A novel role for the nuclear localization signal in regulating hnRNP K protein stability *in vivo*

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ABSTRACT

hnRNP K is a highly conserved nucleocytoplasmic shuttling protein, which associates with RNAs through synergistic binding via its three KH domains. hnRNP K is required for proper nuclear export and translational control of its mRNA targets, and these processes are controlled by hnRNP K's movement between subcellular compartments. Whereas the nuclear export and localization of hnRNP K that is associated with mRNP complexes has been well studied, the trafficking of hnRNP K that is unbound to mRNA has yet to be elucidated. To that end, we expressed an EGFP-tagged RNA binding-defective form of hnRNP K in intact *Xenopus* embryos, and found it was rapidly degraded *in vivo*. Deleting hnRNP K's nuclear localization signal (NLS), which contains two prospective ubiquitination sites, rescued the protein from degradation. These data demonstrate a novel activity for the NLS of hnRNP K in regulating the protein's stability *in vivo* when it is unbound to nucleic acids.

Keywords: hnRNP K; KH domain; RNA-binding protein; *Xenopus*; nuclear localization signal

Abbreviations: hnRNP K, heterogeneous nuclear ribonucleoprotein K; mRNP, messenger ribonucleoprotein; EGFP, enhanced green fluorescent protein; KH, K-homology domain

1. INTRODUCTION

Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is the founding member of the K-homology (KH) domain-containing family of RNA-binding proteins. It plays crucial roles in a myriad of biological contexts, including early embryonic development [1], erythropoiesis [2], developmental and regenerative axonal outgrowth [3-7], long term potentiation [8], neurodegeneration [9; 10], and tumorigenesis [11]. At the molecular level, hnRNP K functions as a central scaffolding component, linking single stranded nucleic acids with proteins that direct RNA trafficking and translation in the cytoplasm and mitochondria, with additional roles in RNA splicing and transcription in the nucleus [12-14]. When bound to mRNAs, hnRNP K undergoes post-translational modifications that control both the subcellular localization and ribosomal association of its mRNA targets [3; 4; 6; 15; 16]. Since the first step in these processes is the binding of hnRNP K to RNA, how this binding affects hnRNP K's subsequent movements through the cell is crucial for understanding its functions.

Current models suggest that hnRNP K initially associates with its target mRNAs as part of a multi-protein mRNP complex in the nucleus, then shuttles with the mRNP to the cytoplasm in response to cell signaling, leading to post-translational modifications of hnRNP K. These modifications include methylation [17] and phosphorylation by ERK [18] of key residues within an RGG motif and nuclear shuttling domain (KNS), respectively. Once in the cytoplasm, hnRNP K initially represses translation [16] until it undergoes additional post-translational modifications, such as phosphorylation by Src [19], JNK [6], ERK [7], and PKC-delta kinases [20]. Once translation is activated, a small portion of cytoplasmic hnRNP K remains associated with polyribosome-bound, actively translated mRNAs [6; 21]. Once released from the mRNP complex, hnRNP K is thought to return to the nucleus to bind additional mRNAs [22]. Thus, one might predict that hnRNP K that cannot associate with RNA will preferentially accumulate within the nucleus.

To test this prediction in an organismal context, we expressed an EGFP-tagged RNA binding-defective mutant hnRNP K in *Xenopus* embryos, wherein hnRNP K is known to regulate translation and turnover of maternally-derived mRNAs at early developmental stages [1], as well as nuclear export and

translation of mRNAs required for axon outgrowth in developing neurons [3; 4; 6; 7]. Instead of accumulating in nuclei, this mutant hnRNP K became prematurely degraded. Surprisingly, deleting the nuclear localization signal (NLS) from the RNA-binding mutant fusion protein restored stability. These data demonstrate a novel role for the NLS— working in conjunction with RNA binding to regulate hnRNP K turnover *in vivo*— with implications for the intracellular trafficking of hnRNP K.

2. MATERIALS AND METHODS

2.1. Plasmid construction and *in vivo* expression

cDNA sequences encoding *Xenopus laevis* EGFP-hnRNP K (“hnRNP K”), which localizes in *Xenopus* cells the same as endogenous hnRNP K and can rescue embryos from the effects of hnRNP K knockdown by morpholino antisense oligonucleotide, and the EGFP control were cloned into a modified pGEM-3Z vector, as described [6]. KH (“KH1-3mut”) and NLS domain (“ΔNLS-KH1-3mut”) mutations were introduced into this plasmid using the QuikChange Lightning Multi and QuikChange II Site-Directed Mutagenesis kits (Stratagene/Agilent), respectively. For DsRed2 expression, the DsRed2 coding region was PCR-amplified (*Pfx* polymerase, Invitrogen) from pDsRed2-C1 (Clontech) and cloned into a modified pGEM-3Z [1]. The relevant oligonucleotide primers (Integrated DNA Technologies) are listed in Table 1.

Plasmids were linearized and transcribed, and RNAs were microinjected into single blastomeres of two-cell stage, periodic albino *Xenopus laevis* embryos, as described [6; 23]. Live embryos were imaged using an Olympus SZX12 fluorescence stereo microscope, a Nikon DS-Ri1 camera, and NIS Elements D software version 3.10. Images were minimally processed for pseudocoloring, brightness, and contrast using Adobe Photoshop CS5 (Adobe Systems). All procedures involving animals were performed in accordance with the Institutional Animal Care and Use Committee guidelines at the University at Albany.

2.2 Sequence alignment and ubiquitination prediction

Possible ubiquitination sites were identified using UbPred [24]. ClustalW2 alignment [25] compared hnRNP K amino acid sequence conservation (GenBank accession numbers: *X. laevis*, AAH44711; chicken, Q5ZIQ3; mouse, P61979; rat, NP_476482; human, AAB20770).

2.3 Western blots

SDS-PAGE was performed on 7.5% polyacrylamide minigels, and transfer to nitrocellulose was performed at 10°C overnight at 60V. Blots were visualized and quantified using a polyclonal antibody against GFP (Rockland) as described [6]. Data from non-saturated Western blots were averaged over separate blots of three biological replicates (n=10 embryos for each replicate), and statistical comparisons between samples were made using two-sided *t* tests. Statistical significance was reached at $P < 0.05$, and the actual values are specified in the text.

3. RESULTS AND DISCUSSION

3.1 Mutations in the KH domains of hnRNP K that abrogated RNA binding led to premature degradation.

The protein structure of hnRNP K is highly conserved among vertebrates. Relevant for this study, the nuclear localization signal (NLS) exhibits 100% identity among the mammalian, chicken and *Xenopus* sequences compared, and the three KH RNA-binding domains each contain only a single amino acid difference between *Xenopus* and the other species. RNA binding, which is mediated cooperatively by all three KH domains, occurs within flexible loop regions between two alpha helices within these domains and depends upon an invariant G-X-X-G motif (Fig. 1); amino acid substitutions that sterically occlude access to these loops (*e.g.*, G → Y) significantly reduce the RNA-binding affinity of the KH domains [26; 27].

To determine how hnRNP K is trafficked within the cell when unbound to nucleic acid, we introduced these same G → Y mutations into all three KH domains of a previously characterized, EGFP-tagged *Xenopus* hnRNP K [6], and compared the expression of the unmutated EGFP-tagged hnRNP K

with this tagged KH domain mutant (KH1-3mut) *in vivo* (Fig. 2A). When RNA encoding unmutated EGFP-hnRNP K was injected into *Xenopus* embryos at the two-cell stage, fluorescence was observed in nuclei as early as 6 h post-injection, and persisted well past 24 h post-injection (Fig. 2B-C and [6]). However, when KH1-3mut RNA was injected, although fluorescence also appeared in nuclei by 6 h post-injection, it disappeared by 24 h (Fig. 2D1,E1). This effect was not due to nonspecific degradation of injected RNAs, because when KH1-3mut RNA was co-injected with RNA encoding DsRed2 fluorescent protein, fluorescent DsRed2 expression was still observed at this time (Fig. 2D2,E2). Thus, inhibiting binding to nucleic acid led to hnRNP K's premature degradation in *Xenopus* embryos.

3.2 Deletion of the NLS stabilized RNA binding-defective hnRNP K.

Because the majority of intracellular proteins are targeted for turnover by ubiquitination at selected lysine residues [28; 29], we used bioinformatic analysis to identify prospective ubiquitination sites within *Xenopus* hnRNP K [24]. Of the 22 lysine residues in *Xenopus* hnRNP K, two sites at K18 and K31, both of which were located within the highly conserved NLS, received markedly high scores (>0.9; Fig. 3A,B).

To determine whether these sites contributed to the higher turnover of hnRNP K unbound to nucleic acid, we deleted the entire NLS (residues 18-34) containing both sites to create the EGFP-tagged *Xenopus* hnRNP K mutant, Δ NLS-KH1-3mut (Fig. 3C). Embryos expressing this fusion protein exhibited more fluorescence through 24 h of development than did those expressing the original KH1-3 mutated hnRNP K (compare Fig. 2D1-E1 to Fig. 3D-E), implicating the NLS in playing an important role in regulating the degradation of RNA binding-defective hnRNP K.

To quantify this effect, we performed Western blots with a GFP antibody on embryos co-injected with equivalent amounts of EGFP RNA (for normalization) and EGFP-tagged forms of either unmutated *Xenopus* hnRNP K, KH1-3mut, or Δ NLS-KH1-3mut RNAs (Fig. 3F-G). At 2 h post-injection, levels of the unmutated hnRNP K protein (normalized against EGFP) were not significantly different from those of

either KH1-3mut ($P = 0.24$) or Δ NLS-KH1-3mut ($P = 0.96$), indicating that all three proteins were initially synthesized at comparable rates. However, at 6 h and 24 h post-injection, KH1-3mut levels were reduced to 21.7% ($P = 0.02$) and 2.7% ($P < 0.01$) of hnRNP K, respectively, whereas levels of Δ NLS-KH1-3mut were not ($P = 0.82$ and $P = 0.60$, respectively). Together, these data indicate that hnRNP K is turned over more rapidly when unbound to nucleic acid, and that the NLS is crucial for mediating this degradation.

This finding of an additional, novel role for the hnRNP K NLS, beyond its traditional role of binding importin proteins to target hnRNP K to the nucleus [30], has implications for how nuclear import might be coordinated with nucleic acid binding to regulate levels of hnRNP K in the cell. For example, as hnRNP K initially emerges from the ribosome during translation, importin binding could occlude access from ubiquitin ligases to its target sites within the NLS, thereby inhibiting hnRNP K's degradation before the newly synthesized protein is delivered to the nucleus. Then, once hnRNP K enters the nucleus and importin is released, hnRNP K would either bind its nascent hnRNA transcript or single-stranded DNA targets, or become ubiquitinated and degraded within the nucleus, thereby maintaining a proper balance between RNA-binding protein and nucleic acid target. As bound hnRNA transcript is further processed, exported to the cytoplasm, and translated, hnRNP K remains associated with the mRNA as part of mRNP complexes [6; 21; 31; 32]. This association may also occlude the ubiquitination sites within the NLS, protecting hnRNP K from degradation.

Given that hnRNP K undergoes multiple post-translational modifications crucial for regulating its nuclear export, RNA binding, and protein-protein interactions, it then seems more parsimonious for the cell to simply degrade hnRNP K as it is released from its mRNAs as translation is completed, rather than to undo these many modifications and recycle the protein to the nucleus. Thus, taken together, this study provides evidence for a new role of the hnRNP K NLS beyond subcellular trafficking, whereby it aids in maintaining the balance between protein stability and turnover.

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Table 1. List of primer sequences.

	Name	Sequences (5'→ 3')
Primers for Site-Directed Mutagenesis	KH1 mutant G56,59Y sense	GCTTCAGAGCAAAAATGCTGGGGCAG TAATCTATAAAGGATATAAAAATAT TAAGGCACTTAGGACAGACTACA
	KH2 mutant G134,137Y sense	GTTATTGATTTCATCAAAGTTTGGCTGG TGGAATTATTTATGTCAAATATGCA AAGATTAAAGAACTTAGAGAGAAA ACG
	KH3 mutant G337,340Y sense	CACAAGTAACCATTCCAAAAGATTTAG CTGGATCAATAATTTATAAAGGATA TCAGAGAATTAAACAAATCCGCCA T
	NLS (del 18-34) sense	GTTGAATTCACAAACACAGAAGCGAA CGGTAACACAGATCTGATGGTTGAG CTGCGTATTC
	NLS (del 18-34) antisense	GCAGAATACGCAGCTCAACCATCAGA TCTGTGTTACCGTTCGCTTCTGTGTT TCACTTAAGTTGG
Primers for DsRed2	DsRed2 sense	ATAAGCGGCCGCGCCACCATGGCCTC CTC
	DsRed2 antisense	AATTGCGGCCGCTGATCAGTTATCTAG ATCCGGTGGATCC

FIGURE LEGENDS

Figure 1. Evolutionary conservation of hnRNP K. Multiple sequence alignment of hnRNP K showed high sequence homology among the indicated species. The nuclear localization signal (NLS) and K-homology (KH) RNA-binding domains are highlighted in blue and yellow, respectively. The G-X-X-G loop within each KH domain, essential for binding nucleic acids, is indicated (black line).

Figure 2. RNA-binding domain mutations in hnRNP K caused its degradation.

(A) Schematic of wild type *Xenopus* hnRNP K highlights locations of the three K-homology RNA-binding domains (KH1, KH2, KH3) and the NLS. The locations of the RNA-binding loop within each wild type KH domain (G-X-X-G), as well as mutations (Y-X-X-Y) inhibiting RNA-binding for the triple mutant, KH1-3mut, are indicated. (B-C) EGFP-tagged, wild type hnRNP K was visualized *in vivo* as early as 6 h following mRNA injection at the two-cell stage (B), and its expression persisted for at least 24 h following mRNA injection (C). (D-E) The EGFP-tagged hnRNP K triple mutant, KH1-3mut (green), as well as a DsRed2 control mRNA (magenta) co-injected at the two-cell stage, were also observed *in vivo* 6 h post-injection (D1-2). However, by 24 h post-injection, KH1-3mut was no longer observed *in vivo* (E1), whereas DsRed2 persisted (E2). Scale bar, 1 mm.

Figure 3. Deletion of the nuclear localization signal rescued expression of the RNA-binding mutant of hnRNP K.

(A) UbPred analysis [24] of the *Xenopus* hnRNP K amino acid sequence. Gray shading and black stars indicate highly probable ubiquitination sites (K18 and K31). (B) Multiple sequence alignment of the evolutionarily conserved nuclear localization sequence (NLS) region of hnRNP K containing predicted ubiquitination sites (black triangles) showed 100% conservation of these sites among the species examined. (C) Schematic highlights the key features of wild type hnRNP K that were mutated in the Δ NLS-KH1-3 mutant, including the three KH domain mutations from KH1-3mut (Fig. 2), from which the nuclear localization signal (NLS) that lies between amino acids 18-34 was deleted. (D-E) EGFP-tagged

Δ NLS-KH1-3mut appeared *in vivo*, similarly to unmutated hnRNP K (Fig. 2), from 6 h through at least 24 h after mRNA injection at the two-cell stage. Scale bar, 1 mm. (F) Representative Western blots of lysates from embryos 2 h, 6 h, and 24 h post-co-injection of EGFP control mRNA with EGFP-tagged unmutated hnRNP K, KH1-3mut, or Δ NLS-KH1-3mut mRNA, probed with an antibody against GFP. (G) Average $\pm$ SEM intensities of indicated bands normalized to EGFP control (three biological replicates of n=10). *, $P < 0.05$; *ns*, non-significant; SEM, standard error of the mean.

Mouse METEQEETFPNTEINGEGKRPAEDMEEQAFKRSRNTDEMVELRILLQSKNAGAVIGKGGKNIKALRTDYNASVSPD 80
 Rat METEQEETFPNTEINGEGKRPAEDMEEQAFKRSRNTDEMVELRILLQSKNAGAVIGKGGKNIKALRTDYNASVSPD 80
 Human METEQEETFPNTEINGEGKRPAEDMEEQAFKRSRNTDEMVELRILLQSKNAGAVIGKGGKNIKALRTDYNASVSPD 80
 Chicken METEQEETFPNTEINGEGKRPAEDMEEQAFKRSRNTDEMVELRILLQSKNAGAVIGKGGKNIKALRTDYNASVSPD 77
 Xenopus METEQEETFPNTEINGEGKRPAEDMEEQAFKRSRNTDEMVELRILLQSKNAGAVIGKGGKNIKALRTDYNASVSPD 77

Mouse SSGPERILSSADHETIGELKKIIPTEELGLQLPSPTATSQLPLESDAVECLNYOHVKGSDFDCELRLLIHQSLAGGII 160
 Rat SSGPERILSSADHETIGELKKIIPTEELGLQLPSPTATSQLPLESDAVECLNYOHVKGSDFDCELRLLIHQSLAGGII 160
 Human SSGPERILSSADHETIGELKKIIPTEELGLQLPSPTATSQLPLESDAVECLNYOHVKGSDFDCELRLLIHQSLAGGII 160
 Chicken SSGPERILSSADHETIGELKKIIPTEELGLQLPSPTATSQLPLESDAVECLNYOHVKGSDFDCELRLLIHQSLAGGII 133
 Xenopus SSGPERILSSADHETIGELKKIIPTEELGLQLPSPTATSQLPLESDAVECLNYOHVKGSDFDCELRLLIHQSLAGGII 133

Mouse GVGAKIKELRENTQTTIKLFQECCHSTDRVVLIGGKPDVVVECIKILDLISESPHKGRAQPYDPNFYDetydyGGFT 240
 Rat GVGAKIKELRENTQTTIKLFQECCHSTDRVVLIGGKPDVVVECIKILDLISESPHKGRAQPYDPNFYDetydyGGFT 240
 Human GVGAKIKELRENTQTTIKLFQECCHSTDRVVLIGGKPDVVVECIKILDLISESPHKGRAQPYDPNFYDetydyGGFT 240
 Chicken GVGAKIKELRENTQTTIKLFQECCHSTDRVVLIGGKPDVVVECIKILDLISESPHKGRAQPYDPNFYDetydyGGFT 213
 Xenopus GVGAKIKELRENTQTTIKLFQECCHSTDRVVLIGGKPDVVVECIKILDLISESPHKGRAQPYDPNFYDetydyGGFT 213

Mouse MMFDDRGRPVGFPMRGRGGFDRMPPGRGGRPMPSRRDYDDMSPRRGPPPPPGRRGGGSRARNLPLPPPPPRGGDL 320
 Rat MMFDDRGRPVGFPMRGRGGFDRMPPGRGGRPMPSRRDYDDMSPRRGPPPPPGRRGGGSRARNLPLPPPPPRGGDL 320
 Human MMFDDRGRPVGFPMRGRGGFDRMPPGRGGRPMPSRRDYDDMSPRRGPPPPPGRRGGGSRARNLPLPPPPPRGGDL 320
 Chicken MMFDDRGRPVGFPMRGRGGFDRMPPGRGGRPMPSRRDYDDMSPRRGPPPPPGRRGGGSRARNLPLPPPPPRGGDL 293
 Xenopus MMFDDRGRPVGFPMRGRGGFDRMPPGRGGRPMPSRRDYDDMSPRRGPPPPPGRRGGGSRARNLPLPPPPPRGG-- 291

Mouse MAYDRRGRPDYDGVVGFSADETWDSDITWSPSEWQAYEPGGSGYDYSYAGGRGSYGLGGPIITTTQVTIPKDLA 400
 Rat MAYDRRGRPDYDGVVGFSADETWDSDITWSPSEWQAYEPGGSGYDYSYAGGRGSYGLGGPIITTTQVTIPKDLA 400
 Human MAYDRRGRPDYDGVVGFSADETWDSDITWSPSEWQAYEPGGSGYDYSYAGGRGSYGLGGPIITTTQVTIPKDLA 400
 Chicken MSYDRRGRPDYDGVVMMQCHVDACD---DMQPPELFE-----GGSGYDYSYAGGRGSYGLGGPIITTTQVTIPKDLA 364
 Xenopus ---DRRGRPDYDGVV-----GGAGY-----GRGSYGLGGPIITTTQVTIPKDLA 333

Mouse SIIGKGGQRIKQIRHESGASIKIDEPLGSEDRITITGTQDQIQNAQLLQNSVKQSGKFF--- 463
 Rat SIIGKGGQRIKQIRHESGASIKIDEPLGSEDRITITGTQDQIQNAQLLQNSVKQSGKFF--- 463
 Human SIIGKGGQRIKQIRHESGASIKIDEPLGSEDRITITGTQDQIQNAQLLQNSVKQSGKFF--- 463
 Chicken SIIGKGGQRIKQIRHESGASIKIDEPLGSEDRITITGTQDQIQNAQLLQNSVKQSGKFF--- 427
 Xenopus SIIGKGGQRIKQIRHESGASIKIDEPLGSEDRITITGTQDQIQNAQLLQNSVKQSEDSYGF 399

Nuclear Localization Signal (NLS)
K-Homology (KH) RNA-binding Domain

Figure 1

Figure 2

Figure 3