Nuclear Import Receptors and hnRNP K Mediates Nuclear and Stress Granule Localization of SIRLOIN

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Abstract

The majority of lncRNAs and a small fraction of mRNAs localize in the cell nucleus to exert their functions. A SIRLOIN RNA motif was previously reported to drive its nuclear localization by the RNA-binding protein hnRNP K. However, the underlying mechanism remains unclear. Here, we report crystal structures of hnRNP K in complex with SIRLOIN, and with the nuclear import receptor (NIR) Impα1, respectively. The protein hnRNP K bound to SIRLOIN with multiple weak interactions, and interacted Impα1 using an independent high-affinity site. Forming a complex with hnRNP K and Impα1 was essential for the nuclear and stress granule localization of SIRLOIN in semi-permeabilized cells. Nuclear import of SIRLOIN enhanced with increasing NIR concentrations, but its stress granule localization peaked at a low NIR concentration. Collectively, we propose a mechanism of SIRLOIN localization, in which NIRs functioned as drivers/regulators, and hnRNP K as an adaptor.

Introduction

Long noncoding RNAs (lncRNAs) are RNA transcripts longer than 200 nt nucleotides without protein-coding ability ¹. They were initially considered unimportant, but recent studies have suggested that some may be as important as protein-coding messenger RNAs (mRNAs) ². The majority of lncRNAs and hundreds of mRNAs predominantly exist in the nucleus ³. The action of RNAs is related to their subcellular location, and dysregulation may be harmful ^{4, 5, 6}.

Yoav et al. reported that RNAs with one or more stretches of 42-nucleotide SINE-derived nuclear RNA localization (SIRLOIN) sequence were significantly more nuclear enriched ⁷. They further demonstrated that hnRNP K was responsible for interaction with SIRLOIN and its nuclear localization ⁷. However, the exact mechanism of hnRNP K-mediated nuclear enrichment of SIRLOIN-containing RNAs remains unknown ⁸.

The ubiquitously expressed RNA-binding protein (RBP) hnRNP K regulates several cellular functions, including gene transcription, splicing, and protein translation ⁹. This protein contains multiple DNA/RNA-binding motifs, including three K homology (KH) domains and an RGG domain ¹⁰. Previous studies have suggested that hnRNP K is a nucleocytoplasmic shuttling protein, and its nuclear localization is mediated by its nuclear localization signal (NLS) and the K nuclear shuttling (KNS) domain ^{11, 12}. If hnRNP K is tightly accompanied by SIRLOIN-containing DNA/RNAs, the nuclear import of hnRNP K may simultaneously mediate the nuclear import of the latter.

When cells are under stress, mRNAs are bound to RBPs to form stress granules (SGs), resulting in halted cellular translation ¹³. Several proteomic studies have indicated that some nucleo-cytoplasmic transport factors, some lncRNAs, and hnRNP K translocate to SGs in stressed cells ^{14, 15, 16}. The excessive SG mislocalization of RBPs disrupts nuclear transport and participates in several neuronal diseases ¹⁷.

Whether SIRLOIN is recruited to SG by interacting with hnRNP K is unclear, and how SG localization is affected by nucleo-cytoplasmic transport factors remains unknown.

Results

SIRLOIN binds to hnRNP K and is imported into the cell nucleus

The electrophoretic mobility of SIRLOIN in the presence of hnRNP K was much slower than in the absence of hnRNP K (Fig. 1A), demonstrating that SIRLOIN bound directly to hnRNP K. There are several DNA/RNA-binding regions in hnRNP K, including three KH domains and an RGG domain (Fig. 1B). The apparent binding affinity between SIRLOIN and hnRNP K was 1.1 μM (Fig. 1C). After deleting the RGG domain, the apparent binding affinity decreased only slightly (Fig. S1, Table 1, 1.9 μM), suggesting that the KH domains are probably the main SIRLOIN-interacting sites in hnRNP K.

Table 1
Summary of ITC data obtained in this study.

SIRLOIN	MBP-hnRNP K	Ratio ^a	K _D ^b (μM)
WT (42 bases)	FL	3:4	1.05 ± 0.306
WT (42 bases)	ΔRGG (Δ220–380)	3:4	1.9 ± 0.34
WT (42 bases)	KH1	N.A.	N.B.
WT (42 bases)	KH1-KH2 ^{G157R}	1:4	4.9 ± 1.5
WT (42 bases)	KH2	1:4	2.4 ± 0.9
WT (42 bases)	KH2 ^{G157R}	N.A.	N.B.
WT (42 bases)	KH3	1:4	2.8 ± 1.04
CTR2 (GATTCTCCTGC)	KH1-KH2 ^{G157R}	1:1	61.4 ± 2.2
CTR2 (GATTCTCCTGC)	KH2	1:1	22.2 ± 2.2
CTR2 (GATTCTCCTGC)	KH3	1:1	37.5 ± 1.2
CTR3 (CAGCCTCCCGA)	KH1-KH2 ^{G157R}	1:1	35.5 ± 2.5
CTR3 (CAGCCTCCCGA)	KH2	1:1	43.8 ± 8.5
CTR3 (CAGCCTCCCGA)	KH3	1:1	18.3 ± 3.0
TCCC	KH3	1:1	54.2 ± 4.7
CCTC	KH3	1:1	24.4 ± 5.3
^a The best assumed binding ratio between SIRLOIN and hnRNP K (refer to text). N.A. not applicable.			
^b Except the last two rows, these values represent apparent K _D because there are multiple sites or multiple binding modes. N.B. no binding.			

SIRLOIN contains three C/T-rich regions (CTRs), named CTR1, CTR2, and CTR3 (Fig. 1B), which are supposed to bind to KH domains¹⁸. A SIRLOIN mutant (M0), which was mutated at all three CTRs, lost binding to hnRNP K (Fig. 1D), suggesting that these CTRs are indispensable for the hnRNP K interaction. To investigate whether this interaction contributed to the subcellular localization of SIRLOIN, Cy3-labeled wild-type (WT) SIRLOIN or M0 was directly transfected into HeLa cells, and subcellular localization was observed after 6 h. M0 did not display any nuclear localization, but WT SIRLOIN entered the nucleus in about 16% of transfected cells (Fig. 1E). The nuclear import of WT was potentiated by fusing two SIRLOINs in tandem, both in terms of percentage of cells exhibiting nuclear import (28%) and in terms of nuclear Cy3 brightness, but a tandem M0 sequence did not enter the nucleus. In conclusion, the hnRNP K-interacting C/T-rich motifs of SIRLOIN played a role in its nuclear import.

The multivalent interaction between hnRNP K and SIRLOIN

To explore the contribution of the three C/T-rich regions (CTRs) to hnRNP K binding, six mutants of SIRLOIN (M1-M6) were constructed (Fig. 2A). The EMSA showed that all CTRs are involved in hnRNP K binding, but the second and the third CTRs contributed slightly more towards hnRNP K binding (Fig. 2B). Isothermal titration calorimetry (ITC) was used to measure the binding affinity between SIRLOIN and the three KH domains. KH1, KH2, and KH3 displayed apparent binding affinities of 4.9 μ M, 2.4 μ M, and 2.5 μ M, respectively (Table 1 and Fig. S1). As the CTR1 sequence is the same as CTR3, we further compared the binding affinity of the three KH domains towards CTR2 and CTR3. The binding affinities all ranged in the middle μ M region for different combinations (Table 1, Fig. S2). Considering that ITC is generally less accurate when binding affinity is weaker than 10 μ M, the observed small differences in affinity may not be sufficient to deduce any specific recognition pattern between CTRs and the KH domains, but was likely a weak and multivalent interaction that is often observed in membrane-less organelles (see later sections) ^{19,20}.

Crystal structure of KH3 in complex with CTR3

Several attempts were made to crystallize the full length or segments of hnRNP K with SIRLOIN, and a crystal structure of the KH3 domain in complex with a ssDNA spanning CTR3 was successfully obtained. The structure was solved by molecular replacement using an ssDNA:KH3 structure (PDB 1zzi). The crystal was scaled to space group P6₂22, containing one complex of protein-DNA per asymmetric unit (Fig. 2C). Except for four nucleotides (CCTC) that interact with KH3, four more nucleotides (TCAG) at the 5' of CCTC have good electron densities (Fig. S3). The extra nucleotides are observed because of crystal packing, whereby TCAGC imperfectly base-paired with another TCAGC from an adjacent asymmetric-unit ssDNA (Fig. 2D).

After structural alignment with other KH3-DNA structures, a high DNA binding plasticity was noticed, although all involved four C/T nucleotides and that all backbone phosphates are oriented towards a basic patch in KH3 (Fig. S5). A comparison with the most closely related CCCC binding motif shows that this T is marginally tilted and the second C is slightly pushed away when T is present at the third position (Fig. 2E). This conformational change is likely necessary to accommodate the extra methyl group present in T. The essential hydrogen bonds between DNA and KH3 are conserved, as KH3 adapted to the base changes accordingly (Fig. 2E). These observations suggest that a T residue could be allowed at the third position. The ITC results confirmed that CCTC was able to bind to KH3, comparable to the KH3 affinities of CTR2, CTR3, and the TCCC motif in CTR3 (Table 1, Fig. S4).

A previously neglected CCTC motif between CTR2 and CTR3 was detected in the SIRLOIN sequence (Fig. 2A). To test whether this C/T rich region (CTR4) was involved in hnRNP K binding, two more mutations were introduced into SIRLOIN (Fig. 2A). The EMSA showed that a mutation in the linker between CTR1 and CTR2 (M7) did not change hnRNP K binding, but a CTR4 mutation (M8) reduced

hnRNP K binding (Fig. 2F), similar to the effect of the CTR1 mutation (Fig. 2B). Thus, CTR4 was likely another KH-binding CTR motif in SIRLOIN.

Nuclear import of hnRNP K via the classical nuclear import

Our earlier results showed that SIRLOIN was imported into the nucleus and that it bound to hnRNP K. We hypothesized that hnRNP K, by binding to nuclear import receptors (NIRs), functioned as an import adaptor for SIRLOIN. The binding of GST-hnRNP K to several NIRs was tested by pull down assay. GST-hnRNP K specifically retained importin $\alpha 1$ (a.a. 70-C, Imp $\alpha 1$), but not any of the other tested NIRs (Fig. 3A, Fig. S6). Similar to the WT protein, the isolated NLS (18–38) bound tightly to Imp $\alpha 1$ (Fig. 3B). Moreover, deleting NLS (Δ NLS) completely abolished Imp $\alpha 1$ binding, suggesting that NLS is solely responsible for Imp $\alpha 1$ binding. Consistent with the pull down assay, ITC showed that FL hnRNP K and its NLS bound to Imp $\alpha 1$ with comparable affinities (Fig. 3C, 75 and 115 nM).

To compare the role of different domains of hnRNP K in its nuclear import, the subcellular localization of HA-tagged hnRNP K or its mutants was analyzed by confocal microscopy. Unlike hnRNP K which is highly nuclear-localized, a small fraction of Δ NLS was localized to the cytoplasm. In contrast, deleting KNS, KH1, or KH3 did not affect hnRNP K subcellular localization (Fig. 3E). Deleting KH2 also slightly increased its cytoplasmic localization, but this does not indicate that KH2 is involved in nuclear import, rather that deleting KH2 causes the protein to become unstable (proper folding of KH1 depends on KH2, as shown earlier).

Imp $\alpha 1$ has several functionally redundant homologs in cells which all rely on Importin $\beta 1$ (Imp $\beta 1$) and collectively are called the classical NIRs. Small interference of Imp $\beta 1$ was performed to test the import dependency on classical nuclear import. Under Imp $\beta 1$ knockdown, endogenous hnRNP K was localized more in the cytoplasmic than the siControl (Fig. 3F).

Crystal structure of Imp $\alpha 1$ in complex with the NLS of hnRNP K

To analyze the interaction between Imp $\alpha 1$ and the NLS of hnRNP K, their complex crystal structure was determined at 2.8 Å (Table S1). The bipartite NLS binds to the major and minor NLS binding sites of Imp $\alpha 1$ (Fig. 4A). At the minor site, K21 and R22 are inserted in the P1' and P2' pockets, respectively (Fig. 4B). F19 is docked in a nearby hydrophobic pocket. At the major NLS site, K34, R35, and R37 form electrostatic interactions with residues from P2, P3, and P5, respectively (Fig. 4C). Unlike most of the other bipartite NLSs, the NLS density of hnRNP K is also strong in the middle linker region (Fig. S7). In this region, A24 and M27 form several hydrophobic contacts with Imp $\alpha 1$ (Fig. 4D). E28 and E29 form electrostatic interactions with two lysine residues (K392 and K348) in Imp $\alpha 1$. Q31 forms hydrogen bonds with the three polar amino acids S234, Y277, and D270.

Based on the crystal structure, a few hnRNP K mutations were designed to evaluate the critical Imp $\alpha 1$ binding residues. The pull down assay showed that both K21 and R37 were essential for Imp $\alpha 1$ binding (Fig. 4E), suggesting that the interaction was dependent on the major and minor sites. None of the linker

residue mutations reduced Imp α 1 binding dramatically, indicating that these residues were less important in the interaction. A subcellular localization analysis showed that K21A was more cytoplasmic than WT, verifying that K21 was a critical NLS element (Fig. 4F).

The oligomeric complex formed by hnRNP K, SIRLOIN, and Imp α 1

To enter the nucleus via classical nuclear import pathway, hnRNP K and SIRLOIN must be able to form a complex with Imp α 1. Size exclusion chromatography was used to analyze whether such complex could form in solution. The RGG region of hnRNP K is mostly disordered and often cause hnRNP K aggregation, thus a hnRNP K mutant without the RGG domain (Δ RGG) was analyzed. The Δ RGG-Imp α 1 complex was eluted at 15.8 mL peak volume, and SIRLOIN was eluted in two peaks at 16.8 and 17.8 mL (Fig. 5A). Co-eluting Δ RGG-Imp α 1 with two-fold excess of SIRLOIN resulted in a new peak at 13.8 mL (Fig. 5B), but such a new peak was not observed when co-eluting Δ RGG-Imp α 1 and M0 (Fig. 5C). PAGE analysis showed that the 13.8 mL fraction indeed contained Δ RGG, Imp α 1, and SIRLOIN (Fig. 5D). In contrast, the Δ RGG-Imp α 1 and M0 eluted with separate peaks of protein and DNA.

The calculated molecular weight (MW) for the Imp α 1/ Δ RGG/SIRLOIN complex is 409 kD, which is much greater than the theoretical MW of a trimeric complex formed by one copy of each molecule (total 96 kD). Therefore, the complex formed must be a higher oligomeric state. The Imp α 1 to Δ RGG ratio in the complex should theoretically be one, which agreed well with the SDS-PAGE band intensities (Fig. 5D). The relative peak heights suggest that the molar ratio of Imp α 1/ Δ RGG vs. SIRLOIN is probably greater than 1, as the height of the 13.8 mL peak is lower than the DNA peak in Fig. 5B. Our earlier analysis suggested that hnRNP K might bind to SIRLOIN at a 4:3 molar ratio (Fig. 2), with all CTR/KH sites saturated in the complex. The theoretical MW of four copies of Imp α 1/ Δ RGG and three copies of SIRLOIN is 370 kD, largely agrees with the calculated MW of the complex. The data could not exclude the possibility of existing complexes with other molar ratios, e.g. 5:4 (468 kD) and 3:3 (288 kD), but these complexes were unlikely to be predominant species.

The earlier biochemical and structural results showed that hnRNP K uses non-overlapping domains for binding to SIRLOIN or Imp α 1, but whether the two binding events are independent of each other is unclear. ITC revealed that the apparent binding affinity between SIRLOIN and hnRNP K was barely affected by Imp α 1 (compare Fig. 1C and 5E). Similarly, the binding affinity between Imp α 1 and hnRNP K was hardly affected by SIRLOIN (compare Fig. 3C and 5F). Hence, there was no positive or negative cooperativity in binding among the three molecules.

Forming a complex with hnRNP K and Imp α 1 is essential for the nuclear import of SIRLOIN

Although SIRLOIN entered the nuclei of living cells (Fig. 1E) and SIRLOIN formed a complex with hnRNP K and Imp α 1 (Fig. 5), it has not been demonstrated that SIRLOIN enters the nucleus via binding to hnRNP K and via classical nuclear import. Using semi-permeabilized cells, SIRLOIN WT, but not the M0 mutant, entered the nucleus in the presence of NIRs (Imp α 1 and Imp β 1) and hnRNP K (Fig. 6A). When Δ NLS was

added instead of FL hnRNP K, SIRLOIN no longer entered the nucleus, suggesting that the nuclear import of SIRLOIN is dependent on the NLS (Fig. 6B). Thus, disruption of the complex by NLS deletion or CTR mutation all inhibited the nuclear import of SIRLOIN. As expected, the nuclear import of hnRNP K required binding to Imp α 1, but not binding to SIRLOIN (Fig. 6C).

Forming a complex with hnRNP K and Imp α 1 is essential for the SG localization of SIRLOIN

In addition to nuclear import, SIRLOIN and hnRNP K also formed cytoplasmic puncta resembling possible SG sites (Fig. 6A, arrows). Thus, Tia1 was transfected to mark the SGs, and the in vitro nuclear import experiment was re-performed. Interestingly, Tia1 expressing cells were slightly refractory to nuclear import of hnRNP K compared with cells not overexpressing Tia1 (Fig. S8). This inhibition of nuclear import was relieved by increasing the concentration of digitonin, a reagent used to permeabilized cell membranes, suggesting that Tia1 may have inhibited nuclear import by strengthening the integrity of the cell membrane. The Tia1 colocalization were specific for hnRNP K and SIRLOIN as another nuclear import cargo mCherry-NLS^{SV40} did not show such staining (Fig. S9).

At elevated concentration of digitonin, cytoplasmic hnRNP K and SIRLOIN were highly colocalized with Tia1 (Fig. 7A), confirming that they could be incorporated into SGs. The extent of SG localization by M0 was substantially reduced, both in terms of Tia1 colocalization and intensity (Fig. 7A and B). Similarly, SIRLOIN in the presence of Δ NLS was barely localized in SGs. These results suggest that forming a complex with hnRNP K and Imp α 1 is required for SG deposition of SIRLOIN. In contrast, SG localization of hnRNP K was independent of binding to SIRLOIN and only slightly dependent on its NLS (Fig. 7C). The weaker colocalization of Δ NLS in SGs was likely due to the greater extent of non-SG staining (Fig. 7A). As expected, colocalization of hnRNP K and SIRLOIN decreased when the CTRs were mutated (Fig. 7D).

Nuclear and SG localization of SIRLOIN and hnRNP K at different NIR concentrations

The above analysis indicated nuclear and SG localization of SIRLOIN is dependent on the NLS of hnRNP K, but it has not been demonstrated whether the NIRs are required for this process. Thus, localization of hnRNP K and SIRLOIN at different concentrations of NIRs (Imp α 1 and Imp β 1 at 1:1 molar ratio) was investigated. The results showed that the nuclear intensity of hnRNP K and SIRLOIN increased gradually with increasing concentrations of NIRs, as expected (Fig. 8A, B). SG localization of SIRLOIN was reduced at high concentration and with no NIRs, suggesting that NIRs have a concentration-dependent effect (Fig. 8C). SG localization of hnRNP K was less dependent on the NIRs but also displayed a peak at a low NIR concentration (Fig. 8C). These results indicate that localization of SIRLOIN and hnRNP K indeed requires NIRs. Taken together, we propose a mechanism of SIRLOIN localization that is assisted by the adaptor hnRNP K and regulated by the concentration of NIRs (Fig. 8D).

Discussion

The KH domains of hnRNP K bind both DNA and RNA, and several studies have used DNA instead of RNA in their experiments^{21,22}. The hnRNP K-SIRLOIN affinity obtained with DNA in our study was highly similar to a recent reported affinity with RNA (1.1 μ M vs. 0.7 μ M)²³. It has been shown previously that KH1 does not bind to CTRs²³. We also determined that KH1 does not bind to DNA when purified as a single domain (Table 1). However, when properly folded with the nearby KH2 domain, KH1 was active in DNA binding similar to the other KH domains. Thus, proper protein purification/folding should be carefully verified when designing or using protein mutants.

Most reports have considered hnRNP K as a poly-C binding protein^{23,24}. A previous analysis proposed that KH3 binds only to TCCC or CCCC²¹. Our structural and biochemical studies showed that KH3 could bind to a CCTC motif, suggesting that the third position does not need to be a C. For the fourth position, given its high degree of flexibility (Fig. S5), substituting it with a T or U would probably not reduce KH3 binding affinity. Thus, we updated the definition of KH3 recognition (which may also work for KH1 and KH2), i.e., except the second base that is exclusively C²¹, the other three bases could be any pyrimidine (C, T, or U).

This new definition can explain the partial conservation of T/U in hnRNP K-bound sequences, particularly that some of the hnRNP K binding sequences lack poly C patches^{23,24}. Furthermore, this definition predicts that the CTR2 of SIRLOIN may bind to KH domains by two overlapping motifs, being either TCTC or TCCT (CTR2 contains no TCCC or CCCC motif). In solution, it is possible that due to the presence of DNA/RNA secondary structure²³ or steric hindrance only one of the motifs is selectively bound by a KH domain, as in the crystal structure that only CCTC in CTR3 is bound by KH3 (Fig. 2C). It has been previously shown that two tandem unspaced CTR motifs allow simultaneous binding to two KH domains²¹. The fourth identified CTR in SIRLOIN, a CCTC motif, is separated from CTR2 and CTR3 by one and two nucleotides, respectively, rendering SIRLOIN capable to bind four KH domains simultaneously. Together with the crystal structure, ITC, EMSA, and size exclusion results (Fig. 2C, 2F, and 5B, Table 1), it is concluded that SIRLOIN is a tetravalent KH domain binding sequence, instead of a trivalent.

The ITC analysis showed that increasing the number of CTRs increased the binding affinity of each KH domain. This agrees with the avidity theory whereby tethering multiple binding sites creates 'forced proximity'²⁵. However, increasing the number of KH domains did not increase SIRLOIN binding affinity (Table 1). Similarly, deleting KH3 did not abruptly reduce its affinity for different CTRs²³. It has been proposed that the protein hnRNP K functions in chromatin remodeling, and that tandem KH domains in a protein can remodel the RNA structure^{26,27}. Thus, the binding energy gained through tethering multiple KH domains may be counter-balanced by the energy spent on remodeling the DNA/RNA secondary structure (to a higher energy state). In agreement with this speculation, C-rich RNAs without secondary structures have a higher affinity for hnRNP K²³.

Previous reports have shown that hnRNP K contains an NLS and a KNS domain which facilitate its nuclear import^{11,27}. Here, deleting NLS, but not KNS, partially inhibited the nuclear import of hnRNP K.

However, our results do not exclude the function of KNS in nuclear import. The finding that the NLS deletion mutant was also mainly nuclear localized suggests that nuclear import of hnRNP K is redundant, similar to nuclear import of many other proteins, such as FUS^{28,29,30}. In particular, hnRNP K contains several RG/GR motifs that may be recognized by transportin 1^{31,32}. It remains unknown whether other hnRNP K import pathways can simultaneously import SIRLOIN into the nucleus.

Lubelsky reported that SIRLOIN promotes nuclear accumulation of a mRNA when fused to that mRNA⁷. Here, it was demonstrated that SIRLOIN, but not its CTR mutant, entered the cell nucleus when directly added into cells (Fig. 1E). Tethering two SIRLOIN repeats in tandem promoted this process, in line with the report that RNAs containing more SIRLOIN repeats are more nuclear-localized than those with one SIRLOIN⁷. Therefore, besides nuclear retention³³, nuclear import may be another mechanism of nuclear localization for SIRLOIN-containing DNAs/RNAs. Landerer and his colleague found that mitochondrial-encoded lncRNAs could be exported from mitochondria and relocalized into the nucleus³⁴. Thus, there exists a lncRNA nuclear import mechanism in cells, and NIRs and hnRNP K may play a role in this step.

A common feature of amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) is the abrupt formation of SGs or cytoplasmic inclusions by different RBPs including FUS, TDP-43, and hnRNP A1^{17,35}. These RNA-binding proteins and hnRNP K have similar characteristics, e.g., all contain RNA-binding domains (also called RNA recognition motifs) and R/G rich disordered domains, but little is known about mislocalization of hnRNP K in the cytoplasm of cells with neuronal disease³⁶. Here, it was found hnRNP K had a high tendency to enter SGs, which was independent of DNA binding but partially dependent on the classical NIRs (Fig. 8). A previous report suggested that SG depletion of hnRNP K prevents SG accumulation of TDP-43, a binding partner of hnRNP K¹⁵. Whether SG localization of hnRNP K plays a role in health and disease warrants further studies.

One of the essential proteins of the nuclear-localized membrane-less organelle paraspeckles is hnRNP K³⁷. The mechanism of hnRNP K SG localization may share some features with its localization in paraspeckles³⁸. The RGG domain has been proposed to non-specifically bind DNA/RNA, and promote its SG (and possibly paraspeckle) localization by interacting with other RNAs or proteins in SGs³⁹. Even without the RGG domain, hnRNP K formed a oligomeric complex with SIRLOIN in the context of size exclusion. The affinities of all CTR-KH domain pair in this complex are in the middle micromolar range (Table 1). Thus, it may not be a specifically configured complex, but rather a complex with dynamic and heterogeneous configurations, featuring a weak multivalent interaction that is often observed in membrane-less organelles^{19,20}. At higher concentrations of hnRNP K and SIRLOIN, particularly in SGs, hnRNP K and SIRLOIN may not form such a 4:3 complex, but rather a more complex network involving other CTRs and KH domains in SGs (beyond the interaction complexity of RGG-mediated networks).

The recruitment of hnRNP K into SGs co-deposited bound SIRLOIN into SGs. This is analogous to storing mRNAs in SGs by RBPs under cellular stress¹³. The nuclear import and SG deposition phenomenon is probably not limited to SIRLOIN but also applicable to other hnRNP K binding motifs²³. A previous study

suggested that P body (another cytoplasmic membrane-less organelle) association of lncRNA may affect mRNA translation by regulating the types of mRNA associated with P bodies⁴⁰, but the function of lncRNA SG localization is unknown. It remains to be explored how the nuclear and SG localization of SIRLOIN is regulated, but the NIR concentration may be a factor (Fig. 8).

Several importins are demonstrated to prevent RBPs from accumulating in SGs^{30,32,35}. Likewise, our results show that SG association of hnRNP K was reduced by a high concentration of classical NIRs (Fig. 8). However, a low concentration of NIRs enhanced its SG localization. The NIRs are able to equilibrate in and out of the membrane-less organelles including SGs⁴¹. It is reasoned that a low concentration of NIR promoted SG accumulation of hnRNP K via enhancing its SG accessibility, in addition to preventing its non-specific cytoplasmic staining (Fig. 6A). Unlike hnRNP K, SG accumulation of SIRLOIN was more dependent on NIRs. This warrants further investigation to understand why hnRNP K alone is inefficient at importing SIRLOIN into SGs.

Methods

Cloning, protein expression and purification

The hnRNP K gene and its mutants were cloned into a pMAL expression vector incorporating a Tobacco Etch Virus (TEV) cleavable N-terminal maltose-binding protein (MBP) tag. The plasmids were transformed into *Escherichia coli* BL21 (DE3) and grown in LB broth medium. Expression of MBP-tagged hnRNP K was induced by adding 0.5 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), and the culture was grown at 37°C for 4 h. The cells were harvested and sonicated in a buffer containing 50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 5 mM DTT, and 1 mM phenylmethanesulfonyl fluoride (PMSF). The proteins were purified on an MBP column and eluted in a buffer containing 50 mM Tris pH8.0, 200 mM NaCl, 10% glycerol, 5 mM DTT, and 10 mM maltose, followed by anion exchange chromatography (Hitrap Q). The eluates were further purified on a gel filtration column using Äkta Pure (GE Healthcare) in a gel filtration buffer containing 20 mM Tris pH 8.0, 200 mM NaCl, and 2 mM DTT.

Imp α 1 (a.a. 70-C) was cloned into pGEX-4T-1 incorporating a TEV cleavable N-terminal glutathione S-transferase (GST) tag. The plasmid was transformed into *Escherichia coli* BL21 (DE3) and grown in LB broth medium. Expression of GST-Imp α 1 was induced by adding 0.5 mM IPTG, and the culture was grown overnight at 20°C. The cells were harvested and sonicated in a buffer containing 50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 2 mM DTT, and 1 mM PMSF. The protein was purified with a GST-tag and eluted after TEV cleavage in a buffer containing 50 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, and 2 mM DTT. The eluate was purified by anion exchange chromatography (Hitrap Q), and further purified by size exclusion in the gel filtration buffer.

Pull down assay

To assess the different interactions, GST-tagged proteins were immobilized on glutathione Sepharose 4B resin and washed three times immediately after immobilization to remove unbound GST-tagged protein.

Soluble proteins were incubated with the immobilized proteins in a total volume of 0.5 mL at 4°C for 1 h. After three washes, bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and visualized by Coomassie Blue staining. The pull down buffer contained 20 mM Tris pH 8.0, 200 mM NaCl, 10% glycerol, 2 mM MgCl₂, 0.005% Triton X-100, and 2 mM DTT.

Electrophoretic mobility shift assay (EMSA)

Single-stranded DNA (ssDNA) of SIRLOIN and its mutants were purchased from Sangon Biotech (China). SIRLOIN or its mutants (20 pmol) were incubated with or without 60 pmol of hnRNP K (Δ 220–380) in a buffer containing 20 mM Tris pH 8.0, 200 mM NaCl, and 2 mM DTT. The reaction volume was 15 μ L. After incubation at 4°C for 0.5 h, the samples were resolved by 8% (w/v) PAGE, and stained with ethidium bromide.

Crystallization and data collection

The KH3 domain of hnRNP K (10 mg/mL) and the ssDNA (CTCAGCCTCCCGACTC, CTR3 is underlined) were mixed at a 1:2 molar ratio. Diffraction-quality crystals were obtained using hanging-drop vapor-diffusion (18°C) in a solution containing 25 % w/v polyethylene glycol (PEG) 3350, 100 mM Bis-Tris pH 5.5, and 200 mM ammonium acetate. The NLS of hnRNP K and Imp α 1 were mixed at a 2:1 molar ratio, purified by gel filtration, and concentrated to 8–9 mg/mL. Diffraction-quality crystals were obtained using hanging-drop vapor-diffusion (18°C) in a crystallization solution containing 0.1 M ammonium acetate, 0.1 M Bis-Tris pH 5.5, and 17% w/v PEG 10,000. A 20% glycerol solution was supplemented with the crystallization conditions as a cryoprotectant for both crystals. X-ray diffraction data were collected at Shanghai Synchrotron Radiation Facility (SSRF) beamline BL17U1. The data collection statistics are given in Table S1.

Structure solution and refinement

Coordinates of KH3 (pdb code: 1zzi) were used as the search model for the KH3 and CTR3 complex. The coordinates were refined using the Refmac5 program and manually built using the COOT program. Data in the interval 50.00–3.00 Å resolution were used, and the R-value was 0.257 (R_{free} = 0.279) for all reflections at the end of the refinement (Table S1).

Coordinates of Imp α 1 (pdb code: 1pjm) were used as the search model for the NLS and Imp α 1 complex. The coordinates were refined using the Refmac5 program and manually built using the COOT program. Data in the interval 50.00–2.80 Å resolution were used, and the R value was 0.213 (R_{free} = 0.240) for all reflections at the end of the refinement (Table S1).

Isothermal titration calorimetry (ITC)

ITC experiments were conducted at 16°C using the ITC200 (MicroCal, USA) in a buffer containing 100 mM HEPES pH 7.5 and 200 mM NaCl. As several domains were insoluble or unstable, each fragment was fused with an N-terminal maltose-binding protein (MBP) to improve solubility/stability during protein purification. MBP-KH1 displayed a very low purification yield and no binding to SIRLOIN according to ITC

(Fig. S1). However, MBP-KH1-KH2 (residues 18 to 199) was very stable and of high purification yield. As the G157R mutation in MBP-KH2 completely abolished its ability to bind to SIRLOIN (Fig. S1), MBP-KH1-KH2^{G157R} was used as a surrogate of well-folded KH1. Data was analyzed with MicroCal PEAQ-ITC Analysis Software. Data were fitted using a 'one set of sites' model even for binding involving multiples sites, as the binding curves were mostly uniphasic and insufficient to deduce the exact binding affinities for the different sites. The (apparent) binding affinities are reported in Table 1.

Subcellular localization imaging

The pCDNA3.1 plasmid was used to express HA-hnRNP K and its mutants. HeLa cells were maintained in Dulbecco's modified Eagle's medium (Hyclone, USA) supplemented with 10% (v/v) fetal bovine serum (Biological Industries, USA). The cells were transfected with TurboFect transfection reagent (ThermoFisher, USA). For the siRNA study, cells were first transfected with siKPNB1 (GAGATCGAAGACTAACAAA) using Lipo2000 (ThermoFisher). After 24 h, the cells were transfected with different plasmids using TurboFect and visualized 48 h later. Antibodies against HA (Protein Tech, 1:200) and hnRNP K (Novogene, 1:100) were used to detect HA-tagged hnRNP K and endogenous hnRNP K. Images were acquired with an Olympus FV-1000 (Japan) confocal microscope, and were analyzed using NIH ImageJ and GraphPad software.

In vitro nuclear import assay in semi-permeabilized cells

HeLa cells were transfected with FLAG-tagged Tia1 plasmids, and the cell membranes were permeabilized with 0.005% digitonin. The cells were incubated with 2 μ M Cy3-labelled SIRLOIN or its mutants, 2 μ M GST-hnRNP K-HA or its mutants, 1 μ M Imp α 1, 1 μ M Imp β 1, 1 μ M NTF2, 2 μ M Ran, 1 mM ATP, and 0.005% Triton X-100. After a 1 h incubation at room temperature, the cells were washed, fixed, and visualized by immunostaining with antibodies against HA (Protein Tech, 1:200) and FLAG (Protein Tech, 1:300). SIRLOIN was visualized with its Cy3 label. Tandem SIRLOIN ($\times 2$) developed stronger signals than single SIRLOIN and was used in these experiments. Images were acquired with Olympus FV-1000 confocal microscope and were analyzed using NIH ImageJ and GraphPad software.

Declarations

Funding

Not applicable

Conflict of Interest

The authors declare that they have no conflicts of interest with the contents of this article.

Availability of data and material

The structure factor and atomic coordinates have been deposited in the Protein Data Bank (PDB) with accession codes 7CRE and 7CRU.

Code availability

Not applicable.

Author contributions

Conceptualization: QS

Methodology: QS, JY

Investigation: QS, JY

Visualization: QS, JY

Supervision: QS, QZ, HX, DJ

Writing—original draft: QS, JY

Writing—review & editing: QS

Ethics approval

Not applicable.

Consent to participate

Not applicable.

Consent for publication

All authors have read and approved the manuscript for publication.

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Figures

Figure 1

SIRLOIN directly binds to hnRNP K and enters the cell nucleus when transfected. (A) Electrophoretic mobility shift assay (EMSA) of SIRLOIN in the presence or absence of hnRNP K on an 8% (w/v) polyacrylamide gel. The protein-bound SIRLOIN band is marked with an ‘*’. (B) Top: domain structure of hnRNP K. KH1-KH3: three K homology (KH) domains. RG/RGG motifs are represented by purple lines. Bottom: the sequence of SIRLOIN and its mutant M0. The predicted hnRNP K binding sites are colored

red. The mutations are colored blue. (C) Isothermal titration calorimetry of SIRLOIN (200 μ M) into hnRNP K (18 μ M). The bottom panel shows the integrated heat change for each injection and the fitted curve. (D) EMSA of SIRLOIN WT or M0 in the presence or absence of hnRNP K. (E) Nuclear import of Cy3-labelled SIRLOIN WT or M0. HeLa cells were transfected with 0.2 μ M Cy3-SIRLOIN WT or M0 or tandem repeats of these DNA fragments. The bottom panel shows the percent of cells exhibiting nuclear Cy3 signals in each group. Error bars represent standard deviations (SD) of three independent repeats, each with at least 60 cells analyzed for each sample.

Figure 2

The multivalent binding mode between SIRLOIN and hnRNP K. (A) Sequences of SIRLOIN and its mutants. (B) EMSA of SIRLOIN and its mutants in the presence or absence of hnRNP K. The protein-bound SIRLOIN band is marked with an “*”. (C) Crystal structure of hnRNP K KH3 domain (green) in complex with CTR3 DNA (orange). (D) The imperfect double helix formed between two asymmetric unit DNA molecules. The two protein-DNA complexes are colored green and magenta, respectively. (E) Structural alignment with the CCCC-KH3 complex (pdb:1zzj). Only the second and the third nucleotides are displayed for clarity. The protein residues forming intermolecular hydrogen bonds (dashed lines) are displayed as sticks. The red dashed line represents mild steric hindrance (3.2 Å). (F) EMSA of SIRLOIN and its mutants in the presence or absence of hnRNP K.

Figure 3

The classical nuclear import pathway mediates nuclear import of hnRNP K. (A) GST–hnRNP K (GST-K) pull down assay of several purified nuclear import receptors (NIRs) including Impα1, Impβ1, Imp4, and Imp8. (B) GST-tagged hnRNP K or its fragments pull down purified Impα1. (C) ITC titration of Impα1 (200 μM) into hnRNP K (20 μM) or its NLS (20 μM). (D) Regions of hnRNP K that may be involved in its nuclear import and the three KH domains. (E) Subcellular localization of HA-tagged hnRNP K or its deletion

mutants. Arrows indicate cytoplasmic hnRNP K. HeLa cells were stained 48 hours after transfection. Quantification of the cytoplasmic ratio (Cyt/WCE) is displayed at the bottom. Error bars represent SD for at least 30 cells from each sample. The result was statistically compared with WT. (F) Subcellular localization of endogenous hnRNP K in the presence of siControl or siImpβ1.

Figure 4

Structural basis of hnRNP K binding to Impα1. (A) Crystal structure of hnRNP K NLS (cyan) in complex with Impα1 (grey). K21 and R37 are displayed as sticks. (B) Zoom-in view of the minor NLS site. The interacting residues are displayed as sticks. Impα1 is displayed as an electrostatic surface potential map. Main chain atoms are omitted for clarity. Yellow dashed lines represent hydrogen bonds. (C) Zoom-in view of the major NLS site. The main chain atoms, except G150, are omitted for clarity. (D) Zoom-in view of linker region and its interaction with Impα1. (E) GST–hnRNP K or its mutants pull down Impα1. The bound protein is stained with Coomassie Blue. (F) Subcellular localization of hnRNP K and its mutant K21A. Hela cells were transfected with HA-hnRNP K WT or K21A, and visualized 48 hours after transfection. Right panel shows the quantification of the cytoplasmic fraction of hnRNP K (Cyt/WCE). Error bars represent SD for at least 30 cells from each sample.

Figure 5

The oligomeric complex formed by hnRNP K, SIRLOIN, and Impα1. (A) Size exclusion profile of ΔRGG (hnRNP K without a.a. 220-380)/Impα1, SIRLOIN, and M0. The number beside the elution peak indicates the elution volume. (B) Size exclusion elution profile of hnRNP K/Impα1 mixed with two-fold molar excess of SIRLOIN. (C) Size exclusion elution profile of hnRNP K/Impα1 mixed with two-fold molar excess of M0. (D) PAGE (top panel) and SDS/PAGE (bottom panel) analysis of peak fractions from B and C. (E) Integrated ITC heat changes and curve fitting for titrating SIRLOIN (200 μ M) into a preformed complex of hnRNP K (16 μ M) and Impα1 (16 μ M). (F) Integrated ITC heat changes and curve fitting for titrating Impα1 (200 μ M) into a preformed complex of hnRNP K (18 μ M) and SIRLOIN (13.5 μ M).

Figure 6

Nuclear import of SIRLOIN is dependent on its ability to bind hnRNP K and the NLS of hnRNP K. (A) Confocal images of semi-permeabilized HeLa cells treated with the classical NIRs (1 μ M), GST-hnRNP K-HA (2 μ M), and Cy3-SIRLOIN or its mutants (2 μ M). GST-hnRNP K-HA was stained with HA anti-body. (B) Quantification of SIRLOIN nuclear intensity in the different groups, normalized by nuclear DNA intensity (DAPI). Error bars represent SD for at least 30 cells from each group. *** denotes $p < 0.001$. (C) Quantification of hnRNP K nuclear intensity in different groups, normalized by nuclear DNA intensity (DAPI). Error bars represent SD for at least 30 cells from each group.

Figure 7

Stress granule localization of SIRLOIN and hnRNP K in the presence of NIRs. (A) Subcellular localization of GST-hnRNP K-HA (2 μM) and Cy3-SIRLOIN (2 μM) or their mutants (2 μM) in semi-permeabilized Tia1-Flag expressing HeLa cells. NIRs were added to all groups at a concentration of 1 μM. The three channel pixel intensities of the yellow lines are shown in the bottom panels. Pearson's correlation coefficients calculated from the line intensities for SIRLOIN vs. Tia1 (B), hnRNP K vs. Tia1 (C), and hnRNP K vs. SIRLOIN (D). Error bars represent SD for at least 20 cells from each sample. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$.

Figure 8

Nuclear and SG localization of hnRNP K and SIRLOIN at different concentrations of NIRs. (A) Subcellular localization of GST-hnRNP K-HA and Cy3-SIRLOIN at different concentrations of NIRs in semi-permeabilized Tia1-Flag expressing HeLa cells. (B) Nuclear SIRLOIN or hnRNP K intensities, normalized by the respective DAPI intensities. Error bars represent standard error of the mean (SEM) for at least 20 cells from each sample. (C) Line intensity Pearson's correlation coefficients for 'SIRLOIN vs. Tia1' and

'hnRNP K vs. Tia1'. Error bars represent SEM for at least 20 cells from each sample. The result was statistically compared to the 0.3 μ M NIR group. *** $p < 0.001$. ** $p < 0.01$. * $p < 0.05$. (D) A proposed model of SIRLOIN nuclear and stress granule localization, assisted by the hnRNP K adaptor. SIRLOIN is imported into the nucleus and SGs at low NIR concentrations, and preferably imported into the nucleus at high NIR concentrations.

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