The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export

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Background: The human immunodeficiency virus (HIV-1) uses the viral protein Rev to regulate gene expression by promoting the export of unspliced and partially spliced viral transcripts. Rev has been shown to function in a variety of organisms, including *Saccharomyces cerevisiae*. The export activity of Rev depends on a nuclear export signal (NES), which is believed to interact either directly or indirectly with the nuclear pore complex to carry out its export function. Crm1p is a member of the importin-beta protein family, other members of which are known to be directly involved in nuclear import. Crm1p has recently been shown to contribute to nuclear export in vertebrate systems. Here, we have studied this mechanism of nuclear to cytoplasmic transport.

Results: Viable mis-sense mutations in the *CRM1* gene substantially reduced or eliminated the biological activity of Rev in *S. cerevisiae*, providing strong evidence that Crm1p also contributes to transport of Rev NES-containing proteins and ribonucleoproteins in this organism. Crm1p interacted with FG-repeat-containing nuclear pore proteins as well as Rev, and we have demonstrated that the previously described two-hybrid interaction between Rev and the yeast nuclear pore protein Rip1p is dependent on wild-type Crm1p.

Conclusions: We conclude that Crm1p interacts with the Rev NES and nuclear pore proteins during delivery of cargo to the nuclear pore complex. Our findings also agree well with current experiments on Crm1p orthologs in *Schizosaccharomyces pombe* and in vertebrate systems.

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Background

The HIV-1 Rev protein promotes the nuclear export of unspliced and partially spliced viral transcripts. The biological activity of Rev is based on three signals: a *cis*-acting Rev response element (RRE) in the RNA targets and two domains within Rev itself, corresponding to an RNA-binding domain that binds to the RRE and determines RNA target specificity, and an activation or effector domain that has been shown to function as a nuclear export signal (NES) [1]. The NES can function independently of the rest of the Rev protein and resembles other viral and cellular export signals [2–4]. The Rev NES can export RNAs and proteins in *Saccharomyces cerevisiae*, and mutations in the Rev NES have similar effects on the biological activity of Rev in both yeast and mammalian cells [5–7]. Consequently, at least a subset of the Rev-relevant export machinery must be conserved from yeast to man.

One of these export components in yeast is the protein Rip1p, originally identified in a yeast two-hybrid screen with the Rev NES and shown to contribute to Rev-mediated export. Rip1p contains XXFG repeats (single-letter amino acid code) [6]: FG-repeat domains are prominent features of a substantial fraction of nuclear pore proteins and fall into three classes, XXFG, GLFG and FXFG, based on amino acid sequence. Moreover, localization studies indicated that Rip1p is predominantly found at the nuclear pore complex [6,8,9]. More recent experiments on *RIP1* further support the notion that Rip1p is a *bona fide* constituent of the nuclear pore complex [10], consistent with our original postulate that Rip1p might act as a docking site at the nuclear pore complex for Rev NES-containing proteins and ribonucleoproteins (RNPs) in yeast [6,11]. Rip1p is also important for the export of natural yeast transport substrates: a *RIP1*-deletion strain is defective in the export of heat-shock RNAs [10,12].

Experiments in the mammalian system gave rise to a similar but somewhat different picture. An XXFG-repeat-containing protein, hRip1/Rab, was identified in two independent two-hybrid screens with a Rev NES bait [13,14]. The hRip1/Rab protein also had an effect on Rev biological activity in mammalian cells. As hRip1/Rab is predominantly nucleoplasmic, it has been proposed that this protein acts as a carrier to deliver Rev NES-containing cargo from the nucleoplasm to the nuclear pore complex [13,14].

A unique role of Rip1p in yeast or hRip1/Rab in humans was somewhat compromised by the fact that Rev manifested two-hybrid interactions not only with Rip1p but also with a broad range of nuclear pore proteins containing XXFG and GLFG repeats [6,11,15]. Surprisingly, the Rev NES mutant series had the same effect on the interactions between Rev and each of these proteins [11]. Furthermore, we and others have failed to observe a credible interaction *in vitro* between Rev NES and recombinant Rip1p or hRip1/Rab ([11]; data not shown), suggesting that this interaction is indirect or that it requires additional stabilizing factors. An attractive hypothesis therefore emerged: an as yet unidentified transporter contributes to the interaction between Rev NESs and the FG-repeat regions of nucleoporins, both during delivery to the pore complex and in the two-hybrid assays. The putative transporter would, therefore, be analogous to the well characterized importin-beta (also called Kap95p or karyopherin-beta). This protein interacts directly with repeat-containing nuclear pore proteins, both in biochemical experiments and during delivery of import substrates to the cytoplasmic face of the pore [16–20].

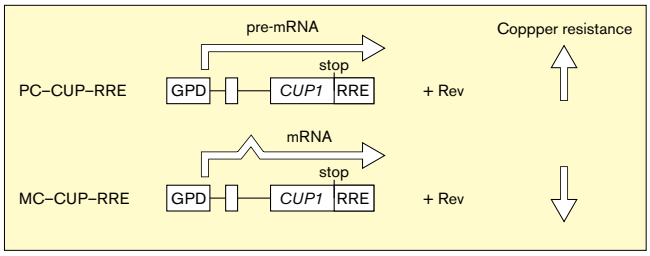
Recently, importin-beta family members have been shown to import distinct classes of substrates [21–24]. The yeast genome project and primary sequence comparisons indicate that importin-beta also has a number of less well characterized relatives [20,25]. Many of these family members share a functional characteristic of importin-beta in that they bind to FG-repeat-containing nucleoporins and to the import–export cofactor Ran [20,22,23,25,26]. Recent work independently identified one of these family members, Crm1p, in a mutant screen for genes that were synthetically lethal in combination with a mutation in the Ran GTPase-activating protein Rna1p. In this study, Crm1p was found to export the transcription factor Yap1p (L.L. and L.I.D., unpublished observations). Human Crm1p (hCrm1p) has also been implicated in Rev-mediated nuclear export in mammalian cells [27].

Here, we provide evidence that Crm1p also contributes to the export of Rev NES-containing proteins and RNPs in yeast. Our results suggest that Crm1p binds to repeat-containing nuclear pore proteins and contributes to the interaction between Rev NESs and nuclear pore proteins, both in the two-hybrid assays and during the actual transport process. Finally, the results agree well with current experiments on Crm1p orthologs in *Schizosaccharomyces pombe* as well as in vertebrates [25,28,29] and raise interesting questions about the relationship between the Rev NES and natural NESs of *S. cerevisiae*.

Results

We assayed the effects of three viable *crm1* mutations on Rev NES-mediated transport from the nucleus to the cytoplasm. The three mutants were isolated in a synthetic lethal screen with the well studied export–import cofactor mutant *rna1-1* (L.L. and L.I.D., unpublished observations). We used the Rev transport assay previously designed for *S. cerevisiae* to study the effects of the *crm1* mutations [5]. The *CUP1* gene, expression of which confers copper resistance in a dose-dependent manner [30,31], was deleted from the three *crm1* mutant strains. The strains were then transformed with a Rev-expressing plasmid and the two Rev-dependent reporter genes (Figure 1). The reporter genes contain the *CUP1* gene and an RRE in the 3′ untranslated region of the transcripts. The *CUP1* open reading frame is interrupted by a small, inefficiently spliced, synthetic intron that lacks stop codons. The two versions of the reporter gene differ in intron reading frame by one nucleotide: in the case of PC–CUP–RRE, only the unspliced pre-mRNA encodes Cup1p and, for MC–CUP–RRE, only the spliced mRNA encodes Cup1p (Figure 1).

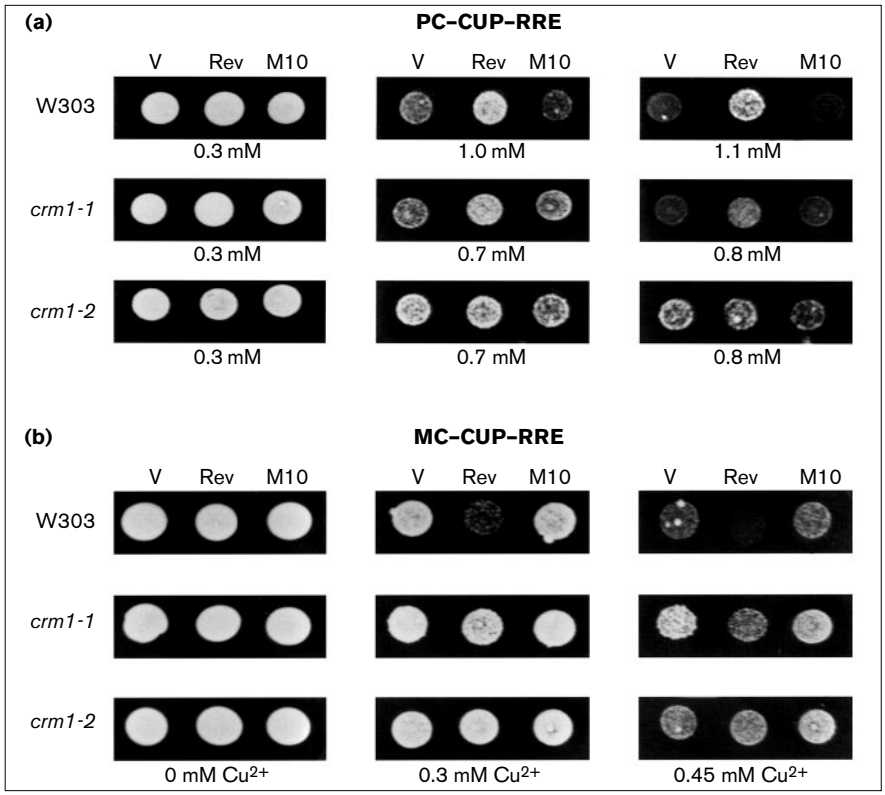
Figure 1



Schematic drawing of the *CUP1* reporter constructs. Details of the CUP1 reporter contructs have been described [5]. Transcription is driven by the glyceraldehyde-3-phosphate dehydrogenase promoter (GPD). In the PC–CUP–RRE construct, Cup1p is encoded by the pre-mRNA only, whereas in the MC–CUP–RRE construct, Cup1p is encoded from the spliced mRNA only. Rev has a positive effect on the cytoplasmic abundance of RRE-containing pre-mRNA transcripts. This effect is measured by growth on copper-containing plates, whereby a strain expressing Rev and containing PC–CUP–RRE shows an increase in copper resistance, whereas a strain containing MC–CUP–RRE shows a decrease. Each strain grows slightly differently on copper due to strain background (see Materials and methods).

The system recapitulates the biological activity of Rev in mammalian systems, namely, there is enhanced Rev-mediated pre-mRNA transport from the nucleus to the cytoplasm. As a consequence, the copper resistance of the strain containing the PC–CUP–RRE reporter gene is increased (Figure 2a; W303 strain, 1.0 mM and 1.1 mM Cu^{2+}). Because of greater pre-mRNA export, there is decreased expression of MC–CUP–RRE, resulting in a lower copper resistance of this strain (Figure 2b; W303 strain, 0.3 and 0.45 mM Cu^{2+}). These changes are dependent on the presence of a RRE as well as Rev itself. Importantly, the changes are also sensitive to mutations in the Rev NES [5–7]. Copper resistance therefore reflects Rev-mediated nuclear export.

Figure 2

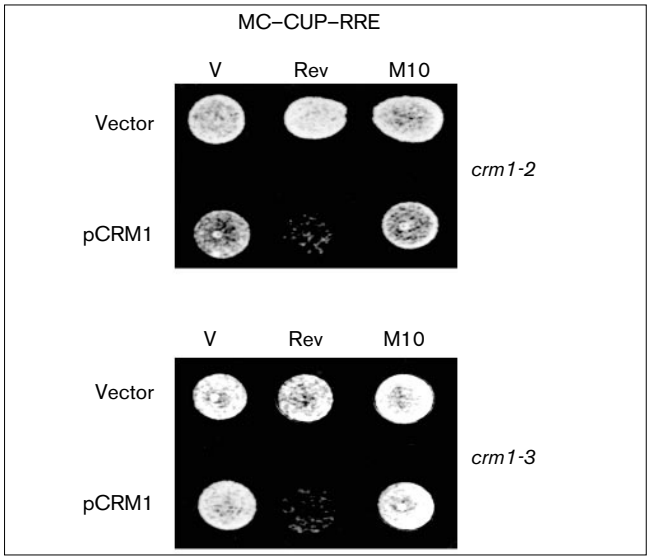


Mutations in *CRM1* inhibit Rev activity in yeast. Wild-type (W303) and *crm1* mutant strains were transformed with **(a)** the PC–CUP–RRE or **(b)** the MC–CUP–RRE reporter constructs (*LEU2*/2μ) as well as with plasmids expressing the wild-type Rev or the Rev M10 mutant (LE78,79DL) or the vector (V) plasmid alone (*TRP1*/*ARS1*-*CEN3*). The growth of each double transformant was examined on selective plates containing increasing copper concentrations as indicated.

All three *crm1* strains have striking effects on Rev biological activity; in the mutant backgrounds, there is much less Rev activity (Figures 2,3), based on a decreased ability of Rev to enhance PC–CUP–RRE-mediated expression (Figure 2a) as well as a decreased ability of Rev to inhibit MC–CUP–RRE-mediated expression (Figures 2b,3). In the case of one mutant, *crm1-2*, Rev biological activity was no longer detectable, particularly evident at 0.3 mM Cu^{2+} in the MC–CUP–RRE assay (Figure 2b). Even the weakest mutant, *crm1-1*, had a stronger effect on Rev than a deletion of the nuclear pore protein Rip1p, previously implicated in Rev-mediated function [6]. The third *crm1* mutant, *crm1-3*, had a comparable effect to *crm1-2* (Figure 3). Rev biological activity was fully restored by transformation with wild-type *CRM1*, indicating that the *crm1* mutations are indeed responsible for inhibition of Rev activity and that the mutant effect is fully recessive (Figure 3).

If Rev were an export substrate of Crm1p, an interaction between the two proteins might be dependent on a functional Rev NES. Indeed, a yeast two-hybrid assay demonstrated that Crm1p and Rev interact with each other; this interaction did not take place when Rev contained the classic M10 null mutation within the NES region (Table 1). A Rev–Rev homotypic interaction occurs through a different region of the Rev protein and was unaffected by the M10 mutation, indicating that M10 Rev is synthesized and

Figure 3



Wild-type Crm1p rescues Rev activity in *crm1* mutant strains. A centromeric plasmid (pRS316; *URA3*/*CEN*) either containing *CRM1* (pCRM1) or lacking *CRM1* (vector control) was introduced into mutant *crm1* strains (*crm1-2*, *crm1-3*); these strains also expressed wild-type Rev (*TRP1*/*ARS1*/*CEN3*) and the MC–CUP–RRE reporter construct (*LEU2*/2μ). The growth of triple transformants was examined on plates containing increasing copper concentrations; the results from plates containing 0.3 mM copper are shown.

Table 1

Crm1p–Rev two-hybrid interaction.

	Prey			
Bait	Rev wt	Rev M10	Rex	Rip1p
Crm1p	++	–	+	+
Rev	+++	++	–	+++
Rip1p	+++	–	++	–

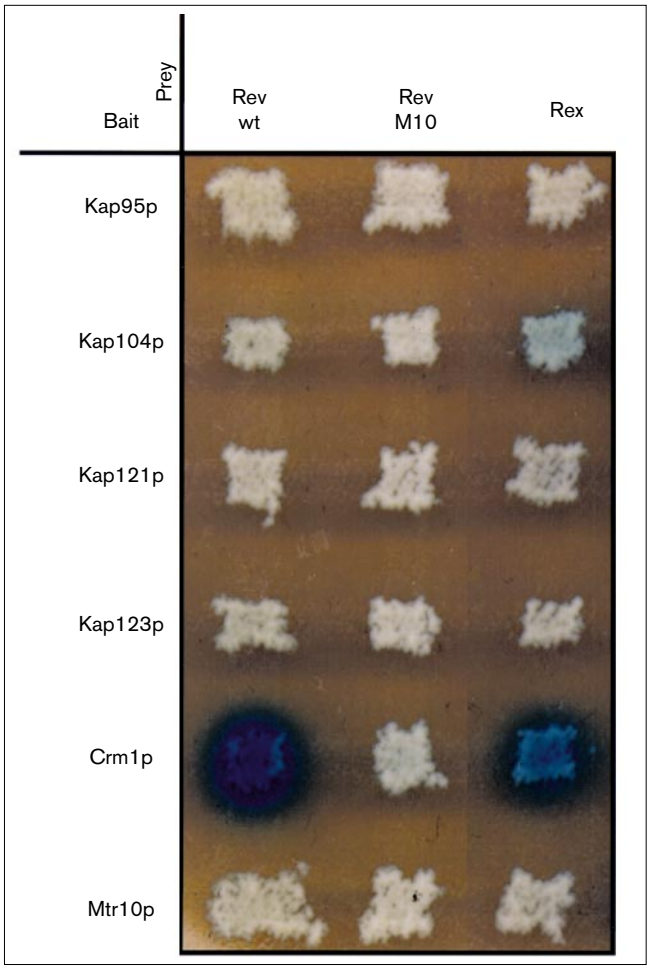
Summary table of the interactions between Crm1p, Rev or Rip1p bait fusions and Rev or Rex prey fusions. The bait constructs express LexA fused to total Crm1p, wild-type Rev or Rip1p (amino acids 121–230 of the Rip1p FG-repeat domain). Each bait construct was tested for interaction with four prey plasmids: wild-type Rev (wt), Rev M10 mutant (amino acids LE78,79DL), wild-type Rex and Rip1p (amino acids 121–230 of the Rip1p FG-repeat domain). The different bait–prey interactions were analyzed in diploids using a mating assay (see Materials and methods). The + symbols correspond to blue color intensity on X-Gal indicator plates as a result of *lacZ* gene activation.

stable in the yeast two-hybrid assay. An interaction was also detected between Crm1p and the HTLV-1 protein Rex, which is functionally related to Rev and has a similar NES ([32]; Table 1).

Crm1p is related to importin-beta [25], so we asked whether an interaction with Rev is a feature of other members of this family, such as Mtr10p as well as the known cytoplasmic transporters Kap95p, Kap104p (yeast transportin), Kap121p (Pse1p) and Kap123p (Yrb4p). Only Crm1p was found to interact with Rev (Figure 4). All of the bait constructs synthesized appropriately sized fusion proteins and were positive in interaction assays with other prey proteins (Table 2; data not shown). Crm1p also interacts with the yeast nucleoporin Rip1p, previously implicated in Rev NES-mediated nuclear export, and with hRip1/Rab as well as a broad set of repeat-containing nucleoporins (Tables 1,2). The interacting proteins contain all three classes of nucleoporin repeats (XXFG, GLFG, FXFG). Some of these interactions may reflect Crm1p re-import rather than export, and others may reflect more generic interactions between the importin-beta family and the nucleoporin repeat regions.

As Rev interacts with both Crm1p and the yeast nucleoporin Rip1p, we suspected that the previously reported Rev/Rip1p interaction [6] might be dependent on Crm1p. This proposal is based on our failure to observe an interaction *in vitro* between the Rev NES and recombinant Rip1p ([11]; data not shown) as well as the absence of any other report documenting a direct biochemical interaction between the Rev NES and recombinant nucleoporin or recombinant nucleoporin-like proteins. This interpretation is also based on a probable direct interaction between Crm1p and Rip1p, because several importin-beta family members are known to bind directly to repeat regions of nuclear pore proteins [22,23,26,33]. To test the possibility

Figure 4



Rev specifically interacts with Crm1p. Mating analysis of diploid strains containing various importin-beta family members as bait constructs and Rev or Rex prey constructs. The bait constructs express LexA fused to full-length Kap95p, Kap104p [22], Kap121p [50], Kap123p [23], Crm1p [34] and Mtr10p [51]. Each bait was tested for interaction with the three prey constructs described above. The results of the mating assay were analyzed on Ura$^-$His$^-$Trp$^-$, 3% galactose/1% sucrose/X-Gal indicator plates.

that Crm1p is important for the apparent Rev–Rip1p association, we assayed this two-hybrid interaction in the viable *crm1-1* mutant background (Figure 5). No interaction was observed, demonstrating that wild-type *CRM1* activity is required for the interaction. This effect was specific, because other two-hybrid interactions were unaffected, such as that between Per and Tim (two *Drosophila* proteins previously shown to interact by two-hybrid analysis; Figure 5), and also because the Rev bait and Rip1p prey interacted well with other proteins in this strain background (data not shown). The results suggest that the Rev–Rip1p two-hybrid interaction has additional requirements, namely, Crm1p normally sits together with these two proteins in a multi-component complex.

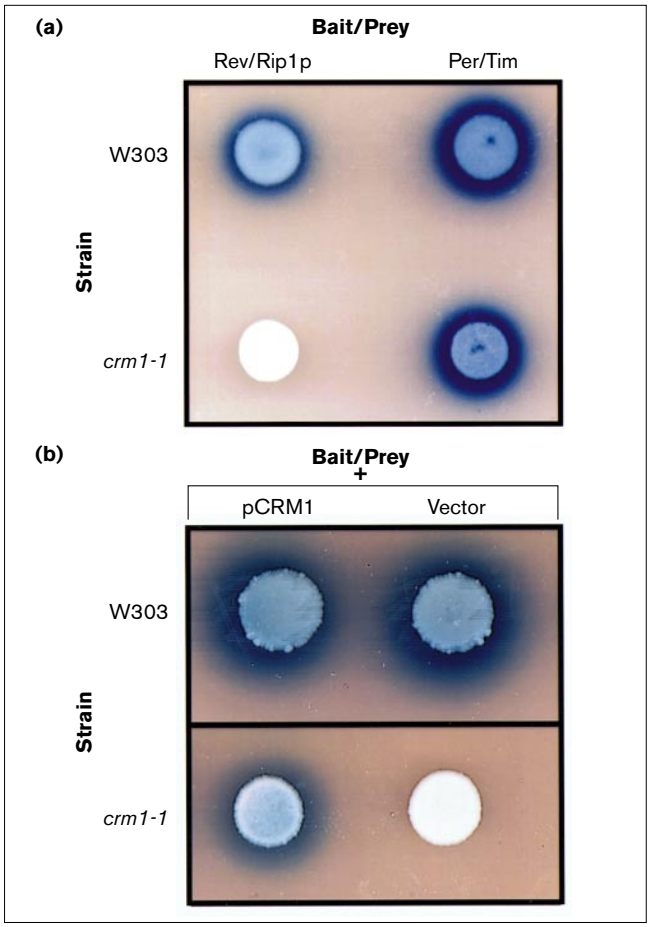
Table 2

Crm1p interacts with multiple nuclear pore proteins.

	Bait					
Prey	Crm1p	Kap95p	Kap104p	Kap121p	Kap123p	Mtr10p
yRip1p	++	+	+++	–	+	++
yNup49p	+++	–	+	–	–	+
yNup57p	++++	–	++++	++	+++	++++
yNup145p	+++	–	+++	–	–	++
yNup1p	++	+++	++++	++	+	++++
hRip1	+++	–	+	–	–	–
hNup98	++++	–	+	–	–	–
hCAN	++++	–	++	–	–	–
yRip1p3′	–	–	–	–	–	–

Summary table of the interactions between various importin-beta family member bait fusion proteins and various nucleoporin prey proteins. The interactions shown are between the bait fusion proteins described in Figure 4 and the following regions of FG-repeat containing nucleoporin prey proteins: yRip1p (121–230), yNup49p (7–239), yNup57p (1–247), yNup145p (24–216), yNup1p (438–737), hRip1 (388–562), hNup98 (41–515), hCAN (1691–1894) and the non-FG-repeat containing prey protein yRip1p3′ (356–430).

Figure 5



The *crm1-1* mutation specifically affects the Rev–Rip1p two-hybrid interaction. **(a)** The wild-type Rev bait construct and the Rip1p prey construct encoding full-length Rip1p were introduced into a wild-type strain (W303) or into the *crm1-1* mutant strain. As a control, the same strains were transformed with two-hybrid constructs encoding a Per bait fusion and a Tim prey fusion [47]. The two-hybrid interactions were examined in the presence of the pSH18-34 *lacZ* reporter construct by spotting the triple transformants on $Ura^{-}His^{-}Trp^{-}$ 3% galactose/1% sucrose/X-Gal indicator plates. **(b)** The interaction between Rev bait and Rip1p prey is restored by the addition of a plasmid expressing wild-type *CRM1* (pCRM1); addition of the empty vector has no effect. The levels of the bait and prey fusion proteins were shown to be similar in all strain backgrounds as assayed by western blotting analysis (data not shown).

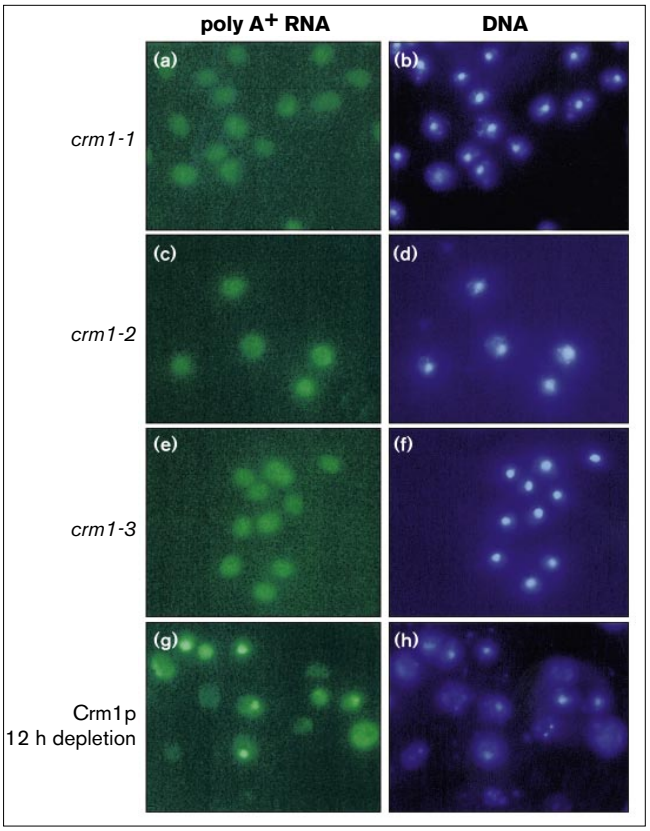
To address the relationship between Rev NES-mediated export and general mRNA export, we assayed the same three viable mutants for nuclear retention of $polyA^{+}$ RNA by *in situ* hybridization (Figure 6). No effect was observed, even after 2 hours incubation at 37°C (Figure 6a–f). Similar negative results were obtained for heat-shock mRNA transport ([10]; data not shown). In contrast, a 12 hour depletion of Crm1p *in vivo* caused substantial retention of nuclear $polyA^{+}$ RNA (Figure 6g). Staining of DNA with the intercalating dye DAPI shows that these cells have deformed nuclear chromosome domains (Figure 6h; [34]), suggesting that the RNA transport defect might be an indirect consequence of cell growth arrest or as a result of other difficulties caused more directly by the prolonged and extensive depletion of Crm1p. Alternatively, a more complete inactivation of Crm1p activity might be required to visualize an effect on $polyA^{+}$ RNA export than that needed to detect an effect on Rev NES activity (see Discussion).

Discussion

Recent results have identified the yeast protein Crm1p and its orthologs as probable transporters of protein and RNA/RNP cargo from the nucleus to the cytoplasm in several systems. Mutations that severely inhibit Crm1p activity lead to a number of dramatic phenotypes [34], although these phenotypes are likely to be secondary consequences of a primary effect on protein/RNA export. Comparable phenotypes have been observed from mutations in other yeast genes involved in transport, including *GSP1* and *RCC1* [35,36].

In *S. pombe*, Crm1p is known to negatively regulate the transcription factor Pap1p [37]. The inhibition of Pap1-regulated gene expression has been shown to be relieved by the anti-fungal agent leptomycin B. Genetic evidence indicates that this relief is a result of leptomycin B binding to Crm1p and blocking its regulation of Pap1p [38]. Interestingly, addition of leptomycin B to a mammalian transport assay system inhibits Rev NES-mediated transport [27], suggesting that mammalian Crm1p plays a role in nuclear export. The human Crm1p homolog is localized to

Figure 6



Cells expressing *crm1* mutants show no polyA+ RNA export defect. The *crm1-1*, *crm1-2*, *crm1-3* mutants were shifted to 37°C for 2 h before analysis. **(a,c,e)** PolyA+ RNA subcellular localization was analyzed by *in situ* hybridization with a digoxygenin-labeled oligo-dT probe and a fluorescein isothiocyanate-conjugated mouse anti-digoxygenin antibody. **(b,d,f)** Visualization of nuclei by DAPI staining of DNA. **(g)** Crm1p depletion induces nuclear accumulation of polyA+ RNA. Cells were depleted for Crm1p by switching a strain expressing only a galactose-inducible *CRM1* gene from galactose-containing to glucose-containing medium. After a 12 h depletion, the localization of polyA+ RNA (g) in most cells correlated with the localization of (h) the DNA note the distorted appearance of the DNA in Crm1p-depleted cells (h).

the nuclear pore complex as well as to the nucleoplasm and interacts with FG-repeat regions of nuclear pore proteins [25]. Very recent results provide more direct evidence for a role of Crm1p in Rev-mediated and uracil-rich small nuclear RNA transport in *Xenopus* oocytes [29].

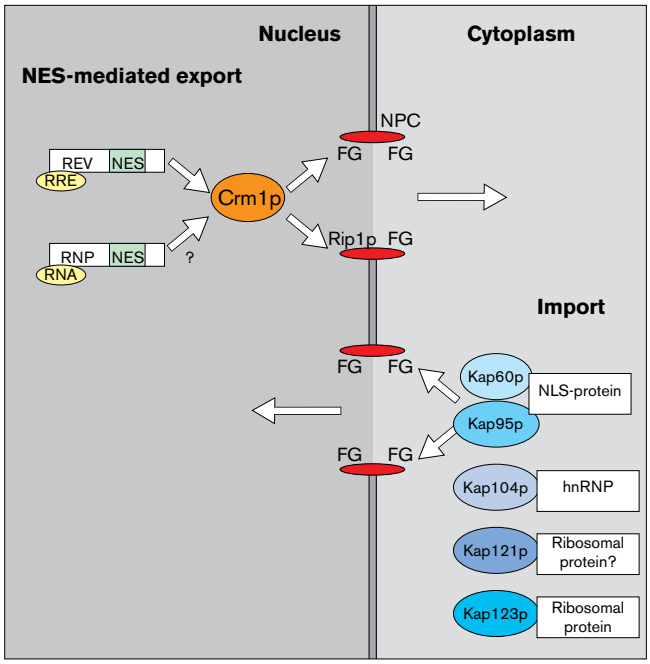
In *S. cerevisiae*, Crm1p has been recognized as an importin-beta family member [20,25], and Crm1p contributes to nuclear export mediated by Yap1p (an AP-1-like transcription factor) as well as Rev NES. Although the three *crm1* mutations have distinguishable effects on Rev and Yap1p export, none of the mutations have an obvious growth phenotype; the effects of the *crm1* mutants on export are, therefore, probably not an indirect consequence of debilitated cell physiology. Generally, the interpretation of viable mutant phenotypes is somewhat more straightforward than that of temperature-sensitive mutant phenotypes, which are more likely to result from indirect effects, such as potent growth inhibition at the non-permissive temperature or strong inhibitory effects on another aspect of transport. Indeed, it is known that effects of mutations on nuclear import and export can be difficult to distinguish [39–41]. But the lack of an effect of the three viable *crm1* mutants on growth and polyA+ RNA transport — indicating that general aspects of transport are not drastically impaired — must be reconciled with the two robust transport phenotypes: the three mutants have profound effects on Yap1p transport (L.L. and L.I.D., unpublished observations) and Rev NES biological activity (Figures 2,3). In the latter case, all of the effects are considerably stronger than those obtained with a *RIP1* deletion [6]; based on our previous standardization [5], we estimate that in the *crm1* mutants residual Rev NES activity is at most 25% of that observed in a wild-type *CRM1* background.

One possible explanation is that Crm1p is specialized for certain substrates, the export of which is not essential for yeast survival and growth; however, *CRM1* is an essential gene. We therefore favor the notion that Crm1p is a more general transporter, perhaps responsible for much or even all NES-mediated traffic. In this case, 10–25% leakage through the pathway may be sufficient for normal growth rates. Another possibility is that the strong allele-specific effects reflect the relative efficiency of different substrates, suggesting that Yap1p and Rev are inefficient substrates compared with most other NES-containing substrates.

For Yap1p, this idea of substrate efficiency has the virtue of facilitating regulation by making the transport of Yap1p sensitive to mild perturbations of the transport machinery. For Rev, the simplest explanation is that the Rev NES is a mammalian signal functioning in yeast and might, therefore, be inefficient because it is modestly different from efficient *S. cerevisiae* export signals. Recent information on natural *S. cerevisiae* NESs suggests that they resemble a canonical Rev-like NES [42–44]. But there are few examples of yeast transport substrates with well characterized NESs. The lack of a viable mutant effect on polyA+ export (Figure 6) may indicate that a low level of leakage through the pathway is also sufficient for near normal RNA export, or that the NESs that govern this pathway may be more efficient than the Rev NES. Alternatively, yeast polyA+ RNA may export via a completely different pathway, which would be consistent with the current view from the *Xenopus* oocyte system [29].

These uncertainties notwithstanding, our data indicate a direct role for Crm1p in Rev function, which agrees well with the leptomycin B-mediated inhibition of Rev activity in mammalian cells and the analysis of Crm1p function in *Xenopus* oocytes [27,29]. Although the yeast Crm1p–Rev

Figure 7



Model for NES-mediated nuclear export. Crm1p is a member of the importin-beta family of proteins, among which Kap95p, Kap104p, Kap121 and Kap123 have been shown to play a role in protein import [19]. Crm1p interacts both with the Rev NES and nucleoporin FG-repeat domains in the two-hybrid assay, suggesting that Crm1p bridges the described two-hybrid interaction between Rev NES and FG-repeat domains [6,13,14]. This latter two-hybrid interaction is inhibited in a *crm1* mutant background, and Rev-mediated RNA export is also abolished in the *crm1* strain, supporting a direct involvement of Crm1p in NES-mediated export. A role of Crm1p in the export of other cellular NES-containing proteins or RNPs is postulated. Abbreviations: FG, FG-domain-containing nucleoporins; NPC, nuclear pore complex.

two-hybrid interaction could be indirect, very recent biochemical experiments indicate a direct association between Rev NES and mammalian Crm1p [29]. Direct binding between yeast nucleoporins and Crm1p is also likely, because a direct interaction between other importin-beta family members and repeat regions of nuclear pore proteins has already been demonstrated [26,33]. In contrast, the interaction between Rev NES and Rip1p is likely to be indirect or have additional components, because it is sensitive to a *crm1-1* mutant background (Figure 5). We have obtained identical results for the effect of *crm1-1* on the previously described interaction between Rev and hRip1/Rab ([13,14]; data not shown), which strengthens the correlation between the mutant effects on Rev biological activity and the two-hybrid results. Other Rev–nucleoporin interactions are insensitive to the *crm1-1* mutation, however (data not shown). The negative results may reflect idiosyncracies of certain two-hybrid combinations or a specific contribution of Crm1p to certain Rev interactions.

Why do the *crm1* mutant strains inhibit Rev activity as well as the two-hybrid interactions? The effects are probably not due to low Crm1p levels, as the levels of all three mutant proteins are indistinguishable from wild-type Crm1p by western blotting analysis (L.L. and L.I.D., unpublished observations). Preliminary experiments indicate that the effects might not be due to defective pairwise interactions, suggesting that mutant Crm1p may be defective in binding both proteins simultaneously. Another possibility is an aberrant association with Ran or Ran GTPase-activating protein [20], which may result in slow recycling and a low nuclear concentration of biologically active Crm1p. In any case, the potent effect of the mutants on the Rev–Rip1p two-hybrid interaction suggests that the *crm1* strains might reveal additional genes important for Rev-mediated export.

Biochemical experiments should be able to verify that the *CRM1* requirement reflects a three-way interaction between Rip1p, Crm1p and Rev. Less likely in our view is a more dynamic explanation, in which Crm1p acts to deliver Rev to Rip1p, allowing Rev and Rip1p to undergo a direct two-way interaction. In either case, the effect of the mutations on Rev suggests that Crm1p interacts with the Rev NES and certain nuclear pore proteins during delivery of Rev-containing proteins and RNPs to the nuclear pore complex (Figure 7). This finding reinforces the strong mechanistic conservation between the export and import processes in all eukaryotes.

Materials and methods

Genotype of crm1 *mutants*

The *crm1* mutants were identified in a screen for mutations that are lethal in combination with *rna1-1*. The genotype of the starting strain was *rna1-1 ade2 ade*3 *ura3*. The *crm1* mutant strains were then backcrossed to wild-type strain W303 (*ade2 his3 leu2 trp1 ura3 MATα*) three successive times. Their resulting phenotypes are as follows: *crm1-1* (*leu2 his3 ade2 trp1 ura3 MATα*); *crm1-2* (*leu2 his3 ade2 ade3 trp1 ura3 MATα*); *crm1-3* (*leu2 his7 ade2 ade3 trp1 ura3 MATα*).

Yeast Rev assay

The yeast Rev assay was carried out in copper-sensitive strains. The *CUP1* gene was disrupted in the wild-type strain W303 (*ade2 his3 leu2 trp1 ura3 MATα*) or in the *crm1* mutant strains using a PCR-based disruption procedure with the kanMX2 template [45] and two oligonucleotides: 5′-TTGAAAAAAATGTATTACTCAAGACATTCGCTTCTAGTCAGTCTGCATAGGCCACTAGTGGATCTG-3′ and 5′-TACCTTTAAAAGACGTTCTCATAATACATTTTAGGATTAATACATCAGCTGAAGCTTCGTACGC-3′. This procedure generated a PCR fragment of ~1.4 kb containing the *Escherichia coli KAN*^{r} (kanamycin resistance) gene flanked by sequences from upstream and downstream of the *CUP1* gene. The plasmid was transformed into yeast and transformants were selected on YPD plates containing 200 mg/l G418 (Geneticin, Gibco BRL) and subsequently replica-plated to copper-containing plates (0.2 mM) to identify the copper-sensitive strains. These copper-sensitive strains were then transformed with the reporter constructs PC–CUP–RRE and MC–CUP–RRE (*LEU2*/2μ) and with the Rev-expressing plasmids pGRev and pGM10 or the empty plasmid pG1 (*TRP1*/*CEN3*/*ARS1*), all previously described [5]; the double transformants were grown to saturation and spotted on copper-containing plates. Growth was examined after 4–5 days at 30°C. The rescue of CRM1 activity in these strains was assayed by transforming the cells with

the *CRM1* expressing plasmid pCRM1/URA or the empty plasmid pRS316 (*URA3*/*CEN*/*ARS*). Triple transformants were assayed again by growth on copper-containing plates.

Two-hybrid cloning and strains

The KAP95, KAP121, KAP104 and KAP123 bait constructs were obtained by cloning the full length coding regions as *Sal*I/*Xho*I PCR fragments into the pEG202+PL vector (*HIS3*/2μ) digested with *Xho*I, thereby creating in-frame fusions with the LexA DNA-binding domain [46]. The CRM1 and the RIP1 bait constructs were obtained by cloning the whole coding region of *CRM1* and codons 121–230 of *RIP1* as *Eco*RI/*Xho*I PCR fragments into pEG202+PL digested with *Eco*RI and *Xho*I. The MTR10 bait construct was obtained by cloning the whole coding region of *MTR10* as a *Bam*HI/*Sal*I PCR fragment into pEG202+PL cut with *Bam*HI and *Xho*I. The Rev bait and the *Drosophila* Per bait constructs have been described earlier [6,47]. The prey constructs used in our analysis were obtained by cloning *Eco*RI/*Xho*I PCR fragments of full-length *RIP1,* codons 121–230 of *RIP1,* wild-type Rev, the Rev M10 mutant, and Rex into pJG4-5 (*TRP1*/2μ) cut with *Eco*RI and *Xho*I. The prey constructs containing the different FG-repeat domains are as described [11]. The Tim prey construct has been described earlier [47]. The strain EGY48 (*MAT*α *trp1 ura3 LEU2*::*plexop6-LEU2*) contains the *lac*Z reporter pSH18-34 on a *URA3*/2μ plasmid, a gift of R. Finley and R. Brent. RFY206 (*MAT*α *his3 ura3 trp1 lys2*), a gift from R. Finley, also contains the *lac*Z reporter pSH18-34.

Two-hybrid interaction assays

Bait constructs were transformed in the strain RFY206 containing the *lac*Z reporter pSH18-34 on a *URA3*/2μ plasmid (gift from R. Finley) and transformants selected on Ura^-Trp^- medium and mated to the strain EGY48 containing the appropriate prey constructs described above. Diploids were selected by growth on $Ura^-His^-Trp^-$ medium and tested for galactose-dependent activation of the reporter genes. Detection of interactions in the mutant *crm1-1* background were carried out as follows (Figure 5): both triple transformations of Crm1p bait, Rip1p total prey, pSH18-34 and Per bait, Tim prey, pSH18-34 were performed in W303 and *crm1-1* strains, and transformants were selected on $Ura^-His^-Trp^-$ medium. Transformants were tested for galactose-dependent activation of the reporter genes. The effects of wild-type *CRM1* expression in these strains was seen by transforming in a wild-type *CRM1*-expressing plasmid (p*CRM1*/*LEU2*) or the empty plasmid pRS315 (*LEU2*/*CEN*/*ARS*).

In situ *hybridization assay*

In situ hybridization to detect $polyA^+$ RNA was performed as described previously [48,49], except that the digoxygenin-labeled oligo-dT probe was used at 500 ng/ml. Cells expressing *crm1* mutants were grown to mid-log phase and shifted from 25°C to 37°C for 2 h before analysis. Crm1p was depleted from strain LLY113 (*ade2 his3 leu2 trp1 ura3 MATa CRM1*::*KAN*^{r}) containing a galactose-inducible *CRM1* expression plasmid (GAL-HACRM1/*URA3*/*CEN*). Cells were grown to mid-log phase in selective medium containing 3% galactose/1% sucrose. Cells were then washed, diluted in selective medium containing 2% glucose, grown at 30°C for 12 h to an OD_{600} of 0.5 and processed for *in situ* hybridization analysis.

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