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The Brain-specific Double-stranded RNA-binding Protein Staufen2

NUCLEOLAR ACCUMULATION AND ISOFORM-SPECIFIC EXPORTIN-5-DEPENDENT EXPORT*[S]

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The mammalian double-stranded RNA-binding proteins Staufen (Stau1 and Stau2) are involved in RNA localization in polarized neurons. In contrast to the more ubiquitously expressed Stau1, Stau2 is mainly expressed in the nervous system. In *Drosophila*, the third double-stranded RNA-binding domain (RBD3) of Staufen is essential for RNA interaction. When conserved amino acids within the RBD3 of Stau2 were mutated to render Stau2 defective for RNA binding, the mutant Stau2 proteins accumulate predominantly in the nucleolus. This is in contrast to wild type Stau2 that mostly localizes in the cytosol. The nuclear import is dependent on a nuclear localization signal in close proximity to the RBD3. The nuclear export of Stau2 is not dependent on CRM1 but rather on Exportin-5. We show that Exportin-5 interacts with the RBD3 of wild type Stau2 in an RNA-dependent manner *in vitro* but not with mutant Stau2. When Exportin-5 is down-regulated by RNA interference, only the largest isoform of Stau2 ($Stau2^{62}$) preferentially accumulates in the nucleolus. It is tempting to speculate that $Stau2^{62}$ binds RNA in the nucleus and assembles into ribonucleoparticles, which are then exported via the Exportin-5 pathway to their final destination.

The double-stranded RNA-binding protein family Staufen (Stau)[1] is essential for the localization of mRNAs in diverse cell types in *Drosophila* and mammals (1–3). Whereas only one Staufen gene exists in *Drosophila*, mammals possess two genes encoding for Stau1 and Stau2 proteins. Due to alternative splicing, three Stau2 isoforms with molecular masses of 62, 59, and 52 kDa exist (4). In contrast to the more ubiquitously expressed Stau1, Stau2 is mainly present in the nervous system. Both Staufen proteins, however, are predominantly found in the cytoplasm around the perinuclear region and associated with the endoplasmic reticulum in either untransfected or transfected cell lines (4, 5). In hippocampal neurons, Staufen proteins additionally appear within distinct RNA-containing particles in distal dendrites (4, 5). Recent work suggested that the brain specific Stau2 plays an important role in the delivery of (yet unidentified) RNAs into dendrites of hippocampal neurons (6).

RESULTS AND DISCUSSION

To address the question whether RNA binding determines the subsequent cellular localization of Stau2, we introduced specific point mutations into the RBD3 that render Stau2 incompetent for RNA binding *in vitro* and we analyzed the subsequent cellular localization. Based on the work performed in *Drosophila* by Ramos *et al.* (7), two mutations were generated by replacing "$H^{203}MK^{205}$" to "AMA" ($Stau2_{AMA}$). In a second mutant, four mutations were introduced by replacing "$H^{203}MK^{205}$ . . . $K^{225}K^{226}$" to "AMA . . . AA" and the quadruple mutant was named "IV" ($Stau2_{IV}$) (supplemental Fig. S1*A*). The two clusters of mutations are located in loop2 and loop 4 of the $\alpha\beta\beta\beta\alpha$ structure of RBD3, respectively (7). The two mutant GST-$Stau2_{RBD3}$ fusion proteins showed no residual RNA binding activity compared with the wild type protein *in vitro* (supplemental Fig. S1*B*). The same results were obtained using exogenously expressed full-length proteins in BHK cells (data not shown). To test whether other mutations in the same region of the RBD3 would yield similar results, the conserved methionine (M^{204}) between the amino acids histidine 203 and lysine 205 was mutated to alanine (supplemental Fig. S1*A*; control mutant $Stau2_{HAK}$). This mutation did not affect the RNA binding capacity of the resulting dsRBD3, suggesting that this methionine, like in *Drosophila* Stau, is not involved in the binding of double-stranded RNA (see Fig. 4*A*, data not shown).

Stau-2 Mutants Accumulate in the Nuclear Compartment—BHK cells were transfected and GFP fluorescence was analyzed 16–18 h after transfection. The majority of the GFP-$Stau2_{WT}$ fusion protein was detected in the cytoplasm, associated with the endoplasmic reticulum and ribosomes (Fig. 1*A*; see also Ref. 4). An additional, yet weak, fluorescence was sometimes detected in the nuclear compartment, probably due to high levels of expression. In contrast, BHK cells expressing either GFP-$Stau2_{AMA}$ or GFP-$Stau2_{IV}$ (data not shown) mutant recombinant proteins showed a drastically altered localization pattern; they accumulated in the nucleus and, to a higher degree, in nucleoli-like structures. The observed fluorescence was confirmed by immunostaining using a Stau2-specific antibody (supplemental Fig. S2*A*). To exclude that any of the observed phenotype might be due to a possible truncation

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[S] The on-line version of this article (available at http://www.jbc.org) contains Experimental Procedures and supplemental Figs. S1–S3.

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[1] The abbreviations used are: Stau, Staufen; RNP, ribonucleoparticle; RBD, RNA-binding domain; GFP, green fluorescent protein; YFP, yellow fluorescent protein; EYFP, enhanced YFP; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; Exp, exportin; GST, glutathione *S*-transferase; BHK, baby hamster kidney; HA, hemagglutinin; miRNA, micro-RNA; siRNA, small interfering RNA.

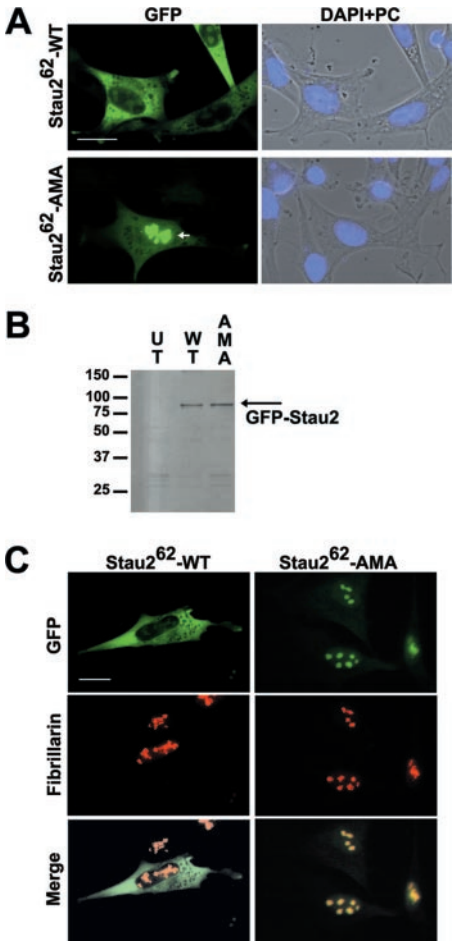

FIG. 1. **Staufen2 mutants accumulate in the nuclear compartment.** *A*, BHK cells were transfected with the indicated constructs to express the long isoform of either wild type GFP-Stau2^{62} (*WT*) or mutant GFP-Stau2^{62} (*AMA*) and their intracellular localization was analyzed by fluorescence microscopy. On the *right side*, the corresponding phase contrast images and the superimposed DAPI staining are shown. The *arrow* indicates accumulation of the RNA-binding-deficient Stau2$_{AMA}$ in the nuclear compartment. *B*, Western blot of BHK cell extracts upon transfection with either GFP-Stau2$_{WT}$ or GFP-Stau2$_{AMA}$ or mock-treatment (*UT*) and detection of the full-length fusion proteins with anti-GFP antibodies. *C*, nucleolar localization of the GFP-Stau2$_{AMA}$ mutant. BHK cells were transfected as indicated, and the signals of the same cell were recorded for GFP fluorescence (in *green*) and for fibrillarin immunostaining (in *red*). The *right panels* show the overlay of both signals (*Merge*). *Bar*, 10 μm.

of the resulting fusion proteins, we performed Western blot analysis of extracts from BHK cells transfected with either wild type or mutant GFP-Stau2 proteins. Fig. 1*B* shows that the full-length fusion proteins (63 + 25 kDa) were not degraded. We also tested whether the observed pattern could either be due to the addition of the GFP tag or to its relative position within the fusion protein. We therefore introduced the same mutations into a cDNA encoding for Stau2 tagged with hemagglutinin (Stau2-HA). Cells were transfected with the resulting constructs and immunostained with an anti-HA monoclonal antibody. Wild type Stau2-HA yielded a pattern similar to GFP-Stau2, whereas cells transfected with any of the RNA-binding-deficient Stau2 mutants showed loss of the cytoplasmic localization and nuclear accumulation (supplemental Fig. S2*B*).

To identify whether the Stau2 mutants indeed accumulated in the nucleolus, BHK cells expressing GFP-Stau2$_{WT}$ or mutant GFP-Stau2$_{AMA}$ were immunostained with anti-fibrillarin antibodies (Fig. 1*C*). In the latter case, the signal from GFP and

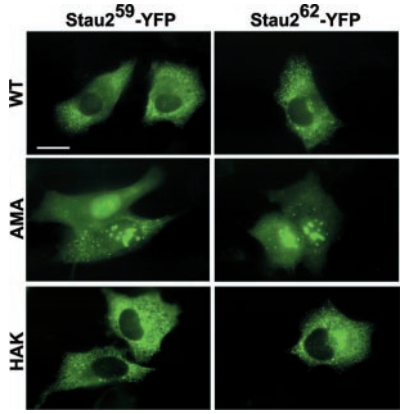

FIG. 2. **Mutated Staufen2 isoforms accumulate in the nuclear compartment of mammalian cells.** Two isoforms of Stau2 tagged with EYFP were expressed in BHK cells. Both 59- and 62-kDa isoforms show the same pattern when their RNA binding activity had been abolished by mutation. In contrast, Stau2 proteins expressing the control mutation (HAK) show an indistinguishable localization from wild type. *Bar*, 10 μm.

fibrillarin displayed identical patterns confirming that the RNA-binding deficient Stau2 mutants reside in nucleoli. Similar results were obtained in BHK cells transfected with the construct coding for Stau2$_{IV}$ (data not shown). Since Stau2^{62} was shown to have a distinct cellular distribution compared with Stau2^{59} and Stau2^{52}isoforms (4), we tested whether Stau2^{59} yielded the same intracellular mislocalization upon mutation of the RBD3. The AMA mutation was therefore introduced into the Stau2^{59} cDNA tagged with EYFP at the C terminus. Both mutant Stau2-EYFP isoforms accumulated into the nuclear compartment, with a prominent signal in the nucleolus (Fig. 2). As a control, BHK cells were transfected with Stau2$_{HAK}$-EYFP; they showed an identical pattern compared with Stau2$_{WT}$-EYFP. This HAK mutant indicated that not all mutations residing within the RBD3 cause a potential misfolding of the resulting fusion protein that could lead to the observed nucleolar localization. We then quantified the nuclear accumulation of the various Stau2 mutant fusion proteins in BHK cells. Whereas only a low percentage of cells transfected with the two wild type Stau2 isoforms showed a significant nuclear and nucleolar accumulation (5% $\pm$ 0.7 for Stau2^{62} and 19% $\pm$ 4 for Stau2^{59}, respectively), the vast majority of cells transfected with the two Stau2$_{AMA}$-EYFP mutants displayed an altered subcellular localization. In contrast, BHK cells transfected with the Stau2$_{HAK}$-EYFP mutant control showed a comparable pattern to the wild type. We then investigated whether this localization pattern was also observed in neurons. When primary cultured hippocampal neurons expressed the Stau2$_{AMA}$-EYFP mutants, a nuclear and nucleolar accumulation was observed (supplemental Fig. S3*A*). These data suggest that nucleolar accumulation of the RNA-binding-deficient Stau2 proteins may be a common phenomenon.

The nuclear and nucleolar accumulation of the various Stau2 mutants prompted us to determine whether Stau2 behaves as a nucleo-cytoplasmic shuttling protein. Computer analysis revealed two possible nuclear localization consensus sequences (NLS) located between the dsRBD3 and dsRBD4 and an additional cluster of basic residues (PRRRR) within the RBD4 (Fig. 3*A*). We cloned GFP in frame with the RBD3 + RBD4 derived from both wild type Stau2 and mutant Stau2$_{AMA}$ and transfected these constructs into BHK cells. Cells expressing GFP-Stau2RBD3$_{WT}$+4 gave a predominantly cytoplasmic localization pattern (see Fig. 2). Additionally, a moderate nucleolar accumulation was observed, probably due to the absence of the other RNA-binding domains. As expected, the mutant GFP-

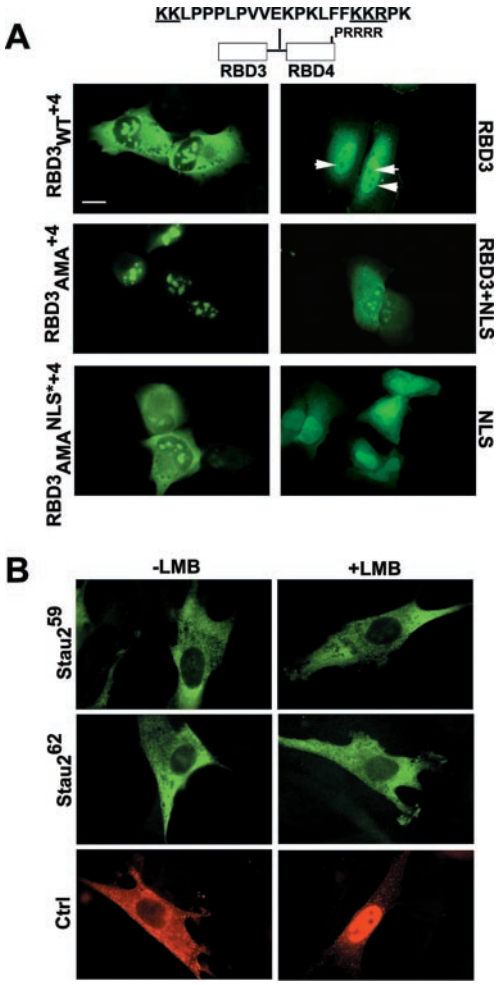

FIG. 3. **Nucleo-cytoplasmic shuttling of Stau2.** *A*, identification of a functional bipartite NLS in Stau2. A sequence located between RBD3 and RBD4 mediates the nuclear import of Stau2. BHK cells were transfected with the following constructs (*left column*): RBD3 + RBD4 of GFP-Stau2 (wild type) ($RBD3_{WT}+4$); GFP-Stau2 (mutant AMA) ($RBD3_{AMA}+4$), or GFP-Stau2 (mutant AMA with a mutagenized NLS; $RBD3_{AMA}NLS^{*}+4$), and their intracellular localization was analyzed by fluorescence microscopy. Due to the small molecular weight of the resulting GFP-Stau2 fusion protein containing just the RBD3 + RBD4, a higher percentage of the reporter is now found in the nucleus. In contrast, the mutant form of the reporter still efficiently accumulates in nucleoli ($RBD3_{AMA}+4$). If the NLS located between the RBD3 and RBD4 had been mutated, the nuclear import of the reporter is strongly reduced ($RBD3_{AMA}NLS^{*}+4$). *Right column*, like GFP, RBD3 can enter the nucleus via diffusion but does not accumulate in nucleoli (indicated by *arrowheads*). The addition of the bipartite NLS (*RBD3+NLS*) causes a selective targeting of the chimeric protein to nucleoli. The NLS sequence alone does not show the same (*NLS*). *B*, nuclear export of GFP-Stau2 is not dependent on the export receptor CRM1. BHK cells were transfected with either wild type 62- or 59-kDa isoforms of Stau ($Stau^{62}$ and $Stau^{59}$, respectively) tagged with HA and then treated with LMB. This did not alter the cytoplasmic localization of Stau2. In contrast, LMB treatment led to nuclear accumulation of axin1-Myc (8), which contains two NES. *Bar*, 10 μm.

$Stau2RBD3_{AMA}+4$ was predominantly nucleolar like the mutant $Staufen2_{AMA}$. To demonstrate that the nuclear localization was indeed mediated by the putative NLS, we introduced specific point mutations into the bipartite NLS in the Stau2 mutant background by replacing the amino acids $KK(X)_{14}KKR$ to $AA(X)_{14}AAA$. The mutated construct $Stau2_{AMA}NLS^{*}+4$ yielded, when expressed in BHK cells, a comparable subcellular localization to wild type $GFP\text{-}Stau2RBD3_{WT}+4$. Consistent with this result, the homologous NLS in Stau1, which shares 63% identity with Stau2, is able to import a cytoplasmic protein into the nucleus in a nuclear import assay.[2] Interestingly, this change in the intracellular localization of Stau is not observed in *Drosophila*, since the Dstau deficient for RNA binding activity remains in the cytoplasm of the germ line cells.[3]

To determine whether the RBD3 plays a role in nucleolar targeting, BHK cells expressing the RBD3 tagged with EYFP were analyzed. Fluorescence signal was detected in the cytoplasm but no significant accumulation in the nucleolus was observed. The nucleoli were free of fluorescence suggesting that RBD3 alone is not responsible for the nucleolar targeting of the fusion protein (Fig. 3*A*, *arrowheads*). In contrast, the addition of the bipartite NLS led a specific nucleolar targeting of the chimeric protein. To verify whether the NLS contained a nucleolar localization signal, cells were transfected with a construct expressing the NLS tagged with YFP. Nuclear, but not nucleolar, accumulation was observed suggesting that the NLS was not responsible for the nucleolar targeting. These results indicate that both RBD3 and NLS are needed for the nucleolar targeting. Taken together, our data suggest that the RNA binding activity in the RBD3 of Stau2 might modulate this nucleolar targeting.

We next investigated whether wild type Stau2 accumulates in the nucleus or in a nucleolar compartment upon addition of leptomycin B (LMB), a specific inhibitor of the CRM1-dependent protein export. BHK cells were transfected with the constructs expressing either long ($Stau2^{62}$-HA) or short ($Stau^{59}$-HA) wild type Stau2, then treated 18 h after transfection with 50 nM of LMB before fixation. As shown in Fig. 3*B*, the addition of LMB did not result in a detectable accumulation of Stau2 proteins in the nucleus. Similar results were obtained even after 10 h incubation with LMB (data not shown). As a positive control, cells transfected with a construct expressing the NES-containing protein axin1 (8) showed accumulation of the reporter protein in the nuclear compartment upon LMB treatment. These results exclude a possible protein export via CRM1 but not via other potential exportins that are insensitive to LMB.

Stau-2 Interacts with the Export Factor Exportin-5—The first experimental evidence for such an additional export pathway for Staufen came from work in mammalian cells identifying exportin-5 (Exp-5) as the export receptor for double-stranded RNA-binding proteins including Staufen (9). Recently, Exp-5 has been also identified as a carrier for tRNA (10–12) and for the nuclear export of pre-miRNAs (13–15). Reverse transcriptase-PCR analysis confirmed that *exportin 5* mRNA was present in both BHK cells and hippocampal neurons (supplemental Fig. S2*C*) indicating that Exp-5 is a candidate export receptor for Staufen proteins in these cell types. We therefore tested whether the RNA-defective Stau2 mutant proteins still interacted with Exp-5 by using an *in vitro* binding assay. The double-stranded RBD3 from either wild type, control mutant HAK, mutant IV, or mutant AA (only "$K^{225}K^{226}$" was changed) fused with GST were incubated in the presence of double stranded RNA. A significant increase in Exp-5 binding was detected for both $GST\text{-}Stau2RBD3_{WT}$ and $GST\text{-}Stau2RBD3_{HAK}$ but not for $GST\text{-}Stau2RBD3_{AA}$ (see scheme in supplemental Fig. S1*A*), when double-stranded RNA was added (Fig. 4*A*, *+dsRNA*). In contrast, mutant GST-Stau2 $RBD3_{AA}$ as well as the quadruple mutant GST-Stau2 $RBD3_{IV}$ did not show any Exp-5 binding. We then went on to verify whether Stau2 directly interacts with Exp-5 via protein-protein interaction or whether RNA mediates this interaction. In the absence of RNA, none of the recombinant proteins bound to

[2] L. DesGroseillers, personal communication.
[3] I. Palacios and D. St. Johnston, unpublished observation.

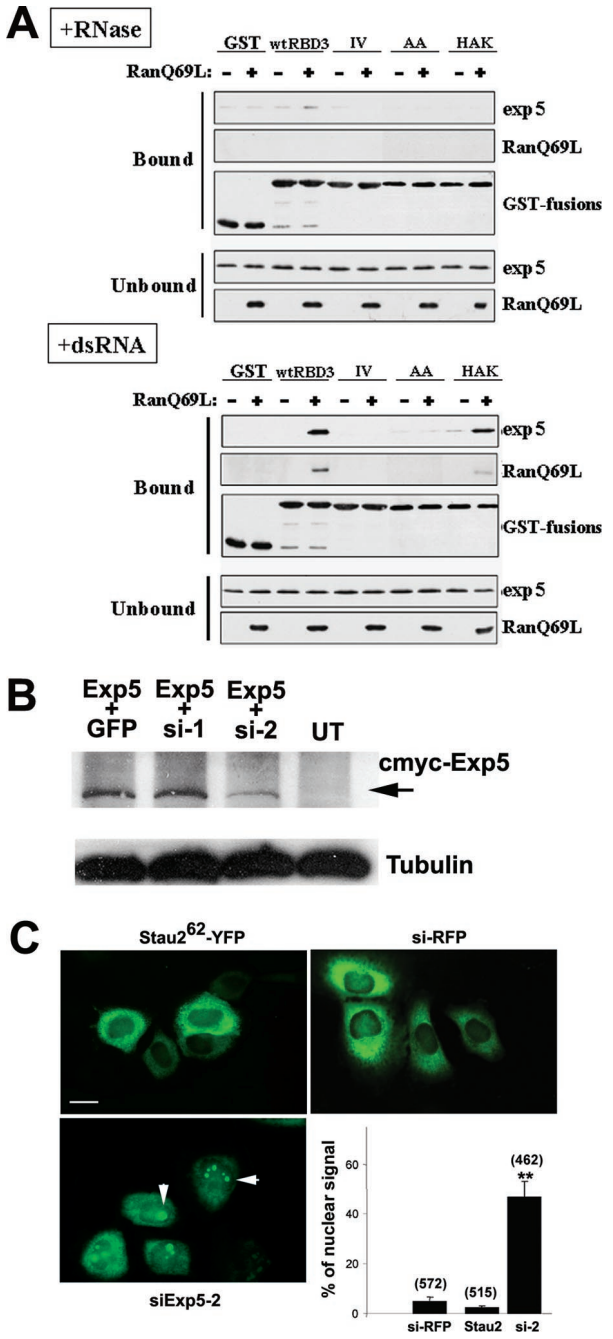

FIG. 4. **Stau2 interacts *in vitro* and *in vivo* with Exportin-5.** *A*, point mutations in Stau2 RBD3 affecting the RNA binding activity interfere with Exp-5 interaction. Both the quadruple mutant IV and another Stau2 RBD3 fusion protein carrying point mutations (mutant AA) are unable to interact with Exp5 *in vitro*. In contrast, the control mutation HAK still efficiently interacts with Exp-5 in the presence of double-stranded RNA. The interaction is greatly diminished upon treatment with RNase. *B*, Exp-5 is efficiently down-regulated by RNA interference in HeLa cells. HeLa were cotransfected with the plasmid expressing a c-Myc-tagged Exp-5 together with either GFP (*lane 1*, mock), siExp5–1 or -2 (si-1 or si-2; *lanes 2* and *3*). Cell lysate from untransfected HeLa cells (*UT*) is shown in *lane 4*. To warrant equal loading, the same blot was decorated with an anti-tubulin monoclonal antibody. C, down-regulation of Exp-5 causes the specific nucleolar localization of Stau2^{62}. When cotransfected with siExp5-2 plasmid, Stau2^{62}-EYFP accumulates in nucleoli (see *arrowheads*). In contrast, si-RFP expression does not alter the cytoplasmic localization of Stau2^{62}-EYFP. The graph shows the quantification of the above experiment. The total number of the counted cells is shown in *brackets*. Down-regulation of Exp-5 leads to an increase of nucleolar accumulation in 49.5% ± 9.2 of the cells ($n = 463$; $p < 0.001$; Student's t test). In contrast, the cytoplasmic localization of Stau2^{59} is not affected under these conditions (data not shown). *Bar*, 10 μm.

Exp-5 in the presence of RanQ69L (Fig. 4*A*, *+RNase*). The fact that Stau2$_{WT}$ needs RNA to bind to Exp-5 *in vitro* suggests that this interaction is not direct but rather mediated by RNA. It is, therefore, tempting to speculate that Exp-5 can potentially discriminate whether Stau2 is bound to RNA or not and specifically exports assembled Stau-containing RNPs into the cytosol.

To verify whether the down-regulation of Exp-5 in mammalian cells would indeed result in an accumulation of Stau2 into the nuclear compartment, we performed RNA interference (16). Constructs expressing siRNA complementary to two different regions of human Exp-5 (si-1 and si-2; target the same regions of Exp-5 as described in Ref. 15) were tested for their ability to reduce the level of the heterologously expressed Exp-5 tagged with c-Myc in HeLa Cells (Fig. 4*B*). In contrast to the si-1 plasmid, the expression of the si-2 construct led to a significant reduction of Exp-5-Myc expression. Moreover, coexpression of si-RFP did not alter the level of Exp-5-Myc-tagged protein (data not shown). To determine the effect of Exp-5 down-regulation on the cellular localization of Stau2 *in vivo*, HeLa cells were then cotransfected with Stau2^{62}-EYFP and si-2 plasmids. Indeed, the expression of si-2 against Exp-5 induced a clear accumulation of the fluorescent fusion protein in the nuclear compartment and in particular in nucleoli (Fig. 4*C*, *siExp5-2*). A cytoplasmic signal was still detectable. This can be explained by residual Exp-5 expression in siRNA-treated cells. The percentage of cells showing enrichment of Stau2^{62}-EYFP in the nuclear compartment was then quantified (Fig. 4*C*, graph; $p < 0.001$ Student's t test). Comparable with the expression of Stau2-EYFP alone, coexpression with si-RFP did not alter the cellular localization of Stau2^{62}. The same results were obtained in hippocampal neurons (supplemental Fig. S3*B*). We then repeated the experiments for the Stau2^{59} isoform. To our surprise, the down-regulation of Exp-5 did not cause a significant nucleolar accumulation of the 59-kDa isoform as demonstrated for the longer isoform (data not shown).

Several studies showed that the nucleolus is not only the compartment where rRNAs assemble with ribosomal subunits but also the place where RNPs assembly occurs (17). Although the functional reason of the observed nucleolar accumulation is still elusive, the mislocalization of the RNA-binding-deficient Stau2 mutants suggests that the interaction of Staufen with either ribosomal subunits or alternatively with (unknown) double-stranded RNAs, *i.e.* tRNAs, rRNA, miRNA, or mRNAs, might occur in the nucleus. The association of Stau2 with ribosomes in a translation-independent manner has been reported recently (4).

Other RNA-binding proteins also accumulate into the nucleus or the nucleolus, such as the fragile X mental retardation protein that is detected in the nucleolar compartment upon treatment with LMB due to the presence of a NES (18). Another example is the human RNA-editing enzyme ADAR1. Interestingly, the nucleo-cytoplasmic distribution of this double-stranded RNA-binding protein is also regulated by its dsRBDs (19) depicting a mechanism similar to the one described here for Stau2. The NLS activity of ADAR1 is also regulated by its main RBD. The import of ADAR1, however, does not require a functional RNA binding activity.

The data presented suggest the following conclusions. First, all isoforms of Stau2 protein have the potential to enter the nucleus via their functional NLS. Second, the binding of Stau2 to RNA interferes with its nucleo-cytoplasmic shuttling. It is tempting to speculate that the RNA binding induces a conformational change in Stau2 that causes masking of the NLS. In contrast, if Stau2 is not bound to RNA, the NLS is exposed, and Stau2 is subsequently imported into the nucleus to bind newly

synthesized RNA. Third, Exp-5 interacts with the Stau2RBD3 in both an RNA- and RanGTP-dependent manner *in vitro* but no longer to RNA-binding-deficient Stau2 mutants. Fourth and most importantly, our data suggest a functional role of Stau2^{62} in the nucleus, where it might bind double-stranded RNA. The role of the Stau2^{59} isoform is much less clear. Future experiments are planned to investigate the different roles of the various Stau2 isoforms within the mammalian cells in more detail and to determine whether Staufen proteins play any role in the transport of distinct RNA species.

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