because the nuclear import of all classical NLS-containing proteins would be disrupted. To test this hypothesis, a diploid strain that was heterozygous for both the imp1Δ mutation and the cut15-85 temperature-sensitive mutation (MATSUSAKA *et al.* 1998) was constructed and grew normally at all temperatures (data not shown). However, by tetrad analysis 37 haploid imp1Δ cut15-85 double mutants were inviable at a range of temperatures from 18° to 36° (data not shown), indicating that these mutations were

synthetically lethal even at the permissive temperature for cut15-85. Therefore, in the absence of Imp1p, cells required a higher level of functional Cut15p than was necessary for viability in its presence, suggesting that they have an overlapping essential function. Microscopic examination revealed that at 36° the double-mutant spores germinated but did not divide (Figure 3D). To analyze the consequences of loss of both Cut15p and Imp1p, a similar heterozygous double-mutant diploid strain that also contained an integrated copy of *cut15* expressed from the high-strength *nmt1* gene promoter was constructed. Haploid double mutants were viable when *cut15* was expressed but died when it was repressed. When the promoter was on, cells divided normally but were slightly elongated and had DNA at the cell tips, characteristic of the telophase delay in the imp1Δ single mutant (Figure 3E, promoter on). In contrast, when the promoter was off, cells exhibited a variety of mitotic abnormalities, including chromosome mis-segregation, lagging chromosomes, cell elongation (which is indicative of a cell cycle block), and the accumulation of "wee" cells (Figure 3E, promoter off), characteristic of cells that enter mitosis prematurely before reaching the critical cell mass (Thuriaux *et al.* 1978; Nurse and Thuriaux 1980). When expression of *cut15* was repressed for 20 hr, 53.5% of cells had mitotic defects compared to only 9.2% when *cut15* was expressed. The observation that loss of both importin-α isoforms causes a terminal phenotype different from either of the single mutants provides further evidence that these two proteins are likely to have some independent functions.

As previously described for Cut15p-GFP (Matsusaka *et al.* 1998), Cut15p-YFP, produced from a single integrated copy of plasmid pDUAL-*cut15*, localized within the nucleus and in a punctate pattern at the nuclear periphery (Figure 3F-3). Imp1p-GFP, produced from the *imp1* promoter at the endogenous locus, had a similar localization pattern (Figure 3F-2) whereas GFP alone equilibrated across the nuclear envelope (Figure 3F-1), as previously reported (Kadura *et al.* 2005).

Using standard nuclear protein import assays, neither *imp1* nor *cut15* was required for nuclear import of an SV40 NLS fusion protein: Although *cut15* is an essential gene, *cut15* temperature-sensitive mutants are competent for classical NLS-dependent import *in vivo* (Matsusaka *et al.* 1998), suggesting that its nuclear import function is not essential *in vivo*. However, like Imp1p, Cut15p could support nuclear protein import *in vitro* (Matsusaka *et al.* 1998).

Because the SV40 NLS is functional in fission yeast, an SV40 NLS-GFP-β-Gal fusion protein was used to assess nuclear protein import competence (Pasion and Forsburg 1999; Fleig *et al.* 2000; Salus *et al.* 2002; Chen *et al.* 2004; reviewed in Yoshida and Sazer 2004). The protein accumulates exclusively in the nucleus of wild-type cells, but mislocalizes to the cytoplasm when the NLS is inactivated by mutation (Pasion and Forsburg 1999; Yoshida and Sazer 2004).

imp1Δ, cut15-85, and wild-type cells producing SV40 NLS-GFP-β-Gal were grown at 25° to midlog phase and then shifted to 36° for 4 hr (Figure 4A). Wild-type and mutant cells accumulated the GFP reporter exclusively in the nucleus at both temperatures, suggesting that they are capable of nuclear protein import.

The ability of cut15-85 to import SV40 NLS-GFP-β-Gal is consistent with the previous report of its ability to import a different SV40 NLS fusion protein (Matsusaka *et al.* 1998). This result was interpreted as meaning that Cut15p is not essential for nuclear protein import *in vivo*. The observation that cells with only Imp1p or only Cut15p could still import an SV40 NLS-containing protein suggests the possibility that in *S. pombe* these two importin-α proteins have partially overlapping functions for the import of this NLS.

Using a more sensitive nuclear protein import assay, efficient nuclear import of an SV40 NLS fusion protein depended upon both Imp1p and Cut15p: One drawback of using SV40 NLS-GFP-β-Gal to monitor nuclear protein import in temperature-sensitive mutant cells is that the protein can accumulate in the nucleus prior to the temperature shift, meaning that an import defect can be detected only by the cytoplasmic accumulation of newly synthesized protein at the restrictive temperature (Yoshida and Sazer 2004). Some groups have attempted to overcome this problem by inducing expression of the reporter and then shifting cells to the restrictive temperature before it accumulates in the nucleus (Matsusaka *et al.* 1998; Chen *et al.* 2004). However, even using this strategy, cut15-85 was competent to import an SV40 NLS cargo (Matsusaka *et al.* 1998). To overcome this complication, nucleocytoplasmic transport in importin-α mutants was tested using a reporter (pR1GLFE1) in which GFP-GST is fused to both the SV40 NLS and the HIV Rev NES (Kudo *et al.* 1998). This protein is expected to continually shuttle between the nucleus and the cytoplasm (Kudo *et al.* 1998). Both cut15-85 and imp1Δ were less efficient at accumulating this reporter in the nucleus than were wild-type cells, while the ability to accumulate an NLS-bearing cargo in the nucleus or to exclude an NES-bearing cargo from the nucleus was identical in the three strains (Figure 4B).

***imp1* and *cut15* were each required for the nuclear import of Pap1p:** Pap1p is an *S. pombe* transcription factor required for the MAP kinase stress response (Shiozaki and Russell 1995; Toone *et al.* 1998). It continually shuttles between the nucleus and the cytoplasm, but is predominantly cytoplasmic in growing cells. Upon stress, such as treatment with hydrogen peroxide, Pap1p nuclear export is blocked and it becomes predominantly nuclear within 15 min. Pap1p-GFP is an ideal physiologically relevant substrate with which to monitor the efficiency of nucleocytoplasmic transport (Yoshida and Sazer 2004). Pap1p contains a complex

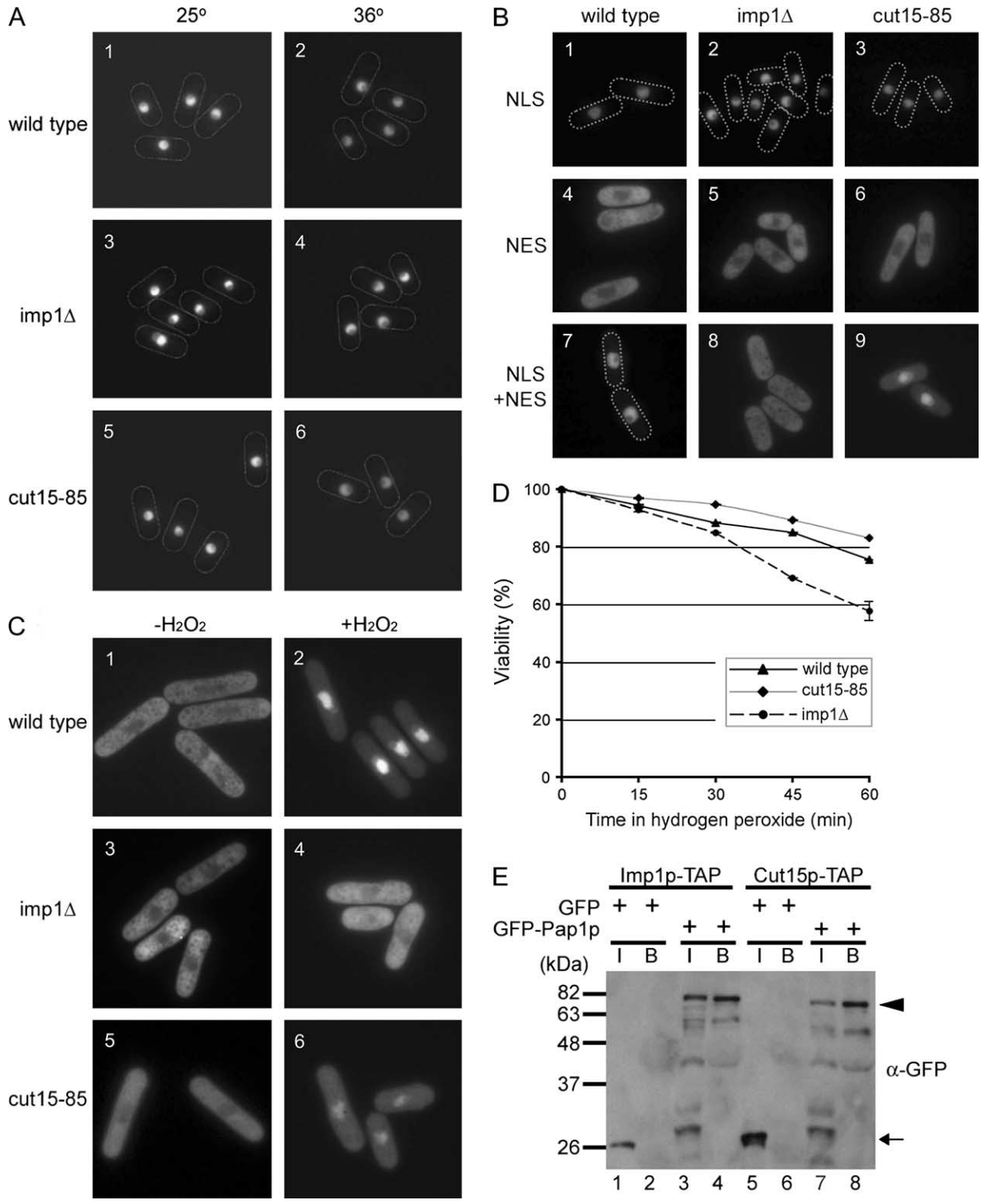


FIGURE 4.—imp1Δ and cut15-85 cells are competent to efficiently import an SV40 NLS-GFP, but neither SV40 NLS-GFP-HIV Rev NES nor Pap1p. (A) Wild-type (1 and 2), imp1Δ (3 and 4), and cut15-85 (5 and 6) cells expressing SV40 NLS-GFP-lacZ were grown at 25° (1, 3, and 5) or shifted to 36° for 4 hr (2, 4, and 6). (B) Wild-type (1, 4, and 7), imp1Δ (2, 5, and 8), or cut15-85^{ts} (3, 6, and 9) cells expressing GST-SV40 NLS-GFP-Rev NES were grown at 25°. (C) Wild-type (1 and 2), imp1Δ (3 and 4), or cut15-85 (5 and 6) cells expressing GFP-Pap1p without (1, 3, and 5) or with (2, 4, and 6) 0.003% hydrogen peroxide for 15 min. (D) Wild-type, cut15-85, and imp1Δ cells treated with 0.003% hydrogen peroxide for 0–60 min, washed, plated to EMM without hydrogen peroxide, and viability was determined by counting colonies after 3 days. (E) TAP-tagged proteins were purified from cells expressing Imp1p-TAP (lanes 1–4) and either GFP (lanes 1 and 2) or GFP-Pap1p (lanes 3 and 4) or from cells expressing Cut15-TAP (lanes 5–8) and either GFP (lanes 5 and 6) or GFP-Pap1p (lanes 7 and 8) and analyzed by Western blot using an anti-GFP antibody. Input (I) (lanes 1, 3, 5, and 7) and bound (B) (lanes 2, 4, 6, and 8) protein samples are shown. GFP-Pap1p (arrowhead), but not GFP alone (arrow), co-immunoprecipitates with both Imp1p-TAP and Cut15p-TAP.

NLS consensus sequence (see MATERIALS AND METHODS) consisting of two overlapping bipartite-type NLSs (*KK*IGRKNSDQEPSS*KRK* and *KRK*AQNRAAQRAF*RKRK*, in which *KK*, *KRK*, and *RKRK* represent conserved basic amino-acid clusters), which would be expected to be transported into the nucleus via importin-α if one or both are functional NLSs. Wild-type, imp1Δ, and cut15-85 strains expressing an integrated copy of the gene encoding Pap1p-GFP were grown at 25° and observed either before or after treatment with hydrogen peroxide (Figure 4C). Neither cut15 nor imp1 mutants were competent to efficiently import Pap1p to the nucleus compared to wild-type cells at 25°, the temperature at which the *cut15-85* and *imp1*Δ mutations are synthetically lethal. However, the import defect, evidenced by the cytoplasmic accumulation of Pap1p fluorescence after hydrogen peroxide exposure, was more severe in imp1Δ cells than in the cut15-85 mutant. In cut15-85, Pap1p failed to accumulate in the nucleus to the wild-type level, but was not excluded from the nucleus as it was in the imp1Δ strain.

imp1Δ and cut15-85 differed in their ability to survive oxidative stress: Because imp1Δ cells were less competent than cut15-85 cells in importing Pap1p to the nucleus in response to stress, we tested the hypothesis that the imp1Δ strain was also less competent to respond to oxidative stress. The two importin-α mutant strains and a wild-type control were exposed to hydrogen peroxide to activate the Pap1p-dependent stress pathway (reviewed in YOSHIDA and SAZER 2004). The cells were then washed, counted, and plated onto supplemented EMM every 15 min for 1 hr, and the viability was assessed after 3 days at 25° (Figure 4D). impΔ cells were more sensitive to this treatment than wild type or cut15-85 mutant cells, dropping to 58% viability after 1 hr, compared to >75% viability in the other two strains. The reduced ability of imp1Δ cells to survive oxidative stress may be due solely to the efficiency of import of Pap1p or there may be other proteins required for survival after stress that also depend primarily on Imp1p for their nuclear import.

Pap1p interacted with both Imp1p and Cut15p: To ask whether Pap1p is a transport cargo of the Imp1p and/or Cut15p transport adaptors, strains expressing TAP-tagged versions of Imp1p or Cut15p along with a GFP-tagged version of Pap1p were constructed (see MATERIALS AND METHODS). The TAP-tagged proteins were purified from cell lysates, and a Western blot was probed with anti-GFP antibody (Figure 4E). Both Imp1p-TAP and Cut15p-TAP copurified with GFP-Pap1p (lanes 4 and 8, arrowhead) but not with GFP (lanes 2 and 6, arrow). These results indicate that the Pap1p nuclear import cargo physically interacts with both the Imp1p and Cut15p transport adaptors.

Strong overexpression of *cut15* or *imp1* was toxic: To test whether increasing the abundance of the two importin-α proteins would affect cell survival, a wild-type copy of either *cut15* or *imp1* under control of the high-, medium-, or low-strength versions of the thiamine-regulatable *nmt1* gene promoter was introduced into wild-type cells. When expression was repressed, all six strains and the empty vector control grew normally (Figure 5A). High-level expression of either *imp1* or *cut15* or medium-level expression of *cut15* inhibited cell growth (Figure 5A) and resulted in the accumulation of elongated cells with a single nucleus (Figure 5B), typical of cell division cycle (cdc) mutants (THURIAUX *et al.* 1978) that are arrested in cell cycle progression.

When expressed from the low level *nmt1* gene promoter, however, neither gene was detrimental to cell cycle progression in wild-type cells (Figure 5A). At this nontoxic level of expression, *imp1* could rescue the temperature sensitivity of the imp1Δ strain (Figure 6D) and *cut15* could rescue the temperature-sensitive lethality of the cut15-85 mutant (Figure 6C).

***imp1* could not fully rescue the temperature-sensitive lethality or the cut phenotype of cut15-85:** To determine whether Cut15p and Imp1p have overlapping functions, *imp1* was expressed from the low-strength *nmt1* gene promoter in the cut15-85 temperature-sensitive mutant (Figure 6, A–C). The viability of this strain was compared to cut15-85 mutant strains with a vector control or with either a plasmid-borne or an integrated copy of *cut15* at a range of temperatures from 25° to 36° (Figure 6C). At 25° to 29° all of the strains grew equally well whether the promoter was on or off. At 32°, a temperature at which the growth of cut15-85 was only slightly reduced compared to 29°, cut15-85 cells expressing *imp1* grew less well than cells containing the vector control, indicating that under this specific condition excess Imp1p was toxic. At 34° or 36° cut15-85 cells containing the vector or the *imp1* plasmid did not form colonies, but the cells grew well when *cut15* was expressed. When these same strains were grown in liquid culture for 4 hr at 36° (Figure 6, A and B), *imp1* partially rescued the mitotic defect in cut15-85, reducing the percentage of cells exhibiting the cut phenotype from 45.3 ± 4.1% in cells with the empty vector to 29.6 ± 2.3%. Expression of *cut15* from a multicopy plasmid or integrated copy of the gene reduced the appearance of cells with mitotic defects to 6.7 ± 2.1% and 2.7 ± 1.2%, respectively. The observation that *imp1* could partially rescue the mitotic defects but not the viability of cut15-85 at the restrictive temperature of 36° indicates that the cut phenotype is only partially responsible for the loss of viability in this strain.

***cut15* could partially rescue the growth defects but not the nuclear position defects of imp1Δ cells:** Using a complementary strategy to further investigate putative overlapping functions of *imp1* and *cut15*, *cut15* was expressed in imp1Δ cells from the low-strength version of the regulatable *nmt1* gene promoter at a level that was not toxic to wild-type cells (see Figure 5A). The transformants were spotted onto supplemented EMM plates, on which the promoter was either on or off,

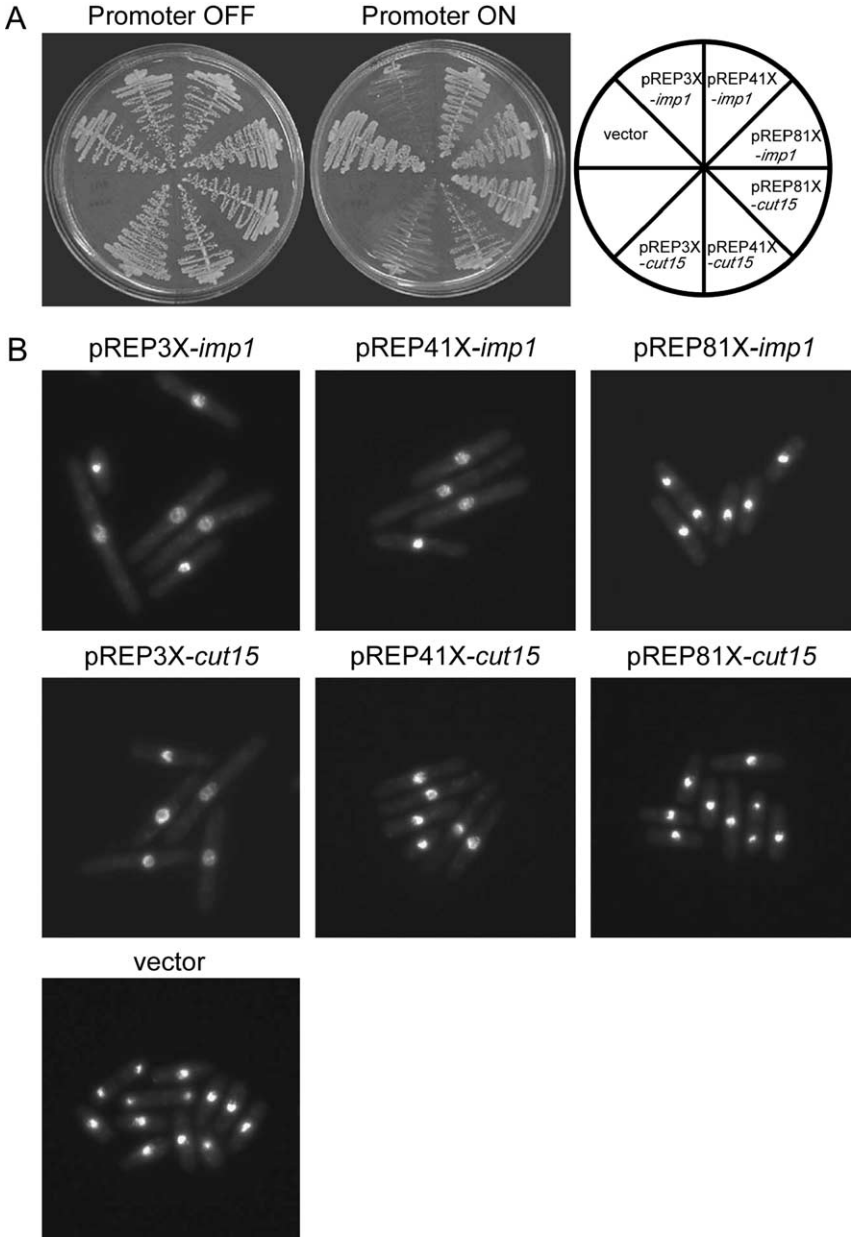


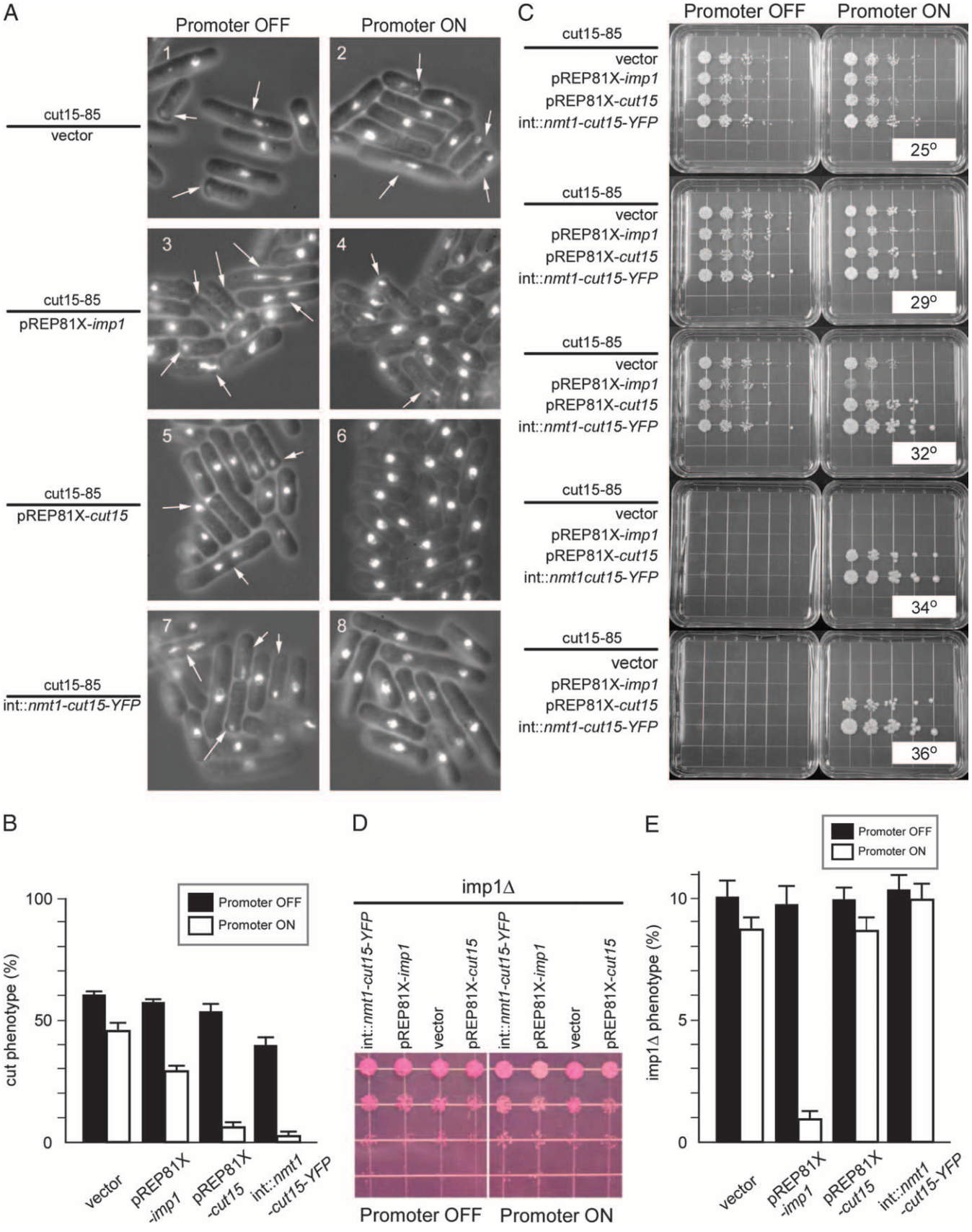
FIGURE 5.—Overexpression of *imp1* or *cut15* is toxic in wild-type cells. Wild-type cells with empty vector or expressing *imp1* or *cut15* from the high- (pREP3X), medium- (pREP41X), or low- (pREP81X) strength *nmt1* gene promoter were (A) streaked to EMM plates with (promoter off) or without (promoter on) thiamine and incubated at 29° for 3 days or (B) grown at 29° without thiamine for 20 hr, fixed in ethanol, and the DNA was visualized with DAPI.

containing the vital dye phloxine B and incubated at temperatures ranging from 25° to 36° (data not shown). At 36° (Figure 6D) when the promoter was repressed, all of the transformant colonies were dark pink, indicating an accumulation of dead cells. When the promoter was derepressed, the imp1Δ cells that expressed *cut15* from either a plasmid or an integrated copy of the gene were intermediate in color between the dark-pink colonies of cells carrying the vector control and the light-pink colonies of cells expressing *imp1* (Figure 6D), indicating a partial rescue of viability.

In the imp1Δ strain, 10.4% of cells had a nuclear position defect in which either the two nuclei in binucleated cells or the single nucleus in mononucleated cells was positioned near the ends of the cell (Figure 6E). When *cut15* was expressed from either a multi-copy plasmid or an integrated copy of the gene, the percentage of cells with mispositioned nuclei was not significantly altered. In contrast, expression of *imp1* reduced the nuclear division defects to <1%. These data indicate that Cut15p can compensate for some functions of Imp1p, but not for the nuclear position defect. They also suggest that the nuclear position defect is not responsible for the slight temperature sensitivity of the imp1Δ strain.

DISCUSSION

***S. pombe* has two importin-α1 proteins, Cut15p and Imp1p, which are required for efficient nuclear protein import:** Animal cells have multiple importin-α proteins, which have been classified into three groups, designated α1, α2, and α3, on the basis of comparisons of the


A
Promoter OFF
Promoter ON
cut15-85
vector
cut15-85
pREP81X-imp1
cut15-85
pREP81X-cut15
cut15-85
int::nmt1-cut15-YFP
1
2
3
4
5
6
7
8
C
Promoter OFF
Promoter ON
cut15-85
vector
pREP81X-imp1
pREP81X-cut15
int::nmt1-cut15-YFP
25°
29°
32°
34°
36°
B
Promoter OFF
Promoter ON
cut phenotype (%)
100
50
0
vector
pREP81X -imp1
pREP81X -cut15
int::nmt1 -cut15-YFP
D
imp1Δ
int::nmt1-cut15-YFP
pREP81X-imp1
vector
pREP81X-cut15
Promoter OFF
Promoter ON
E
Promoter OFF
Promoter ON
imp1Δ phenotype (%)
10
5
0
vector
pREP81X -imp1
pREP81X -cut15
int::nmt1 -cut15-YFP

amino acid sequences of the ARM repeats. ARM repeats constitute the cargo-binding domain of these nuclear import receptors and differences among the three groups are thought to reflect their unique substrate specificities. Genes in the α1 group, found in all eukaryotes, including fission and budding yeast, are believed to have given rise to the metazoan-specific α2 and α3 types, several of which have tissue- and/or developmental-stage-specific roles (reviewed in Miyamoto *et al.* 1997; Kamei *et al.* 1999; Goldfarb *et al.* 2004). The budding yeast *S. cerevisiae* has just one importin-α gene, *SRP1*, but the fission yeast *S. pombe* is unique among the single-celled eukaryotes characterized to date in that it has two importin-α genes, *cut15* and *imp1* (Figure 1). This is somewhat unexpected, since there is no evidence in *S. pombe* for the large-scale genome duplications found in the *S. cerevisiae* genome (Wood *et al.* 2002). Among the known importin-α proteins, Imp1p and Cut15p are most closely related to one another (Kohler *et al.* 1999), and although both are members of the importin-α1 group (Kohler *et al.* 1999; Goldfarb *et al.* 2004), they have distinct roles in nucleocytoplasmic transport and cell cycle progression.

The best-characterized role of importin-α proteins is that of classical NLS-dependent nuclear protein import. Cut15p has previously been shown to support nuclear protein import *in vitro* but cells lacking the protein are, unexpectedly, still able to import a classical NLS-dependent import (Matsusaka *et al.* 1998). Using a more sensitive assay and different import cargos, we found that Cut15p is required for the efficient nuclear import of classical NLS-containing substrates (Figure 4).

Imp1p is also an authentic importin-α, which can support nuclear protein import both *in vivo* and *in vitro* (Figures 2 and 4). In contrast to our results showing that imp1Δ cells, derived from a heterozygous null diploid strain, are viable (Figure 3D-1), *imp1* was recently reported to be essential for viability (Chen *et al.* 2004). However, in our hands the haploid imp1Δ strain reported in this article is viable and has the same telophase delay and growth characteristics as the imp1Δ strain described in this article. We therefore conclude that *imp1* is not essential for vegetative growth.

Cut15p and Imp1p are each required for the efficient nuclear import of the Pap1p component of the MAP kinase stress pathway. Both imp1Δ and cut15-85 are loss-of-function mutations, since the *imp1* gene is deleted and the protein produced by cut15-85 is degraded after 1 hr at the restrictive temperature (Matsusaka *et al.* 1998). However, loss of Imp1p causes a relatively more severe import defect (Figure 4C). imp1 mutant cells are also more sensitive to oxidative stress than are cut15 mutant cells, demonstrating the physiological relevance of the differences in their Pap1p import efficiency (Figure 4D). In contrast to these results, Cut15p and Imp1p bind similarly to Pap1p in the absence of oxidative stress (Figure 4E).

Imp1p and Cut15p are each required for different aspects of cell cycle progression: Although the import cargoes of the fission and budding yeast importin-α proteins remain largely unknown and may not be the same, cell cycle progression in both organisms depends on the proper functioning of the importin-α proteins. The single *S. cerevisiae* importin-α protein, Srp1p, is essential for mitosis (Yano *et al.* 1992, 1994; Kussel and Frasch 1995; Loeb *et al.* 1995). Imp1p and Cut15p are also each required for proper cell cycle progression in *S. pombe* (Figure 3B; see below).

Overexpression of either (Figure 5A) or both (data not shown) importin-α proteins is toxic and cells undergo a classic cdc arrest (Figure 5B). This is perhaps because at a high intracellular concentration the adaptors are more likely to remain bound to their cargoes, thereby preventing these imported proteins from carrying out their normal roles. A screen aimed at identifying genes that cause cell cycle defects when overexpressed also identified *imp1* (Tallada *et al.* 2002).

cut15 and *imp1* are each required for proper mitotic progression but neither gene is transcriptionally regulated during the cell cycle (Rustici *et al.* 2004) upon exposure to a variety of stresses (Chen *et al.* 2003) or during mating, meiosis, and sporulation (Mata *et al.* 2002). Therefore, the functional differences between the two *S. pombe* importin-α genes cannot be attributed to differences in expression pattern.

Imp1p and Cut15p likely interact with both common and unique import cargoes: A relatively small number of importin-α cargo proteins have been identified (reviewed in Jans *et al.* 2000). *In vitro* analysis of importin-α proteins from a variety of organisms showed that the affinities with which they bind to their substrates are

Figure 6.—*imp1* and *cut15* can rescue mutations in *imp1* and *cut15*, respectively, but cannot fully rescue mutations in each other. (A) cut15-85 expressing vector (1 and 2), pREP81X-*imp1* (low-strength promoter) on a plasmid (3 and 4), pREP81X-*cut15* (low-strength promoter) on a plasmid (5 and 6), or *cut15*-YFP (high-strength promoter) integrated (7 and 8) were grown with (promoter off) or without (promoter off) thiamine for 30 hr, shifted to 36° for 4 hr, fixed in ethanol, and the DNA was visualized with DAPI. Arrows indicate cells with cut phenotype. (B) Samples in A were examined microscopically and the proportion of cells with a cut phenotype was quantified. (C) Strains were cultured as described in A and then spotted onto EMM plates with or without thiamine and incubated at the indicated temperature for 5 days. (D) imp1Δ strains expressing the four constructs described in A were grown with thiamine at 25°, washed, spotted onto EMM phloxine B plates with (promoter on) and without (promoter off) thiamine, and incubated at 36°. (E) imp1Δ strains described in D were cultured at 25° with thiamine (promoter off), washed, incubated without thiamine (promoter on) for 20 hr, the last 4 hr of which were at 36°, fixed in ethanol, and the DNA was visualized with DAPI. The proportion of cells with nuclei at the cell tips was quantified.

similar but that *in vitro* import efficiencies vary depending upon whether one or more than one substrate is present (Kohler *et al.* 1999). Furthermore, these *in vitro* analyses do not necessarily reflect *in vivo* import efficiency, because in living cells there is competition among cargo proteins for transport carriers (Kohler *et al.* 1999).

Previous analyses focused on the specialized roles of the importin-α2 and importin-α3 groups in animal cell development (reviewed in Goldfarb *et al.* 2004). Fission yeast cells do not have importin-α2- or -α3-type proteins, but they have two members of the importin-α1 family, which have both common and distinct physiological roles.

Their unique functions are indicated by differences in the phenotypes of cells in which Imp1p and/or Cut15p are mutated. Temperature-sensitive mutants of the essential gene *cut15* arrest in mitosis with decondensed chromosomes and a medial septum that cuts through the undivided chromosomes (Matsusaka *et al.* 1998). *imp1* is not essential for viability but imp1Δ cells are delayed at telophase, have mispositioned nuclei, and are slightly temperature-sensitive (Figure 3B).

The inviability of the cut15-85 imp1Δ double-mutant strain suggests that Imp1p and Cut15p also have common functions and common binding partners. The efficient import of an SV40 NLS-containing cargo and the bipartite NLS-containing endogenous protein Pap1p depend upon each importin-α protein. The *imp1*Δ and *cut15-85* mutations are synthetically lethal, and each importin-α protein can partially rescue some of the defects caused by mutations in the other, providing further evidence that the two *S. pombe* importin-α proteins have some overlapping functions.

However, neither of the *S. pombe* importin-α1 proteins can fully compensate for loss of the other (Figures 5A and 6). Expression of *imp1* rescues the "cut" phenotype but not the loss of viability of cut15-85 at 36°, suggesting that the mitotic defect is not primarily responsible for the inviability of this strain. However, for reasons that are unclear, at the semipermissive temperature of 32°, expression of *imp1* is toxic to cut15-85 cells. Conversely, expression of *cut15* does not rescue the telophase delay but does rescue the temperature sensitivity of the imp1Δ strain, indicating that the mitotic defect is not responsible for the loss of viability at elevated temperature.

Importin-α isoforms in other organisms also have both overlapping and nonoverlapping physiological roles (reviewed in Goldfarb *et al.* 2004; discussed in Kohler *et al.* 1999). Identification of the common and unique interaction partners of Cut15p, Imp1p, and importin-α proteins in other experimental systems will be important for understanding the full implications of these observations.

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