A role for the divergent actin gene, *ACT2*, in nuclear pore structure and function

Catherine Yan, Noah Leibowitz and Teri Mélése^{1,2}

Department of Biological Sciences, Columbia University, New York, NY 10027, USA

¹Present address: Microcide Pharmaceuticals, 850 Maude Avenue, Mountain View, CA 94043, USA

²Corresponding author

We have identified a temperature-sensitive allele of the yeast divergent actin gene *ACT2*, *act2-1*, which displays defects in nuclear pore complex (NPC) structure and nuclear import at the restrictive temperature. Although defective in nuclear import, *act2-1* cells still selectively retain reporter proteins in the nucleus, and by indirect immunofluorescence the actin cytoskeleton appears normal. Previous studies in *Acanthamoeba* and *Saccharomyces cerevisiae* reported that the cellular location of Act2p partially overlaps that of conventional actin, indicating that it has a cytoskeletal function. In this study, both immunofluorescence localization and cellular fractionation of different epitope-tagged versions of Act2p also reveal an association with the nucleus, suggesting an independent nuclear function for Act2p. Analysis of *act2-1* by electron microscopy, 30 min after a shift to the restrictive temperature (37°C), reveals a striking aberration in NPC morphology; NPCs appear as abnormal densities on either side of, rather than spanning, the nuclear envelope. Immunoelectron microscopy confirms that these densities contain XFXFG nucleoporins. *act2-1* is synthetically lethal in combination with a deletion in the XFXFG nucleoporin gene, *NUPI*, or a mutation in the nuclear localization sequence receptor gene, *SRP1*. Act2p and Srp1p co-immunoprecipitate, suggesting that the proteins exist in a complex. Together our data argue that Act2p plays an important role in NPC structure and function.

Keywords: Act2p/actin-related protein/nuclear pore complex/*S.cerevisiae*

Introduction

Nuclear pore complexes (NPCs) are supramolecular structures embedded in the nuclear envelope that have a unique role in facilitating the bidirectional transport of macromolecules between the nucleus and the cytoplasm (Feldherr, 1962; Feldherr *et al.*, 1984). Ultrastructural studies of the NPC show that they have 8-fold symmetry, a central channel thought to be the 'transporter' for incoming nuclear proteins, asymmetrical nuclear and cytoplasmic faces and filaments extending from both sides (Goldberg and Allen, 1995). There is evidence to suggest

that the NPC proteins, or nucleoporins, associate with the filaments (Cordes *et al.*, 1993; Wilken *et al.*, 1993). Colloidal gold-labeled nuclear proteins were followed by electron microscopy and they appear to sit on the filaments prior to moving into the central core of the NPC (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). These observations prompted several investigators to suggest that the filaments at the cytoplasmic face of the NPC might function in the first step of nuclear import to direct incoming proteins to the 'transporter' (Goldberg and Allen, 1995; Melchior and Gerace, 1995).

The majority of proteins entering the nucleus have a well-defined nuclear localization sequence (NLS) (Dingwall and Laskey, 1991). A number of soluble factors involved in nuclear import recently have been identified *in vitro*. A protein complex, known as the NLS receptor, importin or karyopherin, was shown to target substrates that contain a NLS to the NPCs (Adam and Gerace, 1991; Adam and Adam, 1994; Görlich *et al.*, 1995; Imamoto *et al.*, 1995; Moroianu *et al.*, 1995; Radu *et al.*, 1995a). The protein complex is composed of two subunits; karyopherin α mediates recognition of the NLS substrate (Adam and Gerace, 1991; Görlich *et al.*, 1995; Moroianu *et al.*, 1995), whereas karyopherin β functions to dock the karyopherin α -NLS substrate complex to nucleoporins which contain peptide repeats (Moroianu *et al.*, 1995; Radu *et al.*, 1995a,b). The yeast homolog of karyopherin α is Srp1p or Kap60p (Görlich *et al.*, 1994; Moroianu *et al.*, 1995). In support of a role for Srp1p in nuclear import in yeast, there is biochemical and genetic evidence that Srp1p interacts with the nucleoporins, Nup1p and Nup2p (Belanger *et al.*, 1994). Both Nup1p and Nup2p belong to a class of nucleoporins that contain XFXFG repeats which appear to recognize the NLS receptor *in vitro* on ligand blots (Rexach and Blobel, 1995). Mutations in the genes encoding these nucleoporins result in poly(A)⁺ RNA accumulation in the nucleus and the inability to import a NLS reporter protein *in vivo* (Bogerd *et al.*, 1994).

The movement of substrates across the NPC requires the presence of a second protein identified in a different cytosolic fraction, the GTPase Ran (Melchior *et al.*, 1993; Moore and Blobel, 1993, 1994). The cyclic hydrolysis of GTP by Ran is thought to promote nuclear import. The yeast homologs of Ran are Gsp1p and Gsp2p (Belhumeur *et al.*, 1993), the corresponding GTPase-activating protein (GAP) is Rna1p (Bischoff *et al.*, 1995) and the GTP/GDP exchange protein (GEP) for Ran in the nucleus is Prp20p (Aebi *et al.*, 1990). Ran-binding sites are found on the nucleoporin Nup358 which is associated with cytoplasmic filaments (Wu *et al.*, 1995; Yokoyama *et al.*, 1995). The yeast nucleoporin Nup2p binds Srp1p and also contains Ran-binding domains similar to the higher eukaryotic nucleoporin Nup358p (Loeb *et al.*, 1993; Dingwall *et al.*, 1995).

In a screen to identify additional proteins involved in nuclear import, we identified a new allele of the previously described divergent actin gene, *ACT2*. The yeast *Saccharomyces cerevisiae* *ACT2* is an essential gene that encodes a protein belonging to a family of actin-related proteins (Herman, 1993; Clark and Meyer, 1994; Fyrberg *et al.*, 1994; Harata *et al.*, 1994; Muhua *et al.*, 1994; Mullins *et al.*, 1996) and shares 47% identity with the conventional yeast actin gene, *ACT1* (Schwob and Martin, 1992). Previous studies on Act2p in *Acanthamoeba* and *S.cerevisiae* have found that the protein can be co-localized with actin at the cortical patches (Kelleher *et al.*, 1995; Moreau *et al.*, 1996). Act2p was isolated from *Acanthamoeba* by affinity chromatography on profilin-agarose, and found in a complex with six polypeptides; the actin-related protein, Arp3p, and five novel proteins of 40, 35, 19, 18 and 14 kDa (Machesky *et al.*, 1994; Mullins *et al.*, 1997). A similar complex is recruited to the surface of the motile bacteria, *Listeria monocytogenes*, in the cytoplasm of host cells carrying this pathogenic bacterium (Welch *et al.*, 1997). The purified complex is sufficient to promote actin polymerization at the surface of *L.monocytogenes*, but requires the activity of the bacterial surface protein, ActAp (Welch *et al.*, 1997). An Arp3p complex has also been identified in *Schizosaccharomyces pombe*, but Act2p has not been identified in this complex as yet (McCollum *et al.*, 1996). It is proposed that the *S.pombe* Arp3 complex might be involved in two processes; motility of the actin patches during cell growth, and contractile ring formation during septation (McCollum *et al.*, 1996).

Here we report that the *act2-1* mutant has a defect in the structure of the NPC, such that the NPCs are no longer embedded within, but rather lie on either side of, the nuclear envelope. We have found a genetic interaction between Act2p and proteins involved in the initial stages of nuclear import; the nucleoporin Nup1p and the NLS receptor (Srp1p). We also present evidence in support of a physical complex between Act2p and Srp1p. By indirect immunofluorescence and cellular fractionation, Act2p tagged with either a single hemagglutinin (HA) epitope at the N-terminus or six c-Myc epitopes at the C-terminus of the protein is associated with the nucleus.

Results

The divergent actin gene, *ACT2*, is identified in a screen for nuclear import mutants

To obtain mutants in the nuclear import pathway, a temperature-sensitive (*Ts*⁻) lethal yeast collection of 516 mutants (Shuai and Warner, 1991; see Materials and methods and Table I) was screened by indirect immunofluorescence for the cytoplasmic mislocalization of the endogenous nucleolar protein, Nsr1p (Lee *et al.*, 1991; Yan and Melese, 1993). In wild-type controls, Nsr1p was strictly nucleolar 3 h after a shift to the non-permissive temperature of 37°C, but in 40 mutants Nsr1p was mislocalized to the cytoplasm to varying degrees. In separate experiments, those mutants with the strongest mislocalization phenotype also failed to localize a H2B or SNF2 β -galactosidase NLS reporter protein (example shown in Figure 4a–d). In all cases, the *Ts*⁻ lethal and mislocalization phenotypes co-segregated and were

recessive, allowing the assignment of a number of complementation groups, and the mutants were referred to as nuclear import defective or *nid* mutants.

In order to distinguish the *nid* mutants further, we began by examining the time of onset of the nuclear import defect in two of the complementation groups, *nid1* (two alleles) and *nid2* (six alleles). *nid1* and *nid2* were analyzed using indirect immunofluorescence to detect the mislocalization of Nsr1p at varying times after a shift to the non-permissive temperature. We found that the *nid1* mutant had a slow onset of nuclear import that occurred by 2–3 h (data not shown), whereas in the *nid2* mutant, the appearance of Nsr1p in the cytoplasm occurred within 15 min of a shift to 37°C (Figure 2, see anti-Nsr1p staining at 15 min under *act2-1*). To identify the gene responsible for the *nid2* phenotype, *nid2* was transformed with a yeast *CEN4 URA3* library (Thompson *et al.*, 1993) and the gene cloned by complementation of the *Ts*⁻ phenotype (see Materials and methods). The rescuing plasmid (pACT2-1) was isolated from the original *Ts*⁺ colonies. Through subsequent molecular analysis, we isolated the smallest DNA fragment that was able to complement both the *Ts*⁻ growth (data not shown) and the nuclear import defect (Figure 1g–i). Sequence analysis of the rescuing clone showed that it contained the previously identified gene, *ACT2* (Schwob and Martin, 1992). Sequence analysis of the *act2-1* mutation revealed a glycine to valine change at amino acid 161, in a potential ATP-binding site which is conserved in the *ACT1* gene (Kabsch *et al.*, 1990; Wertman *et al.*, 1992).

The actin cytoskeleton appears normal in *act2-1* mutants

The sequence similarity of Act2p to conventional actin raised the possibility that Act2p might play a role in cytoskeletal organization. Thus, the defect in nuclear import observed in *act2-1* might be an indirect result of perturbations in the cytoskeleton. To test this, the actin distribution was analyzed in *act2-1* after a shift to the non-permissive temperature (see Figure 2). Aliquots of cells were taken at 15 min intervals for 3 h and stained with rhodamine-conjugated phalloidin which recognizes actin (Novick and Botstein, 1985; Adams and Pringle, 1991). At the time of onset of the nuclear import defect (15 min), no difference can be detected in actin cable staining in wild-type versus *act2-1* cells (Figure 2), and the distribution of the cortical actin patches in *act2-1* cells appears normal for up to 1.5–2 h at 37°C (Figure 2, actin staining at 1.5 h is shown). To test cell viability of *act2-1* cells, aliquots of cells shifted to the non-permissive temperature were taken at different time points, the OD₆₀₀ of the culture was measured, and a fixed number of cells were plated on YPD (Figure 3B). The number of viable cells remained nearly constant for 2 h and began to decline at an approximate rate of 10%/h, such that by 3 h, 70% of the cells were viable, and by 8 h only 20% were viable (Figure 3B). As also shown in Figure 3, the growth of *act2-1* cells after shifting to 37°C ceased after 4 h (Figure 3A).

We tested strains carrying temperature-sensitive mutations in *ACT1*, *act1-3* and *act1-133* (Shortle *et al.*, 1984; Wertman *et al.*, 1992; Table I, DBY1691 and MSY107) for a defect in nuclear import, and found that

Table I. Yeast strains used in this study

Strain	Source	Genotypes
J1003.1D ^a	Shuai and Warner, 1991	<i>Matα ade2-1, ura3-1, trp1-1, leu2-3,112, can1-100, HIS3</i>
J1003.1E ^a	Shuai and Warner, 1991	<i>Mata ade2-1, ura3-1, TRP1, leu2-3,112, can1-100, his3-111</i>
J1003.1D×1E	Shuai and Warner, 1991	<i>Mata/α ade2-1/ade2-1, ura3-1/ura3-1, TRP1/trp1-1, leu2-3,112/leu2-3,112,can1-100/can1-100, his3-111/HIS3</i>
CTY429	this study	<i>Mata act2-1, ade2-1, ura3-1, TRP1, leu2-3,112, can1-100, his3-111</i>
CTY430	this study	<i>Matα act2-1, ade2-1, ura3-1, trp1-1, leu2-3,112, can1-100, HIS3</i>
SS2	this study	<i>Mata/α act2::HIS3/ACT2, ade2-1/ade2-1, ura3-1/ura3-1, TRP1/trp1-1, leu2-3,112/leu2-3,112,can1-100/can1-100</i>
SS3 ^b	this study	<i>Mata/α act2::HIS3/ACT2, ade2-101/ade2-101, ura3-52/ura3-52, his3Δ200/his3Δ200, tyr1/TYR1, LYS2/lys2</i>
SS4	this study	<i>Mata act2::HIS3, ade2-1, ura3-1, trp1-1, leu2-3,112, can1-100, his3-111, pACT2-6MYC</i>
NOY612	Yano <i>et al.</i> , 1994	<i>Mata srp1-31, ade2-1, ura3-1, trp1-1, leu2-3,112, can1-100, his3-111</i>
NOY613	Yano <i>et al.</i> , 1994	<i>Mata srp1-49, ade2-1, ura3-1, trp1-1, leu2-3,112, can1-100, his3-111</i>
CTY612 ^c	this study	<i>Mata srp1-31, ade2-1, ura3-1,TRP1, leu2-3,112, can1-100, his3-111</i>
CTY613 ^c	this study	<i>Mata srp1-49, ade2-1, ura3-1,TRP1, leu2-3,112, can1-100, his3-111</i>
CTY431 ^d	this study	<i>Mata/α ACT2/act2-1, srp1-31/SRP1, ade2-1/ade2-1, ura3-1/ura3-1, trp1-1/trp1-1, leu2-3,112/leu2-3,112, can1-100/can1-100, his3-111/HIS3</i>
CTY449 ^d	this study	<i>Mata/α ACT2/act2-1, srp1-49/SRP1, ade2-1/ade2-1, ura3-1/ura3-1, trp1-1/trp1-1, leu2-3,112/leu2-3,112, can1-100/can1-100, his3-111/HIS3</i>
LDY461	Kenna <i>et al.</i> , 1996	<i>Mata nup1-2Δ::LEU2, ade2-1, ura3-1, trp1-1, leu2-3,112, his3</i>
CTY461 ^e	this study	<i>Mata/α nup1-2Δ::LEU2/NUP1, ACT2/act2-1, ade2-1/ade2-1, ura3-1/ura3-1, trp1-1/trp1-1, leu2-3,112/leu2-3,112, his3/his3</i>
CTY462 ^e	this study	<i>Mata nup1-2Δ::LEU2, act2-1, ade2-1, ura3-1, trp1-1, leu2-3,112, his3</i>
NUP120-25-3 ^b	Aitchison <i>et al.</i> , 1995	<i>Mata nup120::URA3, ura3-52, leu2-3-112, trp1-1,his3Δ200, lys2-801</i>
CTY435 ^f	this study	<i>Mata/α nup120::URA3/NUP120, ACT2/act2-1, ura3-52/ura3-1, leu2-3-112/leu2-3,112, trp1-1/trp1-1, his3Δ200/his3-111, lys2-80/LYS2, ADE2/ade2-1</i>
Δ133 1.4 ^b	Pemberton <i>et al.</i> , 1995	<i>Mata nup133::HIS3, ura3-52, leu2-3-112, trp1-1,his3Δ200, lys2-801</i>
CTY436 ^f	this study	<i>Mata/α nup133::HIS3/NUP133, ACT2/act2-1, ura3-52/ura3-1, leu2-3-112/leu2-3,112, trp1-1/trp1-1, his3Δ200/his3-111 lys2-80/LYS2, ADE2/ade2-1</i>
DBY1691 ^b	Shortle <i>et al.</i> , 1984	<i>Mata act1-3, his4-619</i>
CTY1691	this study	<i>Mata/α act1-3/ACT1, ACT2/act2-1, ADE2/ade2-1, URA3/ura3-1, TRP1/trp1-1, LEU2/leu2-3,112, his4-619/HIS3</i>
MSY107 ^b	Wertman <i>et al.</i> , 1992	<i>Matα act1-133::HIS3, ade4, ura3-52, leu2-3,112, his3Δ200, tub2-201</i>
CTY107 ^g	this study	<i>Mata/α ACT1/act1-133::HIS3, ade2-1/ade4, ura3-1/ura3-52, leu2-3,112/leu2-3,112, his3-111/his3Δ200, TUB2/tub2-201</i>
BHY32	Haarer <i>et al.</i> , 1993	<i>Mata ade2, ade3, ura3, leu2, his3, lys2, pfy1-116:LEU2</i>
CTY32 ^h	this study	<i>Mata/α ACT2/act2-1, pfy1-116:LEU2/PFY1, ade2/ade2, ade3/ADE3, ura3/ura3, leu2/leu2, his3/his3, lys2/LYS2</i>
CTY33	this study	<i>Mata act2-1, pfy1-116:LEU2, ade2, ade3, ura3, leu2, his3</i>
BHY46	Haarer <i>et al.</i> , 1993	<i>Mata ade2, ade3, ura3, leu2, his3, pfy1-111:LEU2</i>
CTY46 ^h	this study	<i>Mata/α ACT2/act2-1, pfy1-111:LEU2/PFY1, ade2/ade2, ade3/ADE3, ura3/ura3, leu2/leu2, his3/his3, lys2/LYS2</i>
CTY47	this study	<i>Mata act2-1, pfy1-111:LEU2, ade2, ade3, ura3, leu2, his3</i>
CTY102	Shuai and Warner, 1991	<i>Mata prp20, ade2-1, ura3-1, TRP1, leu2-3,112, can1-100, his3-111</i>
DAT1-17 ^b	Amberg <i>et al.</i> , 1992	<i>Matα rat1-1, ura3-52, leu2Δ1, trp1Δ63, his3Δ200</i>

^aParent strains used to generate the Warner temperature-sensitive (Ts) lethal collection are isogenic with W303.

^bNot isogenic with W303.

^cBoth *srp1* conditional alleles were obtained by integration into J1003.1E as described in Materials and methods.

^dConstructed by crossing strain CTY612 or CTY613 to CTY430.

^eThe heterozygous diploid CTY461 was constructed by crossing LDY461 and CTY430. CTY462 is a haploid strain containing both *act2-1* and *nup1-2Δ::LEU2* mutations resulting from tetrad analysis of CTY431.

^fCTY435 was constructed by crossing NUP120-25-3 and CTY430. CTY436 was constructed by crossing Δ133 1.4 and CTY430.

^gConstructed by crossing CTY429 to MSY107.

^hConstructed by crossing strain BHY32 or BHY46 to CTY430.

both endogenous Nsr1p and two NLS β-galactosidase reporter proteins were localized properly to the nucleus at the restrictive temperature in these mutants (data not shown). In addition, we crossed *act2-1* with the two conditional *act1* mutants (*act1-3* and *act1-133*; Table II). The heterozygous diploids grew at 37°C at levels comparable with isogenic wild-type strains. Haploid cells containing double mutations in *act2-1* and *act1-3* or *act1-133* are not synthetically lethal at lower temperatures, suggesting that Act2p might not interact genetically with conventional actin.

Based on a structural model of the *Acanthamoeba* Act2p

homolog, Kelleher *et al.* (1995) proposed that Act2p may contain a conserved profilin-binding site. To begin to test whether Act2p and profilin interact genetically in *S.cerevisiae*, we crossed *act2-1* to two conditional yeast profilin mutants, *pfy1-111* and *pfy-116* (obtained from B.Haarer; Haarer *et al.*, 1993; Table I, BHY46 and BHY32), but no genetic interaction was found (Table II).

The cytoplasmic accumulation of Nsr1p is not due to a lack of retention in the nucleus

A possible explanation for the increased cytoplasmic distribution of the nuclear reporter proteins in *act2-1* cells

Fig. 1. Expression of *ACT2* from a plasmid complements the temperature-sensitive growth and nuclear import defect of *act2-1* cells. *act2-1* cells either lacking (a–f) or bearing a plasmid expressing *ACT2* (pACT2) (g–i) were analyzed for mislocalization of Nsr1p by indirect immunofluorescence at 25°C, and after a 3 h shift to the restrictive temperature (see Materials and methods). mAb 5B5 was used to detect the intracellular localization of Nsr1p. Nsr1p is nucleolar in *act2-1* cells at the permissive temperature of 25°C (a–c), but becomes cytoplasmic after a shift to the restrictive temperature (d–f). However, *act2-1* cells expressing *ACT2* from a plasmid grow at 37°C, and in these cells, Nsr1p is properly localized to the nucleolus at both 25°C (data not shown) and 37°C (g–i). Thus, the nuclear import defect is due to a thermosensitive mutation in the *ACT2* gene. DAPI, anti-Nsr1p and Phase are labeled.

is that the accumulated protein might not be selectively retained in the nucleus. To test this idea, Ura⁺ transformants were grown at 25°C in synthetic complete media lacking uracil and containing 2% galactose (H2B–LacZ hybrid protein expressed) overnight to load the nucleus with the reporter protein. The cells were then washed, resuspended in 2% glucose (to shut off the synthesis of the fusion protein) and then shifted to 37°C. Aliquots were taken at 30 min intervals, and the cellular distribution of the H2B–LacZ hybrid protein was observed by indirect immunofluorescence (Figure 4e–h). After 1.5–2 h at 37°C, *act2-1* cells still retain the H2B–LacZ reporter in the nucleus (Figure 4g–h) at levels comparable with that observed in the wild-type strain (Figure 4e–f). The lack of accumulation of H2B–LacZ reporter in the cytoplasm suggests that the nuclear envelope remains intact. The data above strongly argue that the accumulation of H2B–LacZ in the cytoplasm at early times after shifting to the restrictive temperature is not due to a lack of retention of H2B–LacZ in the nucleus.

Analysis by electron microscopy reveals a striking aberration in the structure of the nuclear pore complex in *act2-1* mutants

Electron microscopy of wild-type yeast cells at 37°C or *act2-1* cells at the permissive temperature of 25°C shows the presence of typical NPCs embedded in the nuclear envelope [Figure 5a–e; arrows point to typical NPCs: note that in b the nucleoplasmic side of the nucleus is labeled (N) and the cytoplasmic side of the nucleus is labeled (C), and the same convention is followed in c–e, g–n and p–q even though they are not labeled]. However, in *act2-1* cells shifted to the non-permissive temperature of

Fig. 2. Analysis of the time of onset of the mislocalization of Nsr1p and of actin staining by indirect immunofluorescence in *act2-1*. As a comparison, the wild-type strain J1003.1D was also analyzed for actin staining. Both *act2-1* and the wild-type strain J1003.1D (wt) were grown to early log phase at 25°C and half of the cultures shifted to the restrictive temperature of 37°C. Aliquots were taken at 15 min intervals for 3 h (0, 15, 30 min and 1.5 h time points are shown), and analyzed by immunofluorescence as described in Materials and methods. *act2-1* cells were stained with mAb 5B5 and an FITC-conjugated goat anti-mouse secondary antibody to visualize Nsr1p staining. Rhodamine-conjugated phalloidin was used to visualize actin staining. The various conditions used are labeled in the figure as follows: actin, anti-Nsr1p and DAPI staining of DNA to visualize the nucleus. In *act2-1* cells, note that Nsr1p is exclusively nucleolar at the 0 min time point, but that cytoplasmic staining becomes evident by 15 min. Actin staining in *act2-1* cells is normal at 1.5 h, as compared with the staining in wild-type cells.

37°C for 30 min, abnormal electron-dense structures are observed which are now on either side of, rather than spanning, the nuclear envelope (Figure 5: in f–j and p, the observed electron densities are indicated by arrows). The shape of the nucleus is not altered in these mutants. When *act2-1* cells are grown at 37°C for 2 h, the aberrant electron-dense structures observed increase in number (Figure 5o; the abnormal electron densities are indicated by arrows) and some become less electron dense. To verify that the electron-dense material observed in *act2-1* represented NPCs, we analyzed the *act2-1* cells by immunoelectron microscopy using mAb414 (Davis and Fink, 1990), which recognizes the class of nucleoporins containing XFXFG repeats (Figure 5k–n and q; see Materials and methods). In *act2-1* cells grown at 25°C, mAb414 staining is visualized as a single gold particle within the NPC (Figure 5: in k and l, the arrows point to the position of the NPC). However, after the cells are shifted to 37°C for 2 h, numerous gold particles are observed that overlap the aberrant densities, strongly arguing that the densities observed are abnormal NPCs (Figure 5: in m, n and q, the arrows point to the normal position of the NPC in the nuclear envelope). In Figure 5m and n, one gold particle resides in the NPC region

Fig. 3. (A) Growth curve of *act2-1* cells. *act2-1* cells were grown at 25°C in YP + 2% glucose medium to early log phase and then diluted to OD₆₀₀ 0.1. Half of the culture was shifted to 37°C, and OD₆₀₀ was measured at 1 h intervals at both 25 and 37°C. OD₆₀₀ versus time (h) was plotted. (B) The viability of *act2-1* cells at 37°C was measured. Aliquots of *act2-1* after the shift to 37°C from 0 to 8 h were plated on YP + 2% glucose at 1:1000 dilution, grown at room temperature and the colonies which grew were counted after 2–3 days. The result is plotted as percentage viability versus time (h). The number of colonies at the 0 time point is plotted at 100% and the subsequent percentages are derived from the number of colonies grown at time (h) divided by the number of colonies grown at the 0 time point.

while two others are present in the nucleoplasm along the inner nuclear membrane. In Figure 5q, a cluster of eight gold particles is observed in the nucleoplasm, adjacent to the NPC. The overall number of aberrant NPCs was quantitated by visual inspection of the electron micrographs and the data are presented in Table III.

To test whether such structures are the result of a defect in the actin cytoskeleton, we analyzed the NPC structure of the temperature-sensitive actin mutant (*act1-3*). When *act1-3* cells were shifted to the non-permissive temperature for 2 h and analyzed by electron microscopy, we could detect no aberrations in NPC or nuclear envelope morphology (data not shown). These experiments strongly suggest that the NPC defect observed in the *act2-1* mutant is not due to non-specific defects in the actin cytoskeleton, suggesting that Act2p plays an important role in maintaining the structural integrity of the NPC.

A change in the stoichiometry of nucleoporins is observed in the *act2-1* mutant

The redistribution of XFXFG nucleoporins at the NPC observed by immunoelectron microscopy of the *act2-1* cells at the non-permissive temperature suggests that there might be a general loss of XFXFG nucleoporins from the NPC. An assembly defect has been reported in cells carrying a deletion of the nucleoporin Nle3p (Nup170p), whereby the stoichiometry of different classes of nucleoporins could be detected by Western blotting (Kenna *et al.*, 1996). To test if this is the case for *act2-1*, total cell extracts of isogenic wild-type and *act2-1* strains were grown at 25°C, shifted to 37°C for 2 h, run on SDS-PAGE and blotted to nitrocellulose. Immunoblots were either probed with mAb 414 (against XFXFG nucleoporins) or mAb 192 (against GLFG nucleoporins) (Figure 6A). *act2-1* cells shifted to the non-permissive temperature showed a decrease in the overall amount of nucleoporins (Figure 6A, panels mAb414 and mAb192, lane 4), in accord with the NPC structural defect observed in *act2-1* analyzed by immunoelectron microscopy (Figure 5m, n and q).

Act2p and Nup1p genetically interact

Our finding that the location of the XFXFG class of nucleoporins is altered in *act2-1* to regions outside that normally observed suggested to us that Act2p might interact with XFXFG nucleoporins (see review by Davis, 1995). To assess whether this was the case, we tested the genetic interaction between *act2-1* and a conditional null allele of *NUP1*. *act2-1* cells were crossed with *nup1-2Δ::LEU2* carrying a full deletion of *NUP1* in a W303 strain background (Table I, LDY461; Kenna *et al.*, 1996). We find that heterozygous diploids recessive for *act2-1* and *nup1-2Δ::LEU2* are lethal at 33–37°C (Table II). Haploids carrying both mutations, although able to grow slowly at 25°C (8 h doubling time), display defects in both nuclear import and RNA export at 25°C, and are dead at 30°C and at temperatures below 18°C (Figure 7A and Table II).

To discern whether the interaction between Act2p and Nup1p is specific, we crossed *act2-1* with strains carrying conditional null alleles in two other nucleoporins that lack peptide repeats, *NUP120* (Aitchison *et al.*, 1995) and *NUP133* (Pemberton *et al.*, 1995). Heterozygous diploids carrying either of the two nucleoporin mutations and the *act2-1* mutation grew normally at 37°C, showing that they do not exhibit unlinked non-complementation (Table II). Our data support a specific genetic interaction either between Act2p and Nup1p, or between Act2p and other proteins that genetically or physically interact with Nup1p.

Act2p and Srp1p genetically interact

The NLS receptor is a heterodimer composed of an α and a β subunit whereby the α subunit recognizes the NLS on incoming nuclear proteins and is directed to the NPC by binding of the β subunit with the XFXFG repeats present in a number of nucleoporins (Görllich *et al.*, 1995; Moroiu *et al.*, 1995; Radu *et al.*, 1995a). The α subunit in yeast is known as Srp1p or Kap60p, and interacts genetically and physically with the nucleoporins, Nup1p and Nup2p (Belanger *et al.*, 1994). Since there was a genetic interaction between *nup1-2Δ::LEU2* and *act2-1*,

Table II. *ACT2* genetically interacts with the nucleoporin, *NUP1*, and the NLS receptor, *SRP1*, but not with 2 conditional alleles of *ACT1* or with the nucleoporins, *NUP120* or *NUP133*

Genotype ^a	Strain ^b	ts/cs (°C) ^c phenotype	Genotype ^a	Strain ^b	ts/cs (°C) ^c phenotype
<i>act2-1</i>	CTY430	37	<i>act2-1</i>	CTY429	37
<i>act1-3</i>	DBY1691	37	<i>act1-133::HIS3</i>	MSY107	37
<i>act1-3xact2-1</i>	CTY1691	grows normally	<i>act1-133::HIS3 xact2-1</i>	CTY107	grows normally
> <i>act1-3 act2-1</i>	CTY1692	37	> <i>act1-133::HIS3 act2-1</i>	CTY108	37
20 tetrads ^d			15 tetrads ^d		
<i>pfy1-111::LEU2</i>	BHY46	37	<i>pfy1-116::LEU2</i>	BHY32	grows normally
<i>pfy1-111::LEU2xact2-1</i>	CTY46	grows normally	<i>pfy1-116::LEU2xact2-1</i>	CTY32	grows normally
> <i>pfy1-111::LEU2xact2-1</i>	CTY47	37	> <i>pfy1-116::LEU2 act2-1</i>	CTY33	37
20 tetrads ^d			20 tetrads ^d		
<i>nup1-2Δ::LEU2</i>	LDY461	37	<i>nup120::URA3</i>	NUP120-25-3	37
<i>nup1-2Δ::LEU2xact2-1</i>	CTY461	33–37	<i>nup120::URA3xact2-1</i>	CTY435	grows normally
> <i>nup1-2Δ::LEU2 act2-1</i> ^e	CTY462	<18, 30	<i>nup133::HIS3</i>	Δ133 1.4	37
40 tetrads ^d			<i>nup133::HIS3xact2-1</i>	CTY436	grows normally
<i>srp1-31</i> ^f	CTY612	37	<i>srp1-49</i> ^f	CTY613	37
<i>act2-1 xsrp1-31</i>	CTY431	33	<i>act2-1 xsrp1-49</i>	CTY449	33
> <i>act2-1 srp1-31</i>		inviable	> <i>act2-1 srp1-49</i>		inviable
> <i>act2-1 srp1-31</i> (pSRP1 TRP1)		viable ura ⁻ Trp ⁺ colonies	> <i>act2-1 srp1-49</i> (pSRP1 TRP1)		viable ura ⁻ Trp ⁺ colonies

^aHeterozygous diploids are indicated by bold lettering and haploids containing double mutations are indicated by (>).^bThe genetic background of strains are listed in Table I. CTY429 (*Mata*) and CTY430 (*Matα*) are the isogenic *act2-1* strains used in all the genetic crosses.^cIndicates the temperature at which a strain displays its mutant phenotype.^dThe number of tetrads analyzed is noted next to each of the haploid strains carrying double mutations.^eThe *nup1 act2* double mutant displays a growth defect at 25°C with a doubling time of 8 h, is dead at 30°C and at temperatures below 18°C.^fPlasmids pNOY172 and pNOY175 were used to integrate *srp1-31* and *srp1-49* respectively into strains isogenic with W303.

we decided to assess whether there was also a genetic interaction between *act2-1* and two different alleles of *SRP1*. Unexpectedly we found that diploids heterozygous for recessive *act2-1* and *srp1* mutations are lethal at 33°C (Figure 7B), and that haploids carrying both these mutations are inviable (Table II).

act2-1 cells were crossed to two strains carrying temperature-sensitive *srp1* mutations, *srp1-31* and *srp1-49* (Yano *et al.*, 1994). All three haploid strains are normally viable at 33°C and die at 37°C. Diploid strains heterozygous for *act2-1* and either of the two *srp1* mutations die at 33°C (Figure 7B and Table II). The Ts⁻ growth in the heterozygous diploid strains can be rescued by a *CEN* plasmid carrying a wild-type copy of either *ACT2* or *SRP1* (Figure 7B).

Our initial attempts to sporulate the heterozygous diploid strains, *srp1-49xact2-1* and *srp1-31xact2-1*, were unsuccessful. To circumvent this problem, we integrated the *srp1-31* and *srp1-49* mutant alleles into *act2-1* using methods described by Rothstein (1983) (see Materials and methods). We were only able to obtain a haploid strain carrying both *srp1* and *act2-1* mutations if we transformed *act2-1* cells with a plasmid containing a wild-type copy of the *SRP1* or *ACT2* gene (Table II). Correct integration into the *SRP1* locus was verified by Southern analysis and PCR. We then integrated both conditional *srp1-31* and *srp1-49* alleles into the wild-type J1003.1E strain background (Table I, CTY612 and CTY613) and crossed them to the *act2-1* mutant. Subsequent tetrad analysis confirmed

that haploids carrying mutations in both *srp1* and *act2-1* were inviable (data not shown). Our data raise the possibility that Act2p and Srp1p functionally interact *in vivo*.

Srp1p and Act2p physically interact

The genetic interaction between Act2p and Srp1p suggested that the two proteins might be physically associated. To ascertain if the two proteins were present in a complex, we carried out co-immunoprecipitations. Fractionated cell extracts from the diploid wild-type strain, J1003.1D×1E, either carrying a plasmid expressing the HA-epitope-tagged version of the Act2p, or containing a plasmid expressing the HA epitope in the absence of Act2p as a control (only genomic Act2p is expressed), were immunoprecipitated with 2625 rabbit anti-Srp1p or anti-HA epitope mAb 16B12 and analyzed by Western blotting (Figure 7C; see Materials and methods). Antibodies against Srp1p co-immunoprecipitate Act2p, and the interaction is only detected in cells expressing the epitope-tagged version of Act2p (Figure 7C, Ip with anti-Srp1p, +HA). Conversely, antibodies against the epitope-tagged version of Act2p co-immunoprecipitate Srp1p (Figure 7C, IP with anti-HA, +HA). To test for specificity and to ask whether the co-immunoprecipitation of Act2p and Srp1p was due to recognition of NLS sequences by Srp1p, the same blots were reprobated with antibody against two nucleolar proteins containing NLSs, Nsr1p (mAb 5B5) and Nop1p (mAb A66), and also conventional yeast actin (C4D6). None of

Fig. 4. Analysis of the ability of *act2-1* cells to import (c and d) or retain (g and h) a H2B-LacZ reporter protein in the nucleus. As a comparison, the wild-type strain J1003.1D was also analyzed, see (a and b) for import and (e and f) for nuclear retention. For both mutant and wild-type cells, the conditions for the nuclear import assay were as follows: Ura⁺ transformants containing pFB1-33C were shifted to the restrictive temperature for 30 min, 2% galactose was then added to induce expression of H2B-LacZ and cells were kept at the restrictive temperature for an additional 1.5 h. To analyze nuclear retention, Ura⁺ transformants were grown in media containing 2% galactose (H2B-LacZ expressed) overnight to pre-load the nucleus with the H2B-LacZ reporter protein. The cells were then switched to media containing 2% glucose (H2B-LacZ expression shut off), and then shifted to the restrictive temperature of 37°C. Aliquots were analyzed by indirect immunofluorescence at 30 min intervals for 3 h (only the 2 h time point is shown). H2B-LacZ and DNA staining are labeled FITC and DAPI, respectively.

the three proteins was detected in the immunoprecipitates (data not shown). To ensure that the precipitation of HA epitope-tagged Act2p only occurs in the presence of anti-HA antibodies, and is not due to non-specific precipitation by protein A beads, yeast extracts of cells expressing HA epitope-tagged Act2p using protein A beads alone was performed (data not shown). In subsequent immunoblots of these precipitates, no HA epitope-tagged Act2p was observed. Thus, our genetic and biochemical data argue strongly that the complex between Act2p and Srp1p is specific.

A nuclear pool of Act2p is found by immunofluorescence and by cellular fractionation

To establish the cellular location of Act2p, either a single copy of a 10 amino acid epitope derived from the HA influenza antigen (HA1) (Green *et al.*, 1982; Field *et al.*, 1988; Materials and methods) was fused to the N-terminus

of Act2p, or six copies (plasmid 6mycD) of a 12 amino acid epitope derived from c-Myc (Roth *et al.*, 1991; Heine *et al.*, 1993) was fused to the C-terminus (Figure 8). Both fusion proteins were expressed under the control of the wild-type *ACT2* promoter and were able to rescue the Ts⁻ phenotype of the *act2-1* mutant (data not shown). Western blot analysis shows that Act2p is present in low abundance in the cell (data not shown). The plasmids carrying the epitope-tagged *ACT2* gene were transformed individually into the diploid wild-type strain J1003.1D×1E (Figure 8A) as well as a diploid strain carrying a wild-type copy of *ACT2* and an *act2::HIS3* disruption (SS2) (Figure 8B, right hand panels which are unlabeled). An *act2* null strain rescued by a plasmid expressing Act2p fused to six copies of the c-Myc epitope (strain SS4, Table I) was also used in these studies (Figure 8B, panels 1 and 2). Cells were subjected to a fast fixation method described by Kilmartin *et al.* (1993) (see Materials and methods). In wild-type cells, both mAb 16B12 against the HA epitope-tagged Act2p (Figure 8A) and mAb 9E10 against the six c-Myc-tagged version of Act2p (Figure 8B) were found at the nucleus. In some instances, preferential staining of the nuclear periphery was observed (Figure 8B). Cells also displayed a punctate cytoplasmic staining which was absent in the wild-type diploid strain lacking the epitope-tagged Act2p. In the *act2* null strain expressing epitope-tagged Act2p, staining is observed at the nucleus and also at cortical actin patches (Figure 8B, panel 2).

To confirm that Act2p is associated with the nucleus, nuclei were isolated according to the procedure of Rout and Blobel (1993), and analyzed for the presence of epitope-tagged Act2p by immunoblotting. Extraction of purified nuclei with various reagents showed that although epitope-tagged Act2p can be partially extracted using DNase or 1 M NaCl, the majority of the protein is only dissociated from the nuclei after treatment with 8 M urea (UR) (Figure 6B, Act2p). These data are consistent with the nuclear association of Act2p observed by indirect immunofluorescence. As a control, the same immunoblots were washed, and then restained with antibody against conventional actin (Figure 6B; actin). In contrast to the extraction properties of Act2p, yeast actin was completely extracted from the nucleus using 1 M NaCl (Na).

To determine whether Act2p is associated with NPCs, we analyzed the location of epitope-tagged Act2p in *nup120* mutants because of the distinct clustering of the NPCs to one side of the nucleus (Aitchinson *et al.*, 1995). The HA-tagged version of Act2p (pACT2HA-HIS3) was transformed into a strain carrying a conditional *nup120* null mutation and the location of the HA-Act2p was analyzed by indirect immunofluorescence. Although we observed an increase in the intensity of nuclear staining, we did not observe NPC staining (Figure 8C).

Discussion

We have found that the divergent actin, Act2p, is required for normal NPC structure and function. Analysis of the conditional *act2-1* mutant by electron microscopy 30 min after a shift to 37°C reveals abnormal discrete densities that lie on the surface but do not traverse the nuclear envelope. Monoclonal antibody against the XFXFG class of nucleoporins stains these aberrant structures, indicating

Fig. 5. Ultrastructural analysis reveals aberrant nuclear pore morphology in the *act2-1* mutant not seen in a conditional mutant in *ACT1*. *act2-1* was grown at 25°C (a–e), and then shifted to 37°C for 30 min (f–j and p) and 2 h (o). Immunoelectron microscopy was performed using mAb 414, an antibody to XFXFG nucleoporins, in *act2-1* at 25°C (k and l) and after a shift to 37°C for 2 h (m, n and q). Arrows in (a–e), (k–n) and (q) point to the position of NPCs embedded in the nuclear envelope. The arrows in (f–j), (o) and (p) point to examples of aberrantly localized NPCs. The nucleus (N), nucleolus (No) and cytoplasm (C) are labeled, and an additional arrow is used to indicate the position of the spindle pole body (Sp) in (f). In (b), the nucleoplasmic face of the nucleus is labeled (N) and the cytoplasmic face of the nucleus is labeled (C); (c–e), (g–n) and (p–q) follow the same convention even though they are unlabeled. Bars: (a, f and o) 0.1 μ m; (b–n and p–q) 0.1 μ m.

that they are morphologically defective NPCs. Most likely, the inability of *act2-1* to import nuclear proteins at the non-permissive temperature is a result of the severe defect

in NPC structure. A direct role in NPC structure and function is strongly suggested by the existence of a nuclear pool of Act2p which was observed by indirect

Table III. Quantitation of abnormal positioning of NPCs observed by electron microscopy in *act2-1* cells^a

Time point	Total number of cells analyzed	Total number of NPCs	Abnormal position of NPCs at the nuclear envelope ^b				Number of normal NPCs	Total % of abnormal NPCs ^d
			Cy face	N face	Cy and N face	↓electron density		
<i>act2-1</i> , 0 min	36	176	0	0	0	1 (0.57%)	175	0.57
<i>act2-1</i> , 30 min	52	361	11 (3.05%) ^c	57 (15.79%)	1 (0.28%)	4 (1.1%)	288	20.22
<i>act2-1</i> , 2 h	12	154	1 (0.65%)	95 (61.69%)	0	14 (9.1%)	44	71.43
<i>act2-1</i> , 3 h	25	255	11 (4.31%)	58 (22.75%)	0	149 (58.43%)	37	85.49

^aQuantitation is based on data from a single experiment in which *act2-1* cells were grown in YPD medium and switched to 37°C for up to 3 h.

^bCy face, cytoplasmic face; N face, nucleoplasmic face; Cy and N face, both cytoplasmic and nucleoplasmic face of the nuclear envelope; decreases (↓) in electron density of NPCs are labeled.

^cAlthough it appears that the total number of NPCs per cell is increasing at later time points, we do not find this to be the case. Rather, the number of NPCs analyzed per cell reflects variations in the plane of section of the nucleus in the electron micrographs.

^dThe percentages obtained are based on the total number of aberrantly localized NPCs divided by the total number of NPCs analyzed.

^eThe percentages within the parentheses are calculated by dividing the fraction of NPCs at the Cy, N, or Cy and N face by the total number of NPCs analyzed.

immunofluorescence and cellular fractionation. Further support comes from the genetic and physical interactions observed between Act2p, the nucleoporin Nup1p and the NLS receptor (Srp1p). *act2-1* is synthetically lethal with a conditional null allele in the nucleoporin gene *NUP1* at temperatures below 18°C and at 30°C. Diploids heterozygous for *act2-1* and either *srp1-31* or *srp1-49* are lethal at 33°C and thus display unlinked non-complementation. Haploids carrying both mutations are synthetically lethal at all temperatures, suggesting that Act2p and Srp1p might function in the same pathway. We also found that Act2p and Srp1p co-immunoprecipitate *in vivo*, supporting the notion that the two proteins are in a complex.

Although we have evidence supporting a role for Act2p in NPC structure, other investigators have suggested that this protein interacts with actin and plays a major role in cytoskeletal function (Machesky *et al.*, 1994; Moreau *et al.*, 1996; Mullins *et al.*, 1997; Welch *et al.*, 1997). Our data argue for an independent role for Act2p in nuclear functions. The mutant allele of *ACT2* that we have isolated confers thermosensitive growth on cells but, at early time points up to 1.5–2 h after a shift to the non-permissive temperature, does not display gross defects in actin or chitin localization, as has been reported for strains carrying mutations in the conventional actin, *ACT1* (Novick and Botstein, 1985). We also observe no defects in spindle positioning or nuclear migration in *act2-1* cells (data not shown). In addition, we have analyzed the conditional *act1-3* for defects in NPC structure or function. Defects in the actin cytoskeleton were observed in the *act1-3* mutant as previously described (Novick and Botstein, 1985), but no defects in nuclear import or nuclear pore morphology were observed. This observation argues that the defect in NPC structure and function observed in *act2-1* cells does not arise from an indirect effect on the actin cytoskeleton. Neither *act1-3* nor *act1-133* were synthetically lethal with *act2-1* (Table II). We favor a specific involvement of Act2p in NPC structure and function because our data provide evidence that aberrations in the morphology of the NPCs and defects in nuclear import occur within 15–30 min after the shift to the non-permissive temperature. During this time period, we found no evidence to suggest a gross alteration in the cytoskeletal organization or nuclear integrity of *act2-1* cells.

The phenotype observed by Moreau *et al.* (1996) for *arp2-H330L* and by our laboratory for *act2-1* at 3 h or

more after shifting to the non-permissive temperature might be due to non-specific effects due to cell death. We have also observed abnormal microtubule structure and an inability of *act2-1* cells to retain NLS reporter proteins in the nucleus during this same time period (3 h), strongly suggesting that the cells are dying. However, this explanation cannot account for the effects on endocytosis observed in *arp2-H330L* at the permissive temperature. Whether Act2p has a direct functional role in endocytosis is unknown, and we are investigating if the observed endocytic defect is allele specific. Since the conditional *ACT2* allele that we have isolated carries a mutation in a conserved ATP-binding domain, and the *arp2-H330L* described by Moreau *et al.* (1996) carries a mutation within a loop at the C-terminus that is divergent from conventional yeast actin, it is possible that these two regions interact with different classes of cellular proteins.

The change in morphology of the NPCs in *act2-1* cells is different from that reported previously for a number of nucleoporin mutants. In these mutants, the NPCs are either clustered together (mutations or deletions of *NUP133/RAT3*, *NUP120/RAT2*, *NUP145* and *NUP159/RAT7*) (Aitchison *et al.*, 1995; Gorsch *et al.*, 1995; Heath *et al.*, 1995; Pemberton *et al.*, 1995; Wentz and Blobel, 1993) or there are gross alterations of the nuclear envelope such that in one case it completely seals the cytoplasmic face of the NPC (deletion in *NUP116*) (Wentz and Blobel, 1994). These mutants also have defects in nuclear import and mRNA export which, like *act2-1*, might be secondary to the gross morphological changes in the NPC. In *act2-1* cells, the NPCs no longer span the nuclear envelope. In *nup120/rat2* and *nup133/rat3* mutants, defects in NPC structure can already be observed at the semi-permissive temperature, but this is not the case for *act2-1*. Both *nup120/rat2* and *nup133/rat3* are conditional deletions such that at all temperatures the nucleoporin is physically absent from the NPC. *act2-1* carries a point mutation in the coding sequence and appears to be a tight temperature-sensitive allele because the NPC defect is only observed after shifting to the non-permissive temperature.

It is striking that a defect in NPC morphology occurs in the *act2-1* mutant as early as 30 min after a switch to the non-permissive temperature. These results suggest that existing pore complexes are collapsing or being released from the nuclear envelope, as opposed to aberrant assembly of newly synthesized pore proteins at improper locations.

Fig. 6. (A) The stoichiometry of nucleoporins in the *act2-1* mutant. Whole cell extracts were prepared, run on an 8% SDS-PAGE and subjected to Western blot analysis using mAb 414 (against XFXGFG nucleoporins) or mAb 192 (against GLFG nucleoporins) as indicated below two of the panels. Wild-type J1003.1D and the *act2-1* mutant were grown at 25°C (panels mAb 414 and mAb 192, lanes 1 and 3) and 37°C (panels mAb 414 and mAb 192, lanes 2 and 4). In the third panel, India ink staining is shown to visualize total protein. Bars on the left correspond to the molecular weight markers (203, 118, 86, 51.6 and 34.1 kDa). **(B)** Nuclear fractionation of six c-Myc epitope-tagged Act2p. An *act2* null strain (SS4) rescued by a plasmid expressing the six c-Myc epitope-tagged *ACT2* (pACT2-6MYC) was grown to early log phase and yeast nuclei were prepared as described by Rout and Blobel (1993). Trapped nuclei and residual cellular debris (L) were first removed from the nuclear fraction by a low speed centrifugation procedure (see Materials and methods) and the resulting pellet fraction was dissolved in Laemmli buffer. The supernatant containing the pure nuclear fraction was fractionated as described by Aris and Blobel (1989, 1991). The nuclei (N) were digested with DNase (Dn) and centrifuged, resulting in a supernatant (S) and pellet (P) fraction. The DNase pellet was extracted with 1 M NaCl (Na), centrifuged and then extracted with 8 M urea (UR). The DNase pellet was also extracted with 1 M NaCl with 1% Triton X-100 detergent (NaTx). Protein concentrations were measured by a Lowry assay, and then samples were loaded evenly, resolved by SDS-PAGE and blotted onto two nitrocellulose filters. The blots were probed with mAb 9E10 (anti-c-Myc antibody) and C4D6 (anti-actin antibody). The second nitrocellulose sheet was stained with India ink, as indicated, to visualize total protein. The wild-type haploid strain J1003.1D lacking the epitope-tagged Act2p (Wt-myc) is shown as a control.

Perhaps Act2p acts as a scaffolding protein, allowing the anchoring of specific nucleoporins to the NPC, either directly or by way of other proteins associated with the filamentous arrays extending from the NPC. One might expect that a collapse of the pore complexes on both sides of the nuclear envelope would not only result in a nuclear import defect, but also in an mRNA export defect, which is not the case for the *act2-1* mutants. Although, poly(A)⁺ RNA accumulation in the nucleus was observed in

control strains carrying mutations in *prp20* and *rat1-1* (see Materials and methods and Table I, CTY102 and DAT1-17), there was no poly(A)⁺ accumulation observed for up to 3 h after a switch to the restrictive temperature in *act2-1* cells (data not shown). Perhaps at early time points, when the number of defective pores is limited, nuclear import is affected to a greater degree than mRNA export.

act2-1 and a null allele (*nup1-2Δ::LEU2*) of the nucleo-

Fig. 7. (A) *act2-1* and a conditional *nup1* null allele are synthetically lethal at 30°C and temperatures below 18°C. Cells were grown to saturation and serial dilutions were spotted onto four YPD plates and grown at 18, 25, 30 and 37°C for 3 days. The results from one set of dilutions from each plate are shown with a comparison with the wild-type haploid strain, J1003.1D. (B) Diploids heterozygous for recessive mutations in *ACT2* and *SRP1* are lethal at 33°C. *act2-1* was crossed to *srp1-49* (data shown in this figure) and *srp1-31* (data not shown). All three haploid strains grow normally at 33°C (*act2-1* and *srp1-49* are shown) and are thermosensitive at 37°C. Heterozygous diploids resulting from a cross to wild-type haploid strains, J1003.1D or J1003.1E, grow normally and are not thermosensitive at 37°C (*srp1-49*×J1003.1D and *act2-1*×J1003.1E are shown). The *srp1-49*×*act2-1* diploid, which is thermosensitive at 33°C, can be partially rescued by a wild-type copy of either *SRP1* or *ACT2*. Plasmid pKBB12 contains a wild-type copy of *SRP1*, and pACT2 contains a wild-type copy of *ACT2*. pCT3 was transformed into the diploid *srp1-49*×*act2-1* strain as a control. Genotypes of strains are shown in Table I. (C) Co-immunoprecipitation of Act2p and Srp1p. Yeast extracts were immunoprecipitated with anti-Srp1p or anti-HA epitope antibodies as described in Materials and methods. Arrows indicate the position of HA epitope-tagged Act2p and endogenous Srp1p. +HA indicates cells expressing HA-tagged Act2p. -HA indicates cells not expressing HA-tagged Act2p.

porin *NUPI* are synthetically lethal at 30°C and at temperatures below 18°C, and only grow poorly at room temperature with an 8 h doubling time. *ACT2* is one of a number of genes that when mutated confers lethality in combination with a null mutation in *NUPI*. Such synthetic lethality suggests a functional or physical association between Act2p and the other Nup1p-interacting proteins, and possibly with Nup1p itself.

Fig. 8. Immunolocalization of HA and c-Myc epitope-tagged versions of Act2p. (A) The wild-type J1003.1D×1E diploid strain bearing a plasmid expressing the HA epitope-tagged *ACT2* (+HA-Act2p) was analyzed by indirect immunofluorescence using anti-HA antibody mAb 16B12 (Cy3) as described in Materials and methods. J1003.1D×E cells lacking pACT2HA were used as a control (-HA-Act2p). (B) A diploid strain carrying a wild-type copy of *ACT2* and an *act2::HIS3* disruption (SS2) (Cy3; right hand panels) and an *act2* null strain (SS4) (see Cy3; panels 1 and 2), both bearing a plasmid expressing the six c-Myc epitope-tagged Act2p, were analyzed by indirect immunofluorescence using anti-c-Myc antibody, mAb 9E10. (C) The localization of the HA epitope-tagged Act2p in the nuclear pore clustering strain *nup120::URA3*. The indirect immunofluorescence was performed as described in Materials and methods. The left panel is Cy3 (antibody staining) and the right panel is DAPI (DNA staining) as labeled in (B).

In support of this idea, we have observed that the *act2-1* mutant does not complement two conditional *srp1* mutants. Although such unlinked non-complementing mutations may identify genes that encode interacting proteins, another alternative is that the *act2-1* mutation in a heterozygous condition might act as a 'weak-link', possibly in the nuclear import pathway (Stearns and Botstein, 1988). In this view, additional stress caused by being heterozygous for mutations in the same pathway, e.g. *srp1*, would cause a block in the nuclear import pathway, leading to cell death. If Act2p is required for the normal distribution of nucleoporins within the NPC, we would expect even less efficient nuclear import when this defect is combined with a defect in the NLS receptor. In this view, the observed genetic interaction between Srp1p and Act2p might be mediated by a common interaction with a nucleoporin. By biochemical analysis, Srp1p and Act2p co-immunoprecipitate, indicating that they do form a complex but it is unclear if they directly interact.

Epitope-tagged versions of Act2p (six c-Myc epitopes at the C-terminus or one HA epitope at the N-terminus) are found associated with the nucleus by indirect immuno-

fluorescence. We also observe Act2p at the nucleus and in cortical actin patches when epitope-tagged Act2p is expressed episomally in an *act2* null strain. In a *nup120* mutant which displays pore clustering to one side of the nucleus, the nuclear staining was more intense, but we did not observe clustering of Act2p. We have also observed that Act2p is enriched in purified nuclei isolated from yeast. Extraction of the purified nuclei with urea is required for full dissociation of Act2p, whereas conventional actin is totally extractable with salt. These data argue that Act2p is tightly associated with the nucleus. The difference in extraction properties of Act2p and conventional actin suggests that this association is likely to be specific and not a result of the collapse of Act2p onto the surface of the nucleus.

A trivial explanation for the interaction between Act2p and Srp1p is that Srp1p is required for import of Act2p into the nucleus. However, as stated in the Results, we were unable to observe two other NLS-containing proteins in the co-immunoprecipitates, the nucleolar proteins Nsr1p and Nop1p, and Act2p does not appear to contain a defined NLS, thus this explanation is unlikely to be the case.

The observed defects in NPC structure and nuclear protein import exhibited by *act2-1* cells at the non-permissive temperature, and the association of Act2p with the nucleus by indirect immunofluorescence and cellular fractionation, argue strongly that Act2p plays an essential role in NPC structure and function. We are now in a unique position to elucidate the specific function of Act2p in these processes by continuing to study its interaction with the NLS receptor, Srp1p, and the nucleoporin Nup1p.

Materials and methods

Strains, media, molecular techniques and miscellaneous chemicals

Escherichia coli strain DH5 α was used for all cloning. The genotypes of yeast strains used in this study are listed in Table I. The Ts⁻ lethal *S. cerevisiae* collection was obtained from Jon Warner (Albert Einstein Medical College, NY) and is described by Shuai and Warner (1991). The genotype of the parent strains, J1003.1D and J1003.1E are listed in Table I. The two conditional mutants in *SRP1*, *srp1-31* and *srp1-49*, were obtained from M. Nomura (Yano *et al.*, 1994). The conditional mutants in *ACT1*, *act1-3* and *act1-133* (described by Shortle *et al.*, 1984; Wertman *et al.*, 1992), were obtained from M.G. Smith and Lisa Pon (Columbia University, NY). The conditional null alleles of *NUP1*, *NUP120* and *NUP133* were obtained from Laura Davis (Brandeis University, MA), John Aitchison and Lucy Pemberton (Rockefeller University, NY) respectively. Standard media preparation and growth of yeast cell cultures were carried out according to Sherman *et al.* (1986). Yeast transformation was done using the lithium acetate method of Ito *et al.* (1983). Molecular biological techniques were carried out according to Sambrook *et al.* (1989). All oligonucleotides used were synthesized and HPLC purified by Operon Technologies Inc. (Alameda, CA). Rhodamine-conjugated phalloidin, used to visualize the actin staining pattern, was obtained from Molecular Probes, Inc. (Eugene, OR). Calcofluor, used to observe chitin staining of bud scars, was obtained from Sigma (Fluorescent Brightener 28, Sigma Chemical Co., St. Louis, MO). Rat anti-tubulin antibody YOL1/34 was obtained from Serotec (Washington, DC). Broad range pre-stained molecular weight standards used for Western blot analysis were obtained from Bio-Rad Laboratories (Hercules, CA).

Plasmids

pCT3 is a *CEN4 URA3* plasmid (provided by Craig M. Thompson, Whitehead Institute, MA) described in Thompson *et al.* (1993). pACT2-1 is a 5 kb *Clal-EcoRI* fragment in pCT3 containing the full *ACT2* gene

and the 3' ends of the *RPK1* and *PRP9* genes (Schwob and Martin, 1992; Poch *et al.*, 1994).

pACT2 was created by inserting a 1.5 kb PCR fragment containing the endogenous *ACT2* promoter and coding regions (+1 to +1501) into the Klenow blunt-ended *BamHI-KpnI* sites of the polylinker region in pCT3. Top strand oligonucleotide, TGAAATCTCTCTGTGATATG and bottom strand oligonucleotide, AATCTATCTGGTACCAAAATTTAGT-CATTGC, in which a *KpnI* site is generated at the end of the gene, were used as primers in the PCR reaction. pACT2-1 was used as template. The nucleotide changes used to generate the *KpnI* site in the bottom strand are shown in bold.

pACT2-6MYC was created by insertion of a *HincII-SmaI* fragment from 6mycD, obtained from P. Dimario (Heine *et al.*, 1993), into the engineered *KpnI* site in pACT2MYC. To create pACT2HA, the *BclI* site of pACT2 was modified to *MluI*, and an influenza virus HA epitope tag (YPYDVPDYAS) engineered with *MluI* ends was inserted into this site. For pACT2HA-HIS3, a blunt-ended *BamHI* fragment containing the *HIS3* selectable marker was inserted into the filled-in *Sall* site in the multiple cloning site in pACT2HA-N.

pKBB12, containing a wild-type copy of the *SRP1* gene, was obtained from Ken Belanger and L.I. Davis (Belanger *et al.*, 1994). pACT2::HIS3 contains a disruption of the *ACT2* gene using the *HIS3* selectable marker. The open reading frame of *ACT2* was removed by *BclI-NdeI* digestion, and a *BamHI* fragment containing the *HIS3* gene was inserted after all sites were made blunt using Klenow.

Integration plasmids pNOY172, carrying the *srp1-31* allele, and pNOY175, carrying the *srp1-49* allele and *URA3* selectable marker, were obtained from Michelle Tabb and M. Nomura.

The following *URA3* plasmids containing different hybrid proteins were used in our studies: the plasmid pFB1-33C containing the NLS of histone H2B fused to β -galactosidase under the *GAL10* promoter (Moreland *et al.*, 1987); and the *SNF2* gene, a transcriptional regulator involved in glucose repression, fused to β -galactosidase under the *GAL10* promoter (Laurent *et al.*, 1991).

Immunofluorescence screen for nuclear import mutants

In a primary screen for nuclear import mutants, yeast strains were analyzed by immunofluorescence after growth at 25°C in YPD medium and after shifting cells to the non-permissive temperature of 37°C for 3 h using established protocols (Pringle *et al.*, 1989). As a primary antibody, we used a 1:2 dilution of a monoclonal antibody against Nsr1p, mAb 5B5, obtained from John Kilmartin (MRC, Cambridge, UK). mAb 5B5 is one pool of mouse monoclonal antibodies raised against a purified spindle pole body preparation from *S. uvarum*; one of the first genes cloned using mAb 5B5 was *NSR1* (J. Kilmartin, unpublished results). Secondary antibody incubations, microscopy and photography are described by Yan and Mélése (1993).

To confirm the primary screen, we used a secondary screen whereby yeast strains harboring pSNF2-LACZ or pFB1-33C were grown and then analyzed by indirect immunofluorescence at 25°C and after shifting cells to the non-permissive temperature for 2 h. Cells were grown to early log phase at 25°C in synthetic complete medium lacking uracil and containing 2% raffinose; 2% galactose was then added to the medium to allow expression of the different fusion proteins. The galactose was added either simultaneously at the time of the shift to the non-permissive temperature, or 30 min after shifting cells to the non-permissive temperature. The cellular distribution of the hybrid proteins was visualized using a monoclonal antibody against β -galactosidase (Promega Corp., Madison, WI) followed by incubation with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch, Inc., West Grove, PA).

Cloning, sequencing and in situ hybridization of *nid2*

In all cases, the Ts⁻ lethal and mislocalization phenotypes co-segregated, allowing the assignment of a number of complementation groups. Due to the early onset of the nuclear import block in the *nid2* mutant, we sought to identify the gene by transforming the cells with a *CEN4 URA3* yeast genomic library (provided by Craig M. Thompson, Whitehead Institute, MA; Thompson *et al.*, 1993). Transformants were tested for growth at 37°C. To confirm that the ability to grow at 37°C was due to the presence of the plasmid, Ura⁺ transformants were replica plated onto 5-fluoro-orotic acid (5-FOA) to select for loss of the plasmid. Ura⁻, 5-FOA^R colonies were patched on to YPD plates and incubated at 37°C to rescreen for Ts⁻ growth. Plasmids from Ura⁺, 5-FOA^S transformants of the original isolates were transformed into the *E. coli* strain DH5 α . Standard molecular biology techniques were used to restriction map and isolate the smallest complementing DNA fragment,

and dideoxy sequencing was carried out using a Sequenase kit (United States Biochemical, Cleveland, OH) according to the manufacturer's procedure. The temperature-sensitive *act2-1* allele was cloned by PCR and sequenced. We identified a mutation at amino acid 161 resulting in a Gly→Val change which is the only mutation in the *ACT2* coding region. This mutation was introduced into the *ACT2* gene in a wild-type haploid strain and found to be Ts⁻ for growth and to mislocalize Nsr1p to the nucleus, confirming that the mutations in *nid2* and *ACT2* are the cause of the observed phenotypes.

To look for a defect in mRNA export, the *nid2* mutant was grown in YPD medium at 25°C, switched to 37°C and samples were taken at 15 min intervals for up to 3 h and fixed. *In situ* hybridization was carried out following the procedure described by Amberg *et al.* (1992). RNA distribution in the cell was visualized after hybridization with digoxigenin-labeled (dT)₅₀ probe and incubation with an FITC-conjugated anti-digoxigenin Fab fragment (Boehringer Mannheim). Two strains which display nuclear retention of poly(A)⁺ RNA were used as controls. *rat1-1* was obtained from David Amberg (Amberg *et al.*, 1992; Table I, DAT1-17) and the *prp20* mutant (Aebi *et al.*, 1990) was identified by us in the Warner temperature-sensitive collection (Shuai and Warner, 1991; Table I, CTY102).

Strain manipulations

Disruptions of a chromosomal copy of *ACT2* in the diploid strains, J1003.1D×1E (SS2) and SS3 (Table I), were accomplished by transformation of a linear *PvuII* fragment from pACT2::HIS3 containing the *HIS3*-disrupted copy of *ACT2*. Transformants were subjected to tetrad analysis. The disruptions were verified by Southern analysis.

Haploid strains carrying both *act2-1* and *srp1* mutations were engineered by integration of conditional *srp1* alleles (using integration plasmids pNOY172 and pNOY175 carrying a *URA3* marker) into either *act2-1* or the isogenic wild-type strain J1003.1E. Plasmids carrying the *srp1-31* allele (pNOY172) or the *srp1-49* allele (pNOY175) were linearized using *EcoRI*, transformed using the standard LiAc method (Ito *et al.*, 1983) and transformants were selected on selective complete medium lacking uracil. Ura⁺ transformants were patched on to 5-FOA plates to select for excision of the *URA3* marker with either the wild-type or conditional allele of *SRP1*, and ura⁻ colonies were then tested for Ts⁻ growth. Proper integrations were verified by both Southern analysis and PCR. In *act2-1* cells, we were unable to obtain Ura⁻, 5-FOA^R colonies unless we first transformed the cells with a plasmid containing either a wild-type copy of *SRP1* or *ACT2*, suggesting to us that *ACT2* and *SRP1* are synthetically lethal. To follow the *act2-1* allele in crosses to *srp1-31* (CTY612) and *srp1-49* (CTY613), non-parental ditype tetrads only displayed two viable wild-type spores, confirming that *ACT2* and *SRP1* are synthetically lethal at all temperatures.

To follow the *act2-1* allele in crosses to the conditional *act1-3* and *act1-133* mutants, tetrad analyses were performed and the Ts⁻ phenotype of the non-parental ditype tetrads were followed. All Ts⁻ spores displayed conditional growth at 37°C but not at lower temperatures, suggesting that *act2-1* was not synthetically lethal with *act1-3* or *act1-133*. In the *act2-1* cross to the *NUP1* null allele, *nup1-2Δ::LEU2*, *act2-1* and *nup1* were followed by their Ts⁻ growth phenotype and the *LEU2* marker in the non-parental ditype tetrads.

Electron microscopy and immunoelectron microscopy

For electron microscopy, *act2-1* was grown in YPD medium to mid-log phase at 25°C, switched to 37°C, and samples were taken at 0 min, 30 min and 2 h. Cells were harvested, and then fixed in pre-warmed 1% glutaraldehyde at 30°C for 10–15 min, spun down for 3 min and washed twice in 1.2 M sorbitol/0.1 M KPO₄ pH 7.4. Fixed cells were spheroplasted and processed as previously described (Reynolds, 1963; Small *et al.*, 1987), and sections were stained with both uranyl acetate and lead citrate. For immunoelectron microscopy, post-immunolabeling using mAb 414 was performed essentially as described by Smith and Blobel (1994).

Immunoprecipitations, nuclear fractionations and immunoblot analysis

The diploid strain, J1003.1D×1E, containing HA epitope-tagged *ACT2* (pACT2HA) under its endogenous promoter was grown to mid-log phase at 30°C in selective medium to maintain the plasmid. For immunoprecipitations, cells were lysed and fractionated as described by Baker *et al.* (1988) or prepared following the procedure for subcellular fractionation of Aris and Blobel (1989). Diploid cells lacking the pACT2HA plasmid were used as a control. The crude nuclear pellet was fractionated further in a buffer containing 0.5 M NaCl as described

by Bogerd *et al.* (1994). The 2652 rabbit anti-Srp1p antibody (obtained from L.I. Davis) and monoclonal antibody against the HA epitope, 16B12 (BAbco), were covalently coupled to protein A-Sepharose using dimethylpimelimidate as described by Harlow and Lane (1988). Beads were incubated with yeast extracts in 50 mM Tris pH 7.5, 5 mM MgCl₂, 500 mM NaCl, 5 mM EDTA, 20 mM HEPES pH 7.3, 1% Triton X-100, 0.25% SDS at 4°C, and washed twice in the same buffer and twice in 10 mM Tris pH 7.5, 50 mM NaCl at 1000 r.p.m. for 10 s. Bound protein was eluted in Laemmli buffer, after boiling for 10 min at 95°C, and subjected to 10.5% SDS-PAGE.

For nuclear fractionations, nuclei from an *act2* null strain containing six c-Myc epitope-tagged Act2p (SS4) were prepared using the method described by Rout and Blobel (1993). The isolated nuclei were resuspended in PSM buffer (20 mM potassium phosphate, pH 7.0, 0.25 M sucrose, 0.1 mM MgCl₂, 1 mM dithiothreitol), frozen in liquid N₂, thawed on ice and then spun at 10 000 r.p.m. for 10 min at 4°C. The pellet containing the nuclei was resuspended in PSM buffer and centrifuged at 3000 r.p.m. to remove any remaining cellular debris. The pellet fraction from this low speed spin (L) was resuspended in Laemmli buffer. The supernatant fraction containing the pure nuclei was fractionated exactly as described by Aris and Blobel (1989, 1991). The purity of the nuclei was verified by phase microscopy and by staining the nuclei with DAPI (data not shown). Protein concentrations were determined by Lowry assay prior to loading. Samples were dissolved in Laemmli buffer and run on a 10.5% SDS-PAGE.

Immunoblotting was performed as described by Lee *et al.* (1991), except gelatin was replaced with 5% non-fat milk for the blocking step. Nitrocellulose blots were probed with mAb 16B12, 2652 rabbit anti-Srp1p antibody, mAb 5B5, mAb A66 (antibody against the nucleolar protein Nop1p, obtained from John Aris; Aris and Blobel, 1988) and anti-actin antibody, C4D6 (obtained from Chloe Bulinski, Columbia University, NY), and protein expression was detected with either alkaline phosphatase-conjugated goat anti-mouse or goat anti-rabbit antibody (Bio-Rad Laboratories, Richmond, CA), or using the ECL system (Amersham).

Immunolocalization of an epitope-tagged version of Act2p

pACT2-6MYC and pACT2HA were both transformed into the wild-type diploid strain, J1003.1D×1E, or into SS2, a diploid strain carrying an *act2::HIS3* null allele, and grown in synthetic medium lacking uracil to maintain the plasmids. An *act2* null strain (SS4) rescued by pACT2-6MYC was also used in these studies. To visualize Act2p, cells were subjected to a fast fixation method (Michael Rout, personal communication; Kilmartin *et al.*, 1993) in 0.1 M KPO₄ pH 6.5 containing 3.7% formaldehyde for 8–30 min (conditions optimized for nucleoporin staining), spheroplasted in 30 µg/ml Zymolase 100T or 1:100 glusulase, 5 µg/ml Zymolase 100T for 1 h at 30°C, and analyzed by indirect immunofluorescence. After cells were adhered to slides, they were permeabilized further in -20°C methanol for 5 min followed by 30 s in acetone, and were blocked with Milk buffer [2% milk, 0.1% Tween-20 in 1× phosphate-buffered saline (PBS)] during all antibody incubations (Rout and Kilmartin, 1990) or were first blocked in 1% Triton buffer containing 1.2 M sorbitol/1× PBS/2% milk for 5 min prior to all antibody incubations. Primary antibody incubations were carried out using a rabbit polyclonal antibody or mAb 16B12 (BAbco) against the HA epitope tag, or mAb 9E10C (Oncogene Science, Inc., Cambridge, MA) against the c-Myc epitope, both at 1 µg/ml concentrations. The cellular distribution of epitope-tagged Act2p was visualized using Cy3-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Jackson ImmunoResearch, Inc., West Grove, PA). The cellular distribution of actin was observed using either rhodamine-conjugated phalloidin, an affinity-purified goat anti-actin polyclonal antibody (obtained from D. Drubin, University of California, Berkeley, CA) or mAb C4D6.

Acknowledgements

We thank Magdi, Rachel Brenner, Jessica Cohen, William Lee, Needhi Bhalla and Yaacov Anziska (Summer Undergraduate Research Fellows at Columbia University) for their assistance during the initial immunofluorescent screen of the thermosensitive yeast mutants, and Jessica Feldman for technical assistance. We are indebted to Helen Shio (Electron Microscopy Service, Rockefeller University, NY) for her excellent work in analyzing the mutants by electron and immunoelectron microscopy. We also thank Jim Erickson for use of his imaging system and helpful discussions, and Laura Davis and Michael Rout for critical reading of the manuscript. We are especially grateful to Jon Warner and

Lenore Neugeborn for providing the thermosensitive yeast collection; John Kilmartin for the mAb 5B5 antibody; Ken Belanger and Laura Davis for providing us with the anti-Srp1p antibody; David Drubin and Chloe Bulinski for anti-actin antibodies; Michelle Tabb and Masayasu Nomura for providing the *srp1* mutant strains and conditional *srp1* integration plasmids; Lisa Pon for the conditional *act1* mutants; B. Haarer for profilin mutants; and John Aitchison and Lucy Pemberton, respectively, for the conditional *nup120* and *nup133* null strains. Additional thanks to Michael Rout in Günter Blobel's laboratory for providing us with reagents and many helpful suggestions concerning the immunofluorescence protocols. This work was supported by a National Institutes of Health (NIH) grant GM-44901-01 to T.M., and C.Y. is supported by a GANN Fellowship from the U.S. Department of Education.

References

- Adam, E.J.H. and Adam, S.A. (1994) Identification of cytosolic factors required for nuclear location sequence-mediated binding to the nuclear envelope. *J. Cell Biol.*, **125**, 547–555.
- Adam, S.A. and Gerace, L. (1991) Cytosolic proteins that specifically bind nuclear location signals are receptors for nuclear import. *Cell*, **66**, 837–847.
- Adams, A.E.M. and Pringle, J.R. (1991) Staining of actin with fluorochrome-conjugated phalloidin. *Methods Enzymol.*, **194**, 729–731.
- Aebi, M., Clark, M.W., Vijayraghavan, U. and Abelson, J. (1990) A yeast mutant, *PRP20*, altered in mRNA metabolism and maintenance of the nuclear structure, is defective in a gene homologous to the human gene *RCC1* which is involved in the control of chromosome condensation. *Mol. Gen. Genet.*, **224**, 72–80.
- Aitchison, J.D., Blobel, G. and Rout, M.P. (1995) Nup120p: a yeast nucleoporin required for NPC distribution and mRNA transport. *J. Cell Biol.*, **131**, 1659–1675.
- Amberg, D.C., Goldstein, A.L. and Cole, C.N. (1992) Isolation and characterization of *RAT1*: an essential gene of *Saccharomyces cerevisiae* required for the efficient nucleocytoplasmic trafficking of mRNA. *Genes Dev.*, **6**, 1173–1189.
- Aris, J.P. and Blobel, G. (1988) Identification and characterization of a yeast nucleolar protein that is similar to a rat liver nucleolar protein. *J. Cell Biol.*, **107**, 17–31.
- Aris, J.P. and Blobel, G. (1989) Yeast nuclear envelope proteins cross react with an antibody against mammalian pore complex proteins. *J. Cell Biol.*, **108**, 2059–2067.
- Aris, J.P. and Blobel, G. (1991) Isolation of yeast nuclei. *Methods Enzymol.*, **194**, 735–749.
- Baker, D., Hicke, L., Rexach, M., Schleyer, M. and Schekman, R. (1988) Reconstitution of *SEC* gene product-dependent intercompartmental protein transport. *Cell*, **54**, 335–344.
- Belanger, K.D., Kenna, M.A., Wei, S. and Davis, L.I. (1994) Genetic and physical interactions between Srp1p and nuclear pore complex proteins Nup1p and Nup2p. *J. Cell Biol.*, **126**, 619–630.
- Belhumeur, P., Lee, A., Tam, R., DiPaola, T., Fortin, N. and Clark, M.W. (1993) *GSP1* and *GSP2*, genetic suppressors of the *prp20-1* mutant in *Saccharomyces cerevisiae*: GTP binding proteins involved in the maintenance of nuclear organization. *Mol. Cell Biol.*, **13**, 2152–2160.
- Bischoff, F.R., Kreber, H., Kempf, T., Hermes, I. and Ponstingl, H. (1995) Human Ran GTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl Acad. Sci. USA*, **92**, 1749–1753.
- Boger, A.M., Hoffman, J.A., Amberg, D.C., Fink, G.R. and Davis, L.I. (1994) *nup1* mutants exhibit pleiotropic defects in nuclear pore complex function. *J. Cell Biol.*, **127**, 319–332.
- Clark, S.W. and Meyer, D.I. (1994) *ACT3*: a putative centractin homologue in *S.cerevisiae* is required for proper orientation of the mitotic spindle. *J. Cell Biol.*, **127**, 129–138.
- Cordes, V.C., Reidenbach, S., Kohler, A., Stuurman, N., van Driel, R. and Franke, W.W. (1993) Intracellular filaments containing a nuclear pore complex protein. *J. Cell Biol.*, **123**, 1333–1344.
- Davis, L.I. (1995) The nuclear pore complex. *Annu. Rev. Biochem.*, **64**, 865–896.
- Davis, L.I. and Fink, G.R. (1990) The *NUP1* gene encodes an essential component of the yeast nuclear pore complex. *Cell*, **61**, 965–978.
- Dingwall, C. and Laskey, R. (1991) Nuclear targeting sequences—a consensus? *Trends Biochem. Sci.*, **16**, 478–481.
- Dingwall, C., Kandels-Lewis, S. and Séraphin, B. (1995) A family of Ran binding proteins that includes nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 7525–7529.
- Feldherr, C.M. (1962) The nuclear annuli as pathways for nucleocytoplasmic exchanges. *J. Cell Biol.*, **14**, 65–72.
- Feldherr, C., Kallenbach, E. and Schultz, N. (1984) Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.*, **99**, 2216–2222.
- Field, J., Nikawa, J.-I., Broek, D., MacDonald, B., Rodgers, L., Wilson, I.A., Lerner, R.A. and Wigler, M. (1988) Purification of a RAS-responsive adenyl cyclase complex from *Saccharomyces cerevisiae* by use of an epitope addition method. *Mol. Cell Biol.*, **8**, 2159–2165.
- Fyrberg, C., Ryan, L., Kenton, M. and Fyrberg, E. (1994) Genes encoding actin-related proteins in *Drosophila*. *J. Mol. Biol.*, **241**, 498–503.
- Goldberg, M.W. and Allen, T.D. (1995) Structural and functional organization of the nuclear envelope. *Curr. Opin. Cell Biol.*, **7**, 301–309.
- Görllich, D., Prehn, S., Laskey, R.A. and Hartmann, E. (1994) Isolation of a protein that is essential for the first step of nuclear protein import. *Cell*, **79**, 767–778.
- Görllich, D., Kostka, S., Kraft, R., Dingwall, C., Laskey, R., Hartmann, E. and Prehn, S. (1995) Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.*, **5**, 383–392.
- Gorsch, L.C., Dockendorff, T.C. and Cole, C.N. (1995) A conditional allele of the novel repeat-containing yeast nucleoporin *RAT1/NUP159* causes both rapid cessation of mRNA export and reversible clustering of nuclear pore complexes. *J. Cell Biol.*, **129**, 939–955.
- Green, N., Alexander, H., Olson, A., Alexander, S., Shinnik, T.M., Sutcliffe, J.G. and Lerner, R.A. (1982) Immunogenic structure of the influenza virus hemagglutinin. *Cell*, **28**, 477–487.
- Haarer, B.K., Petzold, A.S. and Brown, S.S. (1993) Mutational analysis of yeast profilin. *Mol. Cell Biol.*, **13**, 7864–7873.
- Harata, M., Karwan, A. and Wintersberger, U. (1994) An essential gene of *Saccharomyces cerevisiae* coding for an actin-related protein. *Proc. Natl Acad. Sci. USA*, **91**, 8258–8262.
- Harlow, E. and Lane, D. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Heath, C.V., Copeland, C.S., Amberg, D.C., Del Priore, V., Snyder, M. and Cole, C.N. (1995) Nuclear pore complex clustering and nuclear accumulation of poly(A)⁺ RNA associated with mutation of the *Saccharomyces cerevisiae* *RAT2/NUP120* gene. *J. Cell Biol.*, **131**, 1677–1697.
- Heine, M.A., Rankin, M.L. and DiMario, P.J. (1993) The Gly/Arg-rich (GAR) domain of *Xenopus* nucleolin facilitates *in vitro* nucleic acid binding and *in vivo* nucleolar localization. *Mol. Biol. Cell*, **4**, 1189–1204.
- Herman, I.M. (1993) Actin isoforms. *Curr. Opin. Cell Biol.*, **5**, 48–55.
- Imamoto, N., Tachibana, T., Matsubae, M. and Yoneda, Y. (1995) A karyophilic protein forms a stable complex with cytoplasmic components prior to nuclear pore binding. *J. Biol. Chem.*, **270**, 8559–8565.
- Ito, H., Jukuda, Y., Murata, K. and Kimura, A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.
- Kabsch, W., Mannherz, H.G., Suck, D., Pai, E.F. and Holmes, K.C. (1990) Atomic structure of the actin:DNase I complex. *Nature*, **347**, 37–49.
- Kelleher, J.F., Atkinson, S.J. and Pollard, T.D. (1995) Sequences, structural models and cellular localization of the actin-related proteins Arp2 and Arp3 from *Acanthamoeba*. *J. Cell Biol.*, **131**, 385–397.
- Kenna, M.A., Petranka, J.G., Reilly, J.L. and Davis, L.I. (1996) Yeast Nle3p/Nup170p is required for normal stoichiometry of FG nucleoporins within the nuclear pore complex. *Mol. Cell Biol.*, **16**, 2025–2036.
- Kilmartin, J.V., Dyos, S.L., Kershaw, D. and Finch, J.T. (1993). A spacer protein in the *Saccharomyces cerevisiae* spindle pole body whose transcript is cell cycle-regulated. *J. Cell Biol.*, **123**, 1175–1184.
- Laurent, B.C., Treitel, M.A. and Carlson, M. (1991) Functional interdependence of the yeast SNF2, SNF5, and SNF6 proteins in transcriptional activation. *Proc. Natl Acad. Sci. USA*, **88**, 2687–2691.
- Lee, W.-C., Xue, Z. and Mélése, T. (1991) The *NSR1* gene encodes a protein that specifically binds nuclear localization sequences and has two RNA recognition motifs. *J. Cell Biol.*, **113**, 1–12.
- Loeb, J.D., Davis, L.I. and Fink, G.R. (1993). *NUP2*, a novel yeast nucleoporin, has functional overlap with other proteins of the nuclear pore complex. *Mol. Cell Biol.*, **4**, 209–222.
- Machesky, L.M., Atkinson, S.J., Ampe, C., Vandekerckhove, J. and Pollard, T.D. (1994) Purification of a cortical complex containing two unconventional actins from *Acanthamoeba* by affinity chromatography on profilin agarose. *J. Cell Biol.*, **127**, 107–115.

- McCollum, D., Feoktistova, A., Morphey, M., Balasubramanian, M. and Gould, K.L. (1996) The *Schizosaccharomyces pombe* actin-related protein Arp3, is a component of the cortical actin cytoskeleton and interacts with profilin. *EMBO J.*, **15**, 6438–6446.
- Melchior, F. and Gerace, L. (1995) Mechanisms of nuclear protein import. *Curr. Opin. Cell Biol.*, **7**, 310–318.
- Melchior, F., Paschal, B., Evans, J. and Gerace, L. (1993) Inhibition of nuclear protein import by nonhydrolyzable analogues of GTP and identification of the small GTPase Ran/TC4 as an essential transport factor. *J. Cell Biol.*, **123**, 1649–1659.
- Moore, M.S. and Blobel, G. (1993) The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. *Nature*, **365**, 661–663.
- Moore, M.S. and Blobel, G. (1994) A G protein involved in nucleocytoplasmic transport: the role of Ran. *Trends Biochem. Sci.*, **5**, 211–216.
- Moreau, V., Madania, A., Martin, R.P. and Winsor, B. (1996) The *Saccharomyces cerevisiae* actin-related protein Arp2 is involved in the actin cytoskeleton. *J. Cell Biol.*, **134**, 117–132.
- Moreland, R.B., Langevin, G.L., Singer, R.H., Garcea, R.L. and Hereford, L.M. (1987) Amino acid sequences that determine the nuclear localization of yeast histone 2B. *Mol. Cell. Biol.*, **7**, 4048–4057.
- Moroianu, J., Hijikata, M., Blobel, G. and Radu, A. (1995) Mammalian karyopherin $\alpha 1\beta$ and $\alpha 2\beta$ heterodimers: $\alpha 1$ or $\alpha 2$ bind nuclear localization signals and β interacts with peptide repeat containing nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 6532–6536.
- Muhua, L., Karpova, T.S. and Cooper, J.A. (1994) A yeast actin-related protein homologous to that in vertebrate dyactin complex is important for spindle orientation and nuclear migration. *Cell*, **78**, 669–679.
- Mullins, R.D., Kelleher, J.F. and Pollard, T.D. (1996) Actin like actin? *Trends Cell Biol.*, **6**, 208–211.
- Mullins, R.D., Stafford, W.F. and Pollard, T.D. (1997) Structure, subunit topology, and actin-binding activity of the Arp2/3 complex from *Acanthamoeba*. *J. Cell Biol.*, **136**, 331–343.
- Newmeyer, D.D. and Forbes, D.J. (1988) Nuclear import can be separated into distinct steps *in vitro*: nuclear pore binding and translocation. *Cell*, **52**, 641–653.
- Novick, P. and Botstein, D. (1985) Phenotypic analysis of temperature-sensitive yeast actin mutants. *Cell*, **40**, 405–416.
- Pemberton, L.F., Rout, M.P. and Blobel, G. (1995) Disruption of the nucleoporin gene *NUP133* results in clustering of nuclear pore complexes. *Proc. Natl Acad. Sci. USA*, **92**, 1187–1191.
- Poch, O., Schwob, E., de Fraipont, F., Camasses, A., Bordonne, R. and Martin, R.P. (1994) *RPK1*, an essential yeast protein kinase involved in the regulation of the onset of mitosis, shows homology to mammalian dual-specificity kinases. *Mol. Gen. Genet.*, **243**, 641–653.
- Pringle, J.R., Preston, R.A., Adams, A.E.M., Stearns, T., Drubin, D.G., Haarer, B.K. and Jones, E.W. (1989) Fluorescence microscopy methods for yeast. *Methods Cell Biol.*, **31**, 357–435.
- Radu, A., Blobel, G. and Moore, M.S. (1995a) Identification of a protein complex that is required for nuclear protein import and mediates docking of import substrate to distinct nucleoporins. *Proc. Natl Acad. Sci. USA*, **92**, 1769–1773.
- Radu, A., Moore, M.S. and Blobel, G. (1995b) The peptide repeat domain of nucleoporin Nup98 functions as a docking site in transport across the nuclear pore complex. *Cell*, **81**, 215–222.
- Rexach, M. and Blobel, G. (1995) Protein import into nuclei: association and dissociation reactions involving transport substrate, transport factors, and nucleoporins. *Cell*, **83**, 683–692.
- Reynolds, E.S. (1963) The use of lead citrate at high pH as an electron opaque stain in electron microscopy. *J. Cell Biol.*, **17**, 208–213.
- Richardson, W.D., Mills, A.D., Dilworth, S.M., Laskey, R.A. and Dingwall, C. (1988) Nuclear protein migration involves two steps: rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. *Cell*, **52**, 655–664.
- Roth, M.B., Zahler, A.M. and Stolk, J.A. (1991) A conserved family of nuclear phosphoproteins localized to sites of polymerase II transcription. *J. Cell Biol.*, **115**, 587–596.
- Rothstein, R.J. (1983) One-step gene disruption in yeast. *Methods Enzymol.*, **101**, 203–211.
- Rout, M.P. and Blobel, G. (1993) Isolation of the yeast nuclear pore complex. *J. Cell Biol.*, **123**, 771–784.
- Rout, M.P. and Kilmartin, J.V. (1990) Components of the yeast spindle and spindle pole body. *J. Cell Biol.*, **111**, 1913–1917.
- Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Schwob, E. and Martin, R.P. (1992) New yeast actin-like gene required late in the cell cycle. *Nature*, **355**, 179–182.
- Sherman, F., Hicks, J.B. and Fink, G.R. (1986) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
- Shortle, D., Novick, P. and Botstein, D. (1984) Construction and genetic characterization of temperature-sensitive mutant alleles of the yeast actin gene. *Proc. Natl Acad. Sci. USA*, **81**, 4889–4893.
- Shuai, K. and Warner, J.R. (1991) A temperature sensitive mutant of *Saccharomyces cerevisiae* defective in pre-rRNA processing. *Nucleic Acids Res.*, **19**, 5059–5064.
- Small, G.M., Imanaka, T., Shio, H. and Lazarow, P.B. (1987) Efficient association of *in vitro* translation products with purified stable *Candida tropicalis* peroxisomes. *Mol. Cell. Biol.*, **7**, 1848–1855.
- Smith, S. and Blobel, G. (1994) Colocalization of vertebrate lamin B and lamin B receptor (LBR) in nuclear envelopes and in LBR-induced membrane stacks of the yeast *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **91**, 10124–10128.
- Stearns, T. and Botstein, D. (1988) Unlinked noncomplementation: isolation of new conditional-lethal mutations in each of the tubulin genes of *Saccharomyces cerevisiae*. *Genetics*, **119**, 249–260.
- Thompson, C.M., Koleske, A.J., Chao, D.M. and Young, R.A. (1993) A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. *Cell*, **73**, 1361–1375.
- Welch, M.D., Iwamatsu, A. and Mitchison, T.J. (1997) Actin polymerization is induced by Arp2/3 protein complex at the surface of *Listeria monocytogenes*. *Nature*, **385**, 265–269.
- Wente, S.R. and Blobel, G. (1993) A temperature-sensitive *NUP116* null mutant forms a nuclear envelope seal over the yeast nuclear pore complex thereby blocking nucleocytoplasmic traffic. *J. Cell Biol.*, **93**, 63–75.
- Wente, S.R. and Blobel, G. (1994) *NUP145* encodes a novel yeast glycine-leucine-phenylalanine-glycine (GLFG) nucleoporin required for nuclear envelope structure. *J. Cell Biol.*, **125**, 955–969.
- Wertman, K.F., Drubin, D.G. and Botstein, D. (1992) Systematic mutational analysis of the yeast *ACT1* gene. *Genetics*, **132**, 337–350.
- Wilken, N., Kossner, U., Senecal, J.-L., Scheer, U. and Dabauvalle, M.-C. (1993) Nup180, a novel nuclear pore complex protein localizing to the cytoplasmic ring and associated fibrils. *J. Cell Biol.*, **123**, 1345–1354.
- Wu, J., Matunis, M.J., Kraemer, D., Blobel, G. and Coutavas, E. (1995) Nup358, a cytoplasmically exposed nucleoporin with peptide repeats, Ran-GTP binding sites, zinc fingers, a cyclophilin A homologous domain, and a leucine-rich region. *J. Biol. Chem.*, **270**, 14209–14213.
- Yan, C. and Mélése, T. (1993) Multiple regions of NSR1 are sufficient for accumulation of a fusion protein within the nucleolus. *J. Cell Biol.*, **123**, 1081–1091.
- Yano, R., Oakes, M.L., Tabb, M.M. and Nomura, M. (1994) Yeast Srp1 has homology to armadillo/plakoglobin/beta-catenin and participates in apparently multiple nuclear functions including the maintenance of nucleolar structure. *Proc. Natl Acad. Sci. USA*, **91**, 6880–6884.
- Yokoyama, N. et al. (1995) A giant nucleopore protein that binds Ran/TC4. *Nature*, **376**, 184–188.

Received on September 10, 1996; revised on November 18, 1996