Staufen1 is imported into the nucleolus via a bipartite nuclear localization signal and several modulatory determinants

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Mammalian Stau1 (Staufen1), a modular protein composed of several dsRBDs (double-stranded RNA-binding domains), is probably involved in mRNA localization. Although Stau1 is mostly described in association with the rough endoplasmic reticulum and ribosomes in the cytoplasm, recent studies suggest that it may transit through the nucleus/nucleolus. Using a sensitive yeast import assay, we show that Stau1 is actively imported into the nucleus through a newly identified bipartite nuclear localization signal. As in yeast, the bipartite nuclear localization signal is necessary for Stau1 nuclear import in mammalian cells. It is also required for Stau1 nucleolar trafficking. However, Stau1 nuclear transit seems to be regulated by mechanisms that involve cytoplasmic retention and/or facilitated nuclear export. Cytoplasmic retention is mainly achieved through the action of dsRBD3, with dsRBD2 playing a supporting role in this function. Similarly, dsRBD3, but not its RNA-binding activity, is critical for Stau1 nucleolar trafficking. The function of dsRBD3 is strengthened or stabilized by the presence of dsRBD4 but prevented by the interdomain between dsRBD2 and dsRBD3. Altogether, these results suggest that Stau1 nuclear trafficking is a highly regulated process involving several determinants. The presence of Stau1 in the nucleus/nucleolus suggests that it may be involved in ribonucleoprotein formation in the nucleus and/or in other nuclear functions not necessarily related to mRNA transport.

Key words: mRNA localization, nuclear import, nuclear localization signal, ribonucleoprotein, RNA-binding protein, Staufen.

INTRODUCTION

mRNA localization is an important mechanism allowing precise spatial and temporal expression of proteins. Many biological processes such as learning and memory, synaptic plasticity, axis formation, cell motility and asymmetric cell division were shown to depend on local transport and/or translation of specific mRNAs (see [1,2] for reviews). Models of mRNA localization involve several common steps: (i) recognition and binding of target mRNA by specific subsets of RNA-binding proteins and associated cofactors to form RNP (ribonucleoprotein) particles, (ii) active transport of translationally repressed mRNPs (messenger RNPs) along the cytoskeletal network, (iii) anchoring of the mRNPs at specific sites and (iv) local translation of mRNAs following specific signals.

Recent evidence suggests that the initial steps of RNA recognition by specific proteins and RNP formation take place in the nucleus. In *Drosophila*, Sqd, a member of the hnRNP (heterogeneous nuclear RNP) family, is required for grk mRNA localization at the dorsal pole during oogenesis [3]. Sqd has been suggested to bind grk mRNA in the embryonic nucleus. In mammals, recognition of MBP (myelin basic protein) mRNA by hnRNP A2 first occurs in the nucleus [4,5]. MBP mRNA/hnRNP A2-containing complexes then exit the nucleus and localize to the myelin compartment of oligodendrocytes [6,7]. Similarly, the cytoplasmic RNA-binding protein ZBP (zipcode-binding protein), which is involved in β-actin mRNA localization in chicken fibroblasts and neurons, was shown to shuttle through the nucleus by means of specific nuclear import and export signals and to bind β-actin mRNA at its transcription site [8–10].

Stau1 (Staufen1) is a double-stranded RNA-binding protein that was first identified in *Drosophila* where it plays essential roles in mRNA localization [11,12]. Like its *Drosophila* orthologue, mammalian Stau1 is a modular protein composed of several dsRBDs (double-stranded RNA-binding domains) [13–16]. The major determinant for RNA-binding activity is dsRBD3, although a weak RNA-binding activity is also associated with dsRBD4 [15]. Co-sedimentation and co-localization experiments indicated that mammalian Stau1 localizes to the RER (rough endoplasmic reticulum) and associates with ribosomes [14,15]. Direct interaction between Stau1 and ribosomal subunits was also demonstrated [17–19]. This association is mediated by two molecular determinants, dsRBD3 and dsRBD4-TBD (tubulin-binding domain), and probably involves both RNA-binding and protein–protein interaction.

Mammalian Stau1 is thought to be involved in mRNA localization. In neurons, a Stau1–YFP (yellow fluorescent protein) fusion protein rapidly associates with RNA-containing granules [20]. These granules migrate along the microtubule network of dendrites at an average speed of 6.4 μm/min. Down-regulation of Stau1 through RNAi (RNA interference) suppresses the dispersed distribution of the CaMKIIα3′ (calcium/calmodulin-dependent protein kinase IIα3′)-UTR (untranslated region) mRNA in dendrites of neurons [21]. Biochemical and proteomics characterization of Stau1-containing granules revealed a heterogeneous population of granules and particles associated with mRNA, ribosomes and/or other protein cofactors [18,19,22–24]. However, the mechanism and the subcellular site of RNP assembly remain unknown. The presence of putative NLS (nuclear localization signals) in Stau1, its accumulation in nucleolus upon

Abbreviations used: ADAR1, adenosine deaminase that acts on RNA; CaMKIIα3′, calcium/calmodulin-dependent protein kinase IIα3′; CRM, chromosomal region maintenance; DAPI, 4,6-diamidino-2-phenylindole; dsRBD, double-stranded RNA-binding domain; RNP, ribonucleoprotein; hnRNP, heterogeneous nuclear RNP; mRNP, messenger RNP; LMB, leptomycin B; MOI, modulation of import; MBP, myelin basic protein; NLS, nuclear localization signal; RER, rough endoplasmic reticulum; RNAi, RNA interference; RSV, Rous sarcoma virus; siRNA, small interfering RNA; Stau1, Staufen1; TBD, tubulin-binding domain; UTR, untranslated region; YFP, yellow fluorescent protein; ZBP, zipcode-binding protein.

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Table 1 Oligonucleotide primers used in PCR amplification to construct different Stau mutants and/or fusion proteins

The restriction enzyme sequences are italicized in the sequences.

Name	Template	Oligonucleotide sequences	Restriction sites
pNIA-Stau1	RSV-Stau1	5′-AATT*GGTACC*TGCACTGTGCGTGAAACTTGGA 5′-TATA*GTCGAC*GGCCAGAAAAGGT	KpnI SalI
PNIAE2-Nterm	RSV-Stau1	5′-ACCAATGTATAAGCCTGTTGACCCTTAC 5′-TCAAGAACAGCTATGGCATTTT	– –
pNIAE2-NLSs	RSV-Stau1	5′-CGCCATAGCTGTTCTTGA 5′-TTTCCAACCTTCACCTG	– –
pNIAE2-NLSbipartite	RSV-Stau1	5′-CGCCATAGCTGTTCTTGA 5′-GGGCTTGTCTGTGGCTTGACTATGGGTT	– –
pNIAE2-NLSbasic	RSV-Stau1	5′-GCCAGAGTACACGCTCCTCACAGAG 5′-TTTCCAACCTTCACCTG	– –
pNIAE2-Cterm	RSV-Stau1	5′-CACTGCAGAAGGAACGGGCACCAAC 5′-CACCTCCCACACACAGACATTGGTCC	– –
RBD2–YFP	RSV-Stau1	5′-TAGATATC*GAATTC*GCCGCCATGGGATACCCATTTCCAG 5′-TATACTCGAG*GATATC*CAGGATCCTCAA	EcoRI EcoRV
RBD3–YFP	RSV-Stau1	5′-TAGATATC*GAATTC*GCCGCCATGGGAAAATCTGAAATAAGT 5′-TATACTCGAG*GATATC*AACTGCAGGCAGG	EcoRI EcoRV
RBD3^{*F}–YFP	RSV-St/3^{*F}	5′-TAGATATC*GAATTC*GCCGCCATGGGAAAATCTGAAATAAGT 5′-TATACTCGAG*GATATC*AACTGCAGGCAGG	EcoRI EcoRV
RBD4–YFP	RSV-Stau1	5′-TAGATATC*GAATTC*GCCGCCATGGGAAATCCGATTAGC 5′-TATACTCGAG*GATAT*CAAGGATCTCCAG	EcoRI EcoRV
RBD3^{*F}/RBD4–YFP	RSV-St/3^{*F}	5′-TAGATATC*GAATTC*GCCGCCATGGGAAAATCTGAAATAAGT 5′-TATACTCGAG*GATAT*CAAGGATCTCCAG	EcoRI EcoRV
RBD2/RBD3	RSV-Stau1	5′-TAGATATC*GAATTC*GCCGCCATGGGATACCCATTTCCAG 5′-TATACTCGAG*GATATC*AACTGCAGGCAGG	EcoRI EcoRV
St/3^{*F}/ΔN–YFP	St/3^{*F}–YFP	5′-TA*GATATC*GAATTCGCCGCCATGGGAAAATCTGAAATAAGT 5′-TATACTCGAG*GATATC*GCACCTCCCACACAC	EcoRV
St/3^{*F}/Δ2–YFP	Stau1–YFP	5′-TATA*GGTACC*GCCACCATGGCGTTGAGGATC 5′-TATACTCGAGGATATCGCACCTCCCACACAC	KpnI –

overexpression [14,25] and its interaction with the telomeric RNA [25,26] suggest that Stau1 may enter the nucleus where it might play a role in mRNA selection and/or RNP assembly. Recently, mammalian paralogous Stau2 isoforms were shown to shuttle through the nucleus and exit via exportin-5 and/or CRM1 (chromosomal region maintenance 1)-dependent mechanisms [27,28].

In the present study, we identify a functional bipartite NLS that allows Stau1 nuclear/nucleolar trafficking. In addition, we show that several molecular determinants are involved in the modulation of nuclear import. Since these determinants overlap with those involved in Stau1 cytoplasmic retention and in nucleolar trafficking, these results support the notion that Stau1 shuttling through the nucleus may be regulated.

EXPERIMENTAL

Construction and molecular cloning of fusion proteins

pNIA-Stau1 was obtained by PCR amplification of Stau1 cDNA using the Vent DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.) and specific oligonucleotides (Table 1). The resulting product was digested with KpnI and SalI restriction enzymes and cloned in the pNIA vector [29] that was previously digested with the same enzymes. Mutants containing different regions of Stau1 were cloned in pNIAE2 as follows: PCR amplification of each Stau1 region (see Table 1 for oligonucleotide primer sequences and templates), phosphorylation of resulting products with T4 polynucleotide kinase and cloning in SmaI-digested pNIAE2 [29].

Constructions containing one or more Stau1 domains fused to YFP were obtained by PCR amplification (see Table 1 for specific oligonucleotide primers and templates) followed by digestion with the specified restriction enzymes and cloning in YFP-Topaz vector (Packard Bioscience/PerkinElmer LifeSciences, Woodridge, ON, Canada) previously digested with the same enzymes.

St/3^{*F}–YFP and St/3^{*F}/Δ4–YFP were generated by digestion of RSV (Rous sarcoma virus)-St/3^{*F} and RSV-St/3^{*F}/Δ4 [17] respectively with NotI. The resulting fragments were end-blunted with T4 DNA polymerase, digested with KpnI and cloned in SmaI–KpnI-digested YFP-Topaz vector. The St/3^{*K4}–YFP mutant was produced in three steps: (i) Lys^{153} and Lys^{154} of RSV-Stau1 were changed to alanine residues by PCR; (ii) one mutated clone was PCR-amplified, digested with EcoRV and ligated in the EcoRV-digested YFP-Topaz vector, creating St/3^{*K2}–YFP; and (iii) this construct was used as a template to replace His^{131} and Lys^{133} by alanine residues (see Table 2 for sequences of oligonucleotide primers). St/3^{*F}/ΔN and St/3^{*F}/ΔH1 were obtained by PCR amplification, followed by digestion with DpnI and treatment with T4 polynucleotide kinase before being digested with EcoRV and self-ligated. Primers and templates are listed in Tables 1 and 2 respectively. St/3^{*F}/Δ2–YFP was produced by the same procedure (see Table 1 for primers) using Stau1–YFP

Table 2 Oligonucleotide primers used in PCR amplification to construct different Staufen mutants

Mutations are shown in boldface in the sequence and the restriction enzyme sequences (EcoRV) are italicized.

Name	Template	Oligonucleotides sequences
St/3*^{K4}–YFP	(i) RSV-Stau1	5′-AGC**GC**G**GC**GATTTCAAAGAAAAATGCC 5′-AATC**GC**C**GC**GCTTTTCCCTTCACC
	(ii) RSV-St/3*^{K2}	5′-TATAT*GATATC*ACCAATGTATAAGCCT 5′-TATACTCGAG*GATATC*GCACCTCCCACACAC
	(iii) St/3*^{K2}–YFP	5′-GTGGCCCACCC**GC**CATG**GC**GAACTTTGTGACC 5′-GGTCACAAAGTTC**GC**CATG**GC**GGGTGGGCCAC
St/3*^{F*NLS}–YFP	St/3*^{F}–YFP	5′-GTTTTGTTTTCTT**A**TTGAT**G**CTAGG**A**TTTACTCGTTCAAC 5′-GTTGAACGAGTAAA**T**CCTAG**C**ATCAA**T**AAGAAAACAAAACCC
St/3*^{F}/ΔH1–YFP	St/3*^{F}–YFP	5′-TA*GATATC*GAATTCGCCGCCATGGGAAAATCTGAAATAAGT 5′-TATACTCGAG*GATATC*CAGGATCCTCAA

as a template. The resulting fragment was digested with KpnI and SmaI and cloned in the KpnI/SmaI-digested St3*^{F}–YFP vector. Point mutations in the bipartite NLS of St/3*^{F}–YFP (St/3*^{F*NLS}–YFP) were introduced by PCR using specific oligonucleotide primers (Table 2).

Cell culture and microscopy

COS1 cells were grown to 60–80 % confluence on coverslips in Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum and penicillin/streptomycin before transfection with Fugene transfection reagent (Roche, Laval, QC, Canada). Plasmid DNA (2–4 μg) was transfected. Cells were fixed with 4 % (w/v) paraformaldehyde 16–24 h post-transfection and stained with DAPI (4,6-diamidino-2-phenylindole; 300 nM). Fluorescence was visualized using a Nikon TE2000U microscope.

LMB (leptomycin B) treatment and RNAi

LMB experiments were performed as described in [27]. Briefly, cells were transfected with calcium phosphate and then treated with LMB (50 nM) and cycloheximide (30 μg/ml) for 4 h. pSUPER vector producing siRNA (small interfering RNA) against human exportin-5 has been previously described [27,30]. HeLa cells were co-transfected with calcium phosphate and fixed 24 h after transfection.

Yeast assay

Saccharomyces cerevisiae strain L40 was used in all experiments as described in [29]. Introduction of plasmid DNA in yeast was performed following the LiAc TRAFO method as described in [31]. For quantification of growth, yeast were grown in tryptophan dropout minimal medium (6.7 g/l yeast nitrogen base, supplemented with 20 mg/l adenine, 20 mg/l arginine, 20 mg/l histidine, 30 mg/l isoleucine, 100 mg/l leucine, 30 mg/l lysine, 20 mg/l methionine, 50 mg/l phenylalanine, 200 mg/l threonine, 30 mg/l tyrosine, 20 mg/l uracil and 150 mg/l valine), harvested and diluted to an absorbance (A_{600}) of 0.5. Serial 5-fold dilutions were prepared from the resulting culture and 5 μl of each dilution was spotted on appropriate selective medium plates (tryptophan dropout or tryptophan/histidine dropout). Liquid β-galactosidase assay was performed as described in [32]. Briefly, yeast were grown in tryptophan dropout minimal medium until the cultures reached an A_{600} of 0.5. Cells were harvested, lysed and incubated for 15 min at 37 °C with freshly made *o*-nitrophenyl β-D-galactopyranoside solution (4 mg/ml). The resulting mixture was analysed in a spectrophotometer set at A_{420}. Units of β-galactosidase activity were calculated according to the formula $(A_{420} \times 1000)/(t \times V \times A_{600})$, where $t = 15$ min and V is the volume of culture in ml.

Northwestern- and Western-blot assays

HEK-293 (human embryonic kidney 293) cells were transfected with 15 μg of plasmid DNA using the calcium phosphate precipitation technique and lysed 16 h post-transfection as described in [15]. Cell extracts were immunoprecipitated using mouse monoclonal anti-Stau1 antibody as described in [33]. The resulting immunoprecipitate was separated by SDS/PAGE and analysed by Northwestern-blot assay using a ^{32}P-labelled bicoid 3′-UTR RNA as probe and by Western-blot assay using rabbit anti-Stau1 antibody as described in [15].

RESULTS

The nucleocytoplasmic shuttling of Stau1 is independent of the CRM1 and exportin-5 pathways

Endogenous Stau1 is mainly expressed in the cytoplasm in association with ribosomes and/or the RER [14,15]. Upon overexpression, transfected Stau1 often starts to accumulate in the nucleolus [14,25]. Computer analysis of the Stau1 sequence reveals the presence of two potential NLSs, a bipartite NLS located at the end of dsRBD3 and a basic NLS located within dsRBD4 (Figure 1). To reveal nuclear shuttling of Stau1 or Stau1-containing RNPs in mammals, we first inhibited different nuclear export pathways and determined whether Stau1 accumulates in the nucleus under these conditions. The CRM1 export receptor is central to the mechanism of export of several proteins [34–36]. To determine whether Stau1 is exported from the nucleus via this receptor, we treated Stau1–YFP-transfected HeLa cells with LMP, a specific inhibitor of CRM1-mediated export. Under these conditions, Stau1–YFP did not accumulate in the nucleus (Figure 2), indicating that Stau1 export is not dependent on CRM1. Similar results were obtained in COS1 cells (results not shown). We also inhibited the exportin-5-dependent nuclear export pathway since Stau1 dsRBD3 was shown to interact with exportin-5 [37]. siRNAs against exportin-5 were used to down-regulate expression of exportin-5 in Stau1–YFP-transfected HeLa cells. Stau1–YFP did not accumulate in the nucleus (Figure 3), indicating that its export is also not dependent on exportin-5. Finally, we inhibited transcription from RNA polymerases I, II and/or III with various concentrations of actinomycin D or DRB

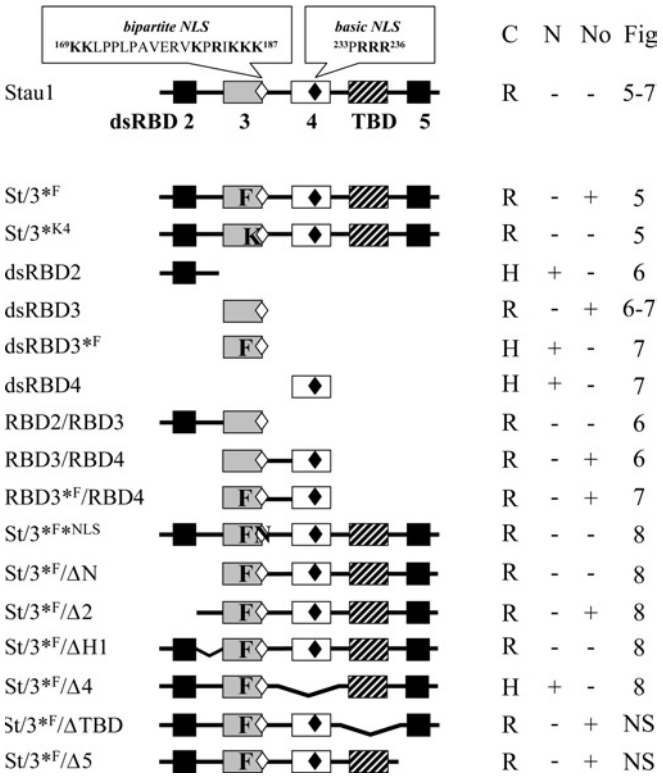

Figure 1 Schematic representation of Stau1 and Stau1 mutants

Left: Stau1 and Stau1 mutants used in the present study are depicted. Grey and white boxes represent the major (dsRBD3) and minor (dsRBD4) RNA-binding domains respectively; black boxes represent regions with RNA-binding consensus sequence but lacking RNA-binding activity *in vitro*. The hatched box indicates the position of the region similar to the MAP1B microtubule-binding domain. The positions of the bipartite (◇) and basic (◆) NLSs are indicated. F, mutation F135A in dsRBD3; K, mutations H131A, K133A, K153A and K154A in dsRBD3; N, mutations K181N, R183S and K185N in the bipartite NLS. Right: summary of the principal results. C, cytoplasmic distribution; N, nuclear distribution; No, nucleolar distribution; Fig, Figure where data can be seen; R, reticular distribution of Stau1 in the cytoplasm; H, homogeneous random distribution of Stau1 in the cytoplasm; +, presence of at least a fraction of Stau1 in the specified organelle; −, absence of Stau1 in the specified organelle; NS, not shown.

(5,6-dichloro-1-β-D-ribofuranosyl-benzimidazol) to test whether the presence of specific RNA species might be required for Stau1 export. These treatments did not induce nuclear accumulation of Stau1 (results not shown).

Stau1 contains a functional NLS

To determine whether Stau1 can actively be imported into the nucleus, we used a sensitive import assay developed in yeasts [29]. This assay is based on the expression of (i) a fusion protein containing a transcription factor lacking a nuclear import activity (pNIA) and (ii) the tested protein carrying putative NLS sequences. Additional fusion of virE2 (pNIAE2) to this protein further prevents passive diffusion in the nucleus. The presence of a functional NLS in the tested protein enables active nuclear import of the fusion protein that then activates transcription of two reporter genes: the *lacZ* gene, encoding the β-galactosidase enzyme, and the *HIS3* gene that enables growth of yeast on histidine-deficient media.

We first fused full-length Stau1 to pNIA and tested whether it allows import of the fusion protein to the nucleus. Expression of pNIA–Stau1 induced significant reporter gene activation (Figure 4), indicating that Stau1 contains a functional NLS. To identify this domain, the ability of several Stau1 fragments fused to pNIAE2 to trigger nuclear import was tested. Interestingly, only fragments containing the bipartite NLS domain yielded activation, suggesting that this domain is responsible for Stau1 import activity

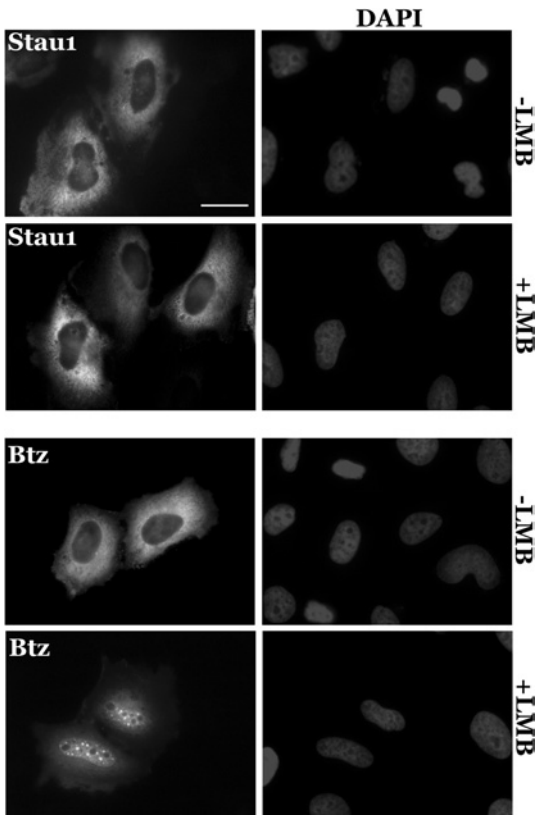

Figure 2 The nucleocytoplasmic shuttling of Stau1 is independent of the CRM1 pathway

HeLa cells were transfected with Stau1–YFP (upper panels) or Barentsz–YFP (lower panels) used as a control [51]. Cells were treated, 24 h post-transfection, with LMB for 4 h (left panels). DAPI staining (right panels) indicates the position of the nucleus. Scale bar, 10 μm.

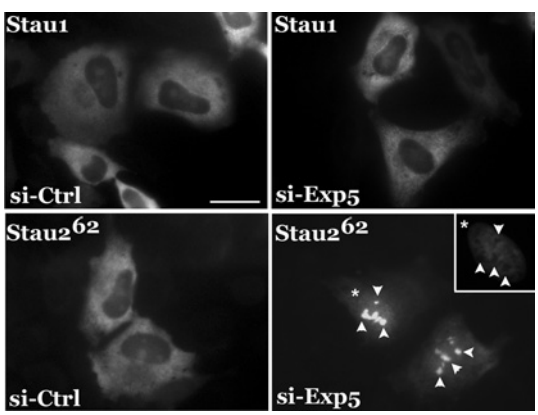

Figure 3 The nucleocytoplasmic shuttling of Stau1 is independent of exportin-5

HeLa cells were co-transfected with Stau1–YFP (upper panels) and with siRNA against a non-silencing exportin-5 siRNA (as control, left panels) or against exportin-5 (right panels). Stau2^{62}–YFP was used as control (lower panels). Inset: DAPI staining showing the position of the nucleoli (arrowheads). Scale bar, 10 μm.

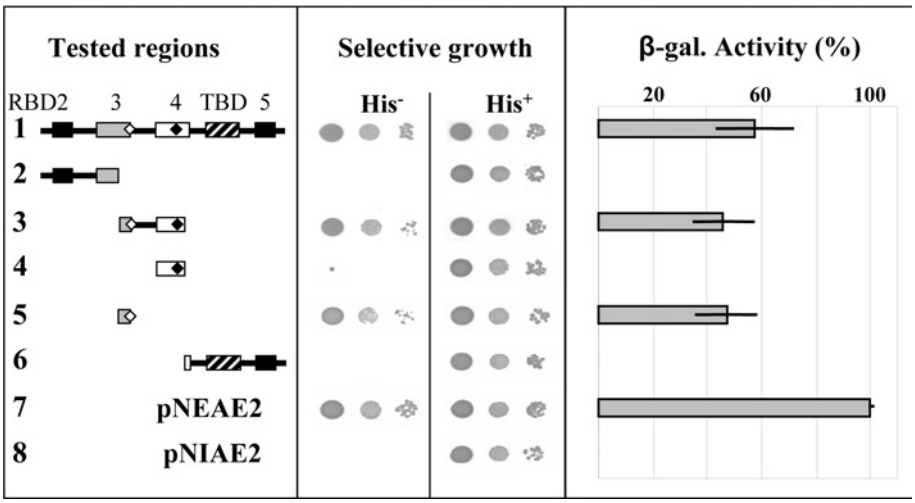

Figure 4 Stau1 contains a functional bipartite NLS

Stau1 and Stau1 domains were fused to the transcription factor LexA-Gal4-virE2 (lacking a functional NLS) and expressed in yeast cells. The presence of a functional NLS in Stau1 should cause import of the fusion protein into the nucleus and activation of the *HIS3*/*lacZ* (middle panel) and *β-galactosidase* (right panel) genes. Selection assays included cell growth on minimal medium deficient (His^-) or not (His^+) for histidine and quantitative β-galactosidase assay in liquid following cell growth in minimal medium. β-Galactosidase activity is shown as a percentage of the maximal activity generated with the SV40 (Simian virus 40)-type NLS (pNEAE2). 1, pNIA-Stau1; 2, pNIAE2-Nterm; 3, pNIAE2-NLSs; 4, pNIAE2-NLSbasic; 5, pNIAE2-NLSbipartite, 6, pNIAE2-Cterm; 7, pNEAE2 (the LexA-Gal4-VirE2 protein fused to the SV40 NLS); 8, pNIAE2 (the LexA-Gal4-VirE2 fusion protein without NLS).

in yeast (Figure 4). These results demonstrate that Stau1 has the ability to transit into the nucleus and that its bipartite NLS is sufficient to import a foreign protein into the yeast nucleus.

MOI (modulation of import) activity

Given the presence of a functional nuclear import domain, why is Stau1 not normally found in the nucleus of mammalian cells? Two non-mutually exclusive phenomena, rapid nucleocytoplasmic shuttling and/or strong retention of the protein in the cytoplasm, may explain this discrepancy. These phenomena were globally referred to as MOI activity by others [38]. Accordingly, the subcellular localization of Stau1 may depend on competition between its nuclear import activity and MOI activity.

dsRBD3 is the major determinant of MOI activity

We hypothesized that mutation/deletion interfering with Stau1 MOI activity should cause the accumulation of the protein in the nucleus. Since Stau1 is an RNA-binding protein, we first tested whether binding to RNA is a determinant for Stau1 MOI activity. Based on the studies in *Drosophila* [39], we introduced specific point mutations in dsRBD3, the major RNA-binding domain: either a single mutation, Phe^{135} (F135A) to generate St/3^{*F}–YFP or mutation of four residues (H131A, K133A, K153A and K154A) to generate St/3^{*K4}–YFP. We chose these because Phe^{32} of *Drosophila* Staufen dsRBD3 (the equivalent of Phe^{135} in human Stau1) was shown to position loop 2 and loop 4 with respect to the RNA. Therefore this mutation is likely to alter the structure of the domain. In contrast, the four equivalent amino acids of His^{131}, Lys^{133}, Lys^{153} and Lys^{154} in *Drosophila* Staufen were shown to be directly in contact with double-stranded RNA and consequently their mutation is not believed to alter the structure of the domain [39].

Next, we performed a Northwestern-blot assay to determine whether the mutants lost their capacity to bind RNA. As expected, the two mutants showed impaired RNA-binding activity *in vitro* (Figure 5A) [17]. Then, we compared their subcellular localization with that of wild-type Stau1–YFP (Figure 5B). In the same way as for the wild-type protein, the two mutants were found to be associated with tubulovesicular structures in the cytoplasm (Figures 5C and 5D); however, a fraction of St/3^{*F}–YFP was also found in the nucleus (Figure 5C). Interestingly, the mutation did not simply cause random accumulation of the protein in the nucleus, but rather specifically targeted the protein to the nucleolus. In contrast, the subcellular localization of St/3^{*K4}–YFP was strictly cytoplasmic (Figure 5D). These results suggest that an intact and correctly folded dsRBD3, but not its RNA-binding activity, is necessary for Stau1 MOI activity.

dsRBD3 MOI activity is regulated by an adjacent domain

To test whether dsRBD3 is sufficient for MOI activity, we created a fusion protein between dsRBD3 and YFP and observed its subcellular distribution. As expected from the observation that the dsRBD3 is required for MOI activity, dsRBD3–YFP showed a typical tubulovesicular and perinuclear staining pattern (Figure 6B). However, its presence in the nucleolus suggests that dsRBD3 is not sufficient for complete MOI activity since it fails to confer a strict cytoplasmic distribution when fused to a heterologous protein. Interestingly, fusion of the N-terminal region of Stau1 to dsRBD3 (RBD2/RBD3–YFP) resulted in an exclusively cytoplasmic localization (Figure 6C). Co-operation between dsRBD2 and dsRBD3 is required for MOI activity since dsRBD2 alone did not prevent the nuclear localization of dsRBD2–YFP (Figure 6D). Furthermore, this co-operation is specific since fusion of dsRBD4 to dsRBD3, instead of dsRBD2, did not strengthen dsRBD3-dependent MOI activity (Figure 6E), with dsRBD3/RBD4–YFP showing both tubulovesicular staining in the cytoplasm and nucleolar staining.

These results highlighted the importance of dsRBD3 for nucleolar association. Indeed, in addition to the typical tubulovesicular and perinuclear staining pattern, dsRBD3–YFP showed a specific accumulation within the nucleolus (Figures 6B and 7B). Other fusion proteins between YFP and individual Stau1 domains did not accumulate in the nucleolus (Figures 6E and 7E and results not shown). Therefore dsRBD3 is the only domain

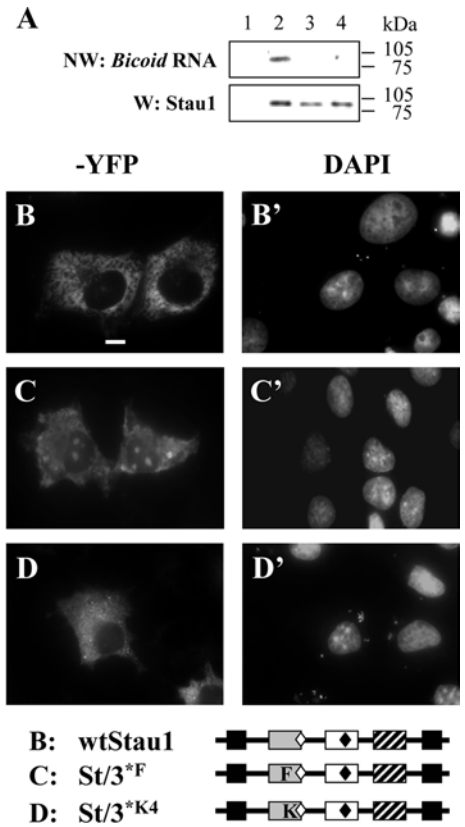

Figure 5 Subcellular localization of the RNA binding-deficient mutants of Stau1

(**A**) RNA-binding activity of Stau1 mutants. Cell extracts from mock transfected cells (lane 1), or cells expressing Stau1–YFP (lane 2), St/3^{*K4}–YFP (lane 3) or St/3^{*F}–YFP (lane 4) were immunoprecipitated with mouse monoclonal anti-human Stau1 antibodies (11C6) and analysed by SDS/PAGE. Upper panel: Northwestern-blot analysis using ^{32}P-labelled bicoid 3′-UTR RNA. Lower panel: Western-blot analysis of the same immunoprecipitates using rabbit anti-Stau1 antibody. (**B**–**D**) COS1 cells were transfected with 2 μg of cDNA coding for Stau1–YFP (**B**, **B′**), St/3^{*F}–YFP (**C**, **C′**) or St/3^{*K4}–YFP (**D**, **D′**). Transfected cells were fixed 16 h post-transfection and the subcellular localization of each construct was monitored by YFP autofluorescence (**B**–**D**). Nuclei were stained with 300 nM DAPI solution (**B′**–**D′**). Scale bar, 20 μm.

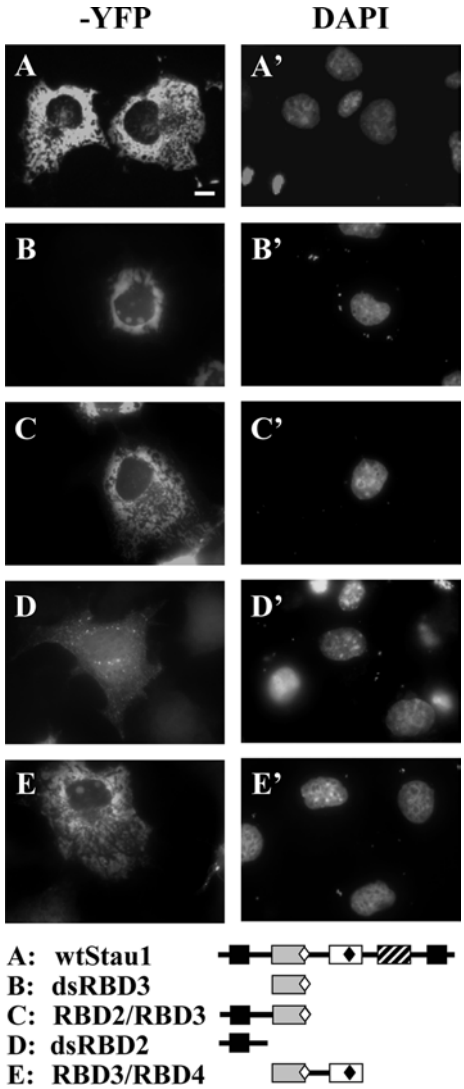

Figure 6 dsRBD3 MOI activity is regulated by adjacent domains

COS1 cells were transfected with 2 μg of cDNA coding for Stau1–YFP (**A**, **A′**), RBD3–YFP (**B**, **B′**), RBD2/RBD3–YFP (**C**, **C′**), dsRBD2–YFP (**D**, **D′**) or RBD3/RBD4–YFP (**E**, **E′**). Transfected cells were fixed 16 h post-transfection and the subcellular localization of each construct was monitored by YFP autofluorescence (**A**–**E**). Nuclei were stained with 300 nM DAPI solution (**A′**–**E′**). Scale bar, 20 μm.

able to interact with the nucleolus. Consistently, when mutated, dsRBD3^{*F}–YFP randomly localized within the cytoplasm and nucleus (Figure 7C). Interestingly, cytoplasmic and nucleolar distributions were rescued by fusing dsRBD4 to dsRBD3^{*F} (Figure 7D), suggesting that although sufficient by itself, dsRBD3 needs co-operation with dsRBD4 to stabilize or strengthen its interaction with cellular components. dsRBD4 did not play such a role by itself (Figure 7E).

Nuclear import of Stau1 requires specific molecular determinants

We took advantage of the nucleolar accumulation of St/3^{*F}–YFP to study the functionality of the NLS in Stau1 in mammalian cells. Point mutations (K181N, R183S and K185N) were first introduced in the bipartite NLS of St/3^{*F}–YFP and the subcellular localization of the resulting protein (St/3^{*F*NLS}–YFP) was examined. Figure 8(A) shows that mutation of the bipartite NLS prevented Stau1 accumulation in the nucleolus. A weak nuclear signal, however, was seen in 30–40 % of the cells, suggesting that this mutation did not completely block import in the nucleus. In these cells, localization in the nucleolus was never observed. This result indicates that the bipartite NLS is important for Stau1 import in mammalian cells; that it plays an additional role in promoting nucleolar targeting; but that other domains may contribute to nuclear localization.

In order to identify other domains that promote nuclear import, specific domains of Stau1–YFP and of St/3^{*F}–YFP were deleted and the subcellular localization of the resulting proteins was determined. Deletion of domain(s) involved in nuclear import should prevent the nucleolar accumulation of the protein. No significant change in the subcellular distribution of the deletion mutants of Stau1–YFP was observed (results not shown), showing that dsRBD3 is central to the mechanism of import/export. In contrast, deletion of the N-terminal extremity of St/3^{*F}–YFP (St/3^{*F}/ΔN) abolished the F135A-induced accumulation in the nucleolus (Figure 8B). Fine deletions within this region indicated that a short sequence located between dsRBD2 and dsRBD3 (named H1 hereafter) contains a molecular determinant for Stau1 import into the nucleus. Indeed, deletion of dsRBD2 alone (St/3^{*F}/Δ2) had no effect on the subcellular localization

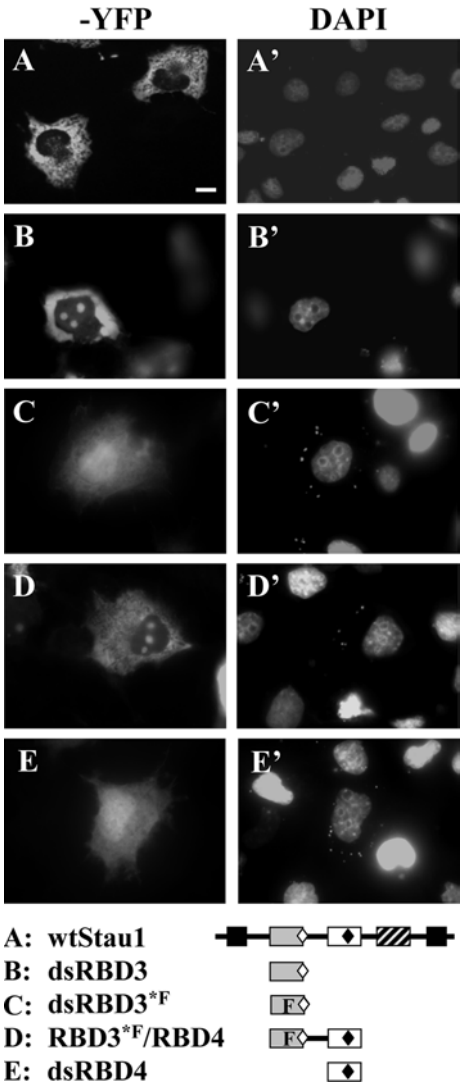

Figure 7 dsRBD4 co-operates with dsRBD3 for nucleolar localization

COS1 cells were transfected with 2 μg of cDNA coding for Stau1–YFP (**A**, **A′**), dsRBD3–YFP (**B**, **B′**), dsRBD3^{*F}–YFP (**C**, **C′**), RBD3^{*F}/RBD4–YFP (**D**, **D′**) or dsRBD4–YFP (**E**, **E′**). Transfected cells were fixed 16 h post-transfection and the subcellular localization of each construct was monitored by YFP autofluorescence (**A**–**E**). Nuclei were stained with 300 nM DAPI solution (**A′**–**E′**). Scale bar, 20 μm.

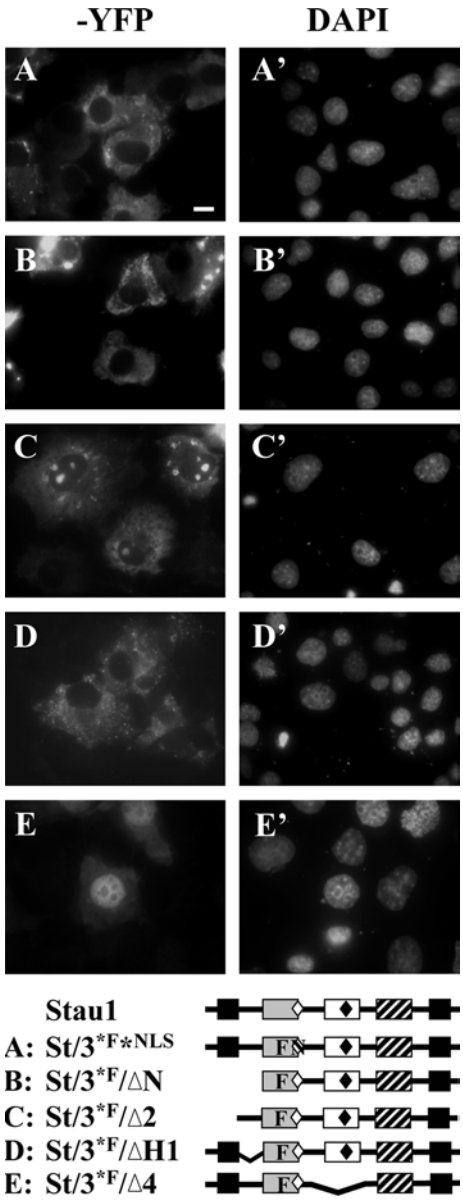

Figure 8 Involvement of the bipartite NLS and H1 regions in nuclear import of Stau1

COS1 cells were transfected with 2 μg of cDNA coding for St/3^{*F*NLS}–YFP (**A**, **A′**), St/3^{*F}/ΔN–YFP (**B**, **B′**), St/3^{*F}/Δ2–YFP (**C**, **C′**), St/3^{*F}/ΔH1–YFP (**D**, **D′**) and St/3^{*F}/Δ4–YFP (**E**, **E′**). Transfected cells were fixed 16 h post-transfection and the subcellular localization of each construct was monitored by YFP autofluorescence (**A**–**E**). Nuclei were stained with 300 nM DAPI solution (**A′**–**E′**). Scale bar, 20 μm.

of the protein, which was still partly found in the nucleolus (Figure 8C), whereas proteins with deletion of the H1 region were no longer seen in the nucleus (Figure 8D). Interestingly, deletion of dsRBD4 (St/3^{*F}/Δ4) causes the protein to accumulate in the nucleus but not in the nucleolus (Figure 8E), again suggesting that dsRBD4 somehow co-operates with dsRBD3 for nucleolar targeting (see above). Deletion of other domains of St/3^{*F} (St/3^{*F}/ΔTBD and St/3^{*F}/Δ5) did not prevent nucleolar accumulation (results not shown). The fact that nuclear import of St/3^{*F}–YFP is dependent on specific molecular determinants suggests that loss of cytoplasmic retention does not simply cause passive diffusion of the protein into the nucleus, but rather makes the protein accessible to the import machinery.

DISCUSSION

mRNA localization is a dynamic process that involves the formation of RNP complexes that are continually modified through binding and/or release of protein partners. The molecular mechanisms responsible for regulating efficient transport and/or translation of RNPs remain unclear, especially the spatial and temporal levels of organization that govern specific interactions between proteins and the transported RNA. The selection of mRNAs to be transported and the initial assembly into RNP complexes are likely to occur in the nucleus. Stau1, a component of moving RNP complexes, is also associated with ribosomes and the RER in the cytoplasm. In the present study, we identified a bipartite NLS at the C-terminal end of dsRBD3 and provided evidence that Stau1 can enter the nucleolus where it might interact with RNPs.

Nuclear import

Our results and published evidence [14,25] are consistent with the existence of an intrinsic import activity in the Stau1 protein. Although passive diffusion could account for the nuclear/nucleolar presence of some of the small fusion proteins (e.g. dsRBD3–YFP), the large size of tagged full-length proteins (> 85 kDa) argues in favour of an active nuclear import mechanism. This is confirmed in yeasts where a bipartite NLS within Stau1 is sufficient to import a foreign protein into the nucleus (Figure 4). This motif is also functional in mammalian COS1 cells since its deletion greatly impairs Stau1 import into the nucleus. Nevertheless, other determinants contribute to full nuclear import activity. Indeed, the partial nuclear accumulation of St/3^{*F} is removed by deletion of the domain between dsRBD2 and dsRBD3. This region is characterized by the presence of several acidic amino acids that are highly conserved in Staufen orthologues from *Drosophila* to mammals. Since most nuclear localization sequences reported to date are basic, the exact function of the acidic H1 region in mediating nuclear import of Stau1 remains unclear.

The bipartite NLS is also involved in Stau1 import into the nucleolus. The basic residues in the bipartite NLS are probably responsible for nucleolar import, as previously reported [40], since mutations of these residues prevent the residual amounts of Stau1 that enter the nucleus being imported into the nucleolus. Nucleolar trafficking is also dependent on a functional dsRBD3, although the RNA-binding activity of this domain is not required (see Figure 5, compare the localization of St/3^{*F} with that of St/3^{*K4}). This contrasts with what was reported for Stau2 where mutations in the four corresponding lysine/histidine residues of dsRBD3 cause accumulation of the protein in the nucleolus [27].

MOI activity: cytoplasmic retention

Why is endogenous Stau1 not normally observed in the nucleus if it contains a functional NLS? Obviously, Stau1 nuclear import/export pathways are highly regulated to prevent massive import and/or nuclear retention that would lead to Stau1 accumulation in the nucleus. Regulation may be achieved by strong anchoring of Stau1 in the cytoplasm, its rapid export from the nucleus following import or both. It is well documented that Stau1 associates with ribosomes and/or cytoskeleton and that these interactions are resistant to detergent, to EDTA and to relatively high concentrations of KCl [15,17]. In addition, deletions/mutations of Stau1 domains that reduce cytoplasmic retention favour its nuclear accumulation. Therefore Stau1 import into the nucleus is dependent on a balance between its nuclear import domains and specific molecular determinants that strongly retain the protein in the cytoplasm. However, it is unresolved whether Stau1 is anchored in the cytoplasm immediately after its translation or whether it transits at least once through the nucleus before being retained in the cytoplasm. In the first case, a competitive balance between anchoring and nuclear import would regulate Stau1's entry into the nucleus, allowing only a regulated and small fraction of the Stau1 pool to enter the nucleus. In the second case, Stau1 may need to associate with nuclear cofactors before being retained in the cytoplasm. Alternatively, we do not completely exclude the possibility that the NLS within Stau1 is not normally accessible to the import machinery and that the protein, anchored in the cytoplasm, is not imported in the nucleus/nucleolus. Fusion of tags to Stau1 and/or deletion of different domains may alter protein folding and favour nuclear import. However, the association of endogenous Stau1 with telomerase RNA and telomerase activity [25,26] is not consistent with this possibility.

MOI activity: nuclear export

Stau1 has no identifiable NES (nuclear export signal) and might exit the nucleus in association with exported cofactors. The results of the LMB treatment indicate that Stau1 does not exit the nucleus by interacting with the CRM1-dependent export machinery or with cofactors that use this pathway. Therefore Stau1 is probably not exported with newly assembled ribosomal subunits known to exit the nucleus via the CRM1-dependent pathway [41,42]. Treatment of the cells with inhibitors of translation suggests that Stau1 does not exit the nucleus with the bulk of mRNA, consistent with the fact that Stau1 binds to a subpopulation of mRNA [43].

Mutations in dsRBD3 result in the retention of Stau1 in the nucleolus, suggesting that dsRBD3 is involved in nuclear export by interacting with nucleolar components. It has been shown that Stau1 dsRBD3 interacts with exportin-5 [37], a factor known to mediate nuclear export of dsRBD-containing proteins in a Ran-dependent but CRM1-independent way. Exportin-5 also exports a subpopulation of RNA containing minihelix and microRNA in association with dsRBD-bearing proteins [30,44,45]. Nuclear export of Stau2 is also mediated by its dsRBD3 in a mechanism that relies on exportin-5 [27], although an additional CRM1-dependent pathway was also reported for a shorter Stau2 isoform [28]. Nevertheless, Stau1 does not accumulate in the nucleus when the exportin-5-dependent nuclear export pathway is down-regulated. It is not clear at this point if MOI activity also involves efficient Stau1 export from the nucleus.

dsRBD co-operation

In addition to its well established function as the major RNA-binding domain [15,46], dsRBD3 plays critical roles in Stau1 cytoplasmic retention, nuclear import and nucleolar trafficking. Although essential, however, dsRBD3 alone is insufficient to promote full MOI activity; its role is modulated by the action of additional dsRBDs. Competition between the functions of dsRBD2 and dsRBD4 may regulate dsRBD3 activity. Whereas dsRBD2 strengthens dsRBD3-dependent MOI activity, dsRBD4 stabilizes dsRBD3-dependent nucleolar trafficking. In *Drosophila*, dsRBD2 is required for the cytoskeleton-dependent transport of *oskar* mRNA [47], suggesting a molecular and/or functional link between this domain and the cytoskeleton. Co-operation between dsRBD3 and dsRBD4 was also documented for Stau1 association with ribosomes [17], where dsRBD3 was shown to be the major determinant involved in ribosome binding while dsRBD4 along with TBD played supporting role(s). Since neither dsRBD2 nor dsRBD4 alone shows any specific localization, their roles are likely to be accessory rather than essential, modulating dsRBD3 activity in a phenomenon defined as dsRBD co-operation. Co-operation between two dsRBD was also described for the human ADAR1 (adenosine deaminase that acts on RNA) protein, another member of the family of dsRBD-containing proteins [38]. In this case, one dsRBD interferes with the NLS function of a second dsRBD for nuclear localization of the protein. However, MOI activity of ADAR1 requires the RNA-binding activity of both dsRBDs that probably leads to masking of the NLS. This is not the case with Stau1 since mutation(s) affecting dsRBD3 RNA-binding activity does not destroy MOI activity.

Role(s) of Stau1 in the nucleus/nucleolus

Many RNA-binding proteins involved in mRNA transport, such as hnRNP A2 and ZBP, are known to shuttle between the cytoplasm and the nucleus to bind specific mRNAs in the nucleus before escorting them to their final destination in the cytoplasm [4,6–10].

Since Stau1 is clearly implicated in RNA transport, its presence in the nuclear compartment may be important for some aspects of the initial steps of RNA recognition and/or packaging of RNP complexes. Alternatively, in addition to its roles in the cytoplasm, Stau1 may be required for unrelated specific nuclear functions. SMD (Staufen-mediated mRNA decay) [43] and regulation of telomerase functions [25,26] are among putative nuclear functions. Furthermore, several studies have uncovered new functions associated with the nucleolus such as gene silencing, cell cycle progression, senescence, mRNA export and assembly of RNPs [48–50]. It is possible that only a small fraction of the Stau1 pool is required for these nuclear functions, explaining the very small steady state level of Stau1 in the nucleus and the putative absence of an identifiable export signal. Further studies will be needed to determine the exact role of Stau1 in the nucleus/nucleolus, the regulation of the import/export mechanisms and its relevance for Stau1-mediated mRNA transport and localization.

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