The EMBO Journal Vol.17 No.24 pp.7416–7429, 1998

Crm1p mediates regulated nuclear export of a yeast AP-1-like transcription factor

Catherine Yan, Linda H.Lee[1] and Laura I.Davis[2]

W.M.Keck Institute for Cellular Visualization, Rosenstiel Center and Department of Biology, MS 029, Brandeis University, 415 South Street, Waltham, MA 02453 and [1]Department of Cell Biology, Duke University Medical Center, Durham, NC 27710, USA

[2]Corresponding author
e-mail: davis@hydra.rose.brandeis.edu

The yeast AP-1-like transcription factor, Yap1p, activates genes required for the response to oxidative stress. Yap1p is normally cytoplasmic and inactive, but will activate by nuclear translocation if cells are placed in an oxidative environment. Here we show that Yap1p is a target of the β-karyopherin-like nuclear exporter, Crm1p. Yap1p is constitutively nuclear in a *crm1* mutant, and Crm1p binds to a nuclear export sequence (NES)-like sequence in Yap1p in the presence of RanGTP. Recognition of Yap1p by Crm1p is inhibited by oxidation, and this inhibition requires at least one of the three cysteine residues flanking the NES. These results suggest that Yap1p localization is largely regulated at the level of nuclear export, and that the oxidation state affects the accessibility of the Yap1p NES to Crm1p directly. We also show that a mutation in RanGAP (*rna1-1*) is synthetically lethal with *crm1* mutants. Yap1p export is inhibited in both *rna1-1* and *prp20* (RanGNRF) mutant strains, but Yap1p rapidly accumulates at the nuclear periphery after shifting *rna1-1*, but not other mutant cells to the non-permissive temperature. Thus, disassembly of export complexes in response to RanGTP hydrolysis may be required for release of substrate from a terminal binding site at the nuclear pore complex (NPC).

Keywords: Crm1p/nuclear export/nucleocytoplasmic transport/transcription/Yap1p

Introduction

Transport of proteins across the nuclear envelope is highly selective and can be temporally regulated. For example, many transcription factors are maintained in an inactive state in the cytoplasm until a signal is received that promotes their translocation into the nucleus, either downstream of a particular signal transduction pathway or at a specific point in the cell cycle (Karin and Hunter, 1995). Until recently such regulation has been attributed to changes in the rate of nuclear import; however, it has now been demonstrated that proteins can also be actively exported from the nucleus to the cytoplasm (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Ossareh-Nazari *et al*., 1997; Stade *et al*., 1997) and that export may also be an important regulatory mechanism by which the steady-state level of a protein within the nucleus can be controlled (Beals *et al*., 1997; Gorner *et al*., 1998; Roth *et al*., 1998; Toyoshima *et al*., 1998; Yang *et al*., 1998).

The master regulator of nuclear transport is a Ras-like GTPase called Ran (Melchior *et al*., 1993; Moore and Blobel, 1993), which is thought to serve as a marker for the nuclear compartment (Izaurralde *et al*., 1997; Görlich, 1998). Although Ran itself is present in both nucleus and cytoplasm, the proteins that regulate its nucleotide-bound state are compartmentalized. The Ran GTPase activating protein (RanGAP/Rna1p; Becker *et al*., 1995; Bischoff *et al*., 1995) is cytosolic (Hopper *et al*., 1990), whereas the guanine nucleotide exchange factor (RCC1/Prp20p; Bischoff and Ponstingl, 1991) is exclusively nuclear (Clark *et al*., 1991). Thus, Ran should be primarily in the GTP-bound form in the nucleus and in the GDP-bound form in the cytoplasm. Indeed, mislocalization of RanGAP to the nucleus, mutation of *PRP20* or *RNA1*, or introduction of GTPase or exchange-defective Ran mutants all inhibit nuclear transport (Palacios *et al*., 1996; Weis *et al*., 1996; Görlich *et al*., 1997; Izaurralde *et al*., 1997; Richards *et al*., 1997; Oki *et al*., 1998).

The targets of Ran are members of a family of proteins referred to as β-karyopherins (also known as β-importins), each of which has a binding site for the GTP-bound form of Ran (Rexach and Blobel, 1995; Lounsbury *et al*., 1996). The karyopherins are soluble cargo receptors that shuttle across the nuclear pore complex (NPC) by binding to specific nucleoporins (Izaurralde and Adam, 1998). The best characterized is Kap95 which, along with an auxiliary protein termed karyopherin α, binds to and imports substrates that contain an SV40-type nuclear localization sequence (NLS) (Görlich *et al*., 1994, 1995a,b; Enenkel *et al*., 1995; Moroianu *et al*., 1995). The GTP-bound form of Ran binds to Kap95 with high affinity and causes it to dissociate from the α subunit and the NLS-bearing substrate (Rexach and Blobel, 1995). Because RanGTP is restricted to the nucleus, cargo should therefore be released preferentially within the nuclear interior, resulting in import against a concentration gradient. Other members of the karyopherin family have since been shown to import distinct classes of substrates, including ribosomal proteins (Rout, 1997), hnRNP proteins (Aitchison *et al*., 1996; Nakielny *et al*., 1996; Bonifaci *et al*., 1997; Fridell *et al*., 1997; Pemberton *et al*., 1997), snRNPs (Huber *et al*., 1998) and tRNA processing factors (Rosenblum *et al*., 1997).

Recently it was shown that three different members of the β-karyopherin family mediate nuclear export rather than import. A sequence necessary and sufficient to promote rapid nuclear export has been identified on several proteins, including HIV Rev, TFIIIA and PKI (Fischer *et al*., 1995; Wen *et al*., 1995; Fridell *et al*., 1996; Figure 10). This nuclear export sequence (NES) consists of several leucine residues with uneven spacing between,

designated $L(X)_{2-3}L(X)_{2-3}LXL$ (Bogerd *et al*., 1996), and is recognized by Crm1p, a member of the β-karyopherin family (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Ossareh-Nazari *et al*., 1997; Stade *et al*., 1997). Another β-karyopherin-like protein, CAS, is responsible for recycling karyopherin α back to the cytoplasm (Kutay *et al*., 1997). The region of karyopherin α that is recognized by CAS has yet to be determined. Finally, Exportin(tRNA) exports tRNA (Arts *et al*., 1998; Kutay *et al*., 1998). In contrast to the karyopherins that mediate import, Crm1p, CAS and Exportin(tRNA) bind to their respective substrates strongly only in the presence of RanGTP, and in each case a cooperative complex is formed between karyopherin, RanGTP and cargo (Fornerod *et al*., 1997; Kutay *et al*., 1997, 1998; Arts *et al*., 1998). Thus, assembly of export complexes would be favored in the nucleus, whereas dissociation would result when cytoplasmic RanGAP promotes hydrolysis of the bound RanGTP.

The elucidation of pathways that mediate nuclear exit suggests an additional mechanism by which the relative steady-state level of proteins in the nuclear and cytoplasmic compartments can be maintained. Thus, what has previously been referred to as 'retention' of proteins within the cytoplasm could reflect an increase in the rate of nuclear export relative to nuclear import, rather than simply an inhibition of nuclear import. In fact, there are few instances in which cytoplasmic retention has been directly attributed to inhibition of nuclear import.

Well before the role of Crm1p in nuclear export was elucidated, a number of *crm1* mutants were identified by virtue of their altered sensitivity to a variety of drugs, including staurosporine, caffeine and leptomycin B (Nishi *et al*., 1994; Turi *et al*., 1994; Kumada *et al*., 1996). The latter is now known to inhibit Crm1p directly (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Ossareh-Nazari *et al*., 1997; Kudo *et al*., 1998), but resistance to other drugs may be the result of deregulated transcriptional activity in *crm1* mutants. Specifically, *Schizosaccharomyces pombe crm1* mutants constitutively express genes activated by the AP-1 transcription factor Pap1p (Toda *et al*., 1992). Pap1p, and the *Saccharomyces cerevisiae* ortholog, Yap1p, is activated by oxidative or toxic stress and mediates transcriptional activation of a number of target genes involved in the stress response (Schnell *et al*., 1992; Wu *et al*., 1993; Gounalaki and Thireos, 1994; Hirata *et al*., 1994; Kuge and Jones, 1994; Toone *et al*., 1998). Interestingly, Yap1p activity is regulated by differential localization; under normal conditions it is maintained in the cytoplasm but it becomes nuclear in response to oxidative stress (Kuge *et al*., 1997). The C-terminal domain containing three critical cysteine residues is required for maintaining Yap1p in the cytoplasm (Kuge *et al*., 1997).

Here we show that under normal conditions Yap1p is restricted to the cytoplasm by virtue of rapid Crm1p-mediated export, rather than by inhibition of import. We also show that Yap1p has an NES embedded within its cysteine-rich domain (CRD) and that Yap1p binding to Crm1p is sensitive to oxidation. Mutational analysis suggests that the cysteine residues serve as redox sensors which regulate the availability of the NES, and thus the activity of the transcription factor. We also provide evidence that RanGAP activity is required to release Crm1p-associated export cargo into the cytoplasm.

Results

We identified the *S.cerevisiae CRM1* gene in a screen for mutations that are lethal in combination with a temperature-sensitive mutation in the yeast RanGAP, *rna1-1*. We obtained three different *crm1* alleles (*crm1-1*, *1-2* and *1-3*; Table I), as shown by complementation and genetic crosses to a marked *CRM1* allele (see Materials and methods). Although *CRM1* is essential (Toda *et al*., 1992), none of our alleles has an appreciable growth phenotype on its own. However, *crm1-3* is resistant to 10 mM caffeine (data not shown).

Maintenance of Yap1p in the cytoplasm requires Crm1p function

Schizosaccharomyces pombe crm1 mutants had been identified previously that constitutively express genes dependent on the AP-1 like transcription factor, Pap1p (Toda *et al*., 1992; Kumada *et al*., 1996). In *S.cerevisiae*, the cellular localization of the Pap1p ortholog (Yap1p) is regulated. Under normal conditions Yap1p is cytosolic, but it becomes nuclear in response to oxidative stress (Kuge *et al*., 1997). Because Crm1p has recently been identified as a nuclear export factor (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Neville *et al*., 1997; Ossareh-Nazari *et al*., 1997; Stade *et al*., 1997), these observations suggested to us that the maintenance of Yap1p in the cytoplasm might rely on rapid Crm1p-dependent export of Yap1p, rather than on inhibition of its nuclear import, as was previously proposed (Kuge *et al*., 1997). To test whether Yap1p localization is altered in our *crm1* mutant alleles, we fused full-length Yap1p to green fluorescent protein (GFP). The Yap1–GFP fusion protein is functional because it is able to suppress the oxidation-sensitive phenotype of yeast cells lacking a functional copy of the *YAP1* gene (Figure 1A). We found Yap1–GFP to be predominantly cytoplasmic in wild-type strains, consistent with previous results (Kuge *et al*., 1997), but largely nuclear in all of our *crm1* mutant strains (Figure 1B and data not shown). The extent of mislocalization was striking considering the lack of any growth phenotype in the *crm1* mutants. We conclude that the cytoplasmic localization of Yap1p is dependent on the export factor Crm1p. Similar results were recently obtained for Pap1p (Toone *et al*., 1998).

Crm1p binds to Yap1p

To determine whether the effect of *crm1* mutants on Yap1p localization is direct, we looked for an interaction between Crm1p and Yap1p. First, we used a bacterially expressed glutathione *S*-transferase (GST)–Yap1 fusion protein to precipitate proteins from cytosolic extracts of yeast strain LDY903, which carries a functional HA epitope-tagged copy of *CRM1* (see Materials and methods). GST–Yap1p did not precipitate Crm1p under these conditions (Figure 2A, lane 6). However, when we added bacterially expressed, GTP-loaded His_6-$Gsp1p^{G21\text{-}V}$ (a yeast Ran mutant, defective for GTP hydrolysis), GST–Yap1p precipitated a small amount of Crm1p (Figure 2A, lane 7). The amount was somewhat less than that precipitated by $GST\text{–}NES^{PKI}$, a known Crm1p substrate (Figure 2A, lane 5), and the significance of this result will be addressed below. In both cases, His_6-$Gsp1p^{G21\text{-}V}$–GTP was also

Table I. Yeast strains and plasmids used in this study

Strain	Source	Genotype
LDY431[a]	this study	*MAT***a** *rna1-1 ade2 ade3 ura3 leu2 his7 lys2*
LDY432[a]	this study	*MAT*α *rna1-1 ade2 ade3 ura3 leu2 trp1*
W303	R.Rothstein	*Mat***a** *ade2-1 ura3-1 trp1-1 leu2-3,112 his3-11, 15 can1-100*
LDY436	this study	*Mat***a**/α *ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 leu2-3, 112/leu2-3, 112 his3-11, 15/his3-11, 15 can1-100/can1-100*
LDY546	this study	*Mat***a** *rna1-1 ade2-1 ura3 trp1-1 leu2-3, 112 his3-11,/15*
LDY846[a]	Amberg *et al*. (1993)	*Mat***a** *prp20 ura3-52 leu2*Δ*1 trp1*Δ*63*
LDY880	this study	*Mat***a**/α Δ*crm1::KAN*^{R}*/CRM1 ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 leu2-3,112/leu2-3,112 can1-100/can1-100*
LDY884	this study	*Mat***a** *ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11, 15 can1-100* <pLDB391 (*CRM1 LEU2*) integrated at CRM1>
LDY885	this study	*Mat*α *ade2-1 ura3-1 trp1-1 leu2-3, 112 his3-11, 15 can1-100* <pLDB391 (*CRM1 LEU2*) integrated at CRM1>
LDY903	this study	*Mat***a**/α Δ*crm1::KAN*^{R}/ Δ*crm1::KAN*^{R} *ade2-1/ade2-1 ura3-1/ura3-1 trp1-1/trp1-1 leu2-3,112/leu2-3,112 his3-11,/15/his3-11,/15 can1-100/can1-100* <pLDB396 (*CRM1*^{HA} *URA3 CEN*)>
LDY924[a]	this study	*Mat*α *rna1-1 crm1-1 ade2 ade3 ura3 trp1 leu2 his3* <pLDB382 (*RNA1 URA3 CEN*)>
LDY928[a]	this study	*Mat*α *rna1-1 crm1-2 ade2 ade3 ura3 trp1 leu2 his3* <pLDB382 (*RNA1 URA3 CEN*)>
LDY932[a]	this study	*Mat***a** *rna1-1 crm1-3 ade2 ade3 ura3 trp1 leu2 his7* <pLDB382 (*RNA1 URA3 CEN*)>
LDY937	this study	*Mat*α *crm1-1 ade2 ura3 trp1 leu2 his3*
LDY939	this study	*Mat*α *crm1-2 ade2 ade3 ura3 trp1 leu2 his3*
LDY940	this study	*Mat***a** *crm1-3 ade2 ade3 ura3 trp1 leu2 his7*
SM13[a]	Wemmie *et al*. (1997)	*Mat***a** Δ*yap1-*Δ*2::hisG ura3-52 trp1-*Δ*901 leu2-3,112 his3-*Δ*200 lys2-801*
EGY48[a]	R.Brent	*Mat*α *ura3 trp1 his3 LEXA*$_{op(X6)}$*-LEU2* <pSH18-34 *URA3* 2μ>
RFY206[a]	R.Brent	*Mat***a** *ura3 trp1 his3 lys2*

Plasmid	Source	Description
pEG202+PL	Gyuris *et al*. (1993)	*LexA*$_{(1–202)}$ *HIS3*
pJG4-5	Gyuris *et al*. (1993)	*B42* HA epitope tag *TRP1*
pSH18-34	Gyuris *et al*. (1993)	*LacZ* reporter gene under the control of eight LexA operators *URA3 Amp*^{r}
pJK19-1	Kahana *et al*. (1995)	'Superglow' GFP (sGFP) in pET12a
pGAD-GFP	Roberts and Goldfarb (1998)	SV40 T-antigen NLS fused to GAL4 activation domain and GFP under control of the *ADH1* promoter <*LEU2* 2μ>
pKW442	Stade *et al*. (1997)	Full-length *CRM1* cloned behind the LexA^{DB} of pEG202
YEp351-*YAP1*	Wu *et al*. (1993)	*YAP1* cloned as a *Sal*I–*Sac*I 2.5 kb fragment into YEp351
pLDB93	this study	*RNA1* in pRS314 <*TRP1 CEN*>
pLDB382	this study	pRS316 <*URA3 CEN*> containing *RNA1* on a *Sac*I–*Xba*I fragment and *ADE3* on a *Sal*I–*Sma*I fragment
pLDB391	this study	*Xho*I–*Aat*II fragment containing *CRM1* ORF cloned into *Hin*dIII site (blunt ended) of pRS305 <*LEU2*>
pLDB392	this study	*Xho*I–*Sal*I fragment containing *CRM1* ORF cloned into *Sal*I–*Xho*I site of pRS315 <*LEU2 CEN*>
pLDB393	this study	*Xho*I–*Sal*I fragment containing *CRM1* ORF cloned into the *Sal*I site of pRS316 <*URA3 CEN*>
pLDB396	this study	*CRM1* in pLDB393 HA tagged at 3′ *Nsi*I site
pLDB432	Neville *et al*. (1997)	*KAP95* gene cloned behind the LexA^{DB} of pEG202+PL
pLDB436	Neville *et al*. (1997)	*KAP123* gene cloned behind the LexA^{DB} of pEG202+PL
pLDB450	J.Booth, M.Sannella, K.Belanger and L.I.Davis (in preparation)	*Gsp1*^{G21V} cloned between *Nhe*I and *Hin*dIII site of pTrcHisA
pLDB455	this study	NES of PKI (amino acids 37–46) cloned between *Eco*RI–*Xho*I sites of pGEX4T-1
pLDB419	this study	*YAP1::GFP LEU2* 2μ plasmid with s*GFP* inserted at *Nde*I site of *YAP1* gene
Derivatives of pLDB419 by site-directed mutagenesis		
pLDB465-yap1$^{Y611\text{-}A,\ I614\text{-}A}$	pLDB470-yap1$^{C620\text{-}A}$	pLDB476-yap1$^{C629\text{-}T}$
pLDB466-yap1$^{I614\text{-}A,\ V616\text{-}A}$	pLDB471-yap1$^{C598\text{-}A}$	pLDB477-yap1$^{H608\text{-}R}$
pLDB467-yap1$^{V616\text{-}A,\ L619\text{-}A}$	pLDB472-yap1$^{C629\text{-}A}$	pLDB516-yap1$^{C598\text{-}T,\ C629\text{-}T}$
pLDB468-yap1$^{L619\text{-}A,\ L623\text{-}A}$	pLDB474-yap1$^{C620\text{-}T}$	pLDB517-yap1$^{H608\text{-}R,\ C620\text{-}A}$
pLDB469-yap1$^{L619\text{-}A}$	pLDB475-yap1$^{C598\text{-}T}$	pLDB518-yap1$^{C598\text{-}T,\ C620\text{-}A,\ C629\text{-}T}$
pLDB439	this study	YAP1-B42^{AD}: *YAP1* full-length cloned between *Eco*RI and *Xho*I sites of pJG4-5
pLDB438	this study	*YAP1*(CRD)-B42^{AD}: *YAP1* <aa 526–650> cloned between *Eco*RI and *Xho*I sites of pJG4-5
Derivatives of pLDB438 by site-directed mutagenesis		
pLDB491-yap1$^{C620\text{-}A}$	pLDB498-yap1$^{L619\text{-}A}$	pLDB506-yap1$^{H608\text{-}R}$
pLDB492-yap1$^{C598\text{-}A}$	pLDB503-yap1$^{C620\text{-}T}$	pLDB507-yap1$^{C598\text{-}T,\ C629\text{-}T}$
pLDB493-yap1$^{C629\text{-}A}$	pLDB504-yap1$^{C598\text{-}T}$	pLDB508-yap1$^{H608\text{-}R,\ C620\text{-}A}$
pLDB494-yap1$^{L611\text{-}A,\ 614\text{-}A}$	pLDB505-yap1$^{C629\text{-}T}$	pLDB593-yap1$^{C598\text{-}T,\ C620\text{-}A,\ C629\text{-}T}$
pLDB462	this study	GST—YAP1: *YAP1* (full length) inserted into *Sma*I–*Eco*RI sites of pGEX2TK
Site-directed GST–yap1 mutant constructs		
pLDB530-yap1$^{L619\text{-}A}$	pLDB558-yap1$^{H608\text{-}R}$ (in at *Bam*HI–*Eco*RI sites of pGEX4T-1)	
pLDB533-yap1$^{C620\text{-}A}$	pLDB560-yap1$^{H608\text{-}R,\ C620\text{-}A}$	
pLDB534-yap1$^{C598\text{-}A}$	pLDB563-yap1$^{C303\text{-}A,\ C310\text{-}A,\ C315\text{-}A}$	
pLDB536-yap1$^{C629\text{-}A}$	pLDB567-yap1$^{C303\text{-}A,\ C310\text{-}A,\ C315\text{-}A,\ C598\text{-}T,\ C620\text{-}A,\ C629\text{-}T}$	
pLDB538-yap1$^{C620\text{-}T}$	pLDB573-yap1$^{C303\text{-}A,\ C310\text{-}A,\ C315\text{-}A,\ C620\text{-}A}$	
pLDB539-yap1$^{C598\text{-}T,\ C629\text{-}T}$	pLDB583-yap1$^{C303\text{-}A,\ C310\text{-}A,\ C315\text{-}A,\ C598\text{-}T,\ C629\text{-}T}$	
pLDB541-yap1$^{C598\text{-}T,\ C620\text{-}A,\ C629\text{-}T}$		

[a]Not isogenic with W303.

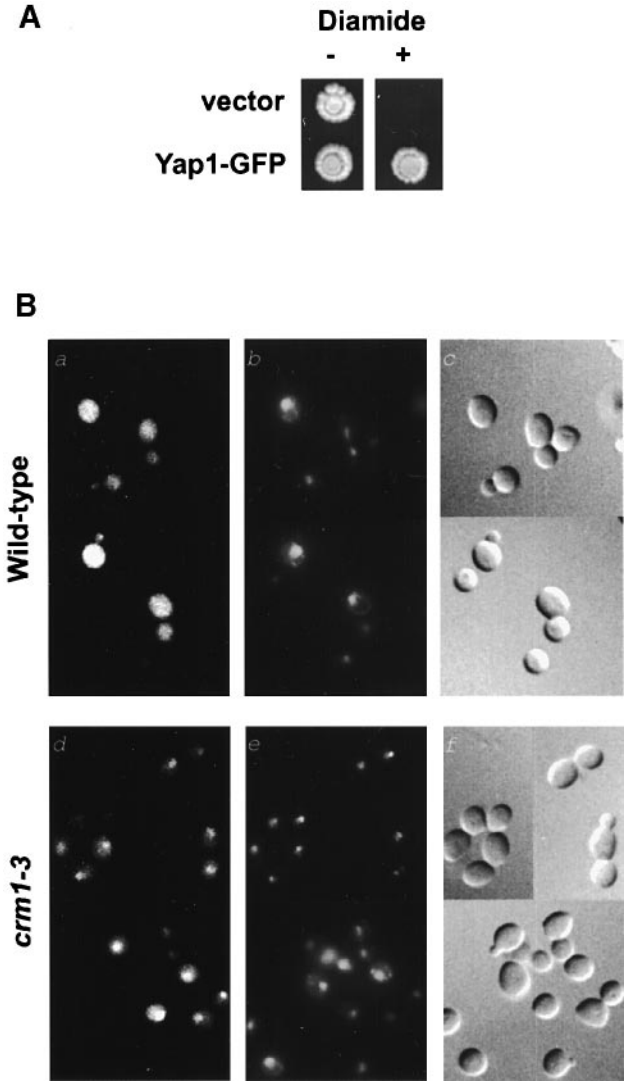

Fig. 1. Yap1–GFP is constitutively nuclear in the *crm1-3* mutant. (**A**) Yeast strain SM13 (Δ*yap1*) containing either pRS315 (empty vector) or pLDB419 (*YAP1–GFP 2*μ *LEU2*) was grown on SC (2% glucose) lacking leucine, in the presence (+) or absence (–) of the oxidant diamide at 1.5 mM. (**B**) Yeast strains W303 [wild-type (a–c)] or LDY940 [*crm1-3*, (d–f)] transformed with pLDB419 (*YAP1–GFP 2*μ *LEU2*) were fixed and DAPI stained (see Materials and methods), and subjected to microscopy. (a and d), GFP fluorescence; (b and e), DAPI; (c and f), Nomarski.

precipitated (Figure 2A, lower part of panel). Thus, Crm1p forms a complex with Yap1p and does so only in the presence of RanGTP. This behavior is what one would expect of an export complex formed by Crm1p (Fornerod *et al*., 1997).

We also tested the interaction between Crm1p and Yap1p in the yeast two-hybrid system (Figure 2B). Full-length *YAP1-B42*^{AD} produced a strong signal in combination with *CRM1-LexA*^{BD}, as well as with *KAP123-LexA*^{BD}, but also showed some background with an empty *LexA*^{BD} plasmid, most probably because of its potential for transactivation. We therefore tested a construct containing only the C-terminal CRD of Yap1p, because this region had previously been shown to mediate the localization of Yap1p (Kuge *et al*., 1997) and should not confer transactivation. *YAP1(CRD)-B42*^{AD} retained a strong interaction with *CRM1-LexA*^{BD}, but not with vector alone or other members of the β-karyopherin family. Thus, Crm1p interacts with Yap1p through the CRD. We also note that the strong interaction between full-length *YAP1-B42*^{AD} and *KAP123-LexA*^{BD} raises the possibility that Kap123p binds to a different region of Yap1p, perhaps one required for import of Yap1p.

The yap1p CRD contains an NES

A canonical NES first described in the HIV-1 Rev and cellular protein kinase inhibitor (PKI) proteins (Fischer *et al*., 1995; Wen *et al*., 1995) has recently been shown to mediate binding of export substrates to Crm1p (Fornerod *et al*., 1997; Fukuda *et al*., 1997; Ossareh-Nazari *et al*., 1997; Stade *et al*., 1997). Inspection of the CRD of Yap1p revealed a sequence rich in hydrophobic residues (**Y**^{611}SD**I**D**V**DG**L**CSE**L**), although not with the consensus spacing [L(X)$_{2–3}$L(X)$_{2–3}$ LXL; Bogerd *et al*., 1996; Figure 10]. To determine whether these hydrophobic residues mediate Yap1p export, we made various single and double point mutations to alanine in the context of the full length Yap1–GFP fusion protein (Figure 3A). All of the fusion proteins were expressed at similar levels (Figure 3C). Yap1$^{Y611-A,\ I614-A}$– GFP showed normal cytoplasmic localization (Figure 3B, panels c and d). In contrast, Yap1^{L619-A}–GFP was constitutively nuclear (Figure 3B, panels g and h), similar to the behavior of wild-type Yap1–GFP in a *crm1* mutant background (Figure 1B). The same phenotype was observed with the Yap1$^{I614-A,\ V616-A}$–GFP double mutant (Figure 3B, panels e and f). Thus, L^{619} and V^{616} are both critical for export. We also note that Yap1^{C620-T}–GFP was shown previously to be constitutively nuclear, whereas Yap1^{C620-A}–GFP was localized normally (Kuge *et al*., 1997). This result could be explained if certain mutations at this position interfere with NES function rather than with regulation of Yap1p localization in response to oxidation, as discussed below.

To determine whether mutations that lead to nuclear accumulation of Yap1–GFP also abrogate binding between Yap1p and Crm1p, we introduced the L619-A and C620-T mutations into the GST–Yap1 fusion protein. Neither mutant fusion protein showed a detectable interaction with Crm1p under conditions in which wild-type GST–Yap1p interacts very efficiently (Figure 4A). As summarized in Table II, we also tested several other mutant fusion proteins. In all cases, the results correlate well with the localization of the various Yap1–GFP mutant proteins.

We also tested the mutants in the two-hybrid assay with *CRM1-LexA*^{BD}. With the *yap1(CRD)*$^{L619-A}$*-B42*^{AD}, *yap1(CRD)*$^{C620-T}$*-B42*^{AD} and *yap1(CRD)*$^{C629-A}$*-B42*^{AD} mutants we observed no detectable signal, whereas all other mutants tested interacted normally (Figure 4B, Table II). Again, the two-hybrid results correlated exactly with the localization of the corresponding GFP fusion protein. We conclude that Yap1p interacts with Crm1p through an NES-like sequence within the CRD, and that this interaction is required for export of Yap1p and its steady-state maintenance in the cytoplasm.

The interaction between Crm1p and Yap1p is regulated by oxidation

The results described above suggest that the steady state cytosolic localization of Yap1p is maintained by rapid export from the nucleus via Crm1p. We sought next to

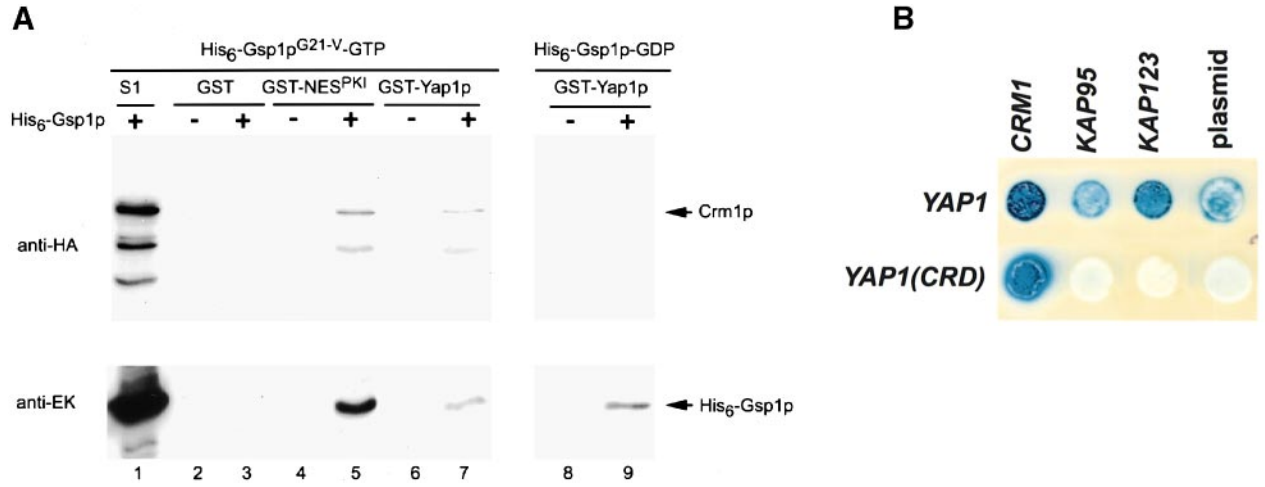

Fig. 2. Crm1p binds to Yap1p in the presence of RanGTP. (**A**) A low-speed supernatant fraction (S1, 1.8 OD_{600} eq per sample) from a lysate of yeast strain LDY903 [Δ*crm1::KAN*/Δ*crm1::KAN* pLDB396 (^{HA}*CRM1 CEN URA3*)] was precipitated with GST fusion proteins expressed in *E.coli* and adsorbed to glutathione–Sepharose, in the presence or absence of 20 μg of purified His_6-$Gsp1p^{G21-V}$-GTP or His_6-Gsp1p-GDP, as indicated. GST alone, lanes 2 and 3; GST–NES^{PKI}, lanes 4 and 5; GST–Yap1p, lanes 6–9. Total S1 (0.2 OD_{600} eq) with 1 μg His_6-$Gsp1p^{G21-V}$ added was loaded in parallel (lane 1). Western blots of precipitated proteins were probed with either anti-HA mAb 12CA5 to visualize $Crm1p^{HA}$ (upper panel) or anti-EK mAb to visualize His_6-Gsp1p (lower panel). The positions of Crm1p and His_6-Gsp1p are shown. (**B**) β-galactosidase production from a LacZ reporter gene was assayed in yeast strain RFY206×EGY48 containing pKW442 (*CRM1-LexA*^{BD}), and either pLDB439 (full-length *YAP1-B42*^{AD}) or pLDB438 [*YAP1(CRD)-B42*^{AD}, amino acids 526–650], as shown at left. For comparison, other karyopherin family members fused to *LexA*^{BD} were also tested with the *YAP1* constructs, as was the empty pEG202+PL vector.

determine whether the accumulation of Yap1p in the nucleus in response to oxidative stress was triggered by inhibiting the binding of Crm1p to Yap1p. The three cysteine residues in and around the Yap1p NES have been shown to be critical for this aspect of Yap1p regulation. If all three are changed to amino acid residues which individually have no effect on Yap1p localization, Yap1p can no longer respond to oxidation and remains cytoplasmic (Kuge *et al*., 1997). An attractive scenario is that oxidation of the cysteine residues somehow masks the NES, thus preventing export of Yap1p and allowing it to accumulate in the nucleus.

In the experiments described above (Figure 2), the interaction between GST–Yap1p and Crm1p was assayed in the absence of reducing agents, conditions that are likely to promote oxidation during the binding step. To determine whether binding might be strengthened under reducing conditions, we repeated the experiment in the presence or absence of 1 mM dithiothreitol (DTT) (Figure 5). We found that ~20-fold more Crm1p was precipitated by GST–Yap1p in the presence of reducing agent (Figure 5, compare lanes 8 and 9). Binding remained completely dependent on added RanGTP. This effect was specific to Yap1p, because GST–NES^{PKI} precipitated somewhat less Crm1p in the presence of reducing agent, perhaps due to competition by endogenous Yap or other redox-sensitive proteins.

To determine whether the cysteine residues in the Yap1p CRD regulate the interaction with Crm1p, these three residues were mutated to alanine or threonine, and the interaction of the mutant GST–Yap1p fusion proteins with Crm1p was assessed *in vitro* (Figure 6). GST–$Yap1p^{C620-A}$ and GST–$Yap1p^{C598-T,\ C629-T}$ mutant proteins precipitated Crm1p in a redox-sensitive manner similar to wild-type GST–Yap1p, consistent with the observation that the localization of the corresponding Yap1–GFP mutants responds to oxidation in a manner indistinguishable from wild-type (Kuge *et al*., 1997). However, the triple GST–$Yap1p^{C598-T,\ C620-A,\ C620-T}$ mutant, which confers constitutive cytoplasmic localization to Yap1–GFP (Kuge *et al*., 1997), bound strongly to Crm1p even in the absence of reducing agents, showing that the interaction is no longer sensitive to oxidation. These results suggest that oxidation of any one of the cysteine residues in the CRD activates Yap1p by masking the NES and preventing Crm1p-mediated nuclear export.

Addition of oxidants, such as diamide, to the growth medium has been shown to cause translocation of Yap1p to the nucleus (Kuge *et al*., 1997). This allowed us to use the two-hybrid assay to test whether the interaction between Yap1p and Crm1p was sensitive to oxidants *in vivo* (Figure 7). Treatment of cells with 0.75 mM diamide inhibited the interaction between wild-type *YAP1(CRD)-B42*^{AD} and *CRM1-LexA*^{BD}, as well as that between *yap1(CRD)*$^{C620-A}$*-B42*^{AD} or *yap1(CRD)*$^{C598-TC629-T}$*-B42*^{AD} and *CRM1-LexA*^{BD}. In contrast, the interaction of *CRM1-LexA*^{BD} with the triple *yap1(CRD)*$^{C598-T,\ C620-A,\ C629-T}$*-B42*^{AD} mutant was completely resistant to diamide. The two-hybrid results exactly parallel those obtained in the *in vitro* binding studies (Figure 6), and taken together they provide strong support for the idea that oxidative stress promotes the accumulation of Yap1p in the nucleus by inhibiting formation of an export complex with Crm1p.

Our results suggest that a single cysteine residue within the CRD is sufficient to confer redox sensitive binding to Crm1p, making it unlikely that masking of the NES requires disulfide linkages between two cysteine residues. However, Yap1p contains three other cysteine residues (C^{303}, C^{310}, C^{315}) outside of the CRD which might participate in the response to oxidation. We therefore mutated these residues in the context of *GST–yap1*$^{C598-T,\ C629-T}$, leaving only a single cysteine residue (C^{620}) remaining. A GST–Yap1 fusion protein carrying the five cysteine mutations displayed DTT-dependent interaction with

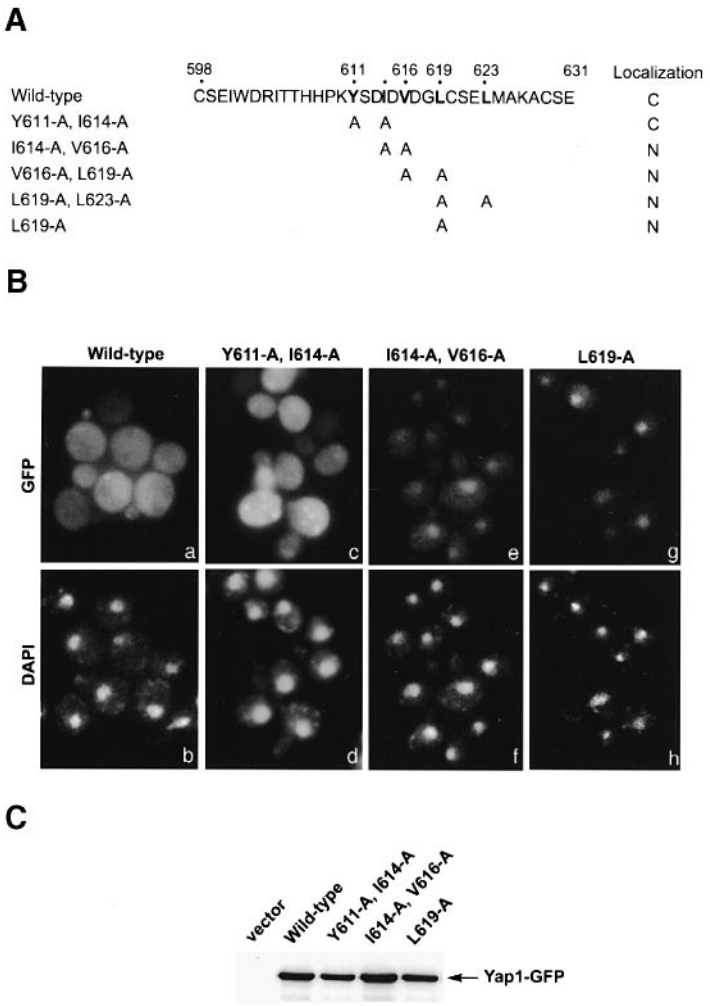

Fig. 3. Yap1p localization is dependent on an NES-like sequence. (**A**) Single and double mutations to alanine residues were introduced at the indicated sites within the Yap1p CRD, in the context of *YAP1–GFP*. The localization of the mutant fusion proteins in cells grown in the absence of oxidative stress is summarized on the right. (**B**) The wild-type and indicated mutant *YAP1–GFP* fusion constructs were transformed into yeast strain SM13 (*yap1-Δ2::hisG*). Transformants were grown in rich medium under normal conditions and the localization of the fusion protein was visualized by fluorescence (a,c,e,g). For comparison, nuclear DNA was visualized by DAPI (b,d,f,h). (**C**) Whole-cell extracts from yeast strain SM13 transformed with a vector control (lane 1) or the indicated wild-type and mutant *YAP1–GFP* fusion constructs (lanes 2–5) were blotted with rabbit anti-GFP antibody.

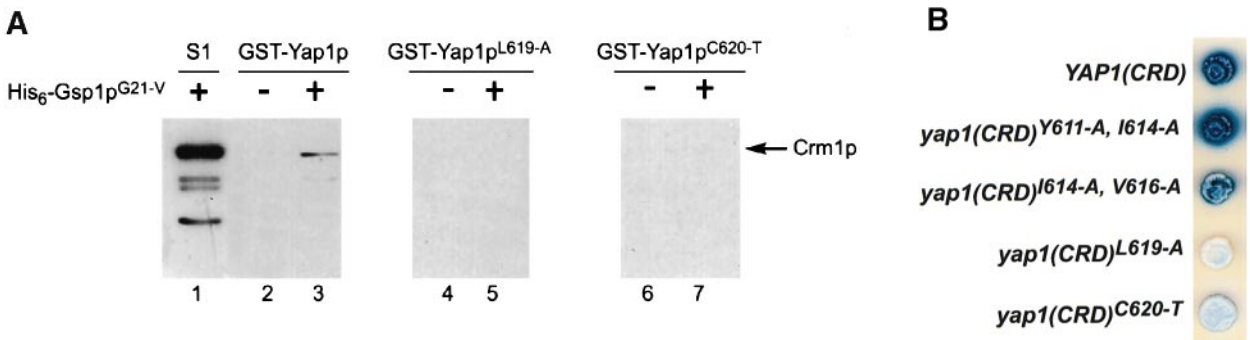

Fig. 4. Mutations in the Yap1p NES prevent binding to Crm1p. (**A**) A low-speed supernatant fraction (S1, 1.8 OD_{600} eq per sample) from a lysate of yeast strain LDY903 [Δ*crm1::KAN*/Δ*crm1::KAN* pLDB396 (^{HA}*CRM1 CEN URA3*)] was precipitated with GST fusion proteins expressed in *E.coli* and adsorbed on glutathione–Sepharose, in the presence (lanes 3, 5 and 7) or absence (lanes 2, 4 and 6) of 20 μg of purified His_6-$Gsp1p^{G21-V}$-GTP. GST–Yap1p, lanes 2 and 3; GST–$Yap1p^{L619-A}$, lanes 4 and 5; GST–$Yap1p^{C620-T}$, lanes 6 and 7. Total S1 (0.2 OD_{600} eq) was loaded in parallel (lane 1). Western blots of precipitated proteins were probed with anti-HA mAb 12CA5 to visualize $Crm1p^{HA}$. The position of Crm1p is shown on the right. (**B**) β-galactosidase production from a *LacZ* reporter gene was assayed in yeast strain EGY48 transformed with the reporter plasmid pSH18-34 and pKW442 (*CRM1-LexA*^{BD}), and either pLDB438 [wild-type *YAP1(CRD)-B42*^{AD}] or *yap1(CRD)-B42*^{AD} mutants containing the indicated amino acid substitutions (Table I).

Table II. Analysis of *yap1* mutant alleles

YAP1 allele	Localization[a]	Growth in 1.5 mM diamide[b]	Two hybrid Crm1p-LexA[c]	Binding to Crm1p[d]
Wild-type	Cyt	+/–	+++	+[e]
Y611-A, I614-A	Cyt	+/–	+++	NT[f]
L619-A	Nuc	+/–	–	–
C598-T	Cyt	+/–	+++	NT
C598-A	Cyt	+/–	++	+[e]
C620-T	Nuc	+++	–	–
C620-A	Cyt	+/–	++++	+[e]
C629-T	Cyt	+/–	++++	NT
C629-A	Nuc	+++	–	–
C598-T, C629-T	Cyt	+/–	+++	+[e]
C598-T, C620-A, C629-T	Cyt	–	+++	+, DTT independent[h]
H608-R	Cyt	+/–	+++	+[e]
H608-R, C620-A	Cyt	+/–	+++	+[e]
C303-A, C310-A, C315-A[g]	NT	NT	NT	+[e]
C303-A, C310-A, C315-A, C620-A[g]	NT	NT	NT	+[e]
C303-A, C310-A, C315-A, C598-T, C629-T[g]	NT	NT	NT	+[e]
C303-A, C310-A, C315-A, C598-T, C620-A, C629-T[g]	NT	NT	NT	+, DTT independent[h]

[a]Localization of wild-type and mutant Yap1–GFP fusion proteins; Cyt, cytoplasmic; Nuc, nuclear.
[b]Growth of SM13 (Δ*yap1*) yeast strain containing *YAP1* alleles fused to GFP on solid YP + 2% glucose medium containing 1.5 mM diamide.
[c]Two-hybrid interaction of yap1(CRD)^{AD} carrying the above mutations with Crm1p-LexA^{BD}.
[d]*In vitro* interaction of GST–Yap1 wild-type and mutant fusion proteins with HA-tagged Crm1p and His_6-Gsp1^{G21-V}-GTP in 1 mM DTT.
[e]Interaction is dependent on presence of DTT.
[f]NT, not tested.
[g]These *YAP1* alleles were constructed only as GST fusions by PCR amplification; plasmids containing wild-type *YAP1* or mutant *YAP1* alleles (carrying mutations in the CRD) fused to GFP were used as template; COL 30, 31, 36 and 37 were used as primers. Cloning was performed exactly as described in the Materials and methods.
[h]Equivalent amount of interaction is observed even in the absence of reducing agent, DTT.

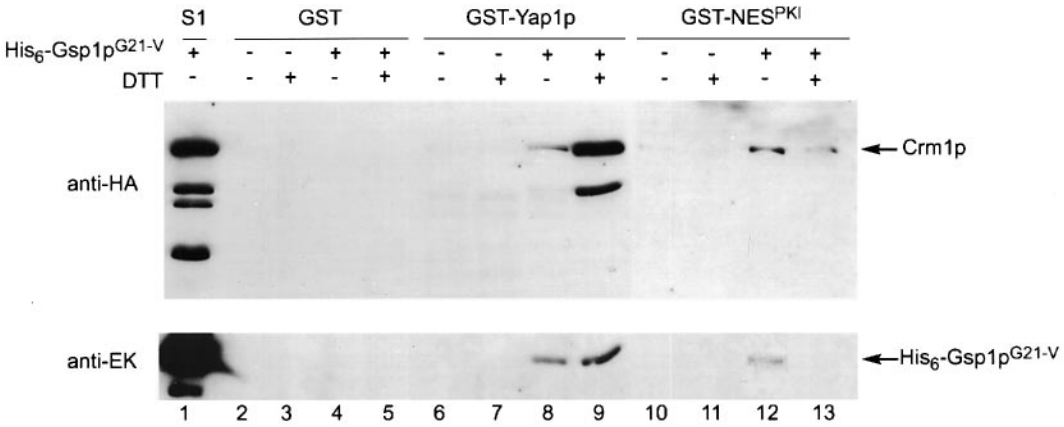

Fig. 5. Binding of Crm1p to Yap1p is sensitive to oxidation. (**A**) A low-speed supernatant fraction (S1, 1.8 OD_{600} equivalents per sample) from a lysate of yeast strain LDY903 [Δ*crm1::KAN*/Δ*crm1::KAN* pLDB396 (^{HA}*CRM1 CEN URA3*)] was precipitated with GST fusion proteins expressed in *E.coli* and adsorbed to glutathione–Sepharose, in the presence or absence (as indicated) of 20 μg of purified His_6-Gsp1p^{G21-V} and 1 mM DTT (the latter added during all steps). GST alone, lanes 2–5; GST–Yap1p, lanes 6–9; GST–NES^{PKI}, lanes 10–13. Total S1 (0.2 OD_{600} eq) with 1 μg His_6-Gsp1p^{G21-V} added was loaded in parallel (lane 1). Western blots of precipitated proteins were probed with either anti-HA mAb 12CA5 to visualize Crm1p^{HA} (upper panel) or anti-EK mAb to visualize His_6-Gsp1p^{G21-V} (lower panel). The positions of Crm1p and His_6-Gsp1p^{G21-V} are shown at the right.

Crm1p, similar to wild-type GST–Yap1p (Figure 6). No difference in binding was observed when the GST moiety was cleaved off, ruling out the possibility that cysteine residues within GST itself can contribute to redox sensitivity (data not shown). We conclude that a single cysteine residue within the CRD is necessary and sufficient to mediate the response to oxidative stress in the context of full-length Yap1p.

Yap1–GFP is concentrated at the nuclear periphery in an rna1-1 mutant

The three *crm1* alleles described here were obtained in a screen for synthetic lethality with the temperature-sensitive RanGAP mutant, *rna1-1*. RanGAP is thought to be required for dissociation of export factors in the cytoplasm and subsequent recycling to the nucleus (Bischoff and Görlich, 1997; Floer *et al*., 1997; Görlich *et al*., 1997; Lounsbury and Macara, 1997). Thus, the export defect of a *crm1* mutant might be exacerbated by inefficient recycling, explaining the synthetic lethality between *crm1* and *rna1-1* mutants. To determine whether the *rna1-1* mutation itself has any effect upon the localization of Yap1p, we transformed strains W303 (wild-type) and LDY546 (*rna1-1*) with *YAP1–GFP*, and localized the fusion protein. At the permissive temperature, Yap1–GFP was localized throughout the cell (Figure 8). However, within 15 min

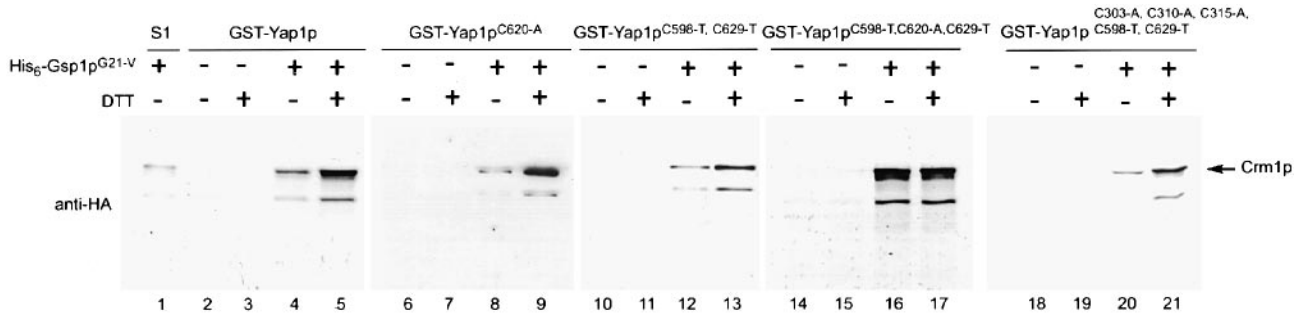

Fig. 6. The sensitivity of Crm1p binding to Yap1p is dependent on the presence of a cysteine residue within the Yap1p CRD. (**A**) A low-speed supernatant fraction (S1, 1.8 OD_{600} eq per sample) from a lysate of a yeast strain LDY903 [Δ*crm1::KAN*/Δ*crm1::KAN* pLDB396 (^{HA}*CRM1 CEN URA3*)] was precipitated with GST fusion proteins expressed in *E.coli* and adsorbed to glutathione–Sepharose, in the presence or absence (as indicated) of 20 μg of purified His$_6$-Gsp1p^{G21-V}, and 1 mM DTT (the latter added during all steps). GST–Yap1p, lanes 2–5; GST–Yap1p^{C620-A}, lanes 6–9; GST–Yap1p$^{C598-T, C629-T}$, lanes 10–13; GST–Yap1p$^{C598-T, C620-A, C629-T}$, lanes 14–17; GST–Yap1p$^{C303-A, C310-A, C315-A, C598-T, C629-T}$, lanes 18–21. Total S1 (0.2 OD_{600} eq) was loaded in parallel (lane 1). Western blots of precipitated proteins were probed with anti-HA mAb 12CA5 to visualize Crm1p^{HA}. The positions of Crm1p are shown on the right.

of shifting *rna1-1* to the non-permissive temperature, Yap1–GFP became predominantly nuclear. Importantly, we observed consistently a distinct nuclear rim staining pattern in many cells, suggesting that Yap1–GFP was accumulating at or near the nuclear envelope. This pattern, as well as the speed with which nuclear accumulation occurred, suggests that dissociation of the export complex by RanGAP may be required to release substrate from the NPC, perhaps at a terminal step of export. Consistent with this idea, we found that the behavior of Yap1–GFP in the *prp20-1* mutant differed from that in *rna1-1*. Although Yap1p-GFP also accumulated in the nucleus at the non-permissive temperature in *prp20–1* mutant cells, it took longer (~45 min) for significant nuclear accumulation to develop. Furthermore, we never observed rim staining similar to that seen with the *rna1-1* mutant (Figure 8). The behavior of the *prp20-1* mutant is consistent with the notion that gradual depletion of the nuclear pool of RanGTP should inhibit formation of export complexes, trapping Yap1–GFP within the nucleus.

Yap1p has a potential NLS at its N-terminus that could mediate its nuclear import, presumably by karyopherin α/β or a related protein. The dependence of karyopherin-mediated nuclear import on Ran has been well documented (Moore and Blobel, 1993; Melchior *et al*., 1995), and is thought to reflect the ability of nuclear RanGTP to dissociate import complexes once they have crossed the NPC (Görlich *et al*., 1996). In spite of this, Yap1p relocated from the cytoplasm to the nucleus in both *rna1-1* and *prp20* mutants, suggesting that import of Yap1p was not affected at early time points. To investigate this question more closely, we took advantage of the *yap1^{L619-A}* mutant, which cannot be exported and is constitutively nuclear (Figure 3), to isolate the import step. We found that Yap1^{L619-A}–GFP relocated from the nucleus to the cytoplasm after shifting *rna1-1* mutant cells to the non-permissive temperature, but did so with somewhat slower kinetics than the block to nuclear export of wild-type Yap1–GFP (Figure 9). The behavior of Yap1^{L619-A}–GFP is identical to that of an NLS–GFP reporter protein examined in parallel. Furthermore, Yap1^{L619-A}–GFP never showed accumulation at the nuclear rim. These results suggest that import of Yap1–GFP is also blocked in the *rna1-1* mutant, but this occurs more slowly than inhibition of export. Furthermore, a functional NES is required to trap Yap1–GFP at the nuclear rim in the *rna1-1* mutant.

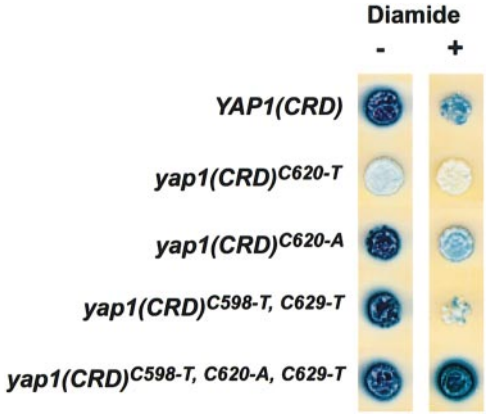

Fig. 7. The interaction between Yap1p and Crm1p is sensitive to oxidation *in vivo*. β-galactosidase production from a *LacZ* reporter gene was assayed in yeast strain EGY48 transformed with pSH18-34 and pKW442 (*CRM1-LexA^{BD}*), and either pLDB438 [wild-type *YAP1(CRD)-B42^{AD}*] or *yap1(CRD)-B42^{AD}* mutants containing the indicated amino acid substitutions (Table I). Each combination was tested for β-galactosidase production in the absence (left) or presence (right) of the oxidizing agent diamide, at 0.75 mM.

Discussion

We have shown that the yeast AP-1-like transcriptional activator, Yap1p, is a regulated substrate of the Crm1p export factor. Yap1p binds to Crm1p via an NES located in the Yap1p CRD. This interaction is inhibited under conditions of oxidative stress, allowing Yap1p to accumulate in the nucleus and activate transcription of genes required for the stress response. The three cysteine residues within the CRD are crucial for this inhibition and may serve as redox sensors that induce masking of the NES upon oxidation. These observations reveal a novel mechanism by which nuclear transport can be temporally regulated.

The Yap1p NES is unusual in that it contains mostly hydrophobic amino acids other than leucine and the spacing between these residues is not canonical (Figure 10). It does have several acidic residues in between the

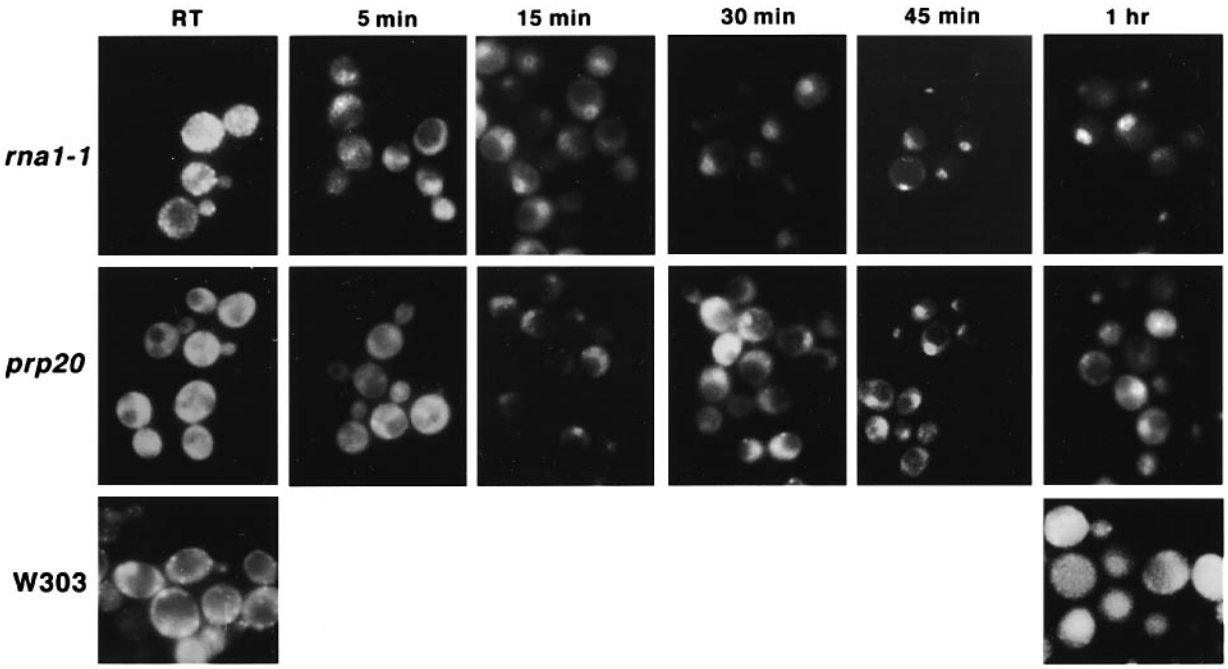

Fig. 8. Yap1–GFP export is dependent on Rna1p and Prp20p. pLDB419 (*YAP1–GFP* 2μ) was transformed into either LDY546 (*rna1-1*) or LDY846 (*prp20-1*). Transformants were grown to mid-log phase in rich medium at 25°C and then shifted to the non-permissive temperature of 37°C for the times indicated. The Yap1–GFP fusion protein was visualized by fluorescence in living cells.

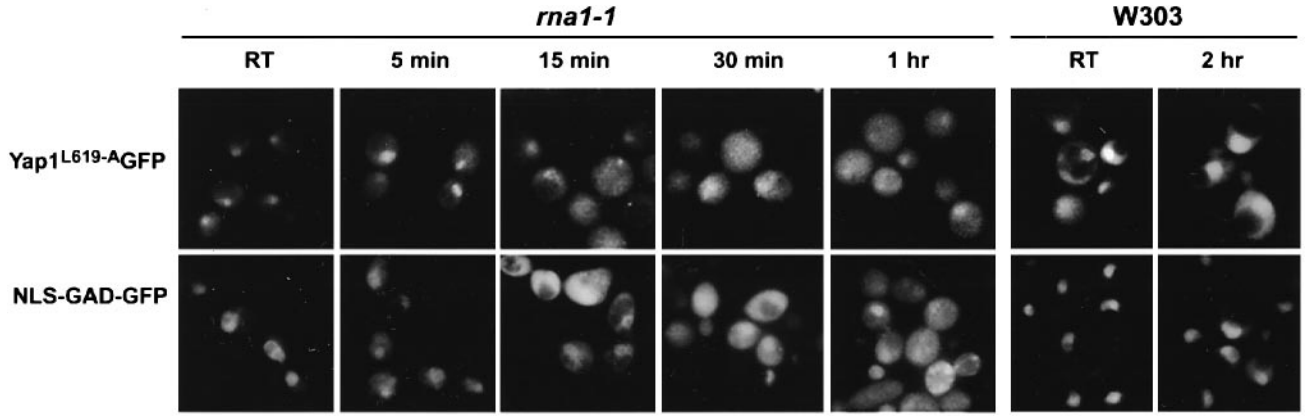

Fig. 9. Nuclear import is also inhibited in the *rna1-1* mutant. Yeast strains LDY546 (*rna1-1*) or W303 (wild-type) were transformed with either pLDB469 (*yap1^{L619-A}-GFP 2*μ *LEU2*, top panel) or NLS-GAD–GFP [*2*μ *LEU2*, (Roberts and Goldfarb, 1998), bottom panel]. Transformants were grown on rich medium at 25°C and then shifted to the non-permissive temperature of 37°C for the times indicated. GFP fluorescence was visualized in living cells.

hydrophobic residues, a feature common to many NESs. This difference may simply reflect the fact that yeast NESs have diverged significantly from their metazoan counterparts. Leptomycin B binds to human and *S.pombe* Crm1p, and inhibits its interaction with the NES, but does not bind to *S.cerevisiae* Crm1p (Fornerod *et al*., 1997). If Leptomycin B acts as a competitor for the NES binding pocket as suspected, this result suggests that this region of Crm1p is significantly divergent in *S.cerevisiae*. Such a difference would also explain why Rev functions only poorly in yeast (Stutz and Rosbash, 1994). An equally plausible explanation is that proteins such as Yap1p, whose binding to Crm1p must be tightly regulated, have NESs that bind less strongly and are thus more sensitive to perturbation. Finally, it is possible that this region of Yap1p is not a bona fide NES, but instead mediates binding to another protein that contains a canonical NES. In this regard, we note that NES-like sequences are very similar to leucine-rich repeats that mediate other protein–protein interactions. The answer to these questions should become clear as more yeast NESs are identified.

In this regard, it is important to note that our *crm1* mutants have no growth phenotype, yet they profoundly mislocalize Yap1p. The essential substrate(s) of Crm1p, which are not yet known, could have more efficient NESs and thus not be mislocalized in our mutants. Alternatively, export of all Crm1p substrates may be very inefficient in the *crm1* mutants, but this may be tolerated under normal conditions. If this is the case, then it is unlikely that negative regulation of proteins like Yap1p is the essential function of Crm1p because our *crm1* mutants are clearly not able to maintain enough export to keep these substrates inactive. Another class of potential Crm1p substrates are proteins that mediate RNA export. Crm1p has been shown

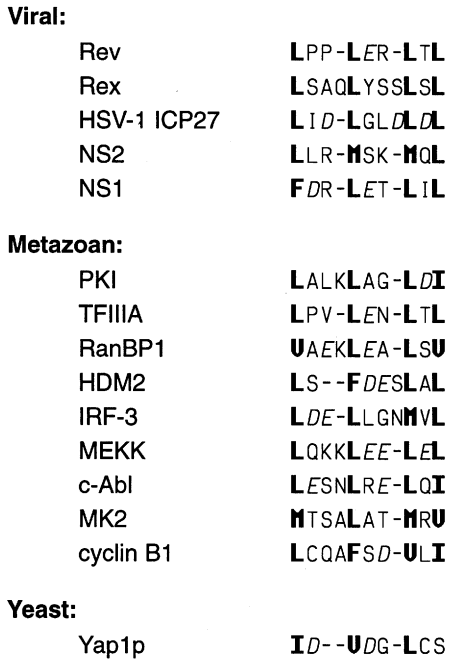

Fig. 10. The Yap1p NES is divergent. NES sequences from viral proteins HIV-1 Rev (Fischer *et al*., 1995), HIV-2 Rex (Palmeri and Malim, 1996), HSV-1 ICP27 (Sandri-Goldin, 1998), flu NS2 (O'Neill *et al*., 1998) and NS1 (Li *et al*., 1998) and metazoan proteins PKI (Wen *et al*., 1995), TFIIIA (Fridell *et al*., 1996), RanBP1 (Zolotukhin and Felber, 1997), HDM2 (Roth *et al*., 1998), IRF-3 (Yoneyama *et al*., 1998), MEKK (Fukuda *et al*., 1996), c-Abl (Taagepera *et al*., 1998), MK2 (Engel *et al*., 1998) and cyclin B1 (Yang *et al*., 1998) are compared with the Yap1p NES. Consensus residues are shown in bold, acidic residues are italicized.

to mediate snRNA export in metazoan cells (Fornerod *et al*., 1997) and may also be required for export of other RNA species, including mRNA (Pasquinelli *et al*., 1997; Stade *et al*., 1997). While absolutely essential, such export may not be limiting under normal laboratory conditions, and thus would need to be only marginally effective to maintain viability.

How do the cysteine residues within the Yap1p CRD mask the NES? The simplest explanation would be that they form intrachain disulfide linkages with one another or with other cysteine residues within Yap1p, thus embedding the NES in a conformation that is not accessible to Crm1p. However, we find that a Yap1p mutant missing all but a single cysteine residue within the CRD still binds to Crm1p in a redox-sensitive manner, suggesting that intrachain disulfide linkages are not required. A different possibility is that the cysteine residues within the Yap1p CRD form linkages with a third protein, as yet unidentified, which serves to mask the NES. This question is currently under investigation.

The behavior of the Yap1–GFP fusion protein in an *rna1-1* mutant provided interesting insights into the requirements for RanGAP in transport. Although RanGAP is required for both import and export, its effect on export appears to be much more direct. Wild-type Yap1–GFP relocated from cytoplasm to nucleus very rapidly after shifting *rna1-1* mutant cells to the non-permissive temperature, suggesting that export was blocked at a time when import was still occurring. Under these conditions, Yap1–GFP was often concentrated at the nuclear rim. In contrast, although Yap1–GFP also accumulated in the nucleus in a *prp20* mutant strain, we never observed rim staining. The simplest interpretation of these results is that RanGTP is required to assemble export complexes that are then targeted to the NPC, whereas RanGAP is required to dissociate these complexes and release substrates, perhaps from the terminal binding site at the cytoplasmic face of the NPC. We envision that the inability to release export complexes would cause subsequent substrates to stall within the NPC, leading to rapid cessation of export and accumulation of Yap1p at the NPC and within the nucleus. Consistent with this view, the Yap1$^{L619\text{-}A}$–GFP mutant, which cannot bind to Crm1p, normally accumulates in the nucleus but never concentrates at the nuclear rim.

RanGAP could be required directly to hydrolyze RanGTP bound to export complexes. Alternatively, RanGAP could function indirectly by releasing Ran from RanBP1. RanBP1 has been shown *in vitro* to remove RanGTP from the CAS/importin α/RanGTP export complex, thereby inducing disassembly of the complex (Bischoff and Görlich, 1997). RanGAP activity may therefore only be required to recycle RanBP1 from the RanGTP-bound state after one round of dissociation. This idea would reconcile our results with those showing that microinjection of a nonhydrolyzable Ran mutant does not inhibit nuclear export (Izaurralde *et al*., 1997; Richards *et al*., 1997).

A growing number of proteins have now been shown to undergo regulated nuclear export. In most cases, the mechanism by which export is regulated has not been fully elucidated, but so far many appear to involve phosphorylation (Beals *et al*., 1997; Engel *et al*., 1998; Yang *et al*., 1998). Direct regulation of Yap1p export by oxidation uncovers a novel mechanism by which cells can sense and respond to environmental changes. However, because we do not yet understand exactly how the cysteine residues within the Yap1p CRD function, we cannot rule out the possibility that phosphorylation is also involved in the oxidation response. In this regard, we note that the *S.pombe* ortholog, Pap1p, is constitutively cytoplasmic in strains carrying a mutation in the stress-activated MAP kinase Sty1p (Toone *et al*., 1998). Thus, phosphorylation by Sty1p may contribute to the inhibition of export upon oxidative stress in some unknown fashion. Alternatively, phosphorylation may regulate Yap1p/Pap1p import by a distinct mechanism.

Given the number of examples already in the literature, it seems likely that regulation of nuclear export is at least as common as that of nuclear import, and that the steady-state localization of many proteins is largely determined by the relative rates of the two processes. Indeed, it may be that apparent exclusion from one compartment or the other is less dependent on a physical barrier to movement across the NPC than on the relative rates of import versus export.

Materials and methods

Reagents

Enzymes for molecular biology were purchased from Boehringer Mannheim (Indianapolis, IN), Pharmacia (Piscataway, NJ) and New England Biolabs (Beverly, MA). Lyticase was purchased from Enzogenetics (Corvallis, OR). 5-fluoroorotic acid (5FOA) was obtained

through the Genetics Society of America consortium. A yeast genomic library carried in pRS314 (C.Connelly and P.Hieter, unpublished) was furnished by Dr Phil Hieter, as were all of the pRS vectors (Sikorski and Hieter, 1989). Anti-GFP antibody was a kind gift from Jason Kahana and Pam Silver.

Strains and microbial techniques

The yeast strains and plasmids used are listed in Table I, and only relevant genotypes are listed in the text. Unless otherwise designated, all yeast strains are derived from W303. Mutants originally present in other strain backgrounds were crossed to W303 at least three times prior to use. Yeast cell culture, media preparation and genetic manipulations were performed essentially according to Sherman *et al*. (1986). Yeast shuttle plasmids and linear fragments were introduced into yeast by lithium acetate transformation (Ito *et al*., 1983). Selection against Ura^+ strains was accomplished by culture on solid synthetic media containing 1 mg/ml 5FOA (Boeke *et al*., 1987). Sectoring assays were performed on solid synthetic media containing 25% of the normal amount of adenine and histidine supplements (SC_{sec}). DNA cloning was performed using standard techniques outlined in Sambrook *et al*. (1989).

Synthetic lethal screen

An *ade2 ade3* sectoring screen (Bender and Pringle, 1991) was used to identify mutants that are lethal in combination with *rna1-1*. Yeast strains LDY431 (MAT**a** *rna1-1 ade2 ade3 ura3 leu2 his7*) and LDY432 (MATα *rna1-1 ade2 ade3 ura3 leu2 trp1*) were transformed with pLDB382 (*RNA1 URA3 ADE3 CEN*) and mutagenized with either EMS (to 80% lethality) or ultraviolet (UV) irradiation (to 50% lethality). Colonies (150 000 total) were screened for the inability to form white sectors indicative of loss of the *ADE3* containing plasmid. Nonsectoring colonies were further screened for sensitivity to 5-FOA, indicating that they could not survive loss of the plasmid-linked *URA3* marker. Of 187 candidates obtained from this analysis, 55 reverted to Sec^+ FOA^r after transformation with pLDB93 (*RNA1 TRP1 CEN*), and 15 of these segregated 2:2 in backcrosses to either LDY431 or LDY432. We refer to these mutants as *rna1-1* lethal, or *rle*. The mutants fell into four complementation groups, the largest being *RNA1* itself. The second largest group contained three members, *rle1-1* to *3* (LDY924, -928 and -932) The *RLE1* gene was cloned by complementation of the non-sectoring, FOA^s phenotype of LDY924 (*rle1-1 rna11 <RNA1 CEN URA3 ADE3*) with a yeast genomic library in pRS314. Three overlapping clones were obtained, all of which contained the *CRM1* gene. An *Xho*I–*Sal*I fragment containing the entire *CRM1* gene cloned into pRS315 (pLDB392) complemented all three *rle1* alleles. Allelism of *rle1* with *CRM1* was confirmed by crossing each of the alleles to LDY884 or LDY885 (*CRM1::LEU2*) and showing segregation opposite the marked *CRM1* allele. A precise disruption of *CRM1* with the bacterial *KAN* gene was made by PCR using the *KAN-MX2* cassette as a template with hybrid oligonucleotides LOL6 and LOL18 (Baudin *et al*., 1993). The resulting fragment was transformed into diploid yeast strain LDY436, yielding strain LDY880. Replacement of the endogenous *CRM1* gene was checked by PCR on genomic DNA, using LOL3 and LOL20. Sporulation and tetrad dissection showed that viability segregated 2:2, and all viable spores were Kan^s.

Plasmid constructions

PCR primers are described in Table III.

YAP1 (CRD)-B42^{AD} and mutations. Full-length *YAP1*, or the CRD alone (amino acid residues 526–650), was PCR-amplified from YEp351-*YAP1* to introduce *Eco*RI and *Xho*I sites at the ends. The resulting fragments were cloned into *Eco*RI–*Xho*I sites of pJG4-5 to create in-frame fusions to the *B42* activation domain (pLDB438 and pLDB439). Site-directed mutations in pLDB438 were generated by two step PCR mutagenesis. In the first step, complementary mutagenic oligonucleotides (Table III) were used in separate PCR reactions with each of two outside primers, homologous to pJG4-5 vector sequences flanking the *YAP1* insert (COL11 and COL12). The resultant PCR fragments overlap 30–50 bp in the region containing the point mutations. The two PCR fragments were gel purified, combined and subjected to five cycles of annealing/extension in the absence of added primers, prior to addition of COL11 and COL12, and continued cycling. The final PCR product was co-transformed into yeast strain SM13 with pJG4-5 that had been linearized with *Eco*RI and *Xho*I. Intact plasmids generated by gap repair were rescued from Trp^+ transformants. Multiple point mutations were generated sequentially, using the same procedure.

YAP1–GFP and mutations. 'SuperGlow' *GFP* (Kahana *et al*., 1995) was PCR-amplified from pJK19-1 using hybrid primers LOL23 and LOL26 that are flanked by sequences homologous to the 3′ end of *YAP1*, to produce a fragment containing *GFP* fused in frame to the last codon of *YAP1*. YEp351-*YAP1* was linearized at the 3′ *Nde*I site of *YAP1* and co-transformed with the PCR product into yeast strain SM13 (*yap1-Δ2::hisG*). The intact plasmid generated by gap repair (pLDB419) was rescued from a Leu^+ transformant expressing *YAP1–GFP*. Site-directed mutations in pLDB419 (Table I) were generated by two-step PCR mutagenesis followed by gap repair, as described above, using the same mutagenic primer pairs with outside primers COL13 and COL14. For co-transformation, pLDB419 was linearized with *Pac*I and *Nco*I.

GST and His_6 fusion proteins. Primers COL30 and COL31 were used to PCR amplify *YAP1* or *yap1* mutants from the *YAP1–GFP* plasmid series described above. The PCR products were digested with *Eco*RV and *Eco*RI and ligated into pGEX2TK between *Sma*I and *Eco*RI, or pGEX4T-1 between *Bam*HI (blunt ended using Klenow) and *Eco*RI to generate the GST–Yap1 series (Table I). To make *GST–NES^{PKI}*, primers MN35 and MN36 were annealed and cloned into *Eco*RI–*Xho*I sites of pGEX4T-1 to generate pLDB455. Construction of *his_6-gsp1$^{G21\text{-}V}$* (pLDB450) will be described elsewhere (J.Booth *et al*., in preparation).

CRM1^{HA}. Oligonucleotide LOL13 was used to introduce the HA epitope into the most 3′ *Nsi*I site of pLDB393 (*CRM1 URA3 CEN*), using the method of Kunkel *et al*. (1987) to generate pLDB396. pLDB396 was transformed into diploid yeast strain LDY880 (*CRM1*/*crm1::KAN*) prior to sporulation and tetrad dissection. Ura^+ Kan^R spores were recovered and grew normally, showing that the epitope-tagged *CRM1* gene is functional.

Yeast cell fractionation, GST–YAP1 precipitation, protein binding assays and immunoblot analysis

Diploid yeast strain LDY903 (*crm1*::*KAN/crm1*::*KAN <CRM1^{HA} URA3 CEN>*) was grown in rich medium to mid-log phase. Cells were spheroplasted and lysed, and the lysate was subjected to low-speed centrifugation as described by Bogerd *et al*. (1994) to yield a supernatant fraction (S1) that contains the bulk of $Crm1p^{HA}$. The S1 fraction was concentrated to one-quarter volume using a Centricon 30 unit. His_6-$Gsp1p^{G21\text{-}V}$ was purified from *Escherichia coli* using Ni-NTA affinity chromatography as recommended by the manufacturer (Qiagen, Santa Clarita, CA). Induction of GST fusion proteins in *E.coli* was performed as described (Smith and Johnson, 1988). Induced cells were washed with ice-cold 0.9% NaCl, collected by centrifugation for 10 min at 5000 r.p.m. in a Sorvall GS-3 rotor and frozen in liquid nitrogen. Pellets were thawed at 37°C, resuspended in Buffer A [2.3 M sucrose, 50 mM Tris–HCl pH 7.5 and protease inhibitiors (PI; Bogerd *et al*., 1994)] and incubated at 4°C for 30 min. Ten milliliters of Buffer B (50 mM Tris–HCl pH 7.5, 10 mM KCl; 1 mM EDTA; 1 mM DTT; PI) containing 3 mg of lysozyme were added, followed by incubation on ice for 1 h. One 1/100 volume of 10% taurodeoxycholic acid, 1/100 volume of 1 M $MgCl_2$ and 0.25 mg DNase were then added to lysed cells, and after incubation for 10–15 min on ice, the lysate was centrifuged at 13 000 r.p.m. for 45 min at 4°C in a Sorvall SS-34 rotor. To purify the GST fusion proteins, crude lysates were incubated with glutathione–Sepharose at 4°C for 1 h in ELB2 (0.2 M NaCl, 0.05 M HEPES pH 7.0, 5 mM $MgCl_2$, 0.1% Tween-20) containing 1 mM DTT and PI. The resin was then washed five times in ELB2 + PI and resuspended to 50% bead volume.

Binding assays were performed in 100 µl volumes containing 60 µl yeast S1 fraction, 20 µl of 4× ELB2 (plus 1 mM GTP and PI, ± 1 mM DTT, ± 20 µg His_6::$Gsp1^{G21\text{-}V}$-GTP) and 20 µl of beads adsorbed with the GST fusion proteins. The mixture was rotated for 2 h at 4°C, and the beads were washed six times using ELB2 plus 0.25 mM GTP and PI, with or without 1 mM DTT. Bound proteins were eluted in Laemmli buffer and heated for 10 min at 95°C prior to SDS–PAGE. Immunoblotting was performed essentially as described (Belanger *et al*., 1994) using mAb 12CA5 (Harvard cell culture facility, Cambridge, MA), anti-EK (Invitrogen, Carlsbad, CA) and anti-GST (Molecular Probes, Eugene, OR) antibodies. Bound antibodies were detected using the ECL detection system (Amersham, Cleveland, OH). In every experiment, blots were stripped and reprobed with anti-GST antibody. In all cases, equivalent amounts of GST fusion protein were present in each lane (data not shown).

For analysis of Yap1–GFP expression, whole-cell lysates were prepared by boiling in SDS sample buffer, as previously described (Yan and Melese, 1993). Lysates were subjected to SDS–PAGE and transferred to nitrocellulose. Blots were probed as described above, using rabbit anti-GFP at a dilution of 1:5000.

Table III. Oligonucleotide primers

Primer#	Sequence
COL1	ATAACAACACATCCGAAAGCCTCAGATGCTGATGTCGATGGTTTATGT Y^{611A},I^{614A} *YAP1* mutations; COL2, reverse.
COL3	CATCCGAAATACTCAGATGCTGATGCCGATGGTTTATGTTCCG I^{614A},V^{616A} *YAP1* mutations; COL4, reverse.
COL5	AATACTCAGATATTGATGCCGATGGTGCATGTTCCGAGCTAATGGC V^{616A},L^{619A} *YAP1* mutations; COL6, reverse.
COL7	GATATTGATGTCGATGGTGCATGTTCCGAGGCAATGGCAAAGGCAAAATG L^{619A},L^{623A} *YAP1* mutations; COL8, reverse.
COL9	GATATTGATGTCGATGGTGCATGTTCCGAGCTAATGGC L^{619A} *YAP1* mutation; COL10, reverse.
COL15	ATTGATGTCGATGGTTTAGCTTCCGAGCTAATGGCAAA C^{620A} *YAP1* mutation; COL16, reverse.
COL17	GAAGGCTCTTTACTAAGGGCTTCGGAAATTTGGGATAG C^{598A} *YAP1* mutation; COL18, reverse.
COL19	CTAATGGCAAAGGCAAAAGCTTCAGAAAGAGGGGTTGTC C^{629A} *YAP1* mutation; COL20, reverse.
COL22	ATTGATGTCGATGGTTTAACTTCCGAGCTAATGGCAAA C^{620T} *YAP1* mutation; COL23, reverse.
COL24	GAAGGCTCTTTACTAAGGACTTCGGAAATTTGGGATAG C^{598T} *YAP1* mutation; COL25, reverse
COL26	CTAATGGCAAAGGCAAAAACTTCAGAAAGAGGGGTTGTC C^{629T} *YAP1* mutation; COL27, reverse.
COL28	GGATAGAATAACAACACGTCCGAAATACTCAGAT H^{608R} *YAP1* mutation; COL29, reverse
COL36	GTTTCGGAGTTTGCTTCGAAAATGAACCAGGTAGCTGGAACAAGGCAAGCTCCCATTCCCAAG C^{303A},C^{310A},C^{315A} *YAP1* mutations; COL37, reverse.
COL11	CCATCATTGAGCAGTTTATCGACT pJG4-5 plasmid; nt 601–624.
COL12	CCTGACCTACAGGAAAGAGTTACTCTC reverse pJG4-5 plasmid; nt 1138–1112.
COL13	AGTCCGGGATCTACCGGCATCGGC *YAP1*; nt +1308 from ATG.
COL14	GGACAGGGCCATCGCCAATTGGAG reverse sGFP; nt +580 from ATG.
COL31	GGCCGATATCG**AGTGTGTCTACCGCCAAGAGG** *Eco*RV site::***YAP1***; nt +4 from ATG.
COL30	CCCGGGGAATTCC**CGCTTTAGTTCATATGCTTATTC** reverse *Eco*RI site::***YAP1***; nt +1952.
LOL3	TAGATTGAGAAATATAGATCCATTTGAG *CRM1*; nt –650 from ATG.
LOL6	**ATGGAAGGAATTTTGGATTTTTCTAACGACTTAGATATCGC**AGCTGAAGCTTCGTACGC ***CRM1***-KAN^R hybrid; *CRM1*; at ATG.
LOL18	**CCGCCTCTAATCATCAAGTTCGGAAGGTTTTAATAACCCA**GCATAGGCCACTAGTGGATCTG reverse ***CRM1***-KAN^R hybrid; *CRM1* nt +3261 from ATG.
LOL13	GCTGAAGATAAAGAGAATGC**GTACCCATACGACGTCCCAGACTACGCT**TTAATGGAACAGAATAGACTA *CRM1*; nt +3150 from ATG, with HA-epitope tag inserted at 3′ *Nsi*I site.
LOL20	GGGATTAATACCACCTGGCACATTATTG reverse *CRM1*; nt +3902 from ATG.
LOL23	**GCTTTGAATAAGCATATGAAC**GCTAGCAAAGGAGAAGAACTC ***YAP1***::sGFP hybrid.*YAP1*; nt +1929 from AUG. sGFP, nt +4 from ATG.
LOL26	**CCAGTTTTCCATAAAGTTCCCGCTTTAG**CAGCCGGATCCTTTGTATAG reverse ***YAP1***::sGFP hybrid. *YAP1*, nt +1977 from ATG.
MN35	**AATTC**AGCAATGAATTAGCCTTGAAATTAGCAGGTCTTGATATCC NES of PKI (amino acids 37–46), with *Eco*RI complementary end.
MN36	GTCGTTACTTAATCGGAACTTTAATCGTCCAGAACTATAG**GAGCT** reverse, NES of PKI, with *Xho*I complementary end.

Two-hybrid assays

pKW442 [*CRM1-LexA*^{BD} (Stade *et al*., 1997)] and other β-karyopherin fusions (Neville *et al*., 1997) were transformed into yeast strain RFY206. LDB432 (*YAP1-B42*^{AD}) and pLDB436 (*YAP1* (*CRD*)-*B42*^{AD}) were transformed into yeast strain EGY48. To test interactions, EGY48 and RFY206 transformants were mated and diploids were selected on synthetic medium (2% glucose) lacking uracyl, tryptophan and histidine. Diploids were tested for LacZ expression by plating on synthetic medium (1% raffinose/2% galactose) lacking uracyl, tryptophan and histidine, containing 80 μg/ml X-gal. To assay the interaction between *CRM1-LexA*^{BD} and the *yap1(CRD)-B42*^{AD} mutants, *CRM1* and *yap1(crd)* constructs were co-transformed, along with the reporter plasmid pSH18-34, into EGY48. Ura^+ Trp^+ His^+ transformants were tested on X-gal-containing plates as described above.

Fluorescence microscopy

To analyze the distribution of GFP fusion proteins, cells were grown in synthetic complete (SC) medium lacking the appropriate amino acid to early log phase, and then shifted to rich medium for 6 h. Cells were washed with phosphate-buffered saline (PBS) and GFP signals were analyzed in living cells. Alternatively, cells were fixed in 75% ethanol for 15 min on ice, washed with PBS and stained using 1 μg/ml DAPI (added directly to fixed cells without further washes). Samples were observed using a Nikon Optiphot fluorescence microscope.

Acknowledgements

We thank Pam Silver, Scott Moye-Rowley, Karsten Weis, Brett Finlay, Anita Hopper and Michael Rosbash for providing strains, antibodies and

plasmids. We are grateful to Melissa Moore, Michael Rosbash, Jim Haber and members of the Rosbash and Davis laboratories for critical reading of the manuscript and for many insightful discussions. We particularly thank Megan Neville for providing the two-hybrid constructs and James Booth for providing the *His$_6$-gsp1^{G21-V}* plasmid construct.

References

Aitchison,J.D., Blobel,G. and Rout,M.P. (1996) Kap104p: a karyopherin involved in the nuclear transport of messenger RNA binding proteins. *Science*, **274**, 624–627.

Amberg,D.C., Fleischmann,M., Stagljar,I., Cole,C.N. and Aebi,M. (1993) Nuclear PRP20 protein is required for mRNA export. *EMBO J.*, **12**, 233–241.

Arts,G.J., Fornerod,M. and Mattaj,I.W. (1998) Identification of a nuclear export receptor for tRNA. *Curr. Biol.*, **8**, 305–314.

Baudin,A., Ozier-Kalogeropoulos,O., Denouel,A., Lacroute,F. and Cullin,C. (1993) A simple and efficient method for direct gene deletion in *Saccharomyces cerevisiae*. *Nucleic Acids Res.*, **21**, 3329–3330.

Beals,C.R., Sheridan,C.M., Turck,C.W., Gardner,P. and Crabtree,G.R. (1997) Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3. *Science*, **275**, 1930–1934.

Becker,J., Melchior,F., Gerke,V., Bischoff,F.R., Ponstingl,H. and Wittinghofer,A. (1995) RNA1 encodes a GTPase-activating protein specific for Gsp1p, the Ran/TC4 homologue of *Saccharomyces cerevisiae*. *J. Biol. Chem.*, **270**, 11860–11865.

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.

Bender,A. and Pringle,J.R. (1991) Use of a screen for synthetic lethal and multicopy suppressee mutants to identify two new genes involved in morphogenesis in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **11**, 1295–1305.

Bischoff,F.R. and Ponstingl,H. (1991) Catalysis of guanine nucleotide exchange of Ran by the mitotic regulator RCC1. *Nature*, **354**, 80–82.

Bischoff,F.R. and Görlich,D. (1997) RanBP1 is crucial for the release of RanGTP from importin β-related nuclear transport factors. *FEBS Lett.*, **419**, 249–254.

Bischoff,F.R., Krebber,H., Kempf,T., Hermes,I. and Ponstingl,H. (1995) Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. *Proc. Natl Acad. Sci. USA*, **92**, 1749–1753.

Boeke,J.D., Truehart,J., Natsoulis,G. and Fink,G.R. (1987) 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.*, **154**, 164–175.

Bogerd,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.

Bogerd,H.P., Fridell,R.A., Benson,R.E., Hua,J. and Cullen,B.R. (1996) Protein sequence requirements for function of the human T-cell leukemia virus type 1 Rex nuclear export signal delineated by a novel *in vivo* randomization-selection assay. *Mol. Cell. Biol.*, **16**, 4207–4214.

Bonifaci,N., Moroianu,J., Radu,A. and Blobel,G. (1997) Karyopherin β2 mediates nuclear import of a mRNA binding protein. *Proc. Natl Acad. Sci. USA*, **94**, 5055–5060.

Clark,K., Ohtsubo,M., Nishimoto,T., Goebl,M. and Sprague,G.F. (1991) The yeast SRM1 protein and human RCC1 protein share analogous functions. *Cell Reg.*, **2**, 781–792.

Enenkel,C., Blobel,G. and Rexach,M. (1995) Identification of a yeast karyopherin heterodimer that targets import substrate to mammalian nuclear pore complexes. *J. Biol. Chem.*, **270**, 16499–16502.

Engel,K., Kotlyarov,A. and Gaestel,M. (1998) Leptomycin B-sensitive nuclear export of MAPKAP kinase 2 is regulated by phosphorylation. *EMBO J.*, **17**, 3363–3371.

Fischer,U., Huber,J., Boelens,W.C., Mattaj,I.W. and Luhrmann,R. (1995) The HIV-1 Rev activation domain is a nuclear export signal that accesses an export pathway used by specific cellular RNAs. *Cell*, **82**, 475–483.

Floer,M., Blobel,G. and Rexach,M. (1997) Disassembly of RanGTP-karyopherin β complex, an intermediate in nuclear protein import. *J. Biol. Chem.*, **272**, 19538–19546.

Fornerod,M., Ohno,M., Yoshida,M. and Mattaj,I.W. (1997) CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell*, **90**, 1051–1060.

Fridell,R.A., Fischer,U., Luhrmann,R., Meyer,B.E., Meinkoth,J.L., Malim,M.H. and Cullen,B.R. (1996) Amphibian transcription factor IIIA proteins contain a sequence element functionally equivalent to the nuclear export signal of human immunodeficiency virus type 1 Rev. *Proc. Natl Acad. Sci. USA*, **93**, 2936–2940.

Fridell,R.A., Truant,R., Thorne,L., Benson,R.E. and Cullen,B.R. (1997) Nuclear import of hnRNP A1 is mediated by a novel cellular cofactor related to karyopherin-β. *J. Cell Sci.*, **110**, 1325–1331.

Fukuda,M., Gotoh,I., Gotoh,Y. and Nishida,E. (1996) Cytoplasmic localization of mitogen-activated protein kinase kinase directed by its NH_2-terminal, leucine-rich short amino acid sequence, which acts as a nuclear export signal. *J. Biol. Chem.*, **271**, 20024–20028.

Fukuda,M., Asano,S., Nakamura,T., Adachi,M., Yoshida,M., Yanagida,M. and Nishida,E. (1997) CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature*, **390**, 308–311.

Görlich,D. (1998) Transport into and out of the cell nucleus. *EMBO J.*, **17**, 2721–2727.

Görlich,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.

Görlich,D., Kostka,S., Kraft,R., Dingwall,C., Laskey,R.A., Hartmann,E. and Prehn,S. (1995a) Two different subunits of importin cooperate to recognize nuclear localization signals and bind them to the nuclear envelope. *Curr. Biol.*, **5**, 383–392.

Görlich,D., Vogel,F., Mills,A.D., Hartmann,E. and Laskey,R.A. (1995b) Distinct functions for the two importin subunits in nuclear protein import. *Nature*, **377**, 246–248.

Görlich,D., Pante,N., Kutay,U., Aebi,U. and Bischoff,F.R. (1996) Identification of different roles for RanGDP and RanGTP in nuclear protein import. *EMBO J.*, **15**, 5584–5594.

Görlich,D., Dabrowski,M., Bischoff,F.R., Kutay,U., Bork,P., Hartmann,E., Prehn,S. and Izaurralde,E. (1997) A novel class of RanGTP binding proteins. *J. Cell Biol.*, **138**, 65–80.

Gorner,W., Durchschlag,E., Martinez-Pastor,M.T., Estruch,F., Ammerer,G., Hamilton,B., Ruis,H. and Schuller,C. (1998) Nuclear localization of the C2H2 zinc finger protein Msn2p is regulated by stress and protein kinase A activity. *Genes Dev.*, **12**, 586–597.

Gounalaki,N. and Thireos,G. (1994) Yap1p, a yeast transcriptional activator that mediates multidrug resistance, regulates the metabolic stress response. *EMBO J.*, **13**, 4036–4041.

Gyuris,J., Golemis,E., Chertkov,H. and Brent,R. (1993) Cdi1, a human G_1 and S phase protein phosphatase that associates with Cdk2. *Cell*, **75**, 791–803.

Hirata,D., Yano,K. and Miyakawa,T. (1994) Stress-induced transcriptional activation mediated by YAP1 and YAP2 genes that encode the Jun family of transcriptional activators in *Saccharomyces cerevisiae*. *Mol. Gen. Genet.*, **242**, 250–256.

Hopper,A.K., Traglia,H.M. and Dunst,R.W. (1990) The yeast RNA1 gene product necessary for RNA processing is located in the cytosol and apparently excluded from the nucleus. *J. Cell Biol.*, **111**, 309–321.

Huber,J., Cronshagen,U., Kadokura,M., Marshallsay,C., Wada,T., Sekine,M. and Luhrmann,R. (1998) Snurportin1, an m3G-cap-specific nuclear import receptor with a novel domain structure. *EMBO J.*, **17**, 4114–4126.

Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) Transformation of intact yeast cells treated with alkali cations. *J. Bacteriol.*, **153**, 163–168.

Izaurralde,E. and Adam,S. (1998) Transport of macromolecules between the nucleus and the cytoplasm. *RNA*, **4**, 351–364.

Izaurralde,E., Kutay,U., von Kobbe,C., Mattaj,I.W. and Görlich,D. (1997) The asymmetric distribution of the constituents of the Ran system is essential for transport into and out of the nucleus. *EMBO J.*, **16**, 6535–6547.

Kahana,J.A., Schnapp,B.J. and Silver,P.A. (1995) Kinetics of spindle pole body separation in budding yeast. *Proc. Natl Acad. Sci. USA*, **92**, 9707–9711.

Karin,M. and Hunter,T. (1995) Transcriptional control by protein phosphorylation: signal transmission from the cell surface to the nucleus. *Curr. Biol.*, **5**, 747–757.

Kudo,N., Wolff,B., Sekimoto,T., Schreiner,E.P., Yoneda,Y., Yanagida,M., Horinouchi,S. and Yoshida,M. (1998) Leptomycin B inhibition of signal-mediated nuclear export by direct binding to CRM1. *Exp. Cell Res.*, **242**, 540–547.

Kuge,S. and Jones,N. (1994) YAP1 dependent activation of TRX2 is essential for the response of *Saccharomyces cerevisiae* to oxidative stress by hydroperoxides. *EMBO J.*, **13**, 655–664.

Kuge,S., Jones,N. and Nomoto,A. (1997) Regulation of yAP-1 nuclear localization in response to oxidative stress. *EMBO J.*, **16**, 1710–1720.

Kumada,K., Yanagida,M. and Toda,T. (1996) Caffeine-resistance in fission yeast is caused by mutations in a single essential gene, *crm1+*. *Mol. Gen. Genet.*, **250**, 59–68.

Kunkel,T.A., Roberts,J.D. and Zakour,R.A. (1987) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Methods Enzymol.*, **154**, 367–381.

Kutay,U., Bischoff,F.R., Kostka,S., Kraft,R. and Görlich,D. (1997) Export of importin alpha from the nucleus is mediated by a specific nuclear transport factor. *Cell*, **90**, 1061–1071.

Kutay,U., Lipowsky,G., Izaurralde,E., Bischoff,F.R., Schwarzmaier,P., Hartmann,E. and Görlich,D. (1998) Identification of a tRNA-specific nuclear export receptor. *Mol. Cell*, **1**, 359–369.

Li,Y., Yamakita,Y. and Krug,R.M. (1998) Regulation of a nuclear export signal by an adjacent inhibitory sequence: the effector domain of the influenza virus NS1 protein. *Proc. Natl Acad. Sci. USA*, **95**, 4864–4869.

Lounsbury,K.M. and Macara,I.G. (1997) Ran-binding protein 1 (RanBP1) forms a ternary complex with Ran and karyopherin β and reduces Ran GTPase-activating protein (RanGAP) inhibition by karyopherin β. *J. Biol. Chem.*, **272**, 551–555.

Lounsbury,K.M., Richards,S.A., Perlungher,R.R. and Macara,I.G. (1996) Ran binding domains promote the interaction of Ran with p97/β-karyopherin, linking the docking and translocation steps of nuclear import. *J. Biol. Chem.*, **271**, 2357–2360.

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.

Melchior,F., Guan,T., Yokoyama,N., Nishimoto,T. and Gerace,L. (1995) GTP hydrolysis by Ran occurs at the nuclear pore complex in an early step of protein import. *J. Cell Biol.*, **131**, 571–581.

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.

Moroianu,J., Blobel,G. and Radu,A. (1995) Previously identified protein of uncertain function is karyopherin alpha and together with karyopherin β docks import substrate at nuclear pore complexes. *Proc. Natl Acad. Sci. USA*, **92**, 2008–2011.

Nakielny,S., Siomi,M.C., Siomi,H., Michael,W.M., Pollard,V. and Dreyfuss,G. (1996) Transportin: nuclear transport receptor of a novel nuclear protein import pathway. *Exp. Cell Res.*, **229**, 261–266.

Neville,M., Stutz,F., Lee,L., Davis,L.I. and Rosbash,M. (1997) The importin-β family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr. Biol.*, **7**, 767–775.

Nishi,K., Yoshida,M., Fujiwara,D., Nishikawa,M., Horinouchi,S. and Beppu,T. (1994) Leptomycin B targets a regulatory cascade of crm1, a fission yeast nuclear protein, involved in control of higher order chromosome structure and gene expression. *J. Biol. Chem.*, **269**, 6320–6324.

O'Neill,R.E., Talon,J. and Palese,P. (1998) The influenza virus NEP (NS2 protein) mediates the nuclear export of viral ribonucleoproteins. *EMBO J.*, **17**, 288–296.

Oki,M., Noguchi,E., Hayashi,N. and Nishimoto,T. (1998) Nuclear protein import, but not mRNA export, is defective in all *Saccharomyces cerevisiae* mutants that produce temperature-sensitive forms of the Ran GTPase homologue Gsp1p. *Mol. Gen. Genet.*, **257**, 624–634.

Ossareh-Nazari,B., Bachelerie,F. and Dargemont,C. (1997) Evidence for a role of CRM1 in signal-mediated nuclear protein export. *Science*, **278**, 141–144.

Palacios,I., Weis,K., Klebe,C., Mattaj,I.W. and Dingwall,C. (1996) RAN/TC4 mutants identify a common requirement for snRNP and protein import into the nucleus. *J. Cell Biol.*, **133**, 485–494.

Palmeri,D. and Malim,M.H. (1996) The human T-cell leukemia virus type 1 posttranscriptional trans-activator Rex contains a nuclear export signal. *J. Virol.*, **70**, 6442–6445.

Pasquinelli,A.E., Powers,M.A., Lund,E., Forbes,D. and Dahlberg,J.E. (1997) Inhibition of mRNA export in vertebrate cells by nuclear export signal conjugates. *Proc. Natl Acad. Sci. USA*, **94**, 14394–14399.

Pemberton,L.F., Rosenblum,J.S. and Blobel,G. (1997) A distinct and parallel pathway for the nuclear import of an mRNA-binding protein. *J. Cell Biol.*, **139**, 1645–1653.

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.

Richards,S.A., Carey,K.L. and Macara,I.G. (1997) Requirement of guanosine triphosphate-bound ran for signal-mediated nuclear protein export. *Science*, **276**, 1842–1844.

Roberts,P.M. and Goldfarb,D.S. (1998) *In vivo* nuclear transport kinetics in *Saccharomyces cerevisiae*. *Methods Cell Biol.*, **53**, 545–557.

Rosenblum,J.S., Pemberton,L.F. and Blobel,G. (1997) A nuclear import pathway for a protein involved in tRNA maturation. *J. Cell Biol.*, **139**, 1655–1661.

Roth,J., Dobbelstein,M., Freedman,D.A., Shenk,T. and Levine,A.J. (1998) Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. *EMBO J.*, **17**, 554–564.

Rout,M.P., Blobel,G. and Aitchison,J.D. (1997) A distinct nuclear import pathway used by ribosomal proteins. *Cell*, **89**, 715–725.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Sandri-Goldin,R.M. (1998) ICP27 mediates HSV RNA export by shuttling through a leucine-rich nuclear export signal and binding viral intronless RNAs through an RGG motif. *Genes Dev.*, **12**, 868–879.

Schnell,N., Krems,B. and Entian,K.D. (1992) The PAR1 (YAP1/SNQ3) gene of *Saccharomyces cerevisiae*, a c-jun homologue, is involved in oxygen metabolism. *Curr. Genet.*, **21**, 269–273.

Sherman,F., Fink,G.R. and Hicks,J.B. (1986) *Laboratory Course Manual for Methods in Yeast Genetics.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Smith,D.B. and Johnson,K.S. (1988) Single step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione *S*-transferase. *Gene*, **67**, 31–40.

Stade,K., Ford,C.S., Guthrie,C. and Weis,K. (1997) Exportin 1 (Crm1p) is an essential nuclear export factor. *Cell*, **90**, 1041–1050.

Stutz,F. and Rosbash,M. (1994) A functional interaction between Rev and yeast pre-mRNA is related to splicing complex formation. *EMBO J.*, **13**, 4096–4104.

Taagepera,S., McDonald,D., Loeb,J.E., Whitaker,L.L., McElroy,A.K., Wang,J.Y.J. and Hope,T.J. (1998) Nuclear-cytoplasmic shuttling of C-ABL tyrosine kinase. *Proc. Natl Acad. Sci. USA*, **95**, 7457–7462.

Toda,T., Shimanuki,M., Saka,Y., Yamano,H., Adachi,Y., Shirakawa,M., Kyogoku,Y. and Yanagida,M. (1992) Fission yeast pap1-dependent transcription is negatively regulated by an essential nuclear protein, crm1. *Mol. Cell. Biol.*, **12**, 5474–5484.

Toone,W.M., Kuge,S., Samuels,M., Morgan,B.A., Toda,T. and Jones,N. (1998) Regulation of the fission yeast transcription factor Pap1 by oxidative stress: requirement for the nuclear export factor Crm1 (Exportin) and the stress-activated MAP kinase Sty1/Spc1. *Genes Dev.*, **12**, 1453–1463.

Toyoshima,F., Moriguchi,T., Wada,A., Fukuda,M. and Nishida,E. (1998) Nuclear export of cyclin B1 and its possible role in the DNA damage-induced G_2 checkpoint. *EMBO J.*, **17**, 2728–2735.

Turi,T.G., Webster,P. and Rose,J.K. (1994) Brefeldin A sensitivity and resistance in *Schizosaccharomyces pombe*. Isolation of multiple genes conferring resistance. *J. Biol. Chem.*, **269**, 24229–24236.

Weis,K., Dingwall,C. and Lamond,A.I. (1996) Characterization of the nuclear protein import mechanism using Ran mutants with altered nucleotide binding specificities. *EMBO J.*, **15**, 7120–7128.

Wemmie,J.A., Steggerda,S.M. and Moye-Rowley,W.S. (1997) The *Saccharomyces cerevisiae* AP-1 protein discriminates between oxidative stress elicited by the oxidants H2O2 and diamide. *J. Biol. Chem.*, **272**, 7908–7914.

Wen,W., Meinkoth,J.L., Tsien,R.Y. and Taylor,S.S. (1995) Identification of a signal for rapid export of proteins from the nucleus. *Cell*, **82**, 463–473.

Wu,A., Wemmie,J.A., Edgington,N.P., Goebl,M., Guevara,J.L. and Moye-Rowley,W.S. (1993) Yeast bZip proteins mediate pleiotropic drug and metal resistance. *J. Biol. Chem.*, **268**, 18850–18858.

Yan,C. and Melese,T. (1993) Multiple regions of *NSR1* are sufficient for accumulation of a fusion protein within the nucleolus. *J. Cell Biol.*, **123**, 1081–1091.

Yang,J., Bardes,E.S.G., Moore,J.D., Brennan,J., Powers,M.A. and Kornbluth,S. (1998) Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. *Genes Dev.*, **12**, 2131–2143.

Yoneyama,M., Suhara,W., Fukuhara,Y., Fukuda,M., Nishida,E. and Fujita,T. (1998) Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300. *EMBO J.*, **17**, 1087–1095.

Zolotukhin,A.S. and Felber,B.K. (1997) Mutations in the nuclear export signal of human ran-binding protein RanBP1 block the Rev-mediated posttranscriptional regulation of human immunodeficiency virus type 1. *J. Biol. Chem.*, **272**, 11356–11360.

Received September 1, 1998; revised and accepted October 23, 1998