Characterizing functional domains of the RNA helicase RHAU involved in subcellular localization and RNA interaction

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SUMMARY

Posttranscriptional regulation of gene expression is an important and highly regulated process in response to developmental, environmental and metabolic signals. During stress conditions such as heat shock (HS), oxidative stress, ischemia or viral infection, the translation machinery of cells is reprogrammed. The majority of actively translated mRNAs is released from polysomes and driven to specific cytoplasmic foci called stress granules (SGs), where dynamic changes in protein-RNA interaction determine the subsequent fate of mRNAs.

In the presented thesis, I show that the DEAH-box RNA helicase RHAU is a novel SG-associated protein and that its N-terminus is necessary and sufficient for localization of RHAU in SGs. While RHAU protein was originally identified as an ARE-associated protein involved in uPA mRNA decay, it was not clear whether RHAU directly interacts with RNA. Here, I demonstrate that RHAU physically interacts with RNA *in vitro* and *in vivo* through the N-terminus. Bioinformatic analysis of the RHAU protein sequence corroborates the experimental data, revealing that the N-terminus of RHAU harbors a unique RNA-binding domain consisting of two abutting motifs: the G-rich region containing one RGG-box and the RHAU specific motif (RSM). It is widely believed that substrate specificity and subcellular localization of RNA helicases is mediated by their less conserved flanked N-/C-terminal domains. As the unique N-terminus of RHAU is essential and sufficient for both subcellular localization and RNA interaction, it most probably determines a functional specificity of RHAU.

I further show that ATPase activity is responsible for the apparent instability of RHAU-RNA complex formation and markedly influences the kinetics of RHAU retention in SGs. The striking difference in SG shuttling kinetics between fully active RHAU protein and its ATPase-deficient mutant triggers the hypothesis that its ATPase activity takes part in energy dependent dynamic remodeling of RNPs in SGs.

In summary, the results presented in this thesis demonstrate that after rck/p54, DDX3 and eIF4A, RHAU is the fourth RNA helicase detected in SGs and that its association with SGs is dynamic and mediated by a RHAU-specific RNA-binding domain.

Additionally, I could show that RHAU is an essential factor for P-body (PB) formation and obtained initial data that RHAU is possibly also involved in the process of translation via its interaction with translation initiation factor eIF3b.

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ABREVIATIONS

aa	amino acid
ARE	AU-rich element
bp	base pair
CCCP	carbonyl cyanide-m-chloro-phenyl-hydrazone
CLIP	cross-linking immunoprecipitation
dsRNA	double stranded RNA
eIF	eukaryotic initiation factor
FCS	fluorescent correlation spectrometry
FISH	fluorescent *in situ* hybridization
FRAP	fluorescent recovery after photobleaching
G4	guanine quadruplex
kb	kilo base
KO	knockout
MEF	mouse embryonic fibroblast
miRNA	micro RNA
NMD	non-sense mediated decay
***O*-GlcNAc**	*O*-linked N-acetylglucosamine
PB	processing body
RBP	RNA binding protein
RNAi	RNA interference
RNP	ribonucleoprotein complex
RSM	RHAU specific motif
SG	stress granule
shRNA	short hairpin RNA
siRNA	small interfering RNA
UTR	untranslated region

1. INTRODUCTION

1.1. Regulation of mRNA stability

Living systems depend on the proper tuning of gene expression to regulate processes in response to developmental, environmental and metabolic signals (Garneau et al., 2007). Control of gene expression can be divided into three main sections: transcriptional, post-transcriptional and post-translational control. All these steps are strongly regulated and there is evidence of communication between them. Interestingly, microarray analysis has revealed that an increase in mRNA concentration over a short time is caused by an elevation in the transcription rate, and *vice versa* that a decrease in mRNA concentration is mostly driven by post-transcriptional regulation (Fan et al., 2002; Perez-Ortin, 2007). A recent, more detailed, genome-wide analysis has revealed that post-transcriptional gene regulation is a complex and multilateral network.

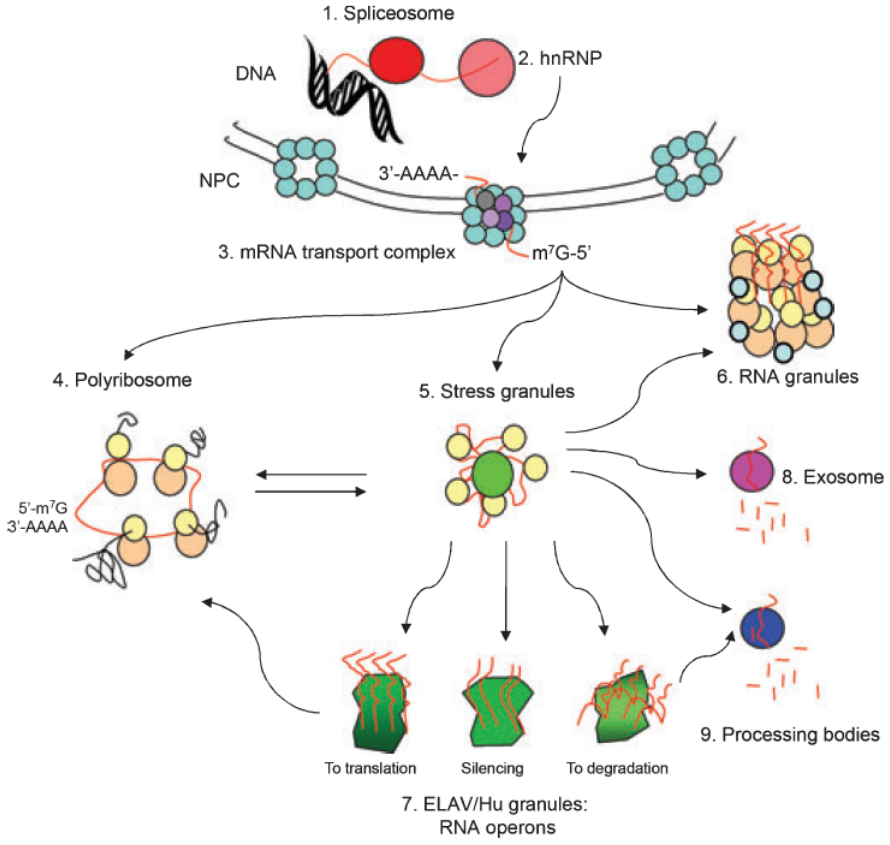


FIGURE 1. RNA from "birth to death". Processing of mRNA transcripts (red lines) occurs at spliceosomes and at hnRNPs that cap and add the poly(A) tail. Transport complexes move mature mRNA through the nuclear pore complex (NPC) to the cytoplasm. In polysomes, mRNAs are translated (40S, yellow; 60S orange circles). Stress granules route mRNAs to other mRNPs. In exosomes and P-bodies, mRNAs are degraded. RNA granules route mRNA and ribosomes to synapses. In ELAV/Hu granules, mRNAs are sequestered together into structural and functional groups of RNA operons that are silenced, translated or degraded. (Degracia et al., 2008)

It has been shown that mRNAs encoding functionally related proteins are controlled by specific RNA-binding proteins and/or non-coding RNAs that bind to specific sequence or structural elements in the RNAs (Halbeisen et al., 2008). This network of mRNA regulators is very important, especially during a stress response when remodeling of mRNA-associated proteins or non-coding RNAs results in changes in mRNA turnover, translation and localization within the cytoplasm. As shown in Figure 1 and discussed in the paragraphs below, from "birth to death" mRNA molecules interact with various proteins affecting their fate and subcellular localization (Degracia et al., 2008).

1.2. mRNA degradation machinery

Eukaryotic mRNA molecules are protected from the degradation machinery by the 5′-cap and 3′-poly(A) tail that are both incorporated concomitantly or immediately after transcription. Furthermore, to protect mRNA messages from exonucleases in the cytoplasm, the 5′-cap and 3′-poly(A) tail interact with proteins such as the cap-binding protein eIF4E and the poly(A)-binding protein (PABP), respectively. To induce mRNA decay, one of these structures must be removed. In eukaryotes, the mRNA level is regulated by three pathways: deadenylation-dependent mRNA decay; deadenylation-independent mRNA decay; and endonuclease-mediated mRNA decay.

The deadenylation-dependent mRNA decay pathway initiates decay of most mRNAs by shortening the poly(A) tail: this is also often the rate-limiting step of degradation. Therefore, transcripts still bearing the correct "protein signals" can be readenylated and returned to polysomes. In most eukaryotes there are three independent complexes possessing poly(A)-specific 3′-exoribonuclease activities: CCR4-CAF1 (complex of nine proteins); PAN2-PAN3; and cap-dependent PARN. PAN2-PAN3 is a PABP-dependent poly(A) nuclease that is involved in the first step of poly(A) shorting, usually shortening to ~80 nucleotides, when the CCR4-CAF1 complex takes over the rest of deadenylation (Yamashita et al., 2005). In contrast to PAN2-PAN3, CCR4-CAF1 activity is inhibited by PABP (Tucker et al., 2002). On the other side, PARN is a unique deadenylase that has been implicated in the deadenylation of maternal mRNAs in *Xenopus leavis* oocytes during maturation (Korner et al., 1998), but also with ARE-dependent deadenylation (Lai et al., 2003).

Subsequently, the deadenylation induces either 5′-cap or 3′-end rapid exonucleolytic decay. The 5′-to-3′ decay pathway starts with cap removal by decapping protein 2 (DCP2) with the assistance of other activators including DCP1, LSM1-7 complex and Pat1. Following decapping, 5′-to-3′ exoribonuclease Xrn1

digests the mRNA body (Wilusz et al., 2001). In the 3′-to-5′ decay pathway, the process is mediated by a large complex known as an exosome. The exosome consists of 9 to 11 subunits with 3′-to-5′ exonuclease activity that forms a donut-like structure (Liu et al., 2006). Although it has been generally agreed that mRNA decay in yeast is mostly mediated via Xrn1 and in mammalian cells via the exosome, recent data has indicated that both 5′-to-3′ and 3′-to-5′ pathways can complement each other. For example, it has been shown that both Xrn1 and the exosome are involved in ARE-mediated mRNA decay in mammalian cells (Stoecklin et al., 2006). However, degradation of mRNAs and pre-mRNAs also occurs to some extent in the nucleus, where the 3′-to-5′ mRNA turnover pathway is implicated in the decay of pre-mRNAs in yeast nuclei (Bousquet-Antonelli et al., 2000).

Although deadenylation-dependent exonucleolytic decay is the major mRNA degradation pathway in eukaryotes, two unrelated transcripts, RPS28B and EDC1 mRNAs, bypass the deadenylation step by direct decapping. Likewise, mRNAs such as insulin-like growth factor 2 (IGF2), c-myc, CLB2 and transferrin receptor escape deadenylation-dependent decay by endonucleolytic cleavage that is followed by 5′-to-3′ and 3′-to-5′ digestion (Gill et al., 2004; Scheper et al., 1996) (Bernstein et al., 1992) (Binder et al., 1994). Furthermore, endonuclease cleavage using Ago2 followed by 5′-to-3′ and 3′-to-5′ decay has been shown to be also involved in siRNA-mediated decay (Sontheimer, 2005).

1.2.1. *Cis*-elements and *trans*-factors in mRNA stability regulation

Stability of eukaryotic mRNA is controlled by regulatory *cis*-acting elements or transcripts and corresponding *trans*-acting proteins or recently reported non-coding small RNAs (Filipowicz et al., 2005; Guhaniyogi and Brewer, 2001). Even though *cis*-acting elements could be found in both the 5′ untranslated region (UTR) and coding region, they are more frequently present in the 3′ UTR, including AU-rich elements (ARE; a destabilizing element) (Chen and Shyu, 1995), iron-response elements (IRE; an iron-regulatory element also found in the 5′UTR) (Thomson et al., 1999), constitutive decay elements (CDE, a destabilizing element) (Stoecklin et al., 2003), pyrimidine-rich elements (stabilizing elements of α-globin, β-globin and α-collagen)(Kiledjian et al., 1995; Lindquist et al., 2004; Yu and Russell, 2001), and the recently identified siRNA/miRNA (Valencia-Sanchez et al., 2006). Each *cis*-element associates with specific binding partners (*trans*-factors) that can recruit or avoid associating mRNAs to/from degradation complexes (such as PBs), depending on the cellular conditions.

1.2.2. Processing bodies (P-bodies, PBs or GW182 bodies)

P-bodies (PBs) are cytoplasmic aggregates of mRNPs where translational repression and mRNA turnover may occur (Bruno and Wilkinson, 2006). Although PBs were discovered approximately 5 years ago as a site where components of the miRNA machinery accumulate, the complete protein composition of PBs has not yet been determined. However, currently known PB-associated proteins may be divided in two main groups: core components and additional components. The core components consist of proteins and enzymes involved in deadenylation, decapping and the 5′-to-3′ turnover pathway. The additional components are proteins involved in miRNA- or siRNA-mediated translation repression or mRNA decay, proteins involved in non-sense mediated decay (NMD), proteins affecting viral function and also proteins that are not involved in RNA metabolism at all such as FAST (Parker and Sheth, 2007). Therefore, PBs are connected with many different mRNA metabolism pathways. Nevertheless, PBs do not contain proteins involved in 3′-to-5′ mRNA decay. Actually, the exosome components were detected in different cytoplasmic foci that did not co-localize with PBs or stress granules (SGs; will be discussed below) (Lin et al., 2007). Furthermore, the protein composition of PBs differs depending on environmental and cell conditions, leading to the conclusion that PBs do not form uniform cytoplasmic foci.

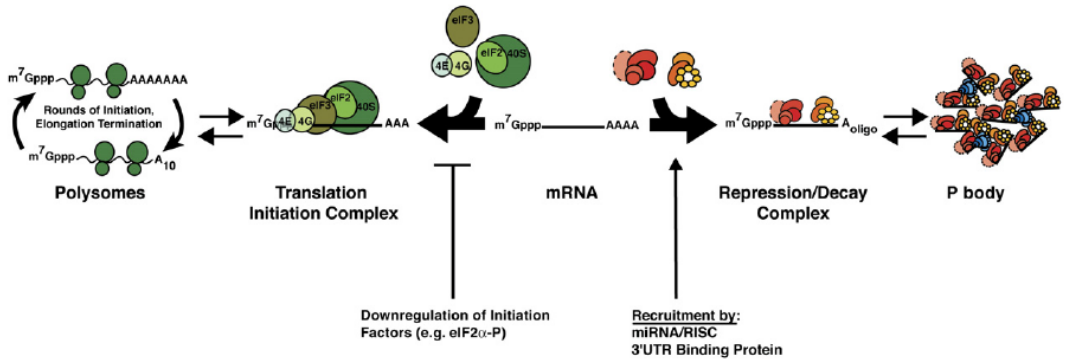


FIGURE 2. Function of mRNAs most likely reflect competition between assembly of translation initiation complexes and translation repression complexes. (Parker and Sheth, 2007)

Although several observations have indicated that mRNA molecules associated with PBs have been decapped and degraded, other observations have shown that, on the contrary, transcripts which were translationally repressed and recruited to PBs could be returned to actively translated pools in polysomes (Figure

2). For example, Bhattacharyya et al. showed that, during normal conditions, CAT1 mRNA is translationally silenced and localizes to PBs by its association with miR-122 (Bhattacharyya et al., 2006). In response to stress, HuR, an ARE-binding protein, translocates from the nucleus to the cytoplasm where it can bind to CAT1 mRNA, and thereby induce CAT1 mRNA release from PBs and its translational de-repression. This experiment showed for the first time that mammalian PBs are places of mRNA storage. Indeed, during normal (basal) conditions, PBs are in finely tuned equilibrium with polysomes (Brengues et al., 2005; Parker and Sheth, 2007).

Importantly, the number and size of PBs are increased in response to stress (Kedersha et al., 2005). In mammalian cells, other cytoplasmic foci known as stress granules (SGs) are formed next to PBs. Interestingly, SGs have not been detected in yeast cells. In sharp contrast to SGs, PB assembly does not require eIF2a phosphorylation. Likewise, PBs are also present during normal conditions. Nevertheless, PBs and SGs share several, but not all, protein and mRNA components. Furthermore, during stress condition PBs and SGs physically associate with each other *in vivo* (Kedersha et al., 2005).

1.3. Reprogramming mRNA translation during stress

In mammalian cells, adverse environmental conditions, collectively called cellular stresses, such as toxic chemicals, heat shock, oxidative stress, ischemia and viral infection, cause damage in proteins, promote their misfolding and interfere with their maturation processes (Brostrom and Brostrom, 1998). These conditions trigger so-called stress responses in cells by radically reprogramming mRNA translation, which involves massive rearrangement of actively translated mRNAs, translation initiation arrest and ribosome run-off. The most prominent cytological change induced by cellular stresses at the subcellular level is the appearance of cytoplasmic foci termed stress granules where translation-arrested mRNAs are accumulated (Anderson and Kedersha, 2002). Importantly, defects in this stress response have been implicated in diverse disease processes, including cancer, microbial infection, diabetes and inflammatory disease (Yamasaki and Anderson, 2008).

1.3.1. Stress granules: a historical overview

SGs were first observed in Peruvian tomato cells as phase-dense cytoplasmic granules formed in response to heat shock (Nover et al., 1983). Later, the same granules were observed in the cytoplasm of heat-shocked mammalian cells (Arrigo et al., 1988). A year afterwards, Nover's laboratory identified that plant heat shock granules contained mRNAs encoding constitutively expressed "housekeeping" proteins but not newly synthesized heat shock proteins, leading to the conclusion that translationally active mRNAs were excluded from granules (Nover et al., 1989). Having identified poly(A)$^+$ RNA, but not actively translated hsp70 mRNA, in mammalian SGs, Kedersha and Anderson confirmed Nover's data and suggested that SGs are sites where, in response to stress, translationally repressed mRNAs accumulate (Kedersha et al., 1999). Furthermore, TIA-1 and TIAR were detected as the first SG-associated RNA-binding proteins (RBPs) (Kedersha et al., 1999). In the case of the TIA-1 protein, it was found that the two amino-terminal RNA-binding domains were necessary for protein localization in SGs and that the carboxyl-terminal prion-like domain was required for SG assembly. Thus, the TIA-1 protein has been postulated as an enhancer of SG formation and is considered to be a general marker for SGs in immunofluorescent analyses (Kedersha et al., 2002).

Nowadays, based on many immunofluorescent reports, it is known that SGs contain, besides an increasing number of RBPs, the 48S pre-initiation complex consisting of eukaryotic initiation factors (eIFs) and small ribosomal subunits. Surprisingly, several proteins involved in metabolic signalling pathways have also been detected in SGs, suggesting that SG assembly is tightly connected with cell metabolism and survival in unfavourable conditions (Kim et al., 2005; Li et al., 2004). With the finding of Argonaute proteins in SGs, it has been speculated that SGs are also involved in miRNA-induced translational silencing (Leung et al., 2006). Furthermore, it has been reported that hyperedited double-stranded RNAs (dsRNAs) bind strongly to several SG components and simultaneously inhibit translation initiation. Although there was no direct immunofluorescent evidence that A-to-I dsRNAs induce formation of SGs or are localized in SGs, Scadden proposed a model where editing by adenosine deaminases results in down-regulation of gene expression via SG formation (Scadden, 2007). Likewise, the detection of the cytidine deaminases APOBEC3G (A3G) and APOBEC3F (A3F), which are involved in anti-retroviral and anti-retrotransposon defence, in SGs indicates a connection between these foci with viral infection and antiviral defence (Gallois-Montbrun et al., 2007; Kozak et al., 2006). Interestingly, some viral infections are known to transiently trigger SG formation and, at the same time, some other viruses, such as the polio virus, inhibit SG aggregation (Esclatine et al., 2004; White et al., 2007). Importantly, using a functional RNAi screen, a recent report suggests that SGs are assembled in the eIF3-dependent manner and that *O*-linked *N*-acetylglucosamine (*O*-GlcNAc)

modification of translation-related proteins is required for aggregation of translationally arrested mRNAs into SGs (Ohn et al., 2008). Taken together, the increasing evidence of different SG functions in mRNA metabolism during stress conditions argues against the original assumption that SGs are only non-specific (non-biological) artificial aggregates.

1.3.2. SG assembly in response to stress-activated signalling pathways

Protein translation is regulated at the levels of initiation, elongation and termination. Although stress influences each step of translation, the majority of stress-induced translational silencing is at the initiation step (Holcik and Sonenberg, 2005). As shown in Figure 3, in the absence of stress, translation initiation is regulated by eleven eIFs and is divided into six consecutive steps: (1) eIF2 ternary complex formation, (2) 43S pre-initiation complex formation, (3) mRNA activation, (4) 48S pre-initiation complex formation by 43S and activated mRNA association, (5) scanning for initiation codon, and (6) 80S complex formation (Holcik and Pestova, 2007). Several stress-activated signalling pathways, which are connected with translation initiation arrest, play a role in phosphorylation of eIF2α, eIF4E-BP and ribosomal protein S6.

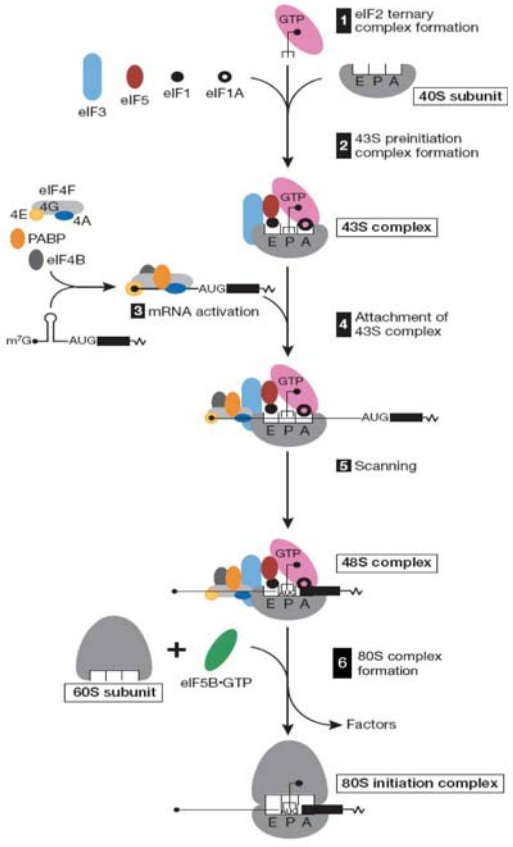


FIGURE 3. Steps of translation initiation.
(Holcik and Pestova, 2007)

The most potent inhibition of translation initiation leading to SG formation is mediated by the phosphorylation of eIF2α, on Ser51. eIF2α is part of the ternary complex eIF2α-GTP-tRNA^{iMet} that recruits the 40S ribosomal subunit to initiate translation. Cells expressing a nonphosphorylatable eIF2α mutant (S51A) do not decrease protein synthesis in response to arsenite, indicating that eIF2α phosphorylation plays a crucial role in translation arrest (Kedersha et al., 1999). Furthermore, cells expressing an eIF2α mutant, which mimics constitutive phosphorylation (S51D) and acts as a dominant inhibitor of

translation, appear to have SGs in non-stressed basal conditions (McEwen et al., 2005). One consequence of eIF2α phosphorylation is a 150-fold increase in the affinity of eIF2α for eIF2B, the eIF2α guanine nucleotide exchange factor (Holcik and Sonenberg, 2005), leading to inhibition of eIF2B function. Inhibition of guanosine diphosphate (GDP) exchange for GTP does not allow ternary complex cycling and results in the accumulation of eIF2-GDP, and thereby effectively halts cap-dependent translation.

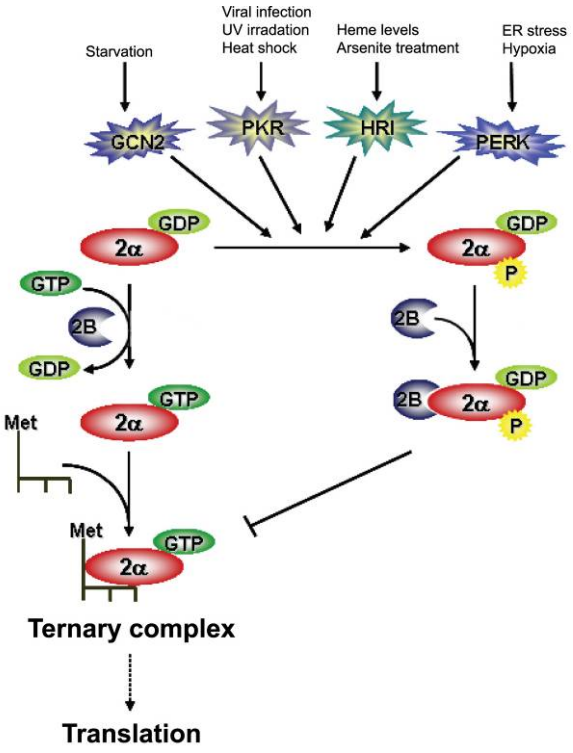


FIGURE 4. Translation initiation arrest via eIF2α phosphorulation.

As shown in Figure 4, phosphorylation of eIF2α is mediated by a family of protein kinases: these are activated by different types of environmental stress (Holcik and Sonenberg, 2005). HRI (heme-regulated initiation factor 2α kinase) is activated by heme during erythrocyte maturation, and by oxidative stress induced by arsenite (Han et al., 2001; McEwen et al., 2005). PERK (PKR-like endoplasmic reticulum kinase) is activated when unfolded proteins accumulate in the ER lumen or by hypoxia (Harding et al., 2000a; Harding et al., 2000b). PKR (protein kinase R) is induced by viral infection, UV irradiation and heat shock (Williams, 1999). GCN2 (general control non-derepressible 2) is activated in starved cells by amino-acid deprivation (Narasimhan et al., 2004). Although there is no clear connection between mTOR signalling and SG assembly, the arrest of translation initiation has been also reported when mTOR complex I activity was reduced, resulting in a decrease in eIF4E-BP and S6K/S6 phosphorylation and thus blocking 4E interaction with 4G because unphosphorylated 4E-BP cannot leave 4E (Proud, 2002). It would be interesting to test whether SGs can be formed in such conditions.

Interestingly, SG formation was observed when RNA helicase eIF4A was inhibited by two compounds, pateamine and hippuristanol (Mazroui et al., 2006). The helicase eIF4A is required for the recruitment of ribosomes to mRNA and during

scanning for a start codon. The binding of pateamine to eIF4A stimulates the enzymatic activities of eIF4A and thereby promotes a stable association between eIF4A and eIF4B leading to the stalling of the initiation complexes on mRNA and SG formation, whereas hippuristanol inhibits eIF4A RNA binding. Independent from the mechanism of the translational arrest, both compounds induce SG formation independent of eIF2α phosphorylation. Therefore, these data have disproved a previous presumption that only eIF2α phosphorylation plays a pivotal role in SG assembly.

In addition, drugs that block protein synthesis at the elongation step by freezing ribosomes on translating mRNA molecules such as cycloheximide or emetin do not induce SG formation, suggesting that 80S complex formation can inhibit SG assembly. In contrast, puromycin, which destabilizes polysomes by releasing ribosomes from mRNA transcripts, induces SGs assembly. Thus, SG formation is solely connected with components involved in translation initiation.

1.3.3. SG-associated proteins

After translation initiation arrest, polysome-free 48S pre-initiation complexes containing initiation factors, small ribosomal subunits and PABP-1 aggregate into SGs (Anderson and Kedersha, 2002; Kedersha et al., 2002). These proteins engaged in the first SG nucleation are called core SG components and are universal markers for all SGs (Figure 5). However, as recently reported, *O*-GlcNAc modification of the translational machinery (e.g. ribosomal protein subunits) is also involve in the SG nucleation (Ohn et al., 2008).

Since TIA-1, TIAR and PABP-1 were detected in SGs, many new SG-associated RBPs have been identified. Under normal conditions, most of these RBPs are involved in various aspects of mRNA metabolism, such as translation (TIA-1, TIAR, PCBP2, Pumilio 2 and CPEB), degradation (G3BP, TTP, Brf1, p54/rck and PMR1), stability (HuR) and specific intracellular localization (ZBP1, Staufen, Smaug, Caprin-1 and FMRP) (see review (Anderson and Kedersha, 2008)). Interestingly, several SG-associated RBPs induce or inhibit SG formation when overexpressed or depleted, respectively. It is presumed that their overexpression interrupts the equilibrium of mRNA distribution between polysomes and polysome-free ribonucleoprotein complexes (RNPs), and thus induces SG formation (Kedersha et al., 2005). These RBPs include those that are able to self-oligomerize, including T-cell internal antigen-1 (TIA-1) or TIA-1-related protein (TIAR) (Gilks et al., 2004), fragile mental retardation protein (FMRP) (Mazroui et al., 2002), Ras-Gap SH3-binding protein (G3BP) (Tourriere et al., 2003), cytoplasmic polyadenylation-binding protein

(CPEB) (Wilczynska et al., 2005), survival of motor neurons protein (SMN) (Hua and Zhou, 2004), smaug (Baez and Boccaccio, 2005) and tristetraprolin (TTP) (Stoecklin et al., 2004). Some RBPs, however, do not induce SG formation upon overexpression, including zipcode-binding protein 1 (ZBP1) (Stohr et al., 2006), hnRNP A1 (Guil et al., 2006) or a poly(A) binding protein 1 (PABP-1) (Kedersha et al., 1999). Nevertheless, these proteins may play other significant roles in SG formation. For instance, ZBP1, which is dispensable during SG formation, is involved in the stabilization of specific target mRNAs under stress conditions by retaining them in SGs. The other example is hnRNP A1, which selectively recruits bound target mRNAs to SGs upon Mnk1/2 phosphorylation (Guil et al., 2006). Therefore, these RNA-binding proteins are most probably involved in SG-mediated mRNA metabolism, thereby influencing the fate of mRNA molecules during stress.

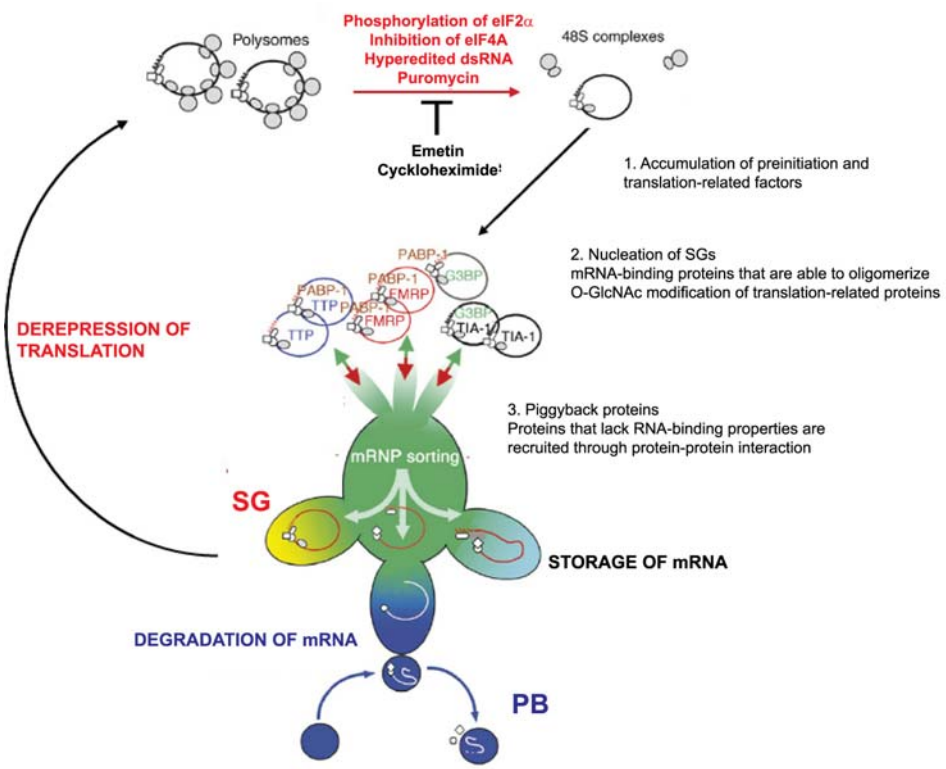


FIGURE 5. SGs biogenesis. (Modified; (Anderson and Kedersha, 2008))

Interestingly, not only RBPs but also proteins that do not directly bind RNA have been found in SGs, including fas-activated serine/threonine phosphoprotein (FAST), tumour necrosis factor receptor-associated factor 2 (TRAF2), plakophilins 3 (PKP3), histone deacetylases 6 (HDAC6) and focal adhesion kinase (FAK). These proteins are mainly involved in signalling pathways, development or adhesion, and are recruited to SGs by protein-protein interaction with another known SG-

associated RBP; e.g. TRAF2 binds to eIF4G, PKP3 interacts with G3BP, FXRP1 and PABP-1 (Hofmann et al., 2006), HDAC6 associates with G3BP (Kwon et al., 2007), and FAK, via growth factor receptor-bound protein 7 (Grb7), interacts with HuR (Tsai et al., 2008). Following these findings, a new role has been proposed for SGs (Anderson and Kedersha, 2008). SGs may actively regulate stress or development responses by sequestering signalling molecules. Although the aggregation of these proteins might be only a consequence of so-called "piggyback" interactions with core SG components without any specific roles in the regulation of signalling pathways, these proteins may still have some unidentified functions in translation and RNA metabolism processes. Accordingly, Kim et al. (Kim et al., 2005) identified TRAF2 as a binding partner of the core SG component eIF4G, and demonstrated that TRAF2 sequestration in heat-induced SGs leads to subsequent blockage of the TNF-α-mediated NF-κB pro-inflammatory response, suggesting that SGs play an important role in breaking the positive-feedback loop of pro-inflammatory signalling. The sequestration of TRAF2 in SGs is most probably not the only mechanism functioning in the anti-inflammatory response.

1.3.4. SG-associated mRNAs

So far, there is no clear evidence that specific mRNA transcripts are recruited to or excluded from SGs. Up to now there has only been one extensive study focused on this topic, where the authors tried to elucidate more about the correlation between ZBP1 mRNA target localization and mRNA stability during stress using FISH and RT-PCR analyses (Stohr et al., 2006). They found that ZBP1 knockdown induced a selective destabilization of target mRNAs during stress, but that ZBP1 was not essential for a specific recruitment of target mRNAs to SGs. ZBP1 target mRNAs are stabilized during stress because they are selectively retained, together with ZBP1, in SGs (Stohr et al., 2006). Likewise, endogenous cellular mRNAs encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β-actin, c-myc, insulin-like growth factor II (IGF-II) and H19 were quantitatively recruited to SGs (Stohr et al., 2006), whereas mRNAs encoding heat-shock protein 70 (hsp70) (Kedersha and Anderson, 2002) and heat-shock protein 90 (hsp90) (Stohr et al., 2006) were largely excluded, indicating that the mRNA recruitment to SGs is selective. Interestingly, hsp90 and hsp70 protein levels increased during stress. Thus, their exclusion from SGs parallels their preferential retention in polysomes. Hsp90 and hsp70 are associated with 3-5% of cellular mRNAs that have been shown to be translated by a cap-independent mechanism, the mechanism first identified for viral mRNAs (Holcik and Sonenberg, 2005). These transcripts mostly contain an internal ribosome entry

site (IRES) or a long structured 5′ UTR that escapes from eIF2α phosphorylation-dependent translation arrest. Furthermore, many cellular IRES-containing mRNAs encode proteins which play roles in proliferation, differentiation and apoptosis, and their protein synthesis occurs predominantly during stress and/or apoptosis (Yamasaki and Anderson, 2008). By sequestering several eIFs that are important for canonical cap-dependent translation, SG formation probably enables translation of normally disadvantaged IRES or highly structured 5′ UTR containing transcripts, thus helping the cell to weather a stress period as safely as possible.

1.3.5. SGs are dynamic foci

Since SGs have not yet been isolated to a significantly pure level, their biochemical analysis is very difficult, leading to retardation of a detailed study of the global SG composition. Nevertheless, based on protein and RNA composition differences in SGs, Anderson has proposed a "triage hypothesis" where the fate of translationally repressed mRNA transcripts is determined by the macroclimate of associated RBPs. Otherwise, SG-associated mRNPs are most probably sorted for decay, storage or translation according to their protein composition. So far, the triage hypothesis has been confirmed only by a combination of indirect studies such as immunofluorescent or fluorescent recovery after photobleaching (FRAP) analyses, mRNA decay assays, polysomal profiles of SG-associated proteins or mRNA transcripts, and RBP immunoprecipitations in normal versus stress conditions.

Importantly, FRAP analysis has revealed that SG-associated proteins behave with differing kinetics in SGs, indicating that SGs are not static aggregations of RNPs, but rather dynamic foci involved in the sorting of individual transcripts for storage, re-initiation or decay. For example, it has been shown that several SG-associated proteins, including TIA-1, TTP, G3BP, PCBP-2, hnRNP A1 and MLN51, are recovered rapidly and completely in SGs within 30s of bleaching (Baguet et al., 2007; Fujimura et al., 2008; Guil et al., 2006; Kedersha et al., 2000; Kedersha et al., 2005), whereas PABP-1 showed only 60% fluorescence recovery after 30s (Kedersha et al., 2005). Interestingly, the FAST protein that is recruited to SGs via TIA-1 exhibited even slower recovery than PABP1, suggesting that it plays a scaffolding role in SGs (Kedersha et al., 2005). Since PABP1 binds very tightly to mRNA, Kedersha et al. have proposed that PABP1 may follow the flux of mRNAs within SGs (Kedersha et al., 2005). While G3BP, TIA-1 and TTP exhibit rapid mobility, they may be involved in RNPs remodelling within SGs, or RNP recruitment to SGs. Therefore, SGs are considered to be sites at which RNPs undergo structural and compositional remodelling and may be temporally stored, returned to polysomes for translation, or

packaged for degradation (Kedersha et al., 2005). Nevertheless, one recent report does not support the current model of SGs as storage sites nor as intermediate locations of mRNA molecules before degradation (Mollet et al., 2008). In this report, the authors claim that mRNA residence time in SGs is brief, in sharp contrast to SG persistence after stress relief, and that this short transit reflects a rapid return to the cytoplasm, rather than a transfer to PBs for degradation. It is clear from the report that mRNA flux in SGs is fast but this observation still does not rule out the possibility that SG-associated mRNA molecules could undergo extensive protein-mRNA complex remodelling. Furthermore, the hypothetical RNP packages do not need to be sent only to PBs for degradation. mRNA degradation also occurs in the cytoplasm. Using FRAP analysis to compare mRNA concentration in SGs and the cytoplasm, they further concluded that most arrested mRNAs are located outside SGs. However, it has to be mentioned that FRAP analysis is not a suitable method for elucidating a real mRNA flux (concentration) in cytoplasmic compartments. Only a more precise method, such as fluorescent correlation spectrometry (FCS), may discover the role of mRNA concentration in cytoplasmic compartments, and thus the correct significance of SGs in mRNA turnover during and after stress. Finally, even though the authors do not agree with the significance of SGs as storage sites, they nicely proved that SGs are dynamic rather static foci.

1.3.6. SG disassembly

In many reports, cell viability and recovery after stress, monitored as SG disassembly, have been linked with several SG-associated RBPs. However, it is not clear whether the sensitivity of cells to stress reflects solely an impairment of SGs. Nevertheless, there are not many reports focused on SG disassembly itself.

Gilks et al. have proposed the mechanism by which SGs are dissolved (Gilks et al., 2004). From the observation that the aggregation of TIA-1 or TIAR was blocked by hsp70 overexpression, they suggested that free hsp70 promotes SG disassembly (Gilks et al., 2004). Stress-induced denaturation of other cytoplasmic proteins mobilizes both hsp70 and ATP for protein renaturation, leading to the deprivation of free hsp70 levels, promoting TIA-1 aggregation and consequent SG formation. Later, the successful refolding of denatured proteins releases hsp70 to its free form resulting in TIA-1 disaggregation and SG disassembly. Hsp72 was likewise reported to disassemble SGs induced in response to proteosome inhibition (Mazroui et al., 2007).

Furthermore, studies focusing on the SG-associated proteins FAK and Grb7 have shown that when cells are released from stress, Grb7 is hyperphosphorylated

by FAK, loses its ability to directly interact with the Hu antigen R (HuR) and is dissociated from SG components, thereby disrupting SGs in recovering cells. Consistently, dominant-negative hypophospho mutants of FAK and Grb7 significantly attenuate SG disassembly during recovery (Tsai et al., 2008). This is the first report showing that signalling molecules actively regulate SG dynamics (Tsai et al., 2008).

1.3.7. SGs in disease and viral infection

Transient assembly of SGs has also been reported during viral infection. The viral replication reprograms the host translation machinery using different mechanisms to manipulate SG assembly. Some observations suggest that SGs function to limit a range of viral infections.

Several viruses have been shown to inhibit SG formation. For instance, during the infection of West Nile virus (WNV) minus-strand 3' terminal stem-loop RNA that binds to TIAR, SG assembly is inhibited and TIAR is sequestered at viral replication foci (Emara and Brinton, 2007). TIAR binding is crucial for the infection because WNV replication is reduced in fibroblasts lacking TIAR. Similarly, Sendai virus encodes an RNA that sequesters TIAR and inhibits SG formation. These results indicate that TIAR plays an important role in SG assembly during viral infection.

In contrast, some viruses induce SG assembly. As shown by White et al., during early poliovirus infection SG formation is induced, but as infection proceeds this ability is lost, and SGs disappear due to the cleavage of G3BP by poliovirus 3C proteinase (White et al., 2007). Interestingly, in this situation TIA-1 and TIAR are not cleaved. Expression of cleavage-resistant G3BP restored SG formation during poliovirus infection and resulted in the significant inhibition of viral replication. SGs are similarly formed, and then dissolved, in cells infected with Semliki Forest virus (SFV) (McInerney et al., 2005). In mouse embryo fibroblasts (MEFs) expressing a non-phosphorylatable mutant of eIF2α, fewer SGs are induced during early SFV infection, resulting in delayed inhibition of host protein synthesis and start of viral RNA replication. Thus, SFV seems to use SGs to regulate its viral gene expression by shutting off host protein synthesis.

Several other viruses have less well established links to SG components. For example, herpes simplex virus 1 (HSV-1) replication is enhanced in MEFs lacking either TIA-1 or TIAR (Esclatine et al., 2004). During HSV-1 infection, TIA-1 and TIAR accumulate in the cytoplasm 6 h post-infection, where they may modulate viral replication or cell survival. No evidence of SG formation has been found under these conditions.

SGs are also thought to contribute to the pathogenesis of several different diseases and have been found in the tissues of stressed animals. In chicken treated with gentamycin, SGs appear in cochlear cells several hours before the onset of apoptosis (Mangiardi et al., 2004). It has also been reported that SGs inhibit the translation of several hypoxia-inducible factor 1 (HIF-1) transcripts during hypoxia to regulate tumour cell survival after irradiation (Moeller et al., 2004). Ischemia/reperfusion (I/R) injury is a major determinant of neural toxicity following cardiac arrest or stroke (Kayali et al., 2005). The delayed and selective vulnerability of post-ischemic hippocampal cornu ammonis 1 (CA-1) pyramidal neurons correlates with a lack of normal protein synthesis recovery (DeGracia et al., 2007). Thus, SG assembly and disassembly might influence the degree of ischemia-induced neuronal damage.

Adaptive immune responses require expansion and differentiation of naive T cells into cytokine-secreting effector cells. Therefore, after initial priming, naive T helper cells express cytokine mRNA but do not secrete cytokine proteins such as interleukin-4 (IL-4) or interferon-γ (INF-γ) without additional T cell receptor stimulation (Scheu et al., 2006). Analysis of the polysome profiles of primed T cells has revealed that cytokine mRNAs are excluded from polysomes. Furthermore, T cell priming induces eIF2α phosphorylation and SG assembly. Restimulation of the cells results in rapid eIF2α dephosphorylation, mRNA translation reinitiation, and cytokine secretion. Therefore, T lymphocytes require components of the integrated stress response and SG formation during T cell differentiation (Scheu et al., 2006). Altogether, these studies indicate that SGs are not *in vitro* artefacts, but are an *in vivo* physiological part of the organism's response to stress.

1.4. RNA helicases

In the last two decades it has become clear that a diverse range of RNAs play critical roles in the regulation of gene expression (Beggs and Tollervey, 2005). It has also become apparent that RNAs hardly ever function alone in a cellular environment. Indeed, immediately after transcription, RNA forms ribonucleoprotein complexes (RNPs) with RBPs: these are dynamic and take part in RNA metabolism (Dreyfuss et al., 2002). The functionality of RNA molecules usually depends on correct folding, but also on the correct set of associated proteins. Furthermore, the function of many small non-coding RNAs involves transient base pairing with a target RNA sequence (Bleichert and Baserga, 2007). All these examples are mainly regulated by a large family of proteins known as RNA helicases that can disrupt RNA-RNA or RNA-DNA base pairing, can dissociate proteins from RNA molecules,

and assist in proper structure formation similar to protein chaperones during protein folding (Bleichert and Baserga, 2007). All these processes, resulting in RNA duplex unwinding, displacement of proteins from RNA, or both, require the energy that is provided by RNA helicases. Traditionally, RNA helicases were defined based on their ability to utilize the energy of NTP binding and hydrolysis to unwind RNA duplexes. However, not all RNA helicases have been shown to unwind double-stranded RNA (dsRNA) in an ATP-dependent manner *in vitro* (Jankowsky et al., 2001; Linder, 2006; Tanner and Linder, 2001), whereas most of them are able to hydrolyze NTP in an RNA-stimulated manner and/or remodel RNPs in an NTP-dependent fashion (Linder, 2006; Mayas et al., 2006; Mazroui et al., 2006; Wagner et al., 1998).

In yeast, almost all RNA helicases are essential for cell viability, and there are orthologs for most of these proteins in mammals (de la Cruz et al., 1999). In humans, 38 DDX-box helicases and 14 DHX-box helicases have been identified so far (Abdelhaleem et al., 2003; Bleichert and Baserga, 2007; Linder, 2006).

1.4.1. Structure

RNA helicases are conserved from bacteria to human and they are surely the largest group of enzymes involved in RNA metabolism, ranging from RNA transcription, RNA editing, mRNA splicing, RNA export, rRNA processing, RNA degradation, and RNA 3′ end formation to translation of mRNA into proteins (Anantharaman et al., 2002; Bleichert and Baserga, 2007). All currently known RNA helicases are divided into the four helicase superfamilies 1-4, but the majority of RNA helicases belong to superfamily 2 (SF2), which also contains a considerable number of DNA helicases. A few RNA helicases belong to helicase superfamily 1 (SF1), including Upf1, an enzyme required for nonsense-mediated decay (NMD). Several viral proteins with RNA helicase activity are classified as SF3 and SF4 proteins (Kadare and Haenni, 1997). Based on protein sequence, SF1 and SF2 helicase groups can be identified by at least seven to nine conserved motifs that are located in two independent helicase core domains that are linked by a flexible loop and form a characteristic cleft for NTP and nucleic-acid (NA) binding (Figure 6A).

These motifs, which are highly conserved among SF1 and SF2 DNA and RNA helicases, are located on the surface of the two core domains as shown in Figure 5B. Based on genetic, biochemical and structural data, different functions have been assigned to these motifs. For instance, they are involved in NTP (mostly ATP) binding (I, II and VI) and hydrolysis (III and V), and in nucleic-acid binding (Ia, Ib, Ic, IV and IVa) (Jankowsky and Fairman, 2007). Interestingly, some RNA helicases

consist of just these core helicase domains, but most of them contain larger characteristic C/N-termini (Tanner and Linder, 2001). SF1 helicases often have essential inserts, which take part in RNA or protein interaction, in each helicase (Figure 6A). In addition, as shown in Figure 6A, SF2 helicases are divided into three subfamilies where the name is derived in single-letter amino acid code from motif II, essential for NTP-hydrolysis: DEAD, DEAH and DExH (Jankowsky et al., 2001; Rocak and Linder, 2004). In humans, DEAD-box proteins have the gene symbol of DDX-, whereas DEAH and DExH-box proteins are designated as DHX- (Abdelhaleem et al., 2003). It is worth mentioning that DEAD-box proteins also contain a Q motif with highly conserved tryptophan that is located several amino acids upstream of motif I and senses just ATP, leading to a preference for ATP hydrolysis rather than NTP. Thus, in comparison to DExH- and DEAH-box proteins, DEAD-box helicases are selective for ATP hydrolysis. Further, in contrast to DEAD and DExH-box protein, DEAH-box helicases also share a high similarity throughout their C-termini. On the other hand, the DExH-box subfamily is the most diverse subgroup, consisting of both RNA and DNA helicases.

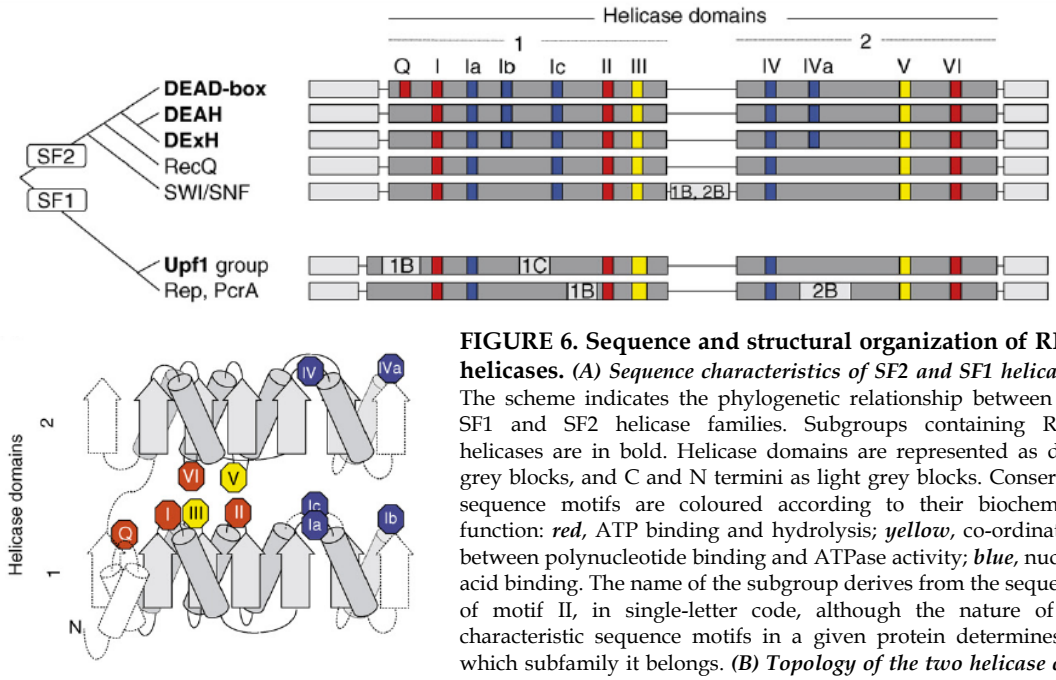


FIGURE 6. Sequence and structural organization of RNA helicases. ***(A) Sequence characteristics of SF2 and SF1 helicases.*** The scheme indicates the phylogenetic relationship between the SF1 and SF2 helicase families. Subgroups containing RNA helicases are in bold. Helicase domains are represented as dark grey blocks, and C and N termini as light grey blocks. Conserved sequence motifs are coloured according to their biochemical function: ***red***, ATP binding and hydrolysis; ***yellow***, co-ordination between polynucleotide binding and ATPase activity; ***blue***, nucleic acid binding. The name of the subgroup derives from the sequence of motif II, in single-letter code, although the nature of all characteristic sequence motifs in a given protein determines to which subfamily it belongs. ***(B) Topology of the two helicase core domains.*** Elements with solid outlines are present in all SF1 and SF2 structures; elements with dashed outlines are not present in all proteins. The position of the conserved sequence motifs is indicated by numbered octagons, coloured as in (A). Domains 1B and 1C of the Upf1 group are inserted before motif I and in between motifs Ib and Ic. (Jankowsky and Fairman, 2007)

However, recent extensive genetic studies have revealed that the classification of RNA helicases by motif II is not so precise, because many SF2 proteins contain a "misleading" motif II which is significantly different in the other motifs. Indeed, several RNA helicases containing DExH motif II, such as RNA helicase A (RHA), share higher similarity with DEAH-box helicases inside, and also outside, the two helicase core domains. Therefore, RHA has been classified as a DEAH-box helicase. Similarly, even though RHAU contains DEIH motif II, it belongs to the DEAH-box protein family, because it shares a higher amino-acid sequence similarity with DEAH-box than with DExH-box proteins. In humans, RHAU has the gene symbol of DHX36.

Structural and single-molecule fluorescence resonance energy transfer (FRET) analysis of RNA helicases has shown that, without ATP or NA, the two helicase core domains are relatively open, especially in DEAD-box proteins (Caruthers et al., 2000; Cheng et al., 2005; Shi et al., 2004; Theissen et al., 2008). ATP and/or NA binding bring the two domains into a more closed defined conformation (Jankowsky and Fairman, 2007). Thus, it is possible that binding to NA promotes ATP binding and hydrolysis and *vice versa*. Many DEAD-box proteins are in fact unable to bind or hydrolyze ATP without RNA (Lorsch and Herschlag, 1998; Polach and Uhlenbeck, 2002; Talavera and De La Cruz, 2005). In contrast, DExH and DEAH proteins already show significant ATP hydrolysis without RNA, although RNA can still stimulate their ATPase activity (Shuman, 1992; Tanaka and Schwer, 2005; Tanaka and Schwer, 2006). This phenomenon could be explained by less dramatic movements, from opened to closed conformations, of the helicase domains seen in the DExH-box protein hepatitis C virus (CV) NS3 upon ATP and NA binding. As helicase structure analysis has revealed, in contrast to the extended shape of the NAs in the HCV NS3, the backbone of the RNA bound to the DEAD-box proteins is severely bent (Bono et al., 2006; Sengoku et al., 2006; Yao et al., 1997). In addition, the DEAD-box proteins bind the RNA exclusively at the sugar-phosphate backbone, whereas DExH-box NS3 helicase also contacts nucleo-bases (Andersen et al., 2006; Bono et al., 2006; Sengoku et al., 2006; Yao et al., 1997).

1.4.2. Mechanism of duplex unwinding and protein displacement from RNA by DEAD- and DExH-box proteins

Originally, RNA helicases were defined as enzymes that use the energy of NTP hydrolysis to move along RNA, leading to duplex unwinding. However, unwinding activity has been shown for only a subset of the RNA helicases, and no general rule can be drawn on how helicase activity is achieved. Although several

DEAD-box proteins unwind blunt-end duplexes too, the majority of RNA helicases require single-stranded RNA overhang. So far there are two main unwinding models proposed: "stepping/inchworm" and "Brownian motor" model (Figure 7A and 7B) (Levin et al., 2005; Patel and Donmez, 2006). The stepping/inchworm model is based on opened and closed conformation of helicases to track along a single-stranded loading RNA and to displace obstacles in front of it [reviewed in (Patel and Donmez, 2006)], whereas the Brownian motor model requires the co-ordination between helicase core domains that alternate in the binding affinities for single-stranded and double-stranded RNA as well as for ATP and ADP (Levin et al., 2005). Thus their reciprocal changes of affinity for the substrate upon ATP binding and hydrolysis lead to helicase translocation along RNA. Based on recent crystal structure data of Vasa in complex with ssRNA poly(U) and the nonhydrolysable ATP analog AMP-PNP, it seems that the inchworm model fits best to DEAD-box proteins (Sengoku et al., 2006).

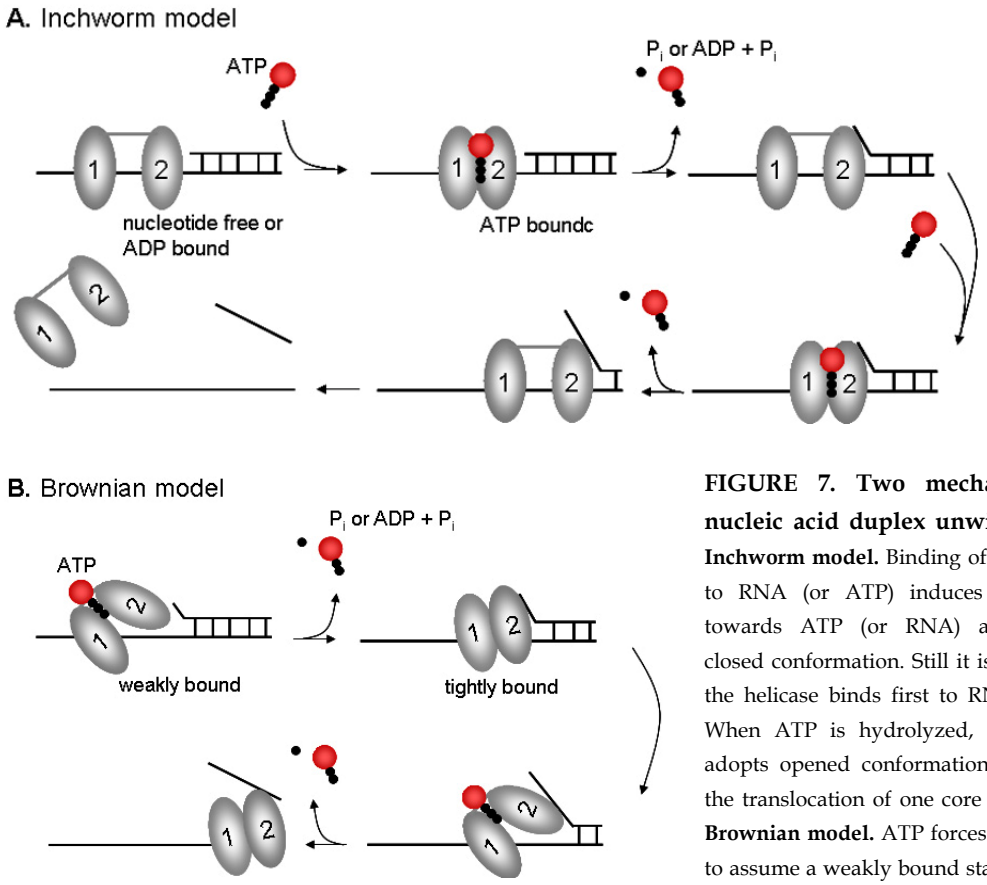


FIGURE 7. Two mechanisms of nucleic acid duplex unwinding. (A) Inchworm model. Binding of the helicase to RNA (or ATP) induces its affinity towards ATP (or RNA) and thereby closed conformation. Still it is not clear if the helicase binds first to RNA or ATP. When ATP is hydrolyzed, the helicase adopts opened conformation that forces the translocation of one core domain. **(B) Brownian model.** ATP forces the helicase to assume a weakly bound state, in which it freely moves between the possible positions along nucleic acids. ATP hydrolysis forces the helicase to bind the nucleic acids tightly, leading to forward movement. The cycle of weak (opened conformation) and tight (closed conformation) interaction is repeated until the helicase releases a template. If a nucleic acid duplex is present, the translocation force can disturb it.

Further, as shown in Figure 8, viral DExH-box protein NS3 unwinds duplexes using not only the D1 and D2 core conserved domains, but also the D3 domain that works in such processes as a ploughshare. As the D1 and D2 motor domains track three base pairs forward, the protein contracts, which pulls the D3 domain towards the D1 and D2 domains, thereby opening the duplex lying between the motor domains and the D3 domain. This hypothetical "spring-like" mechanism of NS3 unwinding is based on FRET analysis (Myong et al., 2007). Although there is no defined crystal structure or FRET data from DEAH-box proteins, it is highly likely that these helicases with their conserved C-termini (possible domain D3) might unwind RNA duplexes or displace proteins from RNA by a similar mechanism to that found in viral DExH helicase NS3.

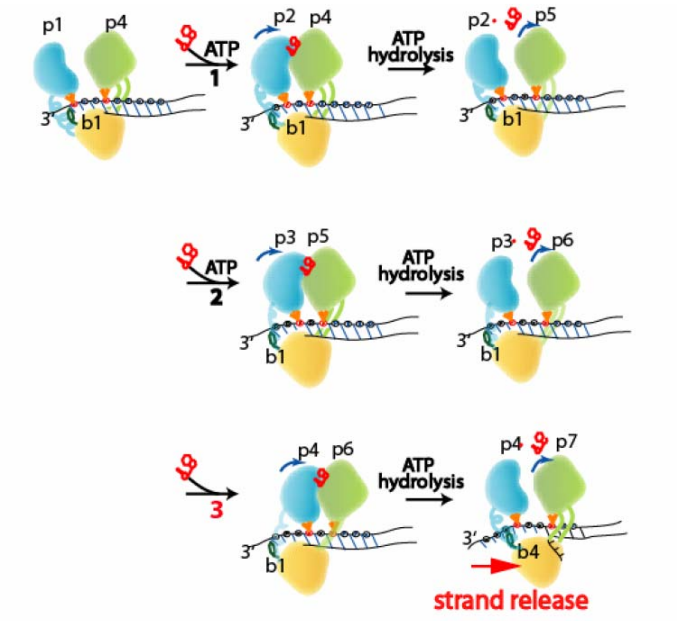


FIGURE 8. The "spring-like" mechanism of NS3 unwinding. By hydrolyzing ATP, the NS3 helicase unwinds three base pairs. Domains 1, 2 and 3 are blue, green and yellow, respectively. Symbols p1-p7 indicate phosphate positions and b1-b4 are base positions (Myong et al., 2007).

In contrast to DNA helicases, RNA helicases are not processive enzymes. In addition, DEAD-box helicases display much lower processivity than viral DExH-box proteins such as NPHII or NS3. Furthermore, it is not yet clear how the ATP hydrolysis cycle is coupled to duplex unwinding or protein displacement.

Since RNA molecules are present in complexes with proteins in living cells, the question is whether RNA helicases unwind the duplex in the presence of tightly bound proteins. Indeed, it has been shown that viral DExH helicase NPH-II induces U1A displacement during RNA duplex unwinding in an ATP-dependent manner. Furthermore, NPH-II processivity has been only partly reduced by U1A, indicating that DExH/D proteins could directly and actively displace stably bound proteins

from RNA in an ATP-dependent reaction without any other cofactors being required (Jankowsky et al., 2001). However, this observation does not rule out the possible requirement of other factors for protein displacement by other DExH/D proteins. It may also be possible that U1A displacement is faster in the presence of further cofactors. In addition, the model of U1A displacement mentioned above does not answer the question of whether dsRNA unwinding is really required in such a process. Thus, two models were designed to answer the question of further cofactors and the need for RNA duplex unwinding. The first model used tryptophan RNA-binding attenuation protein (TRAP), which binds target RNA in a sequence-specific fashion, and the second one used the multi-component exon junction complex (EJC), which interacts with RNA in a non-sequence-specific manner. In both models, single-stranded RNA was used. Strikingly, NPH-II accelerates the dissociation of both the TRAP and EJC in an ATP-dependent manner, indicating that unwinding of RNA duplexes is not required for protein dissociation induced by RNA helicases (Bowers et al., 2006; Fairman et al., 2004). However, the EJC was displaced at a significantly slower rate than TRAP, suggesting that the properties of the RNPs used affect the rate by which they can be remodelled by DExH/D proteins (Fairman et al., 2004). Interestingly, NPH-II increases the dissociation of the U1snRNP complex that consists of both RNA-RNA and RNA-protein interaction mixtures, indicating that the enzyme can actively disrupt a more complex RNA-protein interface (Bowers et al., 2006). Furthermore, it has also been shown that less processive DEAD-box helicase Ded1 from *S. cerevisiae* could dissociate EJC and U1snRNP from RNAs. However, this did not accelerate the displacement of U1A and TRAP from RNA, indicating that different RNA helicases do not necessarily disrupt the same range of RNP substrates in an active fashion (Bowers et al., 2006). Having shown that the "helicase activity", duplex unwinding and/or protein displacement, depends on the microclimate of individual proteins within RNPs, it will be important to focus on how RNA helicases may determined such RNA-protein complexes. One possible explanation might be connected with less conserved N/C-termini of helicases that have been shown to be involved in cofactor and/or nucleic acid interaction.

1.4.3. RNA-helicase functions

Although RNA helicases contain highly conserved helicase core domains that adopt similar three-dimensional folds, they are involved in diverse RNA processes such as transcription, pre-mRNA splicing, ribosome biogenesis, RNA export, translation initiation and RNA decay (Abdelhaleem et al., 2003; Bleichert and Baserga, 2007; Jankowsky and Bowers, 2006). Intriguingly, the majority of RNA

helicases are involved in ribosome biogenesis (20 out of 38 in yeast) or pre-mRNA splicing (8 out of 38) (Bleichert and Baserga, 2007). Unlike their yeast counterparts, the biochemical activities and biological functions of the majority of human RNA helicases are largely unknown (Abdelhaleem, 2004).

Although most RNA helicases exhibit very poor unwinding activity, or none at all, and, most importantly, no RNA substrate specificity, they perform very specific functions *in vivo* and they cannot substitute for each other. How this specificity is accomplished within the cell is not known. Based on genetic or physical interaction studies, it is presumed that the specificity and subcellular localization of RNA helicases is attributed to the less conserved unique N-/C-termini which are probably involved in the interaction of the RNA helicase with cofactors (accessory proteins) (Aratani et al., 2006; Fouraux et al., 2002; Mayas et al., 2006; Mohr et al., 2008; Schneider et al., 2001; Valgardsdottir and Prydz, 2003; Wang and Guthrie, 1998). In general, cofactors could stimulate the ATPase and helicase activities, confer substrate specificity and/or increase the affinity of the helicase for its substrate, or inhibit helicase activity (Cordin et al., 2006), but biochemical *in vitro* confirmation of influence on RNA helicase activity has only been obtained for a few potential cofactors (see an extensive review on cofactors in (Silverman et al., 2003)).

For example, the interactions between cofactors and RNA helicases have been best characterized in the first described RNA helicase, eIF4A. This DEAD-box protein is, together with eIF4G and eIF4E, a component of the eIF4F complex that is required for cap-dependent translation initiation (Rogers et al., 2002). Similarly to other RNA helicases, purified eIF4A shows RNA-stimulated ATPase activity but only non-processive duplex unwinding activity *in vitro* (Korneeva et al., 2005). Nevertheless, it has been shown that the binding of eIF4G to eIF4A stabilizes the active form of eIF4A and thus enhances its helicase activity (Oberer et al., 2005).

Similarly, Dbp5, a DEAD-box helicase involved in mRNA export from a nucleus, directly interacts with Gle1, and this interaction stimulates the ATPase activity of Dbp5 (Alcazar-Roman et al., 2006; Weirich et al., 2006). Interestingly, the optimal stimulation of Dbp5 activity also requires a second cofactor, inositol hexakisphosphate (InsP6) that binds to Gle1 in the presence of Dbp5. Furthermore, Dbp5 has been demonstrated to function as a translation terminator (Gross et al., 2007), suggesting that subcellular localization, together with a different RNP microclimate, has a high impact on helicase function.

Indeed, it has been shown that the function of RNA helicase A (RHA) is dependent on its subcellular localization and/or associated cofactors. For example, in the nucleus this multifunctional helicase interacts with RNA polymerase II and transcriptional regulators such as CBP/p300 (Nakajima et al., 1997), BRCA1 (Anderson et al., 1998) and NF-κB (Tetsuka et al., 2004), as well as p16INK4a and

MDR1 gene promoters (Myohanen and Baylin, 2001; Zhong and Safa, 2004) and activates their transcription. However, RHA is also involved in RNA export mediated by the constitutive transport element (CTE) (Tang et al., 1999; Tang and Wong-Staal, 2000), in RNA splicing by interacting with the survival motor neuron complex (SMN), and in the translation of selected mRNAs (Bolinger et al., 2007; Hartman et al., 2006). Most recently, RHA has been identified in the RNA-induced silencing complex (RISC) in HeLa cells, functioning as an siRNA-loading factor (Robb and Rana, 2007).

Accordingly, it is not surprising that cofactors can also specifically inhibit helicase activity. For instance, the ATPase activity of eIF4AIII, one isoform of eIF4A and a core component of the exon junction complex (EJC), is inhibited by two other EJC components, MAGOH and Y14, thereby locking eIF4AIII and the EJC onto the mRNA until EJC disassembly is triggered by translation (Ballut et al., 2005; Tange et al., 2004). Therefore, the right function of RNA helicases depends mainly on their subcellular localization and cofactor association, rather than on the RNA template itself.

1.5. RHAU: RNA helicase-associated with AU-rich element

RHAU, an RNA Helicase associated with an AU-rich element, was first identified in our laboratory as an ARE-associated factor of uPA mRNA, together with NF90 and HuR (Tran et al., 2004). It was demonstrated that RHAU plays a role in ARE-mediated mRNA decay via its RNA-dependent interaction with ARE-binding protein NF90 (Lattmann, unpublished data). In addition, Tran et al. demonstrated that RHAU physically associates with the human exosome and a poly(A)-specific exoribonuclease (PARN), and that recombinant RHAU protein accelerates deadenylation and, consequently, decay of β-globin-ARE^{uPA} (Tran et al., 2004). In HeLa cells, overexpression of RHAU caused destabilization of both reporter ARE (β-globin mRNA harbouring uPA-ARE) and endogenous uPA mRNA. Conversely, depletion of endogenous RHAU by siRNA stabilized the reporter ARE, indicating that RHAU is a factor promoting degradation of ARE-containing mRNAs. Nevertheless, RHAU may be limited to a very specific group of ARE-containing mRNAs because it does not accelerate ARE-mediated decay of uPA receptor (uPAR) mRNA, which contains a different class of ARE. As mentioned above, the specific function of RNA helicases can be regulated by associated cofactors and, indeed, the interaction of recombinant RHAU with uPA-ARE is strongly increased in the presence of NF90, indicating that NF90 may be a stimulating cofactor of the RHAU-ARE^{uPA} complex (Lattmann and Akimitsu, unpublished data). Nevertheless, RHAU

binds to NF90 only in the presence of ARE^{uPA} but not ARE^{IL2} or mutated ARE^{uPA}, suggesting that not only the cofactor itself plays a role in RNA interaction with RHAU.

In agreement with other known DEAH-box helicases, it has been shown that the ARE-mRNA destabilizing function and nuclear localization of RHAU is dependent on its ability to hydrolyze ATP (Iwamoto et al., 2008; Tran et al., 2004).

1.5.1. RHAU functions as a G4-resolavase

Even though RHAU belongs to the DEAH-box helicases, Akman's group identified RHAU as the major guanine quadruplex (G4) DNA-resolving enzyme (resolvase) in HeLa cell extract (Vaughn et al., 2005). Based on this observation, they called RHAU G4-Resolvase 1 (G4R1).

DNA/RNA G4 structures are composed of several layers of a guanine (G) tetrad in which four G residues are inter- or intra-linked by hydrogen bonding (Maizels, 2006). DNA G4 is a dynamic structure, and its formation depends on the denaturation of the duplex that occurs during replication, transcription or recombination (Maizels, 2006). G4 structures are found or predicted in G-rich regions such as telomeres, ribosomal DNA, and immunoglobulin heavy chain switch regions, as well as in the promoter regions of several proto-oncogenes such as c-myc and c-kit, where G4 structures function as transcriptional repressors (Maizels, 2006) (Shirude et al., 2007; Siddiqui-Jain et al., 2002). Therefore, G4-resolving activity is expected to activate the transcription of genes containing G4 structure in the promoters.

Although initially most of the studies focused on G4 in the DNA, recent studies have also reported G4 structures in the RNA. Using bioinformatics databases, approximately 55,000 G4 structures have been predicted near mRNA splicing and polyadenylation sites in human and mouse (Kostadinov et al., 2006). The 5′ UTRs of several proto-oncogene mRNAs contain G4 structures e.g. NRAS, BCL2, FGR and JUN (Kumari et al., 2007). In the case of NRAS, the presence of the RNA G4 structure in its 5′ UTR represses its translation, indicating that the RNA G4 structure may modulate translation. Interestingly, Alkman's group have also shown that RHAU binds to and resolves RNA G4 structures (Creacy et al., 2008). Furthermore, they demonstrated that RHAU binds more tightly to the RNA G4 than the DNA G4 structure, and that down-regulation of endogenous RHAU reduced the resolution of both RNA and DNA G4 structures, confirming *in vitro* data that RHAU represents the major source of G4 resolvase activity in HeLa cell lysates.

Finding RHAU to be involved in the resolution of DNA/RNA G4 structures, it enlarged its range of activity from an ARE-destabilizing factor to a DNA/RNA G4-resolving enzyme. However, the biological functions of DNA/RNA G4 and G4 resolvases *in vivo* are largely unknown, and the physiological significance of RHAU G4 resolvase activity has also not been defined.

1.5.2. Nuclear localization and possible function of RHAU

Although RHAU was first identified as an ARE-associated factor, it localizes predominantly in the nucleus. Furthermore, a detailed immunofluorescent study has revealed that RHAU does not localize uniformly in the nucleus, but accumulates in nuclear speckles enriched with splicing factors and mRNAs (Iwamoto et al., 2008). Nevertheless, translational arrest altered its localization to nucleolar caps, where RHAU was closely localized with RNA helicases p68 and p72, suggesting that RHAU is involved in translation-related RNA metabolism in the nucleus. Interestingly, RHAU nuclear localization is RNA-dependent (Iwamoto, thesis).

Despite the role of RHAU as a destabilizing factor of uPA mRNA, the global analysis of gene expression in RHAU knockdown cells has revealed that changes in steady state levels of mRNAs are only partially due to mRNA decay regulation (Iwamoto et al., 2008). Indeed, reflected in its nuclear localization and its G4 DNA/RNA-resolving activity (Vaughn et al., 2005), RHAU may also regulate gene expression at various steps other than mRNA decay.

1.5.3. RHAU belongs to DEAH-box RNA helicases

According to amino acid sequence alignment, RHAU (DHX36) belongs to the DEAH-box RNA helicase subgroup that has been studied mostly in yeasts. Interestingly, RHAU does not have a yeast ancestor, but it shares 20% homology with the DEAH-box protein YLR419w, which is similar to another four human RNA helicases (DHX57, DHX30, DHX29 and DHX9). YLR419w has been shown to be disposable in yeasts and, so far, there is no functional information about it.

As mentioned above, DEAH-box helicases have been predominantly studied in yeast, where their function was associated with pre-mRNA splicing. Prp2 and Prp16 are required for the first and second transesterification steps respectively (Kim and Lin, 1996; Wang and Guthrie, 1998). Prp22p and Prp43p are involved in the release of spliced mRNA products from the spliceosome and the disassembly-

recycling of spliceosomal components (Arenas and Abelson, 1997; Schwer and Gross, 1998).

Like other DEAH-box helicases, RHAU is highly conserved, especially among vertebrates, in the central helicase core domains and the C-terminal extremity but not the N-terminus, suggesting that the N-terminal domain is involved in its specific function in higher eukaryotes. Indeed, studies on yeast DEAH proteins have revealed that the N-terminus plays an important role in RNA-dependent ATPase activity and the interaction with spliceosomes or other co-factors. For example, in the case of Prp16, the N-terminus has been demonstrated to also be essential for viability and nuclear localization of proteins. Interestingly, a pull-down assay with a recombinant N-terminus of RHAU identified a lot of new RNA-dependent binding partners of RHAU (Iwamoto, thesis; Pauli, unpublished data), suggesting that the N-terminus is most probably involved in the determination of RHAU specificity. Indeed, in the following results section, I show that the N-terminus of RHAU plays an important role in both RNA interaction and the subcellular localization of protein.

2. MATERIALS & METHODS

2.1. Plasmid constructions

The plasmids pTER-shRHAU and pTER-shLuc were described previously (Iwamoto et al., 2008). Plasmids EGFP-RHAU, EGFP-Nter, EGFP-HCR and EGFP-Cter were based on pEGFP-C1 (Clontech, Mountan View, Calif.) and EGFP-E335A (also termed DAIH in this paper), which was derived from EGFP-RHAU by point mutation in motif II as described previously (Iwamoto et al., 2008). Plasmids RHAU-EGFP, (50:1008)-EGFP, (105:1008)-EGFP, (178:1008)-EGFP, (195:1008)-RGFP, (1:130)-EGFP and (1:105)-EGFP were based on pEGFP-N1 (Clontech, Mountan View, Calif.) by inserting corresponding fragments into the Bgl*II* (XhoI for RHAU full length) and Age*I* sites of the vector. Plasmids EGFP-(50:1008), EGFP-(105:1008) were prepared by inserting the corresponding fragments between Bgl*II* and BamH*I* sites of pEGFP-C1. RHAU-Flag, (50:1008)-Flag, (105:1008)-Flag, (1:105)-Flag and (1:130)-Flag were prepared by inserting corresponding RHAU fragments into the Bgl*II* (Nhe*I* for RHAU full length) and Age*II* sites of pIRES.EGFP-N1-Flag. The vector was prepared by insertion of Flag-tag between Age*I* and Not*I* of pIRES.ECMV-EGFP vector, which was kindly provided by D. Schmitz Rohmer and B.A. Hemmings. Plasmids β-gal-(1:51)-EGFP, β-gal-(1:130)-EGFP, β-gal-(52:200)-EGFP were prepared by inserting corresponding RHAU fragments into EcoR*I* and Sal*I* sites of pβ-gal-EGFP (F. Iwamoto and Y. Fujiki, unpublished). The GST-RHAU vector was designed as described previously (Tran et al., 2004). GST-Nter was prepared by inserting the fragment (1:200aa) between BamH*I* and EcoR*I* sites of pGEX-2T (Amersham, Buckinghamshire, UK).

2.2. Antibodies

Mouse monoclonal anti-RHAU antibody (12F33) was generated against a peptide corresponding to the C-terminus of RHAU, 991-1007 aa, as described in (Vaughn et al., 2005). Commercially obtained antibodies were: mouse anti-GFP (B-2; sc-9996), goat anti-TIA-1 (sc-1751), goat anti-eIF3b (N-20; sc-16377), mouse anti-HuR (3A2; sc-5261), and rabbit anti-eIF2α (FL-315; sc-11386) (Santa Cruz Biotechnology, Carlsbad, Calif.), mouse anti-actin (pan Ab-5; Thermo Fisher Scientific, Fremont, Calif.), rabbit monoclonal anti-eIF2α-P (Ser 51; 119A11; Cell Signaling Technology, Danvers, Mass.), and mouse anti-FLAG M2 (Sigma-Aldrich Co., St. Louis, Mo.). The mouse antibodies were all monoclonal.

2.3. Cell culture, transfection and treatments

HeLa cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal calf serum at 37°C in the presence of 5% CO_2. T-REx-HeLa cells stably transfected with pTER-shRHAU were maintained as described previously (Iwamoto et al., 2008). To induce shRNA expression and consequent depletion of endogenous RHAU, cells were treated with 1 μg/ml of doxycycline (Sigma-Aldrich Co., St. Louis, Missouri) for 7 days. Mouse Embryonic Fibroblasts (MEFs) were cultured in DMEM with 15% fetal calf serum at 37°C in the presence of 5% CO_2. To induce knockout of RHAU in MEFs-CRE ($RHAU^{-/-}$), 2 mM of tamoxifen or EtOH (as a negative control) had been added to medium for 2 days (Lai, unpublished). Then MEFs, treated with tamoxifen (MEF-KO) or EtOH (MEF-WT), were maintained for 3 days in normal condition before actual experiments. As another negative control was used MEFs-GFP ($RHAU^{-/-}$) that after tamoxifen treatment does not induce RHAU knockout. For immunofluorescent analysis, transient transfection of DNA plasmids using FuGENE6 (Roche Applied Science, Rotkreuz, Switzerland) was performed according to the manual provided, using 1 μg plasmid DNA and 3 μl FuGENE6 per 35-mm dish. For immunoprecipitation analysis, cells were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, Calif.) in OPTIMEM-1 medium (Gibco, Invitrogen, Auckland, New Zealand). Briefly, 0.8×10^6 HeLa cells were seeded per 35-mm dish and 24 h later transfected with 4 μg of plasmid DNA and 10 μl of Lipofectamine 2000. The cells were used 24 h later for the following experiments. RNAi silencing was induced by transient transfection of siRNAs with INTERFERin (Polyplus Transfection, New York, NY) following the manual instructions. siRNA was added to give a final concentration 2.5 nM in 2 ml of medium and 8 μl of INTERFERin for transfection of 40% confluent cells per 35-mm dish. To test SG formation, 0.5 mM of sodium arsenite (Sigma-Aldrich Co., St. Louis, Mo) or 1 μM of hippuristanol (kindly provided by J. Tanaka (Mazroui et al., 2006)) was added in conditioned medium for 45 min or 30 min, respectively. To induce SGs with the ionophore carbonyl cyanide-m-chloro-phenyl-hydrazone (CCCP), cells were washed with 1×PBS- and cultured in glucose- and pyruvate-free DMEM containing 1 μM CCCP. Heat shock was performed at 44°C in a 5% CO_2 incubator for 45 min.

2.4. Immunocytochemistry and image processing

At 24 h after transfection by FuGENE6, HeLa cells were re-plated in 12-well dishes with coverslips coated with 0.2% gelatin. The next day, cells were treated with

the indicated stimuli, fixed with 3.8% paraformaldehyde in 1×PBS- for 10 min, permeabilized with 0.2% Triton-X100 in PHEM buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES, 2 mM $MgCl_2$, pH 6.9) for 30 min and blocked with 5% horse serum in PHEM buffer for 1 h. All steps were performed at room temperature. Samples were incubated with primary antibodies [goat anti-TIA-1 (1:200), mouse anti-HuR (1:200), goat anti-eIF3b (1:200)] diluted in the blocking buffer overnight at 4ºC. Coverslips with fixed cells were washed three times with 0.2% Triton-X100 in PHEM buffer and incubated in the dark with the secondary antibody and 500 ng/ml DAPI (Santa Cruz Biotechnology, Carlsbad, Calif.), to identify the nuclei, for 40 min at room temperature. Cy2-, Cy3-, or Cy5-conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA) were used at dilutions of 1:200, 1:2000, or 1:200 with 2.5% horse serum in PHEM buffer, respectively. The cells were mounted in FluoroMount reagent (SouthernBiotech, Birmingham, AL). Fluorescent images were captured with a confocal microscope (LSM 510 META, Carl Zeiss, Jena, Germany) as described previously (Iwamoto et al., 2008), except that a Plan-NeoFluar 100×/1.3 oil DIC objective was used. The data obtained were processed using standard image software (Bitplane Imaris 5.7.1; Adobe Photoshop; Adobe Illustrator). To quantify association of RHAU with SGs, at least 100 transfected cells were analyzed under the wide-spectrum microscope (Axioplan 2; Carl Zeiss, Jena, Germany) and scored as positive when the GFP signal was enriched and co-localized with TIA-1 in SGs. Three (n=3) independent transfections were analyzed to calculate mean percentages and ± standard errors of the means.

2.5. Protein extraction and western blotting

To prepare total cell lysates, cells were lysed with NP40 buffer (50 mM Tris-HCl pH 7.5, 120 mM NaCl, 1% NP-40, 1 mM EDTA, 5 mM Na_3VO_4, 5 mM NaF, 0.5 µg/ml aprotinin, 1 µg/ml leupeptin) on ice for 10 min and centrifuged at 20,000 *g* for 15 min at 4°C to remove cell debris. Typically, 30 µg of the total cell lysate was loaded for Western blotting. The protein bands were visualized with the direct infrared fluorescence method or the chemiluminescence method, as described previously (Iwamoto et al., 2008).

2.6. Cross-linking immunoprecipitation (CLIP)

RHAU and RNA interaction was determined by the previously reported CLIP method with slight modifications (Ule et al., 2005). HeLa cells (0.8×10^6 per 35-

mm dish) were rinsed twice with ice cold PBS and then UV irradiated (400 mJ/cm^2) to induce cross-linking between protein and RNA. Cells were then lysed with 200 µl of RIPA buffer (1% NP-40, 1% DOC, 0.1% SDS, 50 mM NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium vanadate, 100 U/ml aprotinin) per well in a 6-well dish and shaken for 15 min at 4°C. Pooled lysates from 6 wells were treated with 30 µl of RQ1 RNase-free DNase (1 U/µl; Promega, Madison, WI) and with 31 U of RNase A (31 U/µl; USB) as described in the CLIP protocol (Ule et al., 2005). Treated samples were centrifuged at 20,000 *g* for 20 min at 4°C. The supernatants (600 µg) were incubated with 4.5 µg of a mouse anti-RHAU monoclonal antibody (12F33) or 10 µl (bed volume) of anti-FLAG M2 affinity gel (A2220: Sigma-Aldrich Co., St. Louis, MO) with rotation for 2 h at 4°C. Beads were washed twice with RIPA buffer, twice with high-salt washing buffer (5×PBS, 0.1% SDS, 0.5% DOC, 0.5% NP-40) and twice with 1×PNK buffer (50 mM Tris HCl pH 7.4, 10 mM $MgCl_2$, 0.5% NP-40). The associated nucleic acids were radiolabeled with [γ-^{32}P] ATP using T4 polynucleotide kinase PNK (Roche, Mannheim, Germany), as described in the CLIP protocol (Ule et al., 2005)and RHAU-RNA complexes were resolved in NuPage 4-12% Bis-Tris Gel (Invitrogen, Carlsbad, Calif.). Immunoprecipitated RHAU was detected by Coomassie blue staining and in-gel Western blotting according to the manual of Odyssey In-Gel Western detection (LI-COR Biosciences) published on http://biosupport.licor.com/support. Half of the samples were transferred to a PVDF membrane to facilitate better protein detection by Western blotting and to remove free RNA. The proteins were detected by the Odyssey infrared imager as described above. Radiolabeled RNA was detected by a PhosphoImager Thyfoon 9400 (GE Healthcare, UK) and analyzed using the ImageQuant TL program. To test whether RHAU associates with RNA, bound nucleic acids were isolated and radiolabeled according to the CLIP protocol (Ule et al., 2005). Nucleic acids were mixed with increasing amounts of RNase A (0.015; 0.15; 1.5; 15 U) in H_20 and 1U of RQ1 DNase in 1×RQ1 DNase reaction buffer. Reactions were incubated for 30 min at 37°C and resolved by denaturing 8% polyacrylamide gel electrophoresis (PAGE) in 1×TBE buffer.

2.7. Protein purification

E. coli BL21 (DE3), transformed with glutathione S-transferase (GST) or GST-Nter proteins were induced by 1 mM IPTG for 12 h at 25°C and purified by affinity chromatography with glutathione-sepharose 4B (Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. GST-RHAU protein was expressed in

Sf9 cells according to the supplier's instruction (BD Biosciences Pharmingen, San Diego, Calif.) and purified as above. The purity of recombinant proteins was analyzed by 10% SDS-PAGE and Coomassie blue staining.

2.8. Double-filter RNA-binding assay

5 μg of total RNA isolated from HeLa cells was alkali treated with 0.1 M NaOH on ice for 10 min and then EtOH precipitated. Redissolved RNA or poly(rU) (P9528; Sigma-Aldrich Co., St. Louis, Mo.) was 5′-end-labeled using [γ-^{32}P] ATP (Hartmann Analytic GmbH, Braunschweig, Germany) and T4 PNK (Roche, Mannheim, Germany) at 37°C for 30 min and passed through a G-50 column (GE Healthcare, UK) to remove free nucleotides. Reaction mixtures (50 μl) containing varying amounts of recombinant proteins (0-150 nM as specified in text), radiolabeled RNA (poly(rU) 10,000 cpm and total RNA 5,000 cpm) and 2U of RNase Inhibitor (RNAguard; Roche, Mannheim, Germany) in the binding buffer (50 mM Tris-HCl, pH 8.0, 1 mM DTT, 5 mM NaCl) were incubated for 30 min at 37°C. The double-filter RNA-binding assay was performed with a slot-blot apparatus using a 0.45-μm nitrocellulose (PROTAN, Whatman GmbH, Dassel, Germany) and nylon membranes (positively charged; Roche Diagnostics GmbH, Mannheim, Germany) that was pre-soaked in different buffers as described previously (Tanaka and Schwer, 2005). Loaded samples were washed three times with 200 μl of the binding buffer. Retained RNA was detected with a PhosphoImager Thyfoon 9400 and analyzed using the ImageQuant TL program. The nitrocellulose membrane retains only RNA-protein complexes and free RNAs are captured on the nylon membrane. The ratio of RNA that was bound to GST-RHAU or GST-Nter was calculate using the following formula: bound RNA (%) = $100*[(signal_{nitrocellulose})/(signal_{nitrocellulose} + signal_{nylon})]$.

2.9. Bioinformatics

The program RNABindR (http://bindr.gdcb.iastate.edu/RNABindR/) (Terribilini et al., 2007) was used to predict RNA-binding potential in amino acid sequences. Programs RISP and BindN+ were used to confirm the reliability of the RNABindR program: RISP (RNA-Interaction Site Prediction; http://grc.seu.edu.cn/RISP/) (Tong et al., 2008) runs with 72.2% RNA-binding prediction accuracy and BindN+ (http://bioinfo.ggc.org/bindn/) (Jeong et al., 2004) runs with 68% RNA-binding prediction accuracy. For multiple sequence alignments

(MSA) of N-termini, RHAU orthologs were identified by a BLASTP (version 2.2.18+) search of non-redundant protein entries in the NCBI database using the entire sequence of RHAU as a query. MSA was carried out with ProbCons (version 1.12) (Do et al., 2005). Similarity of groups was generated using GeneDoc (version 2.7) with the BLOSUM62 scoring matrix.

2.10. Fluorescence recovery after photo-bleaching (FRAP)

HeLa cells (1.5×10^5 per 35-mm dish) were plated and transfected by FuGENE6 on glass-bottomed dishes (Micro-Dish 35 mm; Fisher Scientific AG, Wohlen, Switzerland). FRAP experiments were carried out with a confocal microscope (LSM 510, Carl Zeiss, Jena, Germany) using a 63×/1.4 NA oil DIC objective. Bleaching was performed using the 488-nm lines from a 40-mW argon laser operating at 75% laser power. Bleaching of circular regions of interest (ROI) was done with 3 scan iterations lasting a total of 1.5 s. Fluorescence recovery was monitored at low laser intensity (approximately 2% of the 40-mW laser) at 0.5-s intervals. Two additional ROI were monitored in parallel to detect fluorescence fluctuations independent of bleaching (ROI of non-bleached SG) and to extract a background (ROI in the cytoplasm). Raw data of one protein were obtained from ≥10 different FRAP analyses from three independent transfections. Fluorescence recovery was normalized with the function $[(I_t-B_t)/(C_t-B_t)]/I_0$, where I_t is the mean intensity in ROI of bleached SGs at time-point t, B_t is the mean intensity in ROI of the background at time-point t, C_t is the mean intensity in ROI of control SGs at time-point t, and I_0 the average intensity in ROI of bleached SGs during pre-bleach. The average fluorescence intensities ± s.e.m from at least ≥10 independent FRAP analyses were extrapolated to the graph as recovery curves.

3. RESULTS

3.1. RHAU protein associates with SGs in response to arsenite-induced stress

Various research groups have shown that different ARE-binding proteins, such as TTP, Brf1, HuR, TIA-1 or TIAR, associate with SGs in cells exposed to environmental stress (Gallouzi et al., 2000; Kedersha et al., 1999; Stoecklin et al., 2004). To determine whether RHAU is also recruited to SGs, HeLa cells were transfected with EGFP-tagged RHAU and treated with arsenite, a typical inducer of SGs. As shown in Figure 9A, the arsenite treatment induced, in addition to diffused nuclear and cytoplasmic localizations, the accumulation of RHAU in distinct cytoplasmic foci that co-localized with TIA-1 protein, an SG marker. Since all available anti-RHAU antibodies, both commercial and our own preparation, were not suitable to visualize of endogenous RHAU by immunofluorescent analysis, we examined the intracellular localization of RHAU by transiently transfecting cells with either EGFP-RHAU or RHAU-EGFP to eliminate unwanted effects of EGFP on RHAU structure and subsequent subcellular localization. Indeed, both EGFP-RHAU and RHAU-EGFP showed the same localization patterns in normal and stress conditions (Figure 9A). EGFP itself did not accumulate in SGs (Figure 9A).

To examine whether the recruitment of RHAU to SGs is restricted to a specific type of stress, EGFP-RHAU transfected HeLa cells were treated with different SG-inducing stimuli, such as arsenite, a mitochondrial inhibitor CCCP, heat shock and hippuristanol, an inhibitor of eIF4A RNA-binding activity known to induce SG aggregation independently of eIF2α phosphorylation (Mazroui et al., 2006). As shown in Figure 9B, EGFP-RHAU co-localized with TIA-1 in SGs in response to all stimuli tested. The phosphorylation of eIF2α was detected after treatment with arsenite, heat shock and CCCP but, as expected, not with hippuristanol (Figure 9B). These results indicate that the formation of SGs, rather than phosphorylation of eIF2α, is required for RHAU protein recruitment to SGs.

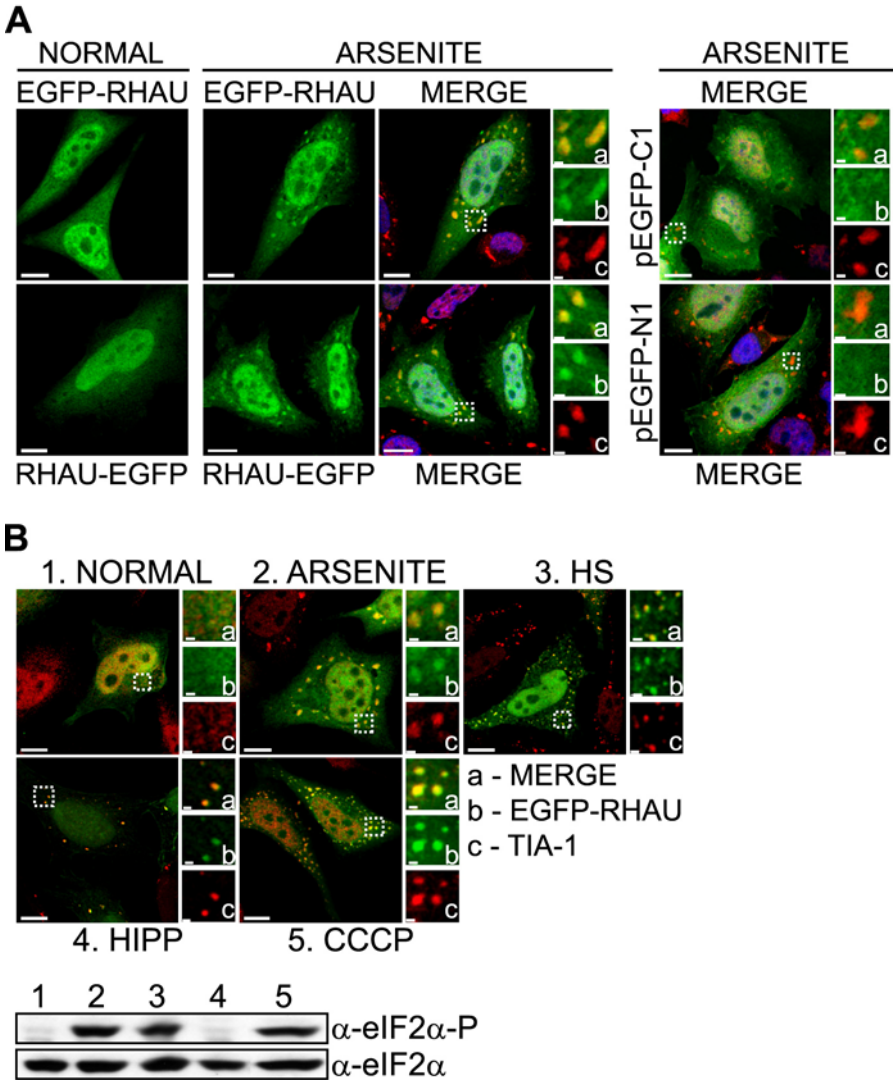


FIGURE 9. RHAU helicase accumulates in SGs in response to all tested stress stimuli. (A) Immunofluorescent analysis of RHAU localization in arsenite-induced SGs. HeLa cells, transiently transfected with EGFP-RHAU or RHAU-EGFP and 48h later treated with 0.5 mM of arsenite for 45 min (ARSENITE) or cultured in normal condition (NORMAL). (*Left*) Distribution of EGFP-RHAU or RHAU-EGFP (green) under normal and arsenite-treated conditions. Zooms of boxed regions are shown on small panels indicating merge (a), EGFP (b), and TIA-1 (c). (*Right*) Merges of DAPI and TIA-1 with empty vectors pEGFP-C1 or pEGFP-N1; zooms of boxed regions are shown on small panels as above. (B) Effects of different stress stimuli on RHAU localization in SGs and eIF2α phosphorylation. EGFP-RHAU transfected HeLa cells were cultured without stress (1) or cultured with stress inducers: 0.5 mM of arsenite for 45 min (2); heat shock at 42°C for 45 min (3); 1 μM of hippuristanol for 30 min (4); 1 μM of CCCP for 90 min (5). The images show merges of EGFP-RHAU (green) and TIA-1 (red). Zooms of boxed regions are shown on small panels indicating merge (a), EGFP (b), and TIA-1 (c). The total and phosphorylated eIF2α were detected by Western blotting; *bar* 10 μm (1 μm in zooms).

3.2. The N-terminal domain recruits RHAU to SGs

RHAU protein consists of three main domains: the unique N-terminus (Nter), the evolutionary conserved helicase core region (HCR), and the C-terminus (Cter) partially conserved among DEAH subfamily members. To identify domains essential for localization of RHAU in SGs, we generated deletion mutants of RHAU: EGFP-Nter (1:200aa), EGFP-HCR (201:650aa), and EGFP-Cter (651:1008aa) (Figure 10A). As shown in Figure 10B, the full-length RHAU (WT) as well as the N-terminus alone (Nter) accumulated in arsenite-induced SGs. Unlike EGFP-WT and EGFP-Nter, EGFP-HCR and EGFP-Cter were excluded from the nucleus and diffused in the cytoplasm as described previously (Iwamoto et al., 2008). After arsenite treatment, they showed slight accumulation in small foci other than SGs (Figure 10B). A similar proportion of cells positive for EGFP signals in arsenite-induced SGs were observed with EGFP-WT and EGFP-Nter transfected cells, namely 78% and 83%, respectively, but no positive EGFP signals in arsenite-induced SGs were detected after transfection with EGFP-HCR and EGFP-Cter (Figure 10C). Taken together, these results indicate that the N-terminus of RHAU is essential for stress-induced accumulation of RHAU in SGs.

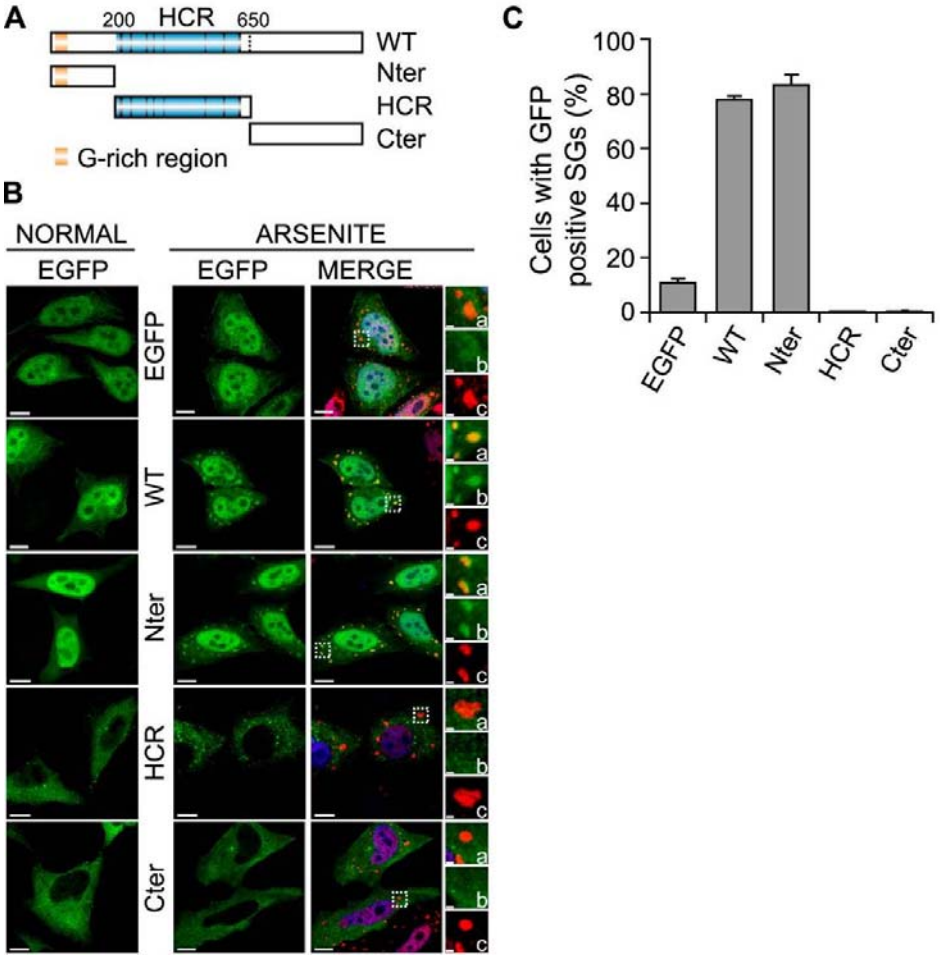


FIGURE 10. The N-terminal domain of RHAU can localize in SGs. (A) Scheme of wild-type RHAU and its fragments. Nter: the N-terminus (1:200aa); HCR: the helicase core region (200:650aa); Cter: the C-terminus (200:1008aa). (B) Intracellular localization of different parts of RHAU. HeLa cells were transfected with an empty pEGFP-C1 vector or plasmids expressing EGFP-fused RHAU (WT) or fragments of RHAU, Nter, HCR and Cter. After 48 h, cells were cultured in normal conditions or treated with 0.5 mM arsenite for 45 min, fixed and stained for TIA-1 (red). Nuclei were visualized with DAPI (blue). Small panels show enlargements of boxed regions: (a) merge, (b) EGFP-fused fragments of RHAU and (c) TIA-1. (C) Data obtained by quantitative immunofluorescence analysis showing the percentage of transfected cells in which EGFP signals were detected in SGs. Values ± s.e.m. were derived from three independent experiments; *bar* 10 μm (1 μm in zooms). (Figure is on a page #43)

Since only the first 200 amino acids of RHAU were necessary for SG localization, we further studied functional domains within the N-terminus. For this we generated two new N-terminal truncated forms of EGFP-fused RHAU by stepwise deletion as depicted in Figure 11A, where the first deletion mutant (50-1008 aa) lacked a G-rich region. These truncated mutants and wild-type RHAU (WT) were transfected into HeLa cells. The association of RHAU and its mutants with arsenite-induced SGs was assessed by immunofluorescence microscopy (Figure 11B) and quantified (Figure 11C). Deletion of the G-rich region significantly decreased RHAU association with SGs, from 81% to 36% of transfected cells. The truncation of amino

acids 1-105 further reduced RHAU accumulation in SGs to 12%. As shown in a graph (Figure 11C), EGFP fusion at the N- or C-terminus with the WT or the mutants did not affect their localization efficiency in SGs. Taken together, these data suggest that the first 105 amino acids of the N-terminal domain are functionally essential for the recruitment of RHAU protein to SGs and that an additional site other than the G-rich region plays a role in this recruitment process.

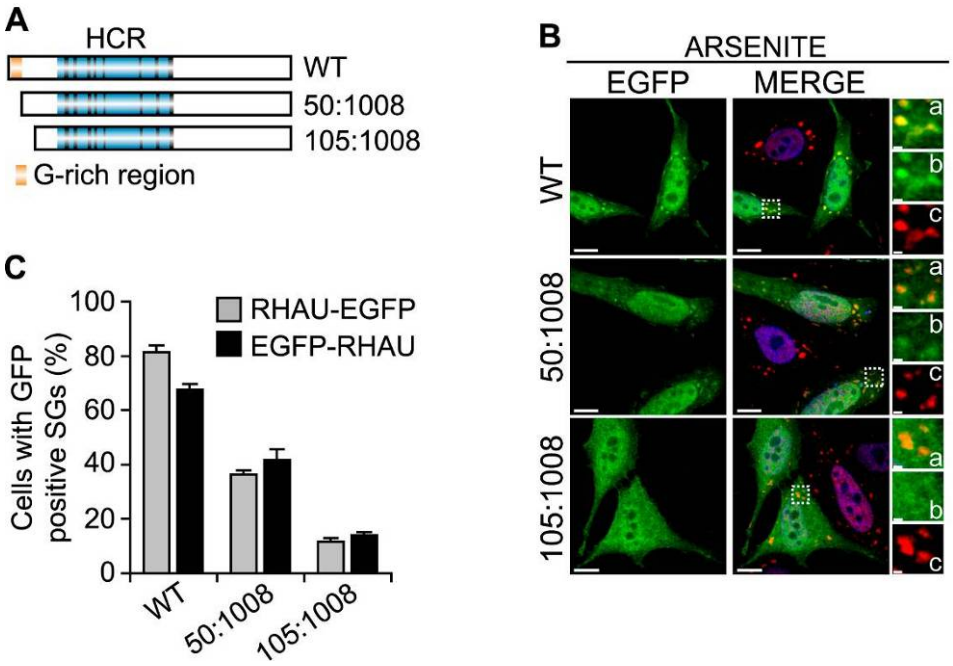


FIGURE 11. The N-terminal domain of RHAU is necessary for RHAU localization in SG. (A) Scheme of wild-type RHAU and its deletion mutants. (B) Intracellular localization of RHAU N-terminal deletion mutants. HeLa cells were transfected with plasmids expressing EGFP-fused RHAU (WT) or N-terminal deletion mutants of RHAU, 50:1008 and 105:1008. After 48 h, cells were treated with 0.5 mM arsenite for 45 min, fixed and stained for TIA-1 (red). Nuclei were visualized with DAPI (blue). (C) Quantitative immunofluorescence analysis showing the percentage of transfected cells in which EGFP signals were detected in SGs. Values ± s.e.m. were derived from three independent experiments; *bar* 10 μm (1 μm in zooms).

3.3. Detection of a potential nuclear localization signal in the N-terminus

Although, our previously published data showed that the truncation of the whole N-terminal domain resulted in a lost of RHAU nuclear localization (Iwamoto et al., 2008), the truncated RHAU mutants, EGFP-(50:1008) and EGFP-(105:1008), still localized in the nucleus such as WT (Figure 11B). Therefore, in addition to (50:1008) and (105:1008) we performed new truncated forms, (178:1008) and (195:1008), in the pEGFP-N1 vector as depicted in Figure 12B to test whether the N-terminus contains next to SG-signal also a potential nuclear localization signal (NLS). As shown in Figure 12A, both new truncated forms of RHAU did not accumulate in SG in response to arsenite treatment. However, they differed in the nuclear localization. While (178:1008)-EGFP mutant localized in the nucleus, (195:1008)-EGFP mutant dramatically reduced its localization in the nucleus. To summarize these data, the N-terminus of RHAU contains two signals within, SG signal and NLS, which do not overlap and have a different impact on RHAU subcellular localization.

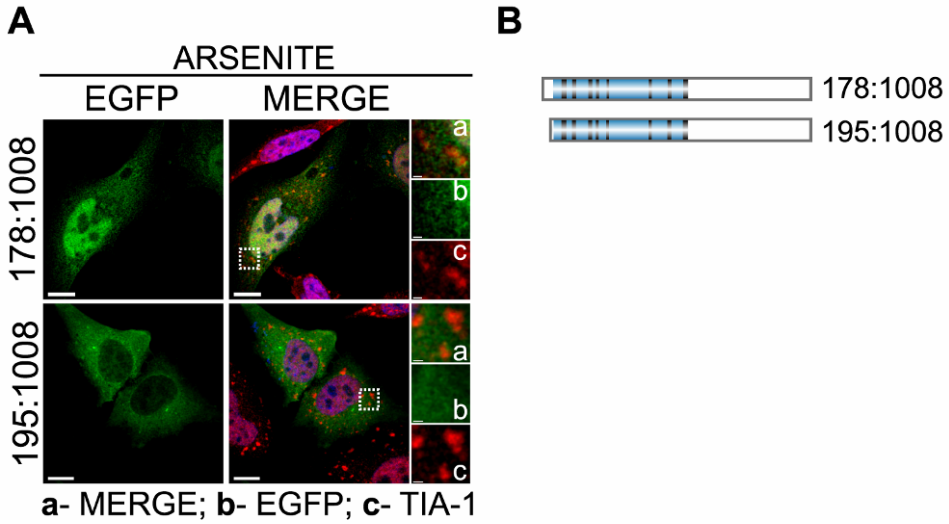
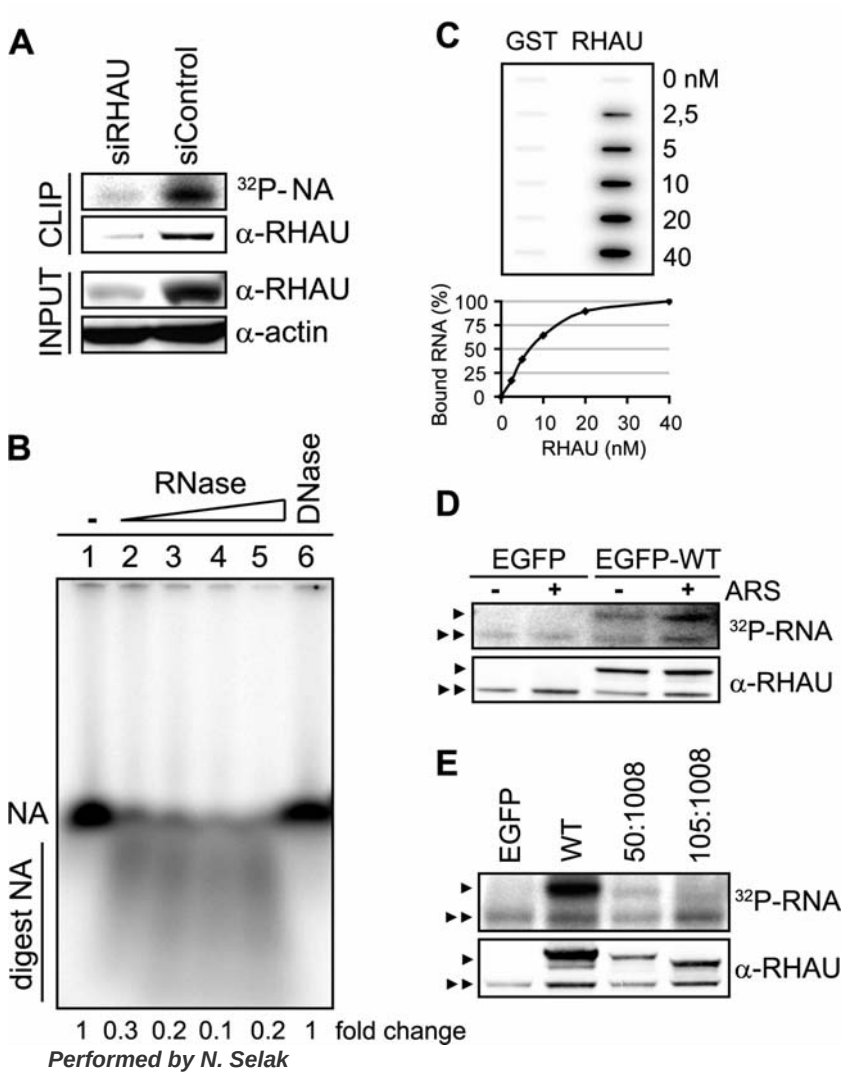


FIGURE 12. The N-terminal domain harbors NLS signal. (A) Intracellular localization of RHAU N-terminal deletion mutants. HeLa cells were transfected with plasmids expressing EGFP-fused N-terminal deletion mutants of RHAU, 178:1008 and 195:1008. After 48 h, cells were treated with 0.5 mM arsenite for 45 min, fixed and stained for TIA-1 (red). Nuclei were visualized with DAPI (blue). Note that EGFP-(195:1008) in sharp contrast to EGFP-(178:1008) does not localize in the nucleaus. (B) Scheme of RHAU deletion mutants; *bar* 10 μm (1 μm in zooms).

3.4. RHAU binds to RNA via the N-terminal domain

The recruitment of proteins to SGs was reported to be mediated by protein-protein or RNA-protein interactions (Anderson and Kedersha, 2008). Since RHAU is an RNA helicase, we first examined whether RHAU binds to RNA and, if so, whether RNA-binding activity is required for its accumulation in SGs. To test this, we used adapted cross-linking immunoprecipitation (CLIP) (Ule et al., 2005), employing irreversible UV cross-linking between protein and RNA to purify protein-RNA complexes. The tightly associated RNA molecules migrating together with immunoprecipitated protein were radiolabeled with [γ-^{32}P] ATP and detected with a PhosphoImager. Endogenous RHAU was immunoprecipitated with monoclonal anti-RHAU antibody. PhosphorImager analysis of radiolabeled nucleic acids showed a prominent signal at 120 kDa corresponding to RHAU (Figure 13A). The signal was absent when RHAU was depleted by RNAi before immunoprecipitation. This indicated that under normal conditions the strong signal at 120 kDa was derived from RHAU-associated nucleic acids. UV irradiation not only crosslinks proteins and RNA, but also proteins and DNA. As the CLIP method includes a stringent DNase treatment prior to immunoprecipitation, it is likely that the detected signals involve RNA. Nevertheless, to test this further, we purified co-immunoprecipitated nucleic acids, radiolabeled them and examined their sensitivity to an increasing concentration of RNAase (lanes 2-5) and 1U of DNase (lane 6). RHAU-associated nucleic acids were digested by RNase but not by DNase (Figure 13B) indicating that RHAU interacts with RNA *in vivo*.

To confirm our in vivo observation, the binding of RHAU to RNA was examined in vitro using purified, recombinant GST and GST-RHAU by double-filter RNA-binding assays (Figure 13C). As we do not know yet *in vivo* RNA targets of RHAU, we incubated radiolabeled, total RNA (5,000cpm) from HeLa extract with increasing amount of GST or GST-RHAU (0 – 40 nM), and filtered the mixture through nitrocellulose and nylon membranes. As shown in Figure 13C, unlike GST itself, GST-RHAU retained on nitrocellulose and moreover, it displayed dose-dependent RNA binding, suggesting that RHAU can directly associate with RNA. We express the relative binding affinity (KD) as the concentration of GST-RHAU at which 50% of RNA was bound. An apparent KD of GST-RHAU was 7 nM (Graph 5C).



Performed by N. Selak

FIGURE 13. RHAU associates with RNA through its N-terminus. (A) CLIP method to identify interactions of RHAU with nucleic acids (NAs). HeLa cells were transiently transfected with siRNAs against RHAU (siRHAU) or luciferase (siControl). Cells were UV-irradiated 72 h later and harvested. Immunoprecipitation was performed with a RHAU antibody. Bound nucleic acids were radiolabeled and detected as a band of approximately 120 kDa corresponding to RHAU (lane siControl). However, this band was strongly reduced when endogenous RHAU was depleted (lane siRHAU). Western blot (INPUT) shows efficient RHAU depletion by siRHAU. Actin was used as a loading control. (B) Identification of associated nucleic acids as RNA. After immunoprecipitation, the nucleic acids bound to RHAU were isolated, radiolabeled and treated with increasing concentrations (0.015; 0.15; 1.5; 15 U) of RNase A and 1 U of RQ1 DNase. Lane 1, isolated and non-treated nucleic acids (NA). Lanes 2-5, isolated NA treated with increasing concentration of RNase. Lane 6, NA treated with DNase. Fold changes in intensity ratio (NA/digest NA) are depicted relative to the intensity of non-treated NA in the lane 1, set as 1. Note that NAs are RNase sensitive but DNase insensitive. (C) RHAU binds to RNA in vitro. 0, 2.5, 5, 10, 20 and 40 nM of GST or GST-RHAU (RHAU) was incubated with 5,000 cpm of 5'-end-labeled RNA for 30 min at 37°C and filtered through nitrocellulose and nylon membranes. The percentage of bound RNA was plotted as a function of increasing concentration of GST-RHAU. Note that dose-dependent RNA binding was seen only with GST-RHAU. (D) Effect of stress on RHAU association with RNA *in vivo*. HeLa cells were transfected with EGFP alone or EGFP-WT (a full-length RHAU). Half were treated 24 h later with arsenite (0.5 mM for 45 min), followed by UV-irradiation and radiolabeling of RHAU-associated nucleic acids. RHAU and associated RNA were analyzed by Western blotting and PhosphoImager to detect levels of protein expression and the amount of radiolabeled RNA, respectively. Note that the arsenite treatment (+) did not abolish or dramatically change the RNA-binding activity of RHAU (E) Comparison of RNA-binding activities of wild-type RHAU and its N-terminal deletion mutants. HeLa cells were transfected with EGFP, EGFP-WT, EGFP-(50:1008) or EGFP-(105:1008). Protein and associated RNA were analyzed as in (A). Radioactivity of bound RNA was normalized to the expression levels of corresponding proteins. In sharp contrast to constant RNA binding of endogenous RHAU in each lane, N-terminal deletion mutants showed stepwise reduction of RNA interaction compared with WT. (►, overexpressed EGFP-RHAU or its N-terminal deletion mutants; ►►, endogenous RHAU)

Next, we examined the influence of arsenite stress on the RHAU-RNA interaction by treating the EGFP- or EGFP-WT-transfected HeLa cells with arsenite before UV irradiation and comparing the RNA-binding activity of RHAU in normal and stress conditions. After immunoprecipitation by a monoclonal anti-RHAU antibody, two radiolabeled RNA bands per the lane were detected; the lower and upper bands corresponding to endogenous RHAU and transfected EGFP-WT, respectively (Figure 13D). The association of endogenous RHAU with RNA was used as a loading control. The intensity of the RNA signals normalized over RHAU protein levels was similar in control and arsenite-treated conditions, suggesting that the amount of RHAU-associated RNA was not influenced by stress. The RNA band migrating in the region corresponding to EGFP-WT was not present in extracts of EGFP-transfected cells, verifying that the RNA signals were derived from RHAU-associated RNA.

Given the apparent significance of the first 105 amino acids for RHAU localization in SGs, we examined whether the N-terminus was also required for RHAU-RNA interaction. After cells were transiently transfected with EGFP-WT and its N-terminal deletion mutants, the extent of RNA binding to RHAU and mutants was examined as described above and normalized over RHAU protein levels. Even though the CLIP method is not a suitable quantitative assay, in six independent assays EGFP-(50:1008) and EGFP-(105:1008) showed a reproducible decrease in RNA-binding activity of approximately 50% and 20 %, respectively, compared with EGFP-WT (Figure 13E). Stepwise decrease in the RNA-binding activity of the two deletion mutants correlated with the decreased RHAU localization in arsenite-induced SGs (see Figure 11C). The small amount of RNA remaining associated with EGFP-(105:1008) may reflect residual RNA-binding activity derived from the helicase core domain of RHAU. Thus, the majority of RNA associated with RHAU is bound to its N-terminus.

3.5. Bioinformatic analysis of the N-terminus revealed a putative RNA-binding domain

Knowing that RHAU interacts with RNA via the first 105 amino acids, we submitted the RHAU protein sequence to RNABindR, a computational program that predicts RNA-binding potential of amino acid residues (Terribilini et al., 2007). This program is focuses on sequence-based prediction and runs on a Naïve Bayes classifier. According to this program, residues from 3 to 75 were highly positive for the RNA interaction (Figure 14A). A similar prediction was obtained by two other programs based on different algorithms, BindN+ and RISP (Jeong et al., 2004; Tong et al., 2008) (Figure 14A).

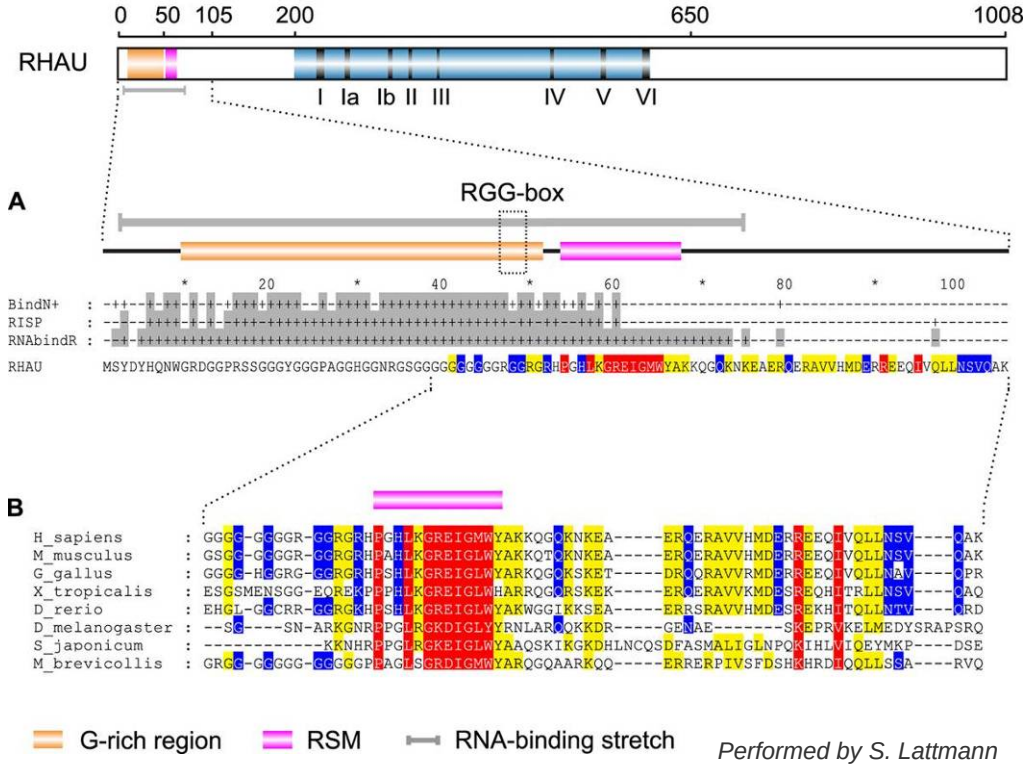


FIGURE 14. Bioinformatic analysis of the RHAU N-terminal domain. (A) Computational analysis for RNA-binding residues within the RHAU N-terminus. The human RHAU amino acid sequence was subjected to three different programs, BindN+, RISP and RNABindR, available on line (http://bioinfo.ggc.org/bindn/; http://grc.seu.edu.cn/RISP/; and http://bindr.gdcb.iastate.edu/RNABindR/, respectively). Residues predicted to be positive for RNA binding are indicated by +and depicted above the RHAU amino acid sequence. The grey shading corresponds to RNA-binding consensus per one amino acid in all three programs. Note that the predicted RNA-binding domain is a long stretch from 3 to 75 aa (grey bar) that overlaps with the G-rich region containing an RGG -box motif and with the highly conserved RSM. (B) Conservation of the RSM through evolution. Orthologs of RHAU used for the multiple sequence alignment were identified as described in Materials & Methods. Similarity of groups was estimated using GeneDoc (version 2.7) with the BLOSUM62 scoring matrix. Red (100-90% similarity); yellow (90-70% similarity) and blue (70-50% similarity). Species and accession numbers are: human (*Homo sapiens*, NP_065916); mouse (*Mus musculus*, NP_082412); chicken (*Gallus gallus*, XP_422834); frog (*Xenopus tropicalis,* XP_422834); zebrafish (*Danio rerio*, CAM56669); fruit fly (*Drosophila melanogaster*, NP_610056); trematoda (*Schistosoma japonicum*, AAW25528); choanoflagellate (*Monosiga brevicollis*, EDQ87802). (*Performed by S. Lattmann*)

Moreover, sequence alignment within the first 105 amino acids of RHAU proteins from choanoflagellate to humans revealed, next to the already known G-rich region (10-50 aa) with one RGG-box motif (47-49 aa), a novel highly conserved domain (54-66 aa) termed RHAU-specific motif (RSM; Figure 14B). While RGG-box motifs have been reported to be involved in RNA binding (Ramos et al., 2003; Solomon et al., 2007), RSM has no similarity to any known functional sequence elements. Thus, RHAU likely harbors an atypical conserved RNA-binding domain in the N-terminus.

3.6. The N-terminal RNA-binding domain is essential and sufficient for RNA interaction and localization of RHAU in SGs

To further test whether these 105 N-terminal residues are sufficient for RNA binding and subsequent RHAU recruitment to SGs, we designed Flag-tagged truncated forms of the RHAU protein: WT-Flag, (50:1008)-Flag, (105:1008)-Flag, (1:105)-Flag and (1:130)-Flag. For the RNA-binding experiment, CLIP analyses were performed using a monoclonal mouse anti-Flag M2 antibody coupled to agarose beads. Western blotting confirmed the successful expression and immunoprecipitation of all fragments, with (1:105)-Flag and (1:130)-Flag at higher protein levels (Figure 15A, left panel). To analyze the RNA-binding activities of these proteins, the measured radiolabeled RNA levels were normalized over protein levels. As shown in Figure 15A (right panel), compared with WT-Flag (lane 2), the amounts of RNA associated with (50:1008)-Flag (lane 3) and (105:1008)-Flag (lane 4) were stepwise reduced to approximately 70% and 30%, respectively. The high levels of RNA-binding (before normalization) with (1:105)-Flag and (1:130)-Flag (lanes 5 and 6) may partly reflect their expression levels (Figure 15A, right panel); however, they still showed approximately 10-fold higher RNA-binding activity than WT-Flag after normalization. Thus, these results demonstrate that the first 105 amino acids are essential and sufficient for RHAU to bind RNA.

Since the N-terminal fragment of RHAU (1:105)-Flag has been shown to bind RNA *in vivo*, we again performed a double-filter RNA-binding assay using the recombinant GST-Nter fragment (1:200aa) to confirm that the N-terminus of RHAU itself can interact with RNA. Purified, recombinant GST and GST-Nter were incubated with 5'-end radiolabled total RNA or with poly(rU) and then filtered through nitrocellulose and nylon membranes. As shown in Figure 15B, the higher dose-dependent amount of RNA, both total RNA and poly(rU), was associated with GST-Nter rather than GST. The apparent affinities of GST-Nter for total RNA and

poly(rU) were 45 and 80 nM, respectively. These data confirmed the in vivo data that the N-terminus is essential and sufficient for RHAU association with RNA.

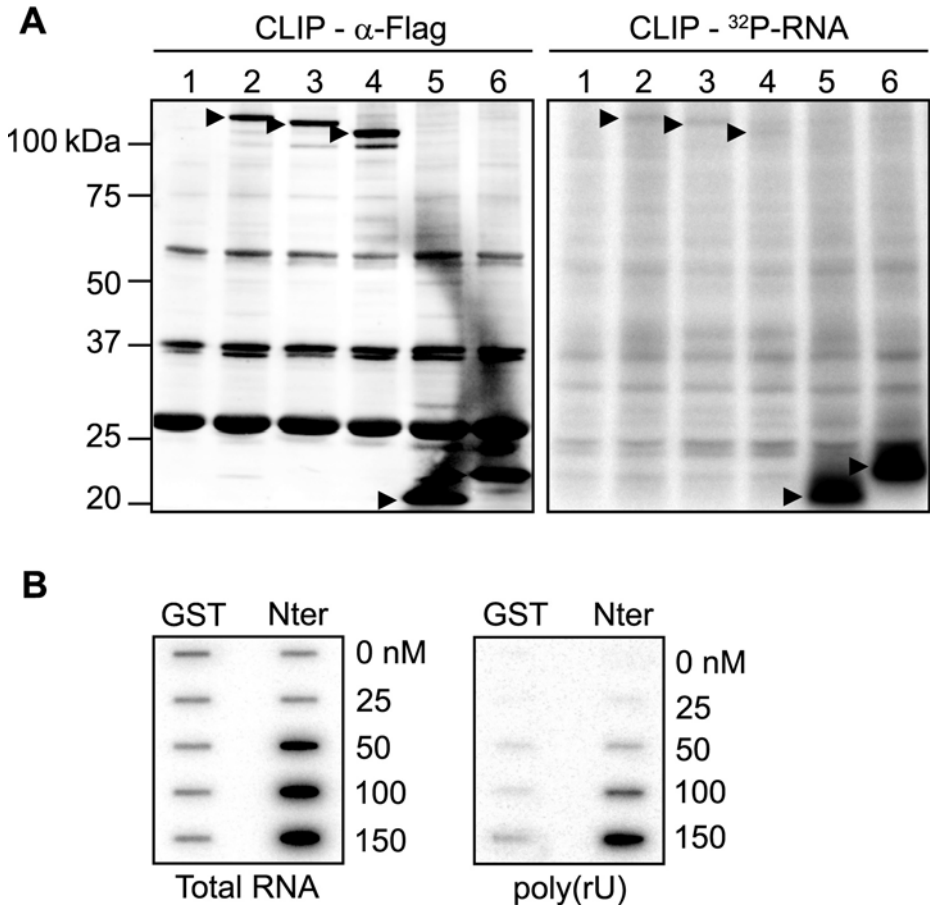


FIGURE 15. The N-terminal RNA-binding domain is essential and sufficient for RNA interaction *in vivo* and *in vitro*. (A) CLIP method to show RNA-binding activity of the N-terminal 105 amino acid residues of RHAU. HeLa cells were transfected with an empty vector, lane 1, or vectors expressing flag-tagged RHAU wild type, lane 2, or its fragments: (50:1008), lane 3; (105:1008), lane 4; (1:105), lane 5; and (1:130), lane 6. Cells were UV-irradiated 24 h later, harvested and immunoprecipitated by Flag antibody. Bound RNA was labeled with [γ-^{32}P]ATP and detected by a PhosphoImager as described in Materials & Methods. *Left panel*, Western blot of immunoprecipitated flag-tagged RHAU and its fragments. *Right panel*, bound RNA. The amount of associated RNA was normalized to the expression level of proteins. (►, flag-tagged RHAU and its fragments). (B) The N-terminus of RHAU binds to RNA in vitro. 0, 25, 50, 100 and 150 nM of GST or GST-Nter (1:200aa) was incubated with 10,000 cpm of 5'-end-labeled total RNA (left panel) or polu(rU) (right panel) for 30 min at 37°C and filtered through nitrocellulose and nylon membranes. Note that the dose-dependent RNA binding, both total RNA and poly(rU), was detected only with GST-Nter.

To determine whether the N-terminus is also sufficient for RHAU recruitment to SG, we transfected HeLa cells with N-terminal mutants fused with the EGFP tag at their C-termini, as shown in Figure 16A. Immunofluorescent analysis revealed that both tested mutants, (1:105)-EGFP and (1:130)-EGFP, co-localized with TIA-1 protein in SGs (Figure 16A). Further, we tested whether the N-terminus can bring about SG localization of a protein that does not normally associate with SGs in response to

stress. We fused different fragments of the RHAU N-terminal domain to β-gal-EGFP constructs (Figure 9B) and transfected them into HeLa cells. In response to arsenite treatment, β-gal-EGFP by itself does not accumulate in SGs, but is diffusely distributed in the cytoplasm as in normal conditions. However, when β-gal-EGFP was fused to the N-terminal 1-130 amino acids, including the complete putative RNA-binding domain with the G-rich region and the RSM, the fusion protein localized to SGs. In contrast, SG localization was not detected when β-gal-EGFP was fused with either the G-rich region [β-gal-EGFP(1-51aa)] or the RSM [β-gal-EGFP(52-200aa)] alone, indicating that only the complete RNA-binding domain is required for RHAU recruitment to SGs.

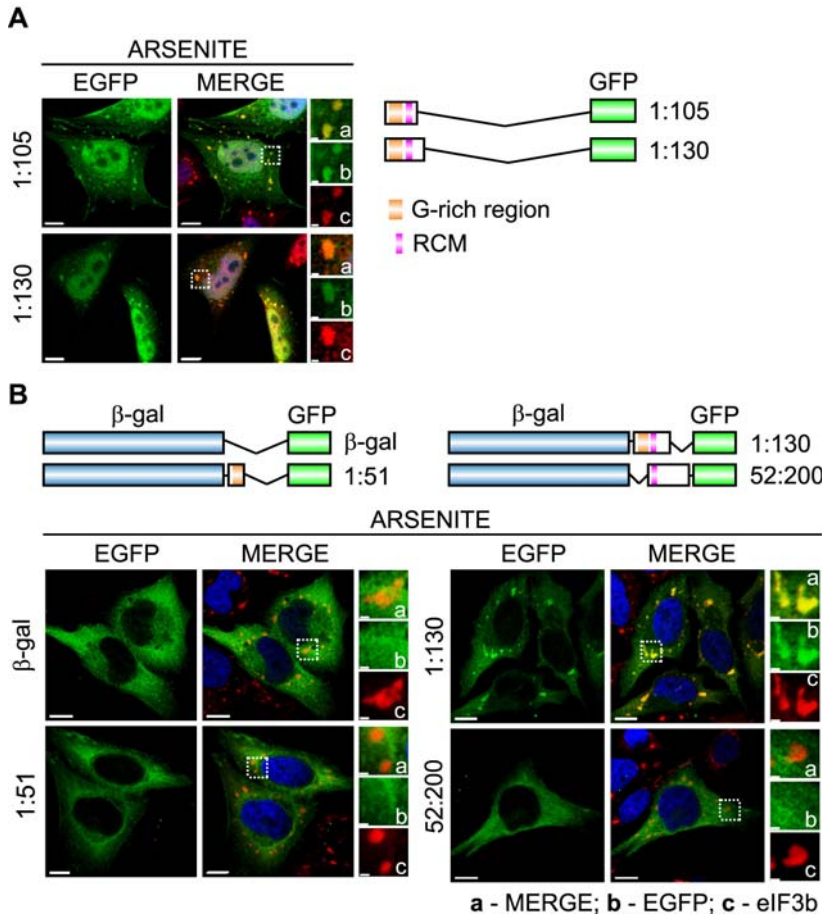


FIGURE 16. The N-terminal RNA-binding domain is also essential and sufficient for SG localization of RHAU. (A) Immunofluorescent images of HeLa cells transfected with vectors expressing EGFP-tagged N-terminal fragments of RHAU, (1:105) and (1:130). After 48 h of transfection, cells were treated with arsenite (0.5 mM, 45 min) to induce SGs. The merge shows the co-localization of EGFP-tagged RHAU fragments (green) and TIA-1 (red). DAPI (blue) stains nuclei. (B) Immunofluorescent images of HeLa cells expressing EGFP-β-galactosidase double-tagged RHAU N-terminal fragments. Cells were treated as above to induce SGs. The co-localization of EGFP (green) and eIF3b (red) is shown in the merge together with DAPI (blue). Note that only the (1:130) fragment containing an intact RNA-binding domain recruits EGFP-β-galactosidase to SGs. Enlargements of boxed regions are shown in small panels indicating (a) merge, (b) EGFP-fused RHAU N-terminal fragment and (c) eIF3b; *bar* 10 μm (1 μm in zooms).

3.7. ATP hydrolysis plays a role in RNA binding and localization in SGs

Previously, we showed that an ATPase-deficient RHAU mutant with a DEIH to DAIH point mutation is unable to accelerate uPA mRNA decay (Tran et al., 2004) and is excluded from the nucleus (Iwamoto et al., 2008). This suggested that the ATPase activity of RHAU has profound effects on its biological activity and subcellular localization. Therefore, we examined the role of RHAU ATPase activity on its RNA-binding activity and its localization in SGs. To elucidate the RNA-binding potential of ATPase-deficient RHAU mutants, we performed CLIP analyses. After UV cross-linking and subsequent immunoprecipitation of EGFP-WT and EGFP-DAIH with an anti-RHAU antibody, the amount of associated RNA was normalized to corresponding protein levels. As shown in Figure 17A, a higher amount of RNA was pulled-down with the ATPase-deficient DAIH mutant than with EGFP-WT. Based on results of Tanaka et al. (Tanaka and Schwer, 2005), where Prp22, a yeast DEAH-box helicase, dissociated from the RNA upon ATP hydrolysis resulting in apparent RNA-binding activity reduction, we concluded that ATPase activity of RHAU is involved in releasing RHAU from RNA rather than in binding to RNA.

Considering the possibility that the N-terminus and ATP hydrolysis contribute to the RNA interaction of RHAU, we investigated whether the N-terminal RNA-binding domain is also required for RNA binding of the DAIH mutant and its accumulation in SGs. For this, we introduced a point mutation (DEIH to DAIH) into motif II of the helicase core region to generate DAIH(50:1008) and DAIH(105:1008) fused with EGFP at their N-termini (Figure 17B). First, the truncated fragments were employed in the CLIP analysis. While being aware of CLIP limitations in quantitative analysis, stepwise deletion of the N-terminal domain of the DAIH mutant to DAIH(50:1008) and DAIH(105:1008) nevertheless decreased the amount of associated RNA to approximately 50% and 15%, respectively, compared with the DAIH full-length mutant (Figure 17C). The stepwise decrease in RNA interaction seen with the two DAIH deletion mutants corresponds to that seen with RHAU deletion mutants (see Figure 13E). Thus, enhanced RNA binding of the DAIH mutant still required the N-terminus.

Having shown that RHAU recruitment to SGs is dependent on its RNA interaction (see Figures 11C and 13E), we tested whether the ATPase-deficient DAIH mutant accumulates in SGs more than wild type. Indeed, the DAIH mutant localized in SGs (Figure 17D) to a greater extent than the wild type, increasing the number of transfected cells with EGFP-positive SGs from 81% to 100%. Unlike DAIH full-length

and DAIH(50:1008), DAIH(105:100) was not detected in SGs. Therefore, RHAU recruitment to SGs is independent of its ATPase activity.

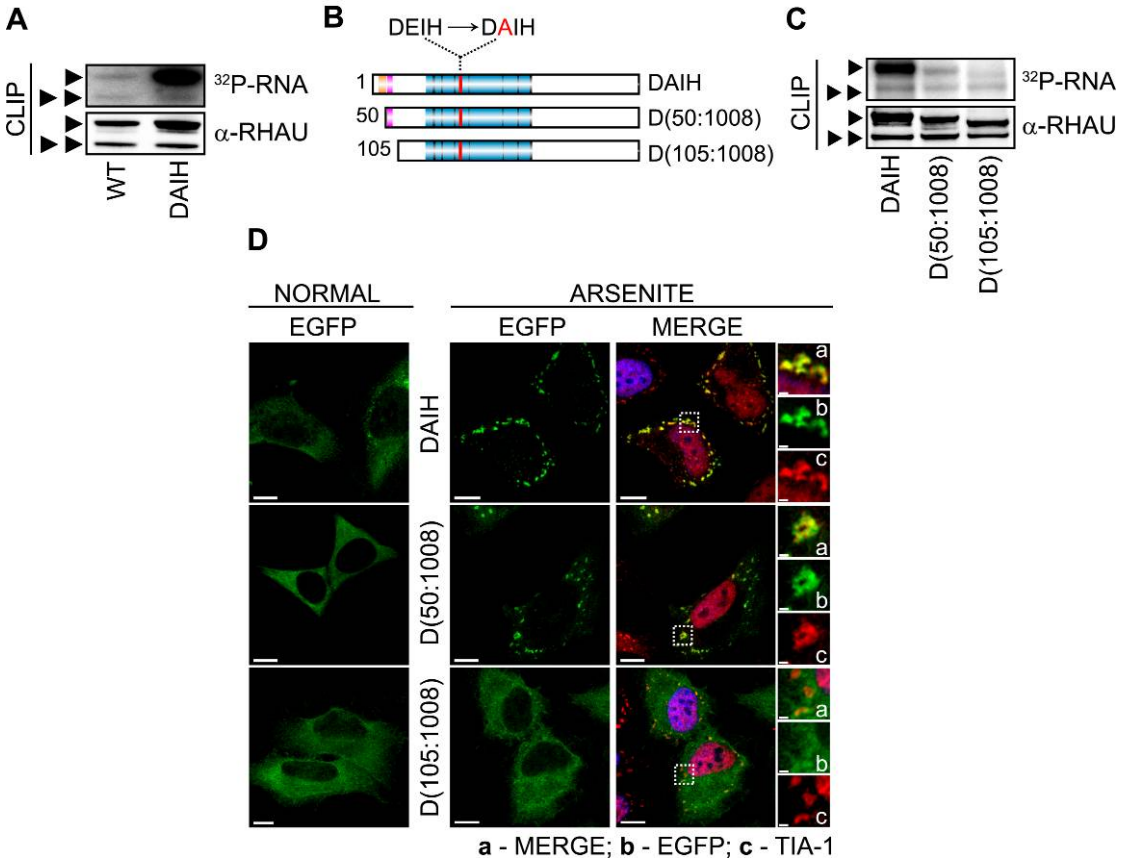


FIGURE 17. Role of RHAU ATPase activity in RNA-binding and retention of protein in SGs. (A) RNA binding activity of wild-type RHAU (WT) and the ATPase-deficient RHAU mutant DAIH. Levels of RHAU-associated RNA were measured by CLIP as described in Materials & Methods 24 h after transfecting HeLa cells with EGFP-fused WT or DAIH. The level of RNA bound to the DAIH mutant was much higher than that bound to WT. Note that the level of RNA bound to endogenous RHAU is comparable in cells expressing exogenous WT or DAIH. (B) Schematic representation of N-terminal deletion, DAIH mutants: EGFP-DAIH, EGFP-D(50:1008) and EGFP-D(105:1008). (C) Comparison of RNA binding between mutants shown in (B). HeLa cells were transfected with vectors expressing EGFP-DAIH, EGFP-D(50:1008) or EGFP-D(105:1008) and 24 h later the levels of RNA associated with these mutants and endogenous RHAU were assessed by CLIP, as in (A). Note that N-terminal deletion mutants show stepwise reduction of RNA binding compared with DAIH full length (such as WT and its deletion mutants in Figure 4E). (D) Intracellular localization of EGFP-tagged DAIH mutants listed in (B) in control and arsenite-treated cells. Transfected HeLa cells were treated without (NORMAL) or with 0.5 mM sodium arsenite (ARSENITE) for 45 min. Images denoted MERGE are the merger of EGFP (green), TIA-1 (red) and DAPI (blue). Enlargements of boxed regions show (a) merge, (b) EGFP-tagged DAIH mutants and (c) TIA-1; *bar* 10 μm (1 μm in zooms). (►, EGFP-tagged DAIH and its deletion mutants; ►►, endogenous RHAU)

3.8. ATP hydrolysis takes part in shuttle kinetics of RHAU into and out of SGs

Having established that both RHAU wild type and the DAIH mutant accumulate in SGs, we used the FRAP method to examine whether the dynamics of shuttling into and out of SGs differs between the two proteins. Cells were transfected with EGFP-WT, EGFP-DAIH, EGFP-G3BP or EGFP-FMRP and the FRAP analyses performed 48 h after transfection. It has been shown that TIA-1 and G3BP recover rapidly and completely in SGs within 30 s after bleaching (Kedersha et al., 2000; Kedersha et al., 2005). In contrast, PABP-1, another marker for mRNA shuttling, showed only 60% fluorescence recovery after 30 s (Anderson and Kedersha, 2002; Kedersha et al., 2005). In our study, we used EGFP-G3BP as a control for the rapid fluorescence recovery (Figure 18A, B). Although FMRP has never been tested by FRAP analysis, we included this protein as a control as it is known to localize in SGs (Mazroui et al., 2002). As shown in Figure 18A, the fluorescence signal of G3BP recovered rapidly and completely within 10 s, as reported (Kedersha et al., 2005), but the FMRP signal did not recover fully within 60 s, suggesting that FMRP is a scaffold SG component. In this analysis, the wild-type and DAIH signals showed different recovery patterns. The former displayed rapid recovery (≥ 90 %) of fluorescence after 8 s, similar to G3BP with the same recovery after 11 s. However, the recovery of the EGFP-DAIH mutant fluorescent signal was much slower, with only 60% of fluorescent recovery after 30 s of bleaching, similar to PABP-1. The recovery rate reflects the strength of binding, whereby slower recoveries correspond to tighter binding. Thus, in comparison with the wild-type, the ATPase-deficient mutant, which cannot release RNA because of its inability to hydrolyze ATP, is retained longer in SGs, resulting in slower recovery kinetics. Combining these results, we conclude that the ATPase activity of RHAU is required for its active shuttling into and out of SGs and may also be important for remodeling and/or recruitment of RNPs in SGs.

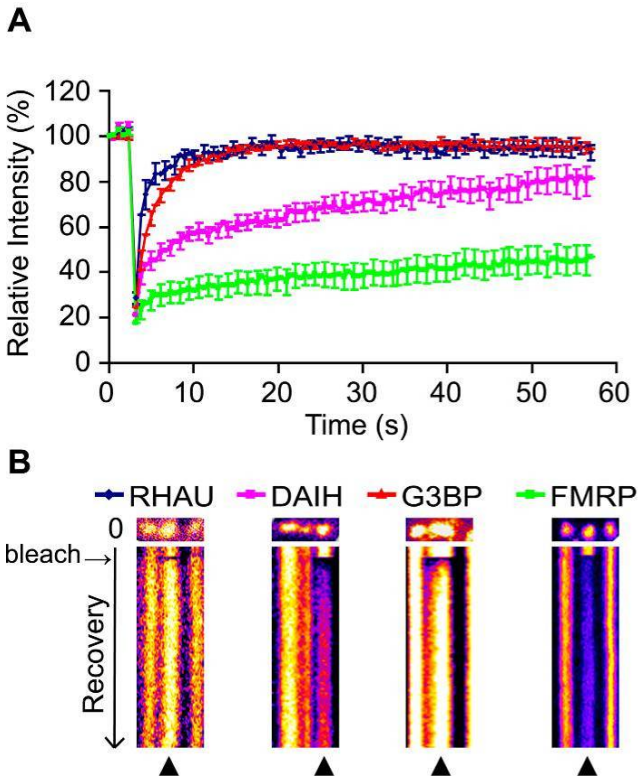


FIGURE 18. ATP hydrolysis takes part in dynamic RHAU shuttling into and out of SGs. (A) Fluorescent recovery patterns of RHAU, ATP-deficient RHAU mutant (DAIH), G3BP and FMRP. HeLa cells transfected with EGFP-tagged RHAU, DAIH, G3BP or FMRP were treated with arsenite and analyzed by the FRAP method. Bleaching of selected SGs was performed with 3 scan iterations for a total of 1.5 s. Fluorescence recovery was monitored at 0.5-s intervals for 50 s and results were analyzed as described in Material & Methods. Data were collected from at least ≥10 independent FRAP analyses and each curve represents average fluorescence intensity ± s.e.m. over time. (B) Representative result of FRAP analysis of EGFP-tagged RHAU, DAIH, G3BP or FMRP. SGs were photobleached and subsequent fluorescence recoveries are shown in a fire mode in the time-scale of recovery. ▲indicates SGs bleaching.

3.9. The initiation factor eIF3b, which physically interacts with RHAU, does not recruit RHAU to SGs

Having shown that RHAU can accumulate in SGs in response to stress, we were interested in whether it would also interact with some SG-associated proteins. Therefore, we compared the list of known SG-associated proteins with our list of potential RHAU binding proteins (Pauli unpublished data). This rough screen allowed us to identify two SG-associated proteins as potential RHAU binding proteins, PABP and eIF3 and by immunoprecipitation analysis using RHAU monoclonal antibody coupled to sepharose beads we confirmed that both proteins interact with RHAU (Figure 19A and B). As shown in Figure 19A, PABP associates with RHAU in an RNA-dependent manner (compare lanes 3 and 4). However, unlike PABP, eIF3b exhibits an RNA-independent interaction with RHAU (Figure 19B, lane

5) and as shown in Figure 19B, this association also remains after arsenite (stress) treatment (lanes 6 and 7). Since our PABP antibody was not suitable for immunofluorescent study, we focused our interest on eIF3b. Remarkably, immunostaining revealed that although RHAU is mostly a nuclear protein, it partly co-localized with eIF3b in the cytoplasm under normal conditions (Figure 19C; NON) and after stress induction it accumulated together with eIF3b in SGs, detected using HuR antibody (Figure 19C; ARS).

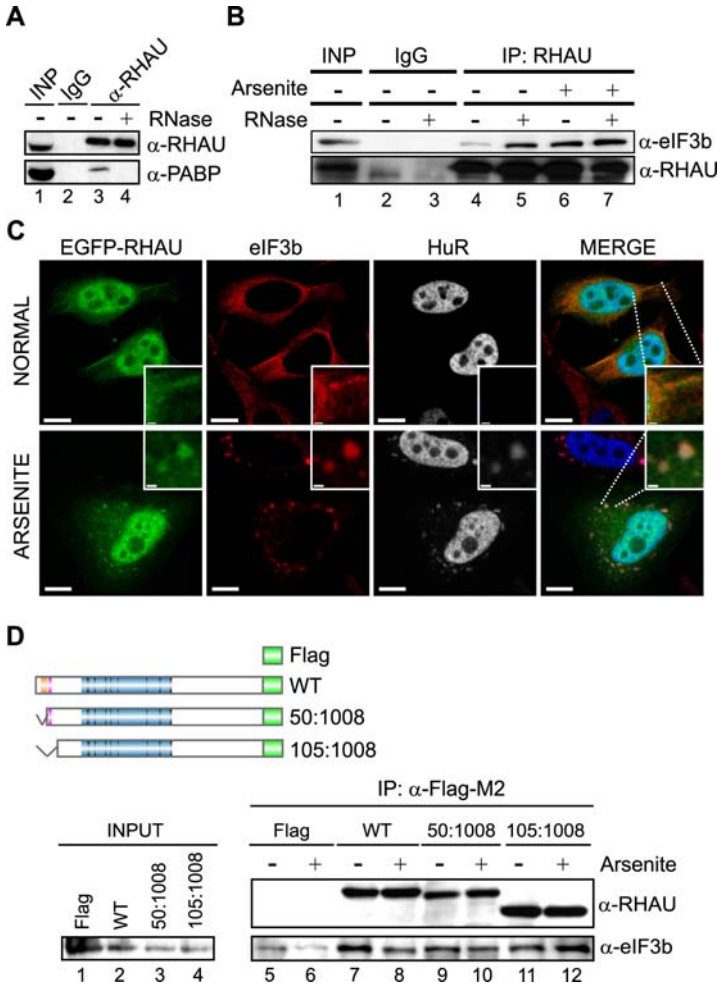


FIGURE 19. RHAU physically associates with eIF3b, and exhibits an RNA-dependent interaction with PABP. (A) Whole lysates prepared from HeLa cells were immunoprecipitated with anti-RHAU (12F33) sepharose beads in the absence (-) or presence (+) of micrococcus nuclease (RNase). Lysates incubated with beads coupled to IgG antibody served as negative controls (lane 2). Immunoprecipitates were analyzed by Western blotting with antibodies against PABP and RHAU. (B) HeLa cells were culture in the presence (+) or absence of arsenite for 1 hour before being harvest. Whole lysates prepare from HeLa cells were processed as described in A. Note that RHAU associates with eIF3b in an RNA-independent manner (lanes 5 & 7) and the interaction remains after arsenite treatment (lanes 6 & 7). (C) Co-localization of RHAU with eIF3b and HuR in arsenite-induced SGs. Before fixation EGFP-RHAU transfected HeLa cells were cultured with or without arsenite (0.5 mM for 45 min). The panels show EGFP-RHAU (green), eIF3b (red) and HuR (white; blue in merge). The two right panels showed the merged image. (D) eIF3b interacts with the truncated form of RHAU that does not localize in SGs. Whole lysates prepared from HeLa cells expressing Flag, Flag-WT, Flag-(50:1008) or Flag-(105:1008) were immunoprecipitated with Flag M2 agarose beads in the absence (-) or presence (+) of arsenite. Lysates with overexpressed Flag served as negative controls (lanes 5 and 6). Immunoprecipitates were analyzed by Western blotting with antibodies against RHAU and eIF3b; *bar* 10 μm (1 μm in zooms).

While eIF3b physically interacts and also co-localizes with RHAU in SGs, it is possible that eIF3b could recruit RHAU to SGs. To investigate this hypothesis, we performed RNAi experiments, in which endogenous eIF3b was depleted by two different short hairpin RNAs. Unfortunately, the down-regulation of eIF3b led to global cell death and thus, we were not able to test a possible role of eIF3b in RHAU recruitment to SGs (data not shown). Futhermore eIF3b has recently been identified as an essential protein for SG and PB assembly (Ohn et al., 2008), therefore the down-regulation of eIF3b would unlikely addressed whether eIF3b could recruit RHAU to SGs, because they would not be formed at all. Therefore, we approached the task by an indirect experiment. Knowing that the truncated mutant of RHAU [Flag-(105:1008)] lacking the first 105 amino acids almost completely lost the ability to accumulate in SGs, we tested whether it also loses the interaction with eIF3b that localizes in SGs. If so, we could speculate that eIF3b may interfere in RHAU recruitment to SGs. Nevertheless, as shown in Figure 19D, both truncated forms of RHAU, Flag-(50:1008) and Flag-(105:1008), associated with eIF3b in normal and stress conditions (lanes 7 - 10) similarly to wild type RHAU. Since eIF3b still associates with the truncated form, which is not able anymore to accumulate in SGs, we concluded that eIF3b does not play a role in the recruitment of RHAU to SGs. Furthermore, these data indicate that eIF3b-RHAU interaction is formed before SG assembly and remains present during stress but does not take part in RHAU accumulation in SGs.

3.10. RHAU influence on SG assembly and disassembly

Importantly, the over-expression of EGFP-RHAU induced spontaneous assembly of SGs in approximately 30% of transfected COS-7 cells (data not shown) suggesting that RHAU may be involved in SG assembly. Therefore we decided to test whether down-regulation of RHAU influences the formation of SGs. The inducible RHAU-knockdown T-REx-HeLa cell line, in which a RHAU-specific short hairpin RNA (shRHAU) is induced by doxycycline, was used in following experiments (Iwamoto et al., 2008). As shown on Western blots in Figure 20A, the maximal down-regulation to 20% of the total RHAU protein level was observed after the 7th day of doxycycline treatment. At this time point, cells were treated with arsenite, fixed and stained for two SG-associated proteins, TIA-1 and HuR, to visualize SGs. Nuclei were detected by DAPI. The immunofluorescent staining demonstrated that SGs were formed in both RHAU expressing (dox -) and RHAU depleted (dox +) cells, indicating that RHAU is not essential for SG assembly (Figure 20A).

Although, the formation of SGs in T-REx-HeLa cells with depleted RHAU protein levels (dox +) was not impaired, we next monitored whether RHAU plays a role in SG disassembly after releasing stress. Therefore, after arsenite treatment, the medium was changed and cells were cultured for 0, 1, 2 and 3 hours in normal culture conditions before fixation. SGs were visualized by TIA-1 antibody and cells forming SGs were quantified. As shown in the graph of Figure 20B, the SG disassembly was enhanced in cells lacking RHAU, especially in the 1st hour after stress relief. Thus, we concluded that even though RHAU is not involved in SG formation it most probably takes part in SG maintenance during and after stress.

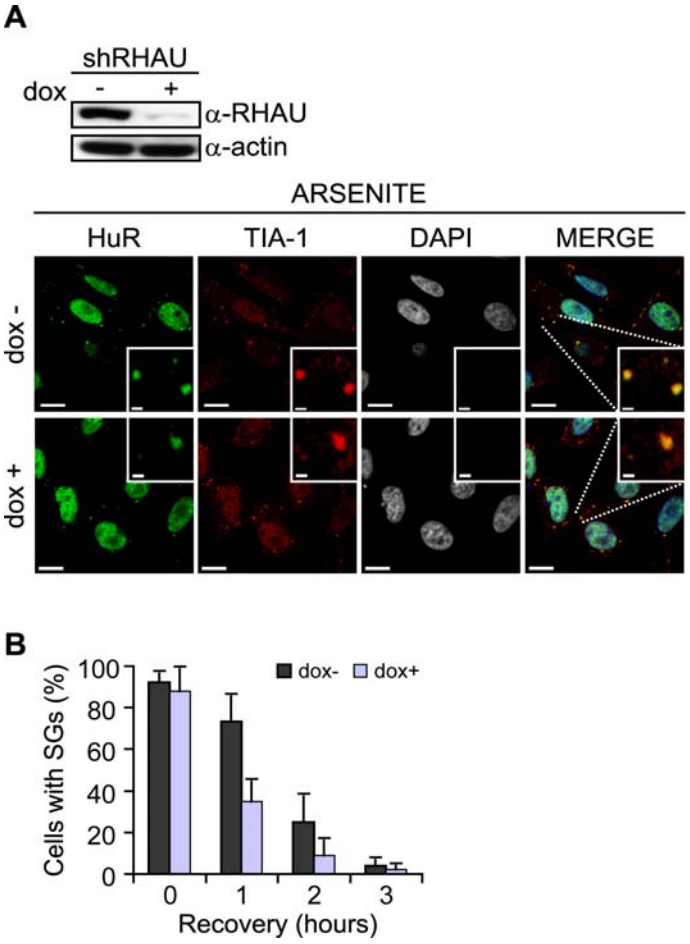


FIGURE 20. RNAi-mediated down-regulation of RHAU did not abrogate SG formation but influenced SG disassembly. (A) SGs assemble in a RHAU-independent manner. Prior to arsenite treatment (0.5 mM for 45 min), T-REx-HeLa cells stably transfected with pTER-shRHAU were maintained in normal (dox -) or in conditioned medium containing 1 μg/ml of doxycycline (dox +) for 7 days to induce shRHAU expression. The depletion of RHAU protein was confirmed by Western blotting using a monoclonal anti-RHAU antibody. After arsenite treatment, cells were fixed and stained with DAPI (white; blue in merge) to visualize nuclei and with anti-HuR and anti-TIA-1 antibodies (green and red) to detect SGs. Note that SGs were formed in both cell lines independently of RHAU depletion. (B) SGs disassemble faster in the absence of RHAU protein. T-Rex-HeLa cells were pre-treated with doxycycline as described in A. After arsenite treatment, the conditional medium was changed to the normal medium. Cells were fixed and stained with TIA-1 to detect SGs in the time scale indicated in the graph. Data obtained by quantitative immunofluorescence showing the percentage of T-REx-HeLa cells with SGs in time (hours) after arsenite treatment. Values ± s.e.m. were derived from two independent experiments; *bar* 10 μm (1 μm in zooms).

3.11. RHAU is essential for PB assembly

Since RHAU has been detected in SGs, we were interested whether the protein may also accumulate in other known cytoplasmic foci that are linked with RNA metabolism. These foci are known as P-bodies (PBs) and they contain proteins involved in diverse posttranscriptional processes, such as mRNA degradation, nonsense-mediated mRNA decay (NMD), translational repression, and RNA-mediated gene silencing (Eulalio et al., 2007b). To determine if RHAU is also recruited to PBs, HeLa cells were transfected with EGFP-tagged RHAU and 48 hours later fixed and stained with antibody against the Dcp1a protein, a general PB marker. As shown in Figure 21A, in addition to diffused nuclear and cytoplasmic localizations, RHAU accumulated in some but not all distinct cytoplasmic foci containing Dcp1a protein. However, the localization of RHAU in PBs was very slight and hard to be detected solely by microscope. Moreover, arsenite treatment, which has been shown to increase number and size of PBs (Kedersha et al., 2005), did not induce the localization of RHAU in PBs (Figure 21A), indicating that RHAU is not a general PB-associated protein.

Nevertheless, to our surprise, we observed a reduced number of PBs per cell in cell lines, where endogenous RHAU was knocked down. These experiments were performed in inducible RHAU-knockdown T-REx-HeLa cell lines (shRHAU1 and shRHAU2) expressing two different short hairpin RNAs against RHAU mRNA, and in inducible control T-REx-HeLa cell line expressing short hairpin RNA against Luciferase (shLuci). As shown in Figure 21B, endogenous RHAU was successfully down-regulated after 7 days of doxycycline pre-treatment. Arsenite treated or non-treated cells were fixed and stained with α-Dcp1a to detect PBs. Interestingly, unlike the control cell line (shLuci), RHAU knockdown cell lines, shRHAU1 and shRHAU2, showed clear reduction of an average number of PBs per cell during normal culture conditions [Figure 21D; NON(dox+)]. The average number of PBs per cell was calculated from a sum of all deteced PBs divided by the number of counted cells. However, when T-REx-HeLa cells (shRHAU1 and shRHAU2) were not pre-treated with doxycycline thus RHAU was normally expressed in these cells, the phenomenon of reduced average PB number per "shRHAU" cell disappeared. Thus, the reduced number of PBs per cell upon RHAU knockdown responds to RHAU protein level and is not an artifact of the cell lines used [Figure 21; NON(dox-)]. Moreover, as shown in Figure 21B, once all tested cell lines were exposed to arsenite before fixation, the number of PBs per cell raised and did not differ between cell lines [Figure 21B; ARS(dox+), ARS(dox-)]. Thus, these data suggest that down-regulation

of RHAU impairs PB assembly during normal culture conditions and that this PB formation arrest can be bypassed by stress.

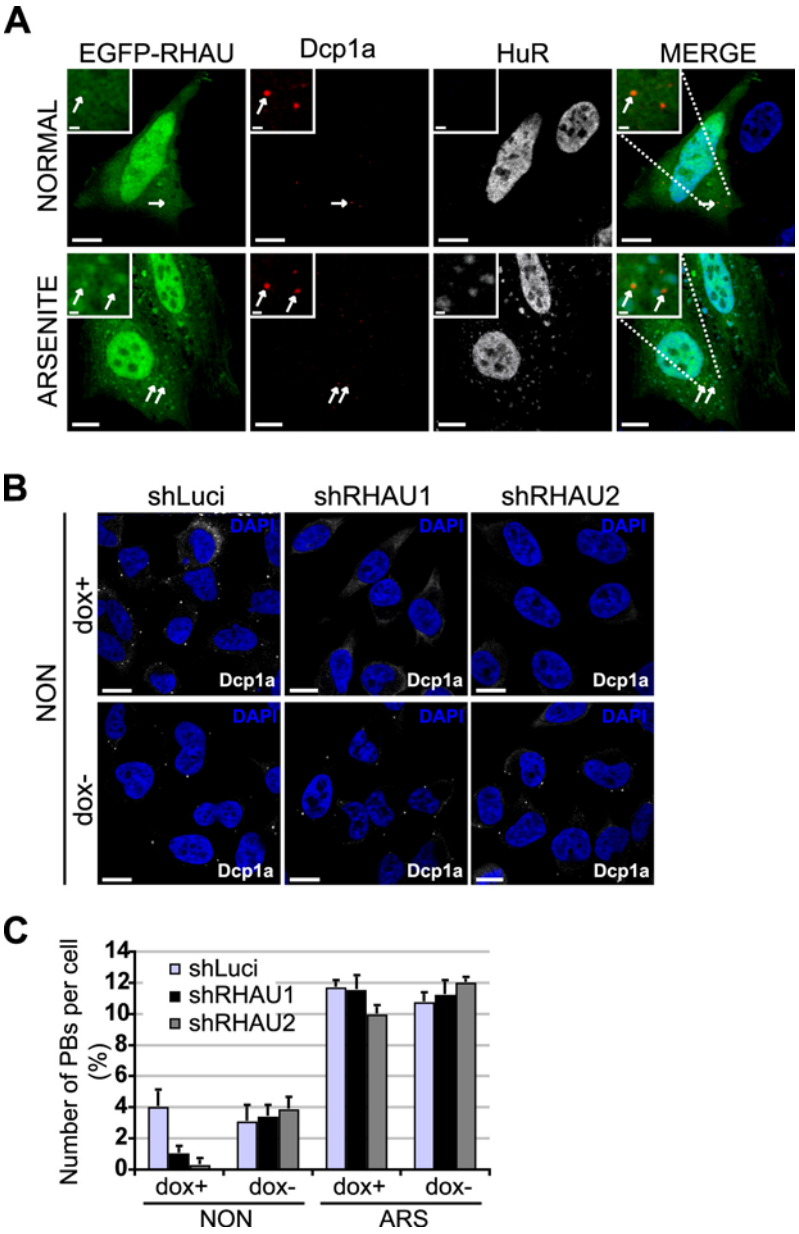


FIGURE 21. RNAi-mediated down-regulation of RHAU leads to arrest of PB assembly under normal culture conditions. (A) Immunofluorescent analysis of RHAU localization in PBs. HeLa cells, were transiently transfected with EGFP-RHAU and 48h later cultured under normal conditions (NORMAL) or treated with 0.5 mM of arsenite for 45 minutes (ARSENITE). Distribution of EGFP-RHAU (green), endogenous Dcp1a (red) and HuR (white; blue in merge), and their merge under normal and arsenite-treated conditions are depicted from left to right; zooms of boxed regions are shown on small panels and white arrows indicate PBs, in which RHAU slightly localizes. (B) PB assembly impairment in a RHAU-dependent manner. T-REx-HeLa cells stably transfected with, pTER-shLuci, pTER-shRHAU1 and pTER-shRHAU2, constructs were maintained in normal (dox-) or in conditioned medium containing 1 μg/ml of doxycycline (dox+) for 7 days to induce construct expression. Half of cells were exposed to arsenite before fixation and α-Dcp1a staining (PB marker). (C) Data obtained by quantitative immunofluorescence analysis showing the average number of PBs per T-REx-HeLa cells in normal and stressed conditions. Note that the average number of PBs per cell was reduced only during normal conditions in cells in which endogenous RHAU was depleted (shRHAU1 and shRHAU2; NON(dox+)). Values ± s.e.m. were derived from at least two independent experiment; *bar* 10 μm (1 μm in zooms).

These results were also confirmed in Mouse Embryonic Fibroblasts (MEFs). As shown in Figure 22B, MEF-KO, which did not contain endogenous RHAU, showed a loss of PBs when compared with wild-type MEFs (MEF-WT). Moreover, after stress, PBs were detected in both MEF-WT and MEF-KO just like in T-REx-HeLa cells reported above, which indicates that RHAU indeed influences PB assembly but only in normal (non-stressed) conditions.

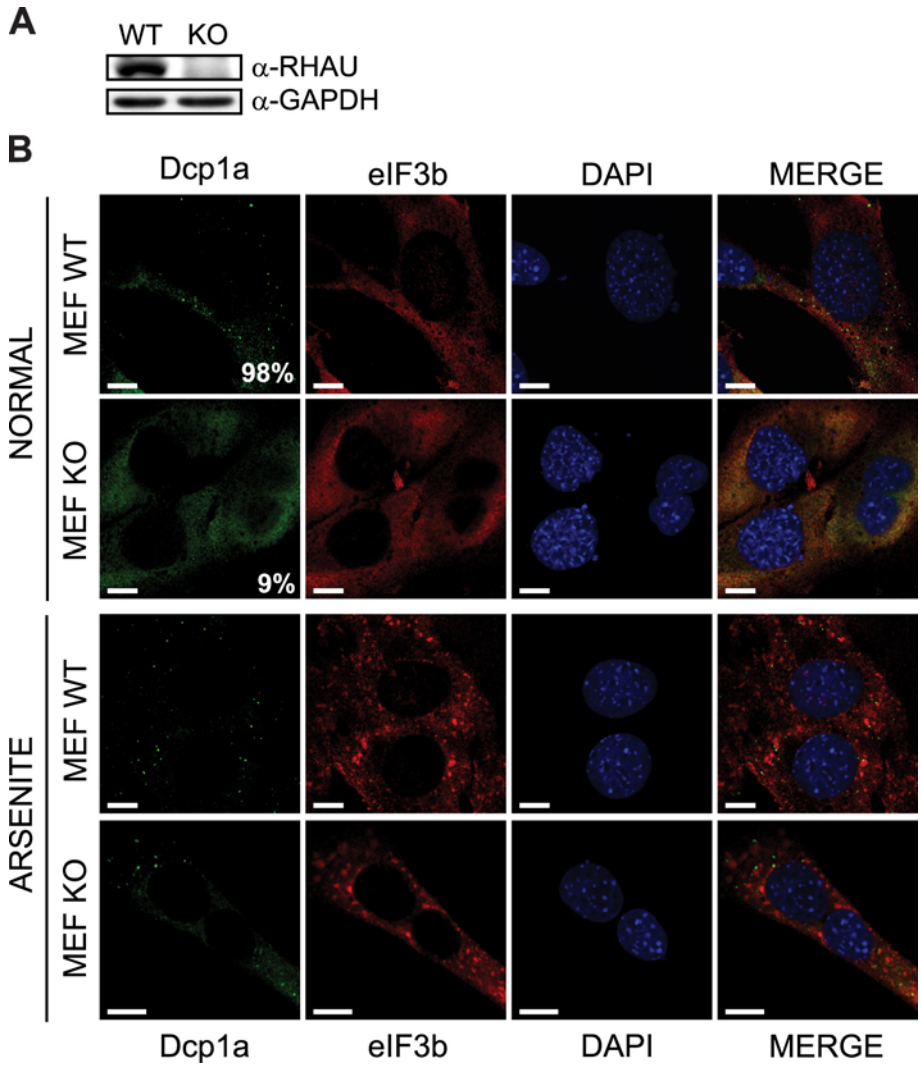


FIGURE 22. In normal conditions RHAU is essential for PB assembly in MEFs. (A) Knockout of RHAU was examined by Western blotting using anti-RHAU antibody. Anti-GAPDH antibody was used as a loading control. (B) PBs are not formed in MEFs with knockout RHAU (MEF-KO). Before fixation, MEFs, both WT (wild type) and KO (RHAU$^{-/-}$), were treated with 0.5 mM of arsenite for 45 min (ARSENITE) or cultured under normal conditions (NORMAL). Distribution of Dcp1a (a PB marker; green), eIF3b (a SG marker; red), DAPI (blue) and their merge under normal and arsenite-treated conditions are depicted from left to right. The numbers, in white, of the first two left panels correspond to the percentage of MEFs with PBs. Values were derived from at least two independent experiment; *bar* 10 μm (1 μm in zooms).

4. DISCUSSION

4.1. RHAU as a component of SGs

The list of proteins identified as components of SGs, co-localizing with known SG-associated proteins such as TIA-1, is still expanding. However, a method for isolating SGs to a significantly pure level has not yet been established, rendering the biochemical analysis of SGs very difficult. For this reason there is currently no consensus on their detailed structure and molecular composition. Furthermore, it has not been examined systematically whether SGs induced by different stresses are qualitatively different. A hint that this could be the case is given by the fact that the recruitment of TTP to SGs is induced by FCCP but not by arsenite, while TIA-1 is recruited to SGs by both stimuli (Stoecklin et al., 2004). Our results clearly identify RHAU as a new SG-associated protein that is recruited to SGs independently of the type of stress. Thus, RHAU appears to be a general SG component like TIA-1 or eIF3b.

Although RHAU is recruited to SGs, RNAi-mediated down-regulation of endogenous RHAU had no apparent effect on arsenite-induced SG formation, indicating that RHAU is not essential for SG assembly. However, after stress release SG disassembly was faster in cells with low level of endogenous RHAU (RHAU knockdown), suggesting that RHAU may somehow participate in SG biogenesis during the recovery phase of the cell. Recent preliminary experiments performed in MEFs, with or without knockout of RHAU protein (MEF-KO or MEF-WT), indicate that complete abolishment of RHAU results in SG assembly retardation and consequent cell viability reduction after stress (Figure 22). Taken together, these data suggest that RHAU may play a physiological role in stress response since its knockout reduces cell recovery after stress release. Although two SG-associated proteins, hnRNPA1 and MLN51, have been found to participate in stress response by increasing cell recovery and viability, a functional link between SG biogenesis and cell viability after stress has not clearly been shown (Baguet et al., 2007; Guil et al., 2006). So far only one indirect link between SGs and cell death via tumor necrosis factor (TNF) signaling has been reported (Kim et al., 2005). In this case, TNF receptor associated factor 2 (TRAF2), a central mediator of NFkB activation, is trapped into SGs which is thought to lead to the prevention of TNF-induced apoptosis under stress conditions. Nevertheless, they did not show whether SG formation and subsequent TRAF2 sequestration are really necessary for TRAF2 cell recovery after stress.

However, the importance of a protein in the SG-context is not solely defined by its ability to influence SG formation. Several SG-associated proteins, e. g. ZBP1, did not induce or inhibit SG formation when overexpressed or knocked down, respectively (Stohr et al., 2006). Nevertheless in the case of ZBP1, its significance for

SGs is attributed to its function in mRNA turnover regulation. Interestingly, the stability of ZBP1 target mRNAs is not regulated by their recruitment to SGs but by their retention in SGs to prevent their rapid translocation and subsequent decay in PBs or by the exosome (Stohr et al., 2006). Knowing that RHAU is a *cis*-acting factor involved in ARE-mediated decay of uPA mRNA and that its association with SGs is mediated by an RNA interaction, we speculate that RHAU may act in SGs in a manner similar to ZBP1.

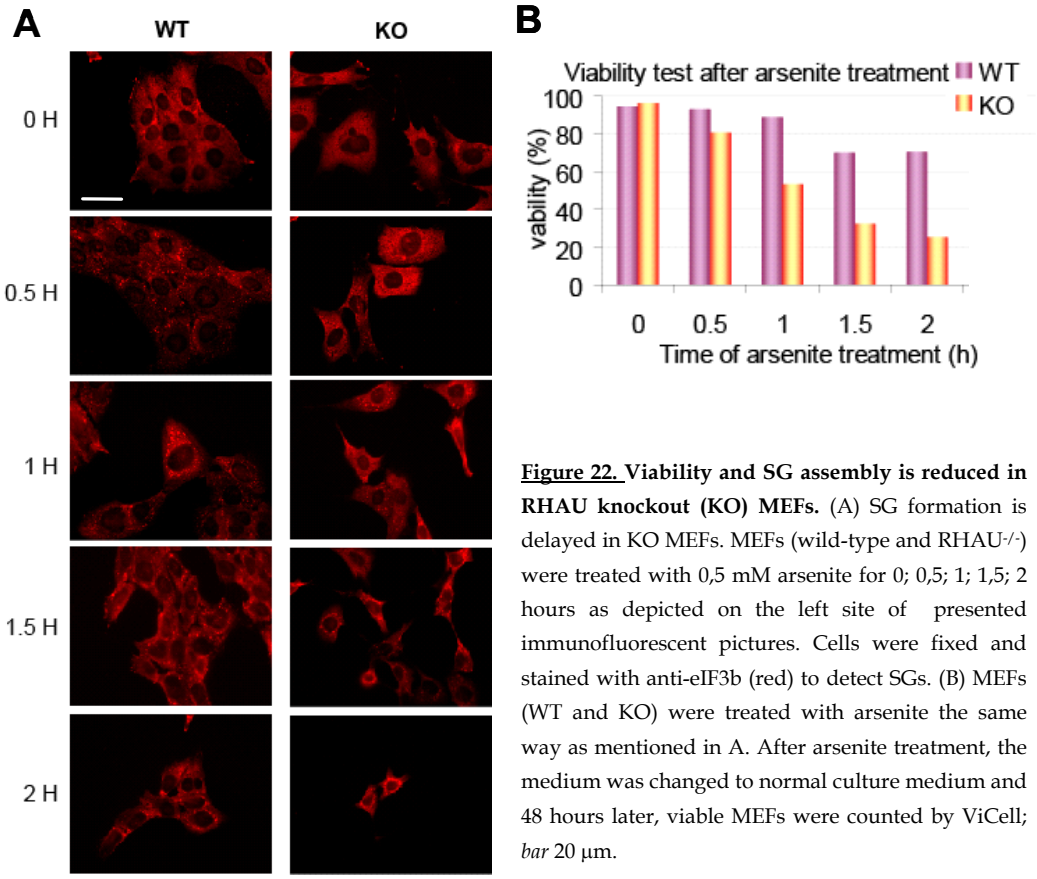


Figure 22. Viability and SG assembly is reduced in RHAU knockout (KO) MEFs. (A) SG formation is delayed in KO MEFs. MEFs (wild-type and RHAU$^{-/-}$) were treated with 0,5 mM arsenite for 0; 0,5; 1; 1,5; 2 hours as depicted on the left site of presented immunofluorescent pictures. Cells were fixed and stained with anti-eIF3b (red) to detect SGs. (B) MEFs (WT and KO) were treated with arsenite the same way as mentioned in A. After arsenite treatment, the medium was changed to normal culture medium and 48 hours later, viable MEFs were counted by ViCell; *bar* 20 μm.

4.2. RHAU interacts with RNA

Even though RHAU was first identified as an ARE-associated protein of uPA mRNA (Tran et al., 2004), its direct interaction with the ARE of uPA was almost not detectable by gel mobility shift assays (Lattmann and Akimitsu, unpublished data). The association between RHAU and ARE of uPA was induced when NF90 was added to the reaction (Lattmann and Akimitsu, unpublished data). These data are in great agreement with the widely accepted theory that substrate specificity of RNA

helicases is mediated indirectly by co-factors rather than directly by a specific RNA sequence (Linder, 2006; Pyle, 2008; Silverman et al., 2003). Nevertheless, up to now we were not able to show that RHAU is indeed an RNA-binding protein. The CLIP results presented here are the first clear data showing that RHAU binds to RNA *in vivo*. The radioactively labeled nucleic acids immunoprecipitated together with endogenous RHAU were identified as RNA since they were sensitive to RNase treatment, suggesting that RHAU preferentially binds to RNA rather than DNA. This preference of RHAU to bind RNA rather than DNA was also observed by our collaborators, Akman's laboratory. They identified RHAU as the main enzyme harboring DNA guanine quadruplex (G4-DNA) resolving activity in HeLa extract (Vaughn et al., 2005). More recently they demonstrated that RHAU can directly interact with and also resolve an RNA guanine quadruplex (G4-RNA) structure. Furthermore, they showed that RHAU forms tighter interaction with G4-RNA than G4-DNA structure (Creacy et al., submitted to JBC) and that the apparent K_D value of RHAU-G4-RNA interaction is ~ 40 pM (Creacy et al., submitted to JBC). Surprisingly, the K_D value is many times smaller than the one detected for the DEAH helicase Prp22 and poly(A) interaction, which ranges in tens of nM (Tanaka and Schwer, 2005), indicating that RHAU may have a preference for G-rich RNA or for the G4 structure itself. However, since RHAU immunoprecipitated RNA molecules have not been sequenced yet, it is not clear whether RHAU can bind to a specific RNA sequence (Selak and Lattmann, unpublished data).

In cells RNA molecules exist in a permanent association with proteins forming ribonucleoprotein (RNPs) complexes. Therefore, CLIP data revealed only that RHAU has the potential to bind RNA but it did not elucidate whether the RHAU-RNA interaction is direct or formed with the help of co-factors. By performing a double-filter RNA-binding assay in a purely *in vitro* system, we confirmed that RHAU itself binds to random RNAs. In addition, the apparent K_D value of RHAU-RNA interaction is ~ 7 nM, which correlates with so far known K_D values of other DEAH-helicases. Thus, this data suggests that RHAU does not necessarily require co-factors for the interaction with RNA. However, the possibility that RHAU-RNA complex formation may be enhanced and/or more specified by co-factors should still be taken into consideration since we know that NF90 can strongly accelerate the RHAU and ARE^{uPA} mRNA interaction.

4.3. The N-terminus, a crucial part of RHAU

According to the classification of putative RNA helicases by Abdelhaleem et al. (Abdelhaleem et al., 2003), RHAU belongs to the DEAH-box family containing a highly conserved C-terminus next to the conserved helicase core region. Studies of DEAH proteins in yeast showed that the specific function of each member is determined mainly by its N-terminus. For example, Prp16, the ortholog of mammalian DHX38, localizes in the nucleus and recognizes the spliceosome through its N-terminus (Wang and Guthrie, 1998). Its C-terminus enhances spliceosome binding, while the helicase core region drives the process by binding and hydrolyzing ATP. Our results show that the RHAU helicase follows the same principle, because its specific localization in SGs and the interaction RNA is governed by its N-terminal domain, where the unique RNA-binding domain is located.

Interestingly, the first 105 amino acids, which were identified to play a pivotal role in RHAU-RNA interaction and RHAU localization in SGs, contains the computationally predicted RNA-binding domain (3-75 aa) composed of an RNA-binding motif not yet characterized. The domain is located in an unstructured und flexible sequence composed of two adjacent motifs, the G-rich region (10-50 aa) and the RSM (54-66 aa). The latter is highly conserved among RHAU orthologs and it was not found in other proteins, suggesting that this motif confers a unique function on RHAU. However, after more detailed alignment, a small part of RSM (54-63 aa) shared quite high similarity with the K homology (KH) domain sequence (Lattmann, unpublished data). The KH module is a widespread RNA-binding motif that has been detected in such proteins as heterogeneous nuclear ribonucleoprotein K (hnRNP K) or FMRP (Darnell et al., 2005; Siomi et al., 1993). In addition, a potential RGG-box motif (47-49 aa), which has been reported previously to be involved in the RNA interaction of several proteins, is present in the G-rich region of RHAU. For example, two RGG-box sequences identified at the C-terminus of the FMRP protein were shown to be necessary for both an RNA and G4-RNA interaction of FMRP and also for the induction of SG formation by EGFP-FMRP overexpression (Mazroui et al., 2002; Ramos et al., 2003). FMRP also contains KH domain that is involved in RNA interaction but does not take part in FMRP localization in SGs (Mazroui et al., 2002). Moreover, Caprin-1, a SG-associated protein, contains three RGG-box motifs in its C-terminus that are also important for its specific RNA interaction and induction of SG assembly (Solomon et al., 2007). Our analyses of the RHAU N-terminus show that the deletion of the G-rich region together with the RGG-box motif reduces RNA-binding activity of RHAU and its accumulation in SGs. This suggests that the RGG-box motif may be involved in the RNA interaction and localization of RHAU in SGs.

However, the G-rich region and the RGG-box motif alone were not sufficient for the recruitment of a fused β-galactosidase reporter protein to SGs (see Figure 16B). Only the complete RNA-binding domain enables RHAU to localize in SGs, indicating that a domain other than the G-rich region and RGG-box motif is responsible for RHAU recruitment to SGs. The detailed nature of the cooperation between the G-rich region and the RSM (KH domain) and the underlying SG-recruitment mechanism are challenging questions for the future.

In addition, the N-terminal deletion mutant of RHAU (105:1008aa), which lacks the ability to associate with RNA, lost also the potential to interact with proteins that bind to RHAU only in the presence of RNA (Pauli, unpublished data). However, the mechanism of how RHAU may associate with proteins in an RNA-dependent manner is not yet clear. It could be that RHAU first binds to RNA via an RNA-binding domain and then recruits "free" proteins on the target RNA or RHAU may interact with already RNA-associated proteins or RHAU actually never associated directly with these proteins and just use the same RNA template as a target. Furthermore, it is possible that the N-terminus itself binds to RNA-associated proteins directly without associating with RNA at all and that this interaction allows RHAU helicase core domains containing RNA-binding motifs to approach RNA and bind to it. Since our double-filter RNA-binding assays have shown that the N-terminal domain (1:200aa) binds to RNA in a purely *in vitro* system without requirement of co-factors, the later option can be excluded. To investigate all possibilities of how the N-terminus is involved in an RNA-dependent interaction of RHAU with its protein partners, more experiments are required.

In vitro assays demonstrated that the N-terminus of RHAU can bind to poly(U) and that this interaction is dose dependent (see Figure 15B), which indicates that RHAU may display a sequence specificity. Similar data have been collected with two other yeast DEAH-helicases, Prp22 and Prp43, concerning their interaction with poly(A) (Tanaka and Schwer, 2005; Tanaka and Schwer, 2006). However, in one report, authors showed that the length rather than the sequence of RNA molecules plays an important role in helicase-RNA interactions (Tanaka and Schwer, 2006). Since we have used a mixture of differently long poly(U) oligomers in our study, we did not distinguish whether the length of RNA or its sequence determines RHAU-RNA complex assembly.

The protein alignment revealed that RHAU also harbors a putative nuclear localization signal (NLS) with PY motif, which for example has been found in HuR, hnRNP D or hnRNP A1 as a target binding sequence of transportin-1 (Imasaki et al., 2007; Lee et al., 2006). Indeed, disruption of this PY motif in RHAU resulted in the lost of its nuclear localization (Figure 12A). The potential interaction between RHAU and transportin-1 has not been yet tested. However, the immunofluorescent study

with different N-terminal truncated forms of RHAU indicates that its N-terminus contains two signals within, SG signal and NLS, which do not overlap and have a different impact on RHAU subcellular localization.

4.4. RHAU interacts with eIF3b in an RNA-independent manner

In the initiation step of protein synthesis, at least 12 different eukaryotic initiation factors (eIFs) interact with Met-tRNAi^{Met}, mRNA and the ribosomal subunits to stimulate the assembly of preinitiation complexes and to ensure that the correct AUG start codon is decoded (Holcik and Pestova, 2007). Evidence now indicates that most of the reactions in the initiation pathway are stimulated by eIF3, including assembly of the eIF2–GTP–Met-tRNAi^{Met} ternary complex, binding of ternary complex and other components of the 43S preinitiation complex to the 40S subunit, mRNA recruitment to the 43S preinitiation complex, and scanning the mRNA for AUG recognition (Hinnebusch, 2006). Upon stress, when translation initiation is arrested and polysomes disassemble, eIF3 plays a pivotal role in mRNP aggregation and subsequent SG assembly (Ohn et al., 2008). Therefore, eIF3 is essential for both translation and assembly of SG and PB *in vivo*. By co-immunoprecipitaition we could demonstrate an interaction between RHAU and eIF3b. The association was RNA-independent, because RNase treatment (micrococcus nuclease) of cell extracts did not disrupt the RHAU-eIF3b complex. This finding led us to speculate that an RNA-dependent localization of RHAU in SGs may be a consequence of eIF3b recruitment. Since RNAi depletion of endogenous eIF3b resulted in cell death, we could not test whether RHAU is recruited to SGs by eIF3b. In addition, a recent report has shown that SG-assembly occurs in an eIF3-dependent manner (Ohn et al., 2008), thus preventing us to determine whether eIF3b plays a role in RHAU recruitment to SGs because down-regulation of eIF3b leads to a global SG assembly arrest. However, upon normal and stress conditions, eIF3b still co-immunoprecipitated with the truncated form of RHAU lacking the first 105 amino acids. As reported above, this deletion mutant does not accumulate in SGs. Thus, it seems that the interaction of eIF3b and RHAU, which is formed already before stress, remains present also during stress but outside of SGs. These data indicate that RHAU is most probably involved in translation or is present together with eIF3b in specific mRNPs that do not accumulate in SGs. Indeed, the polysome profile of RHAU and eIF3b in normal conditions showed that RHAU distributed together with eIF3b in fractions enriched with both mRNPs and the 40S ribosome subunit (Figure 23), indicating that RHAU might be associated with translation initiation complexes. However, unlike eIF3b, RHAU was also detected in fractions of 60S and 80S

ribosome subunits, and partly in polysomes, which implies that RHAU might participate also in the elongation or subsequent steps of translation. Strikingly, translation elongation factors, eEF2 and eEF1, have been detected as binding partners of RHAU by mass spectrometry, and eEF2 was confirmed to associate with RHAU by co-immunoprecipitation assays (Pauli, unpublished data). Thus, RHAU might be involved in translation via its interaction with eIF3b and eEF2.

Two recent reports independently revealed that the DEAD-box RNA helicase DDX3 associates with eIF3 in an RNA-independent manner and one of them suggests that DDX3 facilitates translation by resolving secondary structures of the 5'-untranslated region in mRNAs during ribosome scanning (Lai et al., 2008; Lee et al., 2008). In the other study, DDX3 was further reported to be a cap-dependent translational repressor, and this effect is thought to be mediated by a direct interaction between DDX3 and eIF4E (Shih et al., 2008). Importantly, by mass spectrometry DDX3 was identified as an RHAU binding partner (Iwamoto, thesis). However, the RNA-dependent association between RHAU and DDX3 was confirmed only once by coimmunoprecipitation assay (Iwamoto, unpublished data). To determine whether RHAU like DDX3 is involved in translation of transcripts forming secondary structures in the 5'-untranslated region, further studies have to be performed.

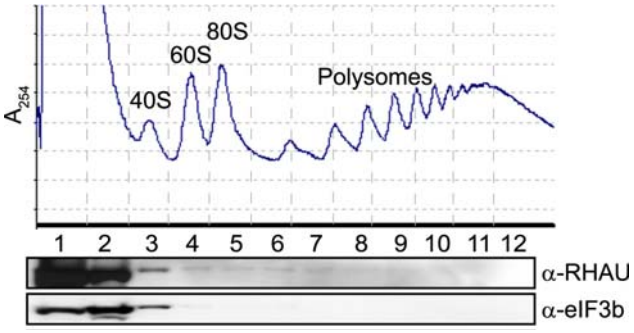


Figure 23. RHAU is distributed mostly in mRNP and 40S ribosome subunit containing fractions like eIF3b. HeLa cytoplasmic extract was centrifuged through a continuous 15–55% sucrose gradient. The polysome profile was plotted by A254 values (top). Proteins were recovered from 12 fractions for analysis. Immunoblotting was performed using anti-RHAU or anti-eIF3b (bottom).

4.5. ATPase deficient mutant of RHAU

Our results, showing that the abrogation of RHAU ATPase activity increases the apparent amount of associated RNA, are in good agreement with generally assumed models of RNA duplex unwinding, where ATPase activity plays a role in a relaxation of the helicase-RNA complex. Based on the FRET analysis and structural information of complete DEAD-box helicases, one model, an inchworm model, is established on closed and open conformation of the helicase in response to ATP

binding and hydrolysis. Thus, the cooperative binding of ATP and RNA induces a closed conformation of helicases that is relaxed upon ATP hydrolysis, leading to the release of bound RNA (Caruthers et al., 2000; Cheng et al., 2005; Shi et al., 2004; Theissen et al., 2008). On the other side, the other model, a Brownian model, is established on different affinities of DExH helicases to RNA upon ATP binding. So far it is not obvious to which model RHAU belongs. Up to now we know that the ATPase deficient mutant cannot hydrolyze ATP (Tran et al., 2004), but it is not clear whether the mutant can bind to ATP. Furthermore, we do not know whether ATP binding can cause changes in the conformation of RHAU. However, our CLIP data clearly showed that the ATPase deficient mutant is able to pull down more RNA than RHAU wild type. Based on the report about Prp22, a DEAH-box helicase, that releases bound RNA upon ATP hydrolysis, thereby reducing its apparent RNA-binding affinity (Tanaka and Schwer, 2005), we favor the idea that the ATPase deficient mutant of RHAU cannot release already bound RNA.

We formerly reported that the ATPase-deficient mutant of RHAU is excluded from the nucleus (Iwamoto et al., 2008). However, the nuclear exclusion of the ATPase-deficient mutant DAIH is not a consequence of it being trapped in the cytoplasm by a strong, irreversible interaction with RNA, as we proposed previously. Since the N-terminus-deleted ATPase-deficient mutant DAIH-(105:1008), which shows markedly reduced RNA interaction, remains mainly in the cytoplasm (Figure 17D), the nuclear-cytoplasmic translocation of RHAU is most probably regulated by its ATPase activity. A similar role for ATPase activity in nuclear export via the CRM1 protein was reported previously for the DEAD-box helicase An3 (Askjaer et al., 2000).

4.6. Kinetics of RHAU shuttling into and out of SGs

Many SG-associated proteins are RNA-binding proteins involved in different intracellular processes mediated by or acting upon mRNA. Several of these proteins, including TIA-1, TTP, hnRNP A1, PCBP-2, MLN51 and G3BP (Baguet et al., 2007; Fujimura et al., 2008; Guil et al., 2006; Kedersha et al., 2000; Kedersha et al., 2005), have been shown by FRAP analysis to rapidly shuttle into and out of SGs. This leads to the notion that SGs are not static storage centers for untranslated mRNA, but rather dynamic structures that sort individual transcripts for storage, re-initiation or decay (Anderson and Kedersha, 2006; Anderson and Kedersha, 2008). FRAP analysis also revealed that proteins behave with differing kinetics. For instance the PABP-1 protein exhibits a slower and only partial recovery (60%) 30 s after bleaching compared with TIA-1, which shows a complete recovery (Baguet et al., 2007;

Kedersha et al., 2000; Kedersha et al., 2005). Like G3BP, wild-type RHAU shows very rapid shuttling into and out of SGs. In striking contrast, the kinetics of the ATPase-deficient mutant were different from RHAU and G3BP: GFP signal recovery in SGs was as slow as for PABP-1, showing that ATP hydrolysis affects the shuttling kinetics of RHAU into and out of SGs. Furthermore, our analysis showed that the FMRP protein is not a member of the group of rapidly shuttling RNA-binding proteins. Rather, it exhibits slow shuttling like the FAST protein and, thus, may play a scaffolding role in SGs (Kedersha et al., 2005). In contrast to FMRP, RHAU exhibits the rapid mobility of G3BP, TIA-1 and TTP, which suggests it may be involved in RNPs remodeling or their recruitment (retention) to (in) SGs, as suggested for TIA-1 and TTP (Kedersha et al., 2000).

4.7. RHAU influence PB assembly under normal conditions

PBs are sites where untranslated mRNAs accumulate as mRNPs. The information about mRNPs fate – translation, silencing or decay – is carried by associated proteins or regulatory RNAs (Eulalio et al., 2007a). Strikingly, all proteins that function in the 5′ to 3′ mRNA-decay pathway have now been shown to localize to PBs (Parker and Sheth, 2007). By contrast, the exosome (3′ to 5′ mRNA-decay) is not detected in PBs but in other cytoplasmic foci, which indicates a degree of compartmentalization of mRNA-degradation pathways in the cytoplasm (Lin et al., 2007). However, depletion of several known PB-associated proteins principally resulted in either PB loss or increase in the size and number of PBs. Although, over-expressed EGFP-RHAU localizes only very slightly in some but not all PBs, knockout of RHAU leads to complete loss of PBs. Interestingly, the average number of PBs per cell, where RHAU was down-regulated to 30% of total protein level by RNAi, is strongly reduced from ~ 4,5 to 1, indicating that even the low level of RHAU protein is efficient in induction of PB assembly. However, RHAU is dispensable for PB assembly when cells are exposed to stress. In these cells, PBs are formed regardless of RHAU, indicating that during stress other signaling pathways, in which RHAU is not involved, induce PB assembly. Finding RHAU to be essential for PB formation upon normal conditions and knowing that miRNA silencing occurs in PBs, we tested whether RHAU is potentially involved in miRNA silencing. Indeed, our data suggest that RHAU is involved in translation repression mediated by let-7 miRNA (Figure 24). Nevertheless, the mechanism of how RHAU regulates PB assembly and de-represses let-7 targets is still unidentified. While RHAU belongs to RNA helicases that unwind RNA duplexes or disrupt protein-RNA interactions, it might be involved in mRNP remodeling after translation arrest leading to the recruitment of

these silenced transcripts to PBs. All together, these data suggest that RHAU might modulate translation and subsequently PB assembly by its association with eIF3b, but the possible role of RHAU in translation still needs to be studied in more detail.

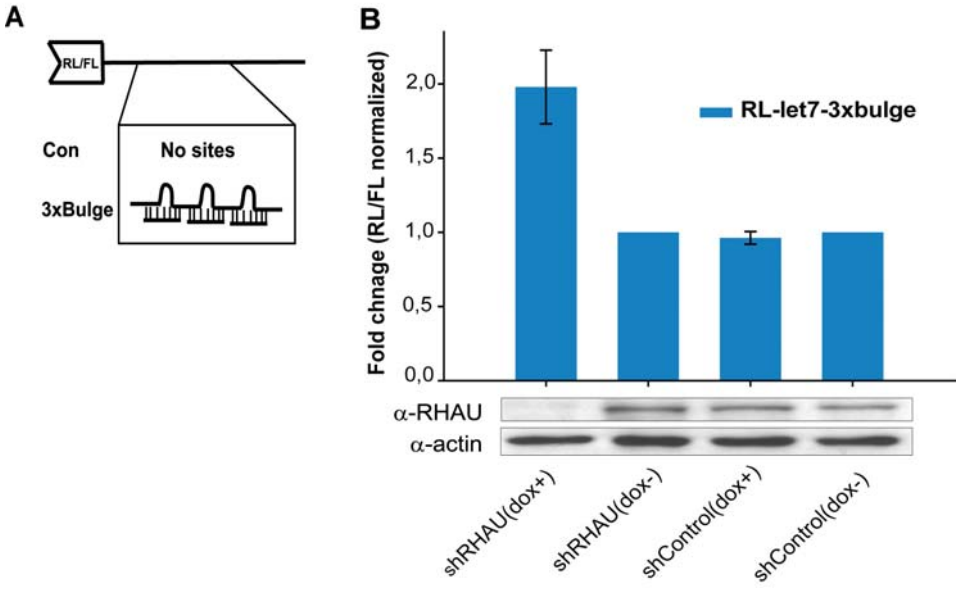


FIGURE 24. RHAU is involved in miRNA-mediated repression. (A) Schematic representation of mRNA reporters used in the experiment (adapted from (Pillai et al., 2005)). (B) T-REx-HeLa cells were pre-treated 7 days in medium with (dox+) or without (dox-) doxycycline to induce knockdown of endogenous RHAU in shRHAU cell lines. The protein level of RHAU in shControl cell lines was not changed after doxycycline treatment. Pre-treated cells were cotransfected with RL-3xBulge reporter and FL-Con luciferase vector as a control. 24 hours later, dual Luciferase assay was performed and the relative Luciferase activity was represented as the ratio of RL/FL, which was set as 1 in doxycycline non-treated cell lines [shRHAU(dox-) and shControl(dox-)]. Mean ± s.e.m. were derived from five independent transfections performed in triplicates.

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ErasmusFellowship (University of Bologna, Italy) Jan. 2002 - Apr. 2002

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PUBLICATIONS

Chalupníková K., Lattmann S., Selak N., Iwamoto F., Fujiki Y., and Nagamine. Y., *Recruitment of the RNA helicase RHAU to stress granules via a unique RNA-binding domain.* (2008), ***J. Biol. Chem.***, (accepted, in press January 2009)

Iwamoto F., Stadler M., Chalupníková K., Oakeley E., and Nagamine.Y., *Transcription-dependent nucleolar cap localization and possible nuclear function of DExH RNA helicase RHAU*. (2008), ***Exp Cell Res.***, (314)1378-1391

CONFERENCES ATTENDED

09/2008	**FMI Annual Meeting**, Grindelwald, Switzerland Oral presentation
09/2007	**FMI Annual Meeting**, Grindelwald, Switzerland Poster presentation
06/2007	**12th Annual Meeting of the RNA Society**, Madison, USA Poster presentation
09/2006	**FMI Annual Meeting**, Murten, Switzerland Poster presentation
03/2006	**FMI and Novartis Epigenetics Meeting 2006**, Les Diablerets, Switzerland Poster presentation
09/2005	**FMI Annual Meeting**, Pontresina, Switzerland Poster presentation
08/2005	**EMBO – Workshop – Mechanisms and Regulation of mRNA Turnover,** Arolla, Switzerland Poster presentation
09/2004	**FMI Annual Meeting**, Crans Monatana, Switzerland Poster presentation

EVENT ORGANIZATION

Member of the Organizing Committee of the Career Guidance Conference in Life Science under the hospices of Novartis, Basel, Switzerland, March 2005