Biological roles of DExH RNA helicase, RHAU

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Fumiko Iwamoto
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In this thesis, I have described the work carried out on a single protein called RHAU, dealing with aspects of protein localization in cells, the regulation of global gene expression by different mechanisms, and cellular stress responses. RHAU, RNA helicase associated with AU-rich element, was originally identified from the results of RNA affinity chromatography using the AU-rich element of uPA messenger RNA. RHAU was characterized as a factor accelerating AU-rich element-mediated mRNA degradation.

The aim of this present study was to investigate possible role(s) of RHAU in mammalian cells. In the first part of the study, dealing with the cellular localization of RHAU using biochemical fractionation and microscopic analysis, I found that RHAU is predominantly localized in the nucleus, despite the fact that mRNA degradation occurs in the cytoplasm. In HeLa cells, RHAU is localized throughout the nucleoplasm with some concentration in nuclear speckles in a manner dependent on ATPase activity. Furthermore, it has been shown that transcriptional arrest changes RHAU localization to nucleolar caps, where it is co-localized with other RNA helicases, p68 and p72. This suggests that RHAU is involved in transcription-related RNA metabolism in the nucleus.

The discovery that RHAU is localized mainly in the nucleus prompted me to consider the nuclear functions of RHAU, which led to a second project using RHAU-knockdown. To see whether RHAU affects global gene expression either transcriptionally or posttranscriptionally, microarray analysis using total RNA prepared from RHAU-depleted HeLa cell lines was performed to measure both the steady-state mRNA level and mRNA half-life by actinomycinD-chase. Most transcripts whose steady-state levels were affected by RHAU knockdown showed no change in half-life, suggesting that these transcripts were the subject of transcriptional regulation.

In cells depleted of RHAU using shRNA, retardation of growth was observed, especially when cells were stressed, for example, by serum-starvation. RHAU indeed affected more genes in starved conditions, suggesting the involvement of RHAU in cellular stress responses in mammalian cells.

Overall, the results suggest that each RNA helicase is involved in various cellular processes. RHAU has dual functions, being involved in both the synthesis and degradation of mRNA in different subcellular compartments. Thus, my work presents a novel view of RNA helicases as proteins with multiple functions in different cellular contexts.
1.01. Regulation of gene expression at different steps

Control of gene expression in eukaryotes is a complex process involving numerous steps, from the binding of transcription factors to their target sequence to the post-translational modification of proteins. Specific factors, either proteins or functional RNAs, are involved at each step to maintain cellular homeostasis in changing environments. Figure 1 illustrates six steps important for the control of gene expression in eukaryotic cells. In principle, all steps leading from DNA to protein can be regulated and will influence total protein activity. The initial transcriptional control is of paramount importance to the expression of most genes because only this step can result in “on” or “off” of RNA synthesis, which dramatically changes protein levels. The subsequent steps 2-4 are regulatory steps acting on mRNA. Many mammalian mRNAs require premRNA splicing and some are processed or edited. These steps, including alternative splicing, contribute to variation in mRNA. Since maturation of mRNAs is required for nuclear export and mRNA stability, malfunction of these mechanisms is coupled to abnormal states of gene expression. The third step of mRNA transport is important for maintaining RNA quality because it ensures that all nuclear metabolic events of mRNAs are completed prior to export from the nucleus. Thus, this step is intimately connected to other steps in RNA metabolism. Some mRNAs must be transported to specific locations in the cytoplasm, resulting in localized protein synthesis (St Johnston 2005). The fourth step of mRNA degradation changes cytoplasmic concentrations of mRNA more directly. This is a highly regulated step responsible for determining mRNA amount and turnover. Gene silencing mediated by small RNAs like micro RNAs (miRNAs) is also based on mRNA degradation as well as the suppression of
translation, both of which have a large impact on total protein. The rate of translation is also regulated and varies depending on cellular conditions. Finally, post-translational modifications, such as phosphorylation, methylation and acetylation, regulate protein activities, and protein degradation, e.g. by ubiquitin-dependent proteolysis, is also highly regulated depending on the cellular status.

It is, therefore, important to consider the regulation of these different steps when investigating control of gene expression. Referring to total mRNA, at least the two different parameters of synthesis and degradation must be considered. As discussed in the next section, not only the rate of transcription but also the regulation of mRNA degradation can greatly affect fine-tuning of gene expression in eukaryotic cells. Importantly, there is evidence for cross-talk between these different steps (Reed 2003). For example, factors of the general transcription machinery, such as the C-terminal domain of RNA polymerase II (CTD) and the transcription elongation factor TAT-SF1, associate with components of the splicing pathway (Abovich and Rosbash 1997; Morris and Greenleaf 2000; Fong and Zhou 2001). mRNA export is also physically and functionally linked to transcription and splicing: export factors are loaded on to synthesized mRNAs co-transcriptionally (Strasser et al. 2002; Vinciguerra and Stutz 2004; Kohler and Hurt 2007). The coupling of cellular pathways requires the interaction of molecules involved in the different pathways and this is achieved by regulated intracellular transport of molecules by energy-dependent mechanisms. In this thesis, I report on the investigation of one particular protein that associates with RNA and various proteins at different cellular locations. The study was initiated with the single question of why this molecule is actively translocated into different cellular compartments. The results I report here do not fully answer the question but suffice to put forward the hypothesis that the protein is transported to fulfil different functions in different intracellular locations and also that it mediates between different steps of RNA metabolism through its association with various molecules.

1.02. Regulation of mRNA stability

1.02.01. Regulation of mRNA stability contributes to changes of gene expression.

Regulation of gene expression at the level of mRNA stability is an important and highly regulated process. Half-lives vary considerably between mRNA species. In yeast, mRNA half-lives range from 1 min to more than 100 min (Gouka et al. 1996) and in mammalian cells from less than 20 min to over 50 h (Stolle and Benz 1988). For a given mRNA molecule, stability changes in response to extracellular stimuli. T cell activation, for example, dramatically stabilizes lymphokine mRNAs, such as IL2, GM-CSF, INF-γ, and TNF-α, contributing to increases in total mRNA (Lindstein et al. 1989). Global gene expression analyses using microarrays have revealed that regulation of mRNA stability accounts for about 50% of all changes in response to cellular signals (Fan et al. 2002; Cheadle...
et al. 2005). These data all clearly suggest the existence of a mechanism to maintain and regulate mRNA stability in cells.

The rapid induction as well as reversal of gene expression allows prompt changes in mRNA steady-state levels in response to changing environmental conditions. Transiently expressed genes, early response genes, are induced by various cellular stimuli, with transcript levels increasing as much as 50-fold in a short time. Change in mRNA stability is especially critical for the rapid reduction of mRNA following induction, as a reduction of transcription rate alone is not sufficient to quickly reduce cellular mRNA levels. Microarray analysis has shown that 74% of mRNAs that declined in response to stress did so not by inactivation of transcription but by mRNA destabilization (Fan et al. 2002). Computational models also predict that the best strategy to reduce cellular mRNA concentration is a decrease in half-life. Conversely, induction of transcription rather than stabilization of mRNAs seems to be effective for increasing mRNA concentration in a short time (Perez-Ortin et al. 2007).

1.02.02. mRNA degradation machinery

Most mRNAs in eukaryotes are 5'-capped and 3'-polyadenylated. These mRNA modifications occur shortly after initiation of transcription and are both important in the protection of mRNAs from exonucleolytic degradation. Poly(A) tail shortening is often the initial and rate-limiting step of mRNA degradation in yeast and mammalian cells. Three independent complexes possessing poly(A)-specific 3'-exoribonuclease activities exist in most eukaryotic cells but yeast and Drosophila contain only two, CCR4-CAF1 and PAN2-PAN3. In mammalian cells, these two complexes represent the major cytoplasmic deadenylation activity (Yamashita et al. 2005). Decay of both stable and nonsense-codon-containing unstable β-globin mRNAs is first initiated by the PAN2-PAN3 complex and then by CCR4-CAF1 in the second phase of deadenylation, which is followed by degradation of the mRNA body (Yamashita et al. 2005). In contrast, higher eukaryote-specific poly(A) exoribonuclease, PARN, seems to deadenylate specific sets of mRNAs, such as those containing AU-rich elements (ARE). It has been shown that PARN catalyses ARE-dependent deadenylation in vitro (Lai et al. 2003).

As soon as poly(A) is removed, mRNA is destined for rapid exonucleolytic decay, either from the 5' or 3' end. The 5'-to-3' decay pathway starts by removal of the cap by decapping protein 2 (DCP2) together with the other activators DCP1, LSM1-7 complex and Pat1. Following decapping, 5'-to-3' exoribonuclease XRN1 digests the mRNA body (Wilusz et al. 2001; Meyer et al. 2004). In the other pathway, deadenylation is followed by 3'-to-5' decay of the RNA body mediated by the exosome. The exosome consists of 9-11 subunits of 3'-to-5' exonucleases
forming a donut-like structure that progressively phosphorylizes the mRNA body from 3' ends (Liu et al. 2006).

Although deadenylation-dependent exonucleolytic decay is the major mRNA degradation pathway in eukaryotes, mRNAs such as for insulin-like growth factor 2 (IGF2), c-myc, and transferrin receptor are degraded by endonucleolytic activities independent of deadenylation (Bernstein et al. 1992; Binder et al. 1994; Scheper et al. 1995; Scheper et al. 1996). This process is mediated by specific endonucleases and particular sets of mRNAs, often in response to extracellular stimuli. Endoribonuclease RNaseL, for example, plays an important role in viral infection and the interferon response (Silverman 1994; Li et al. 1998; Li et al. 2000).

1.02.03. cis-element

mRNA stability is controlled by regulatory cis-acting elements on transcripts and their trans-acting binding proteins (Ross 1995; Guhaniyogi and Brewer 2001). Cis-acting elements are frequently found in 3'-untranslated regions (UTR), for example, of AU-rich elements (ARE; a destabilizing element) (Chen and Shyu 1995), iron-response elements (IRS; an iron-regulatory element also found in 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; Yu and Russell 2001; Lindquist et al. 2004) and others (Guhaniyogi and Brewer 2001). Regulatory elements are also found in the 5'UTR and even in protein-coding regions such as the c-jun response element in the 5'UTR of IL-2 mRNA (Chen et al. 1998) and coding elements of c-myc mRNA, which destabilize the message (Yeilding and Lee 1997). Each element associates with specific binding partners that can recruit or avoid associating mRNAs to/from degradation complexes, depending on the cellular conditions, thus regulating mRNA stability.

1.03. AU-rich element-mediated mRNA decay (AMD)

1.03.01. AU-rich element

By far the best-studied cis-element is ARE, located in the 3'UTR of many transcripts encoding, for example, cytokines, proto- oncoproteins and transcription factors (Khabar 2005). In 1986, a conserved sequence enriched with adenylate and uridylylate was found in the 3'UTR of mRNAs encoding inflammatory mediators (Caput et al. 1986). This sequence was shown later to be responsible for the instability of granulocyte macrophage-colony stimulating factor (GM-CSF) message since it elicited the rapid decay of otherwise stable β-globin reporter
mRNA (Shaw and Kamen 1986). Subsequently, the ARE has been characterized as an instability regulatory element for numerous mRNAs encoding proteins with diverse cellular functions. A database of ARE-containing mRNAs predicts that 5–8 % of human genes encode transcripts containing AREs (Bakheet et al. 2006). AREs vary in sequence and length but most contain one or more copies of the octamer UUAUUUAU, the AUUUA core sequence of which is essential for the mRNA destabilization elicited by AREs. According to the classification by Chen and Shyu (Chen and Shyu 1995), class I AREs contain 1–3 non-tandem copies of the pentanucleotide AUUUA embedded within a U-rich region. Class II AREs contain two or more reiterated copies of this motif and class III ARE, exemplified by that found in c-jun mRNA, are U-rich sequences lacking AUUUA motifs (Peng et al. 1996).

1.03.02. Degradation machinery and AUBPs

To elicit rapid degradation, ARE must be recognized by the mRNA degradation machinery. ARE-RNA itself can interact with the exosome component PmScl-75 (Mukherjee et al. 2002) and the in vitro reconstituted exosome drives efficient degradation of AU-containing RNA but not the generic RNA without ARE (Liu et al. 2006). This suggests that ARE-RNA has a higher affinity for the exosome than other stable mRNAs. However, the in vitro reconstituted exosome does not degrade poly(A)-tailed mRNA efficiently (Liu et al. 2006), whereas the immunopurified exosome from HeLa cells can degrade ARE-RNA with poly(A) tails (Chen et al. 2001), suggesting that further factors in addition to the exosome are required for efficient deadenylation and decay of ARE-RNA. A group of proteins termed ARE-binding proteins (AUBPs) with affinity for ARE has been shown to mediate AMD. Three AUBPs, 37-kDa isoforms of AUF1, KSRP, and tristetraprolin (TTP), have affinity for the exosome and the latter two factors are required for exosome-mediated AMD. TTP and its binding partner BRF1 are also involved in the 5′-to-3′ decay pathway, since they interact with a decapping complex (Kedersha et al. 2005; Lykke-Andersen and Wagner 2005). It has been shown that ARE stimulates decapping activity in HeLa cells (Gao et al. 2001). Furthermore, ARE-RNA is detected in cytoplasmic processing bodies (P-bodies), colocalizing with TTP, BRF1, and DCP1. P-bodies are cytoplasmic foci containing many components of 5′-to-3′ decay pathways, including XRN1, DCP1, and the LSM complex but not the exosome (Franks and Lykke-Andersen 2007), which suggests that ARE-RNA is degraded in P-bodies by the 5′-to-3′ decay machinery. However, Lin et al. reported that ARE-RNA is also present in distinct cytoplasmic granules containing the exosome (Lin et al. 2007). Furthermore, a significant but diffuse amount of ARE-RNA is found in the cytosol, suggesting that P-bodies may not be the only site of AMD (Lin et al. 2007).

It is currently unclear which pathways of exonucleolysis, either from the 5′ or 3′ end, contribute to AMD in mammalian cells. In siRNA approaches to
downregulate individual decay factors in HeLa cells, two groups (Stoecklin et al. 2006; Lin et al. 2007) showed that knockdown of the 5’-to-3’ pathway components XRN1 and LSM1 as well as the exosome components PmScl-75 and Rrp46 impaired AMD. Downregulation of two factors, one involved in 5’-to-3’ and another in 3’-to-5’ decay, produced the largest impairment of AMD, implying that both pathways are active in AMD and that some are not redundant. In addition to these two distinct pathways, miRNA-mediated gene silencing has also been implicated in AMD in *Drosophila* and HeLa cells. RISC complexes, required for miRNA and siRNA-mediated gene silencing, are directed to ARE-RNA by imperfect base pairing between miR-16 and AREs, which eventually facilitates degradation of ARE-RNA (Jing et al. 2005). Most recently, ARE has been implicated in upregulation of translation by fragile-X mental-retardation-related protein 1 (FXR1) and an essential miRNA-loading factor, Argonaute2 (Vasudevan and Steitz 2007). A relationship between ARE and translational regulation has also been described in another pathway, in which HuR (an AUBP) suppresses miRNA-mediated translational repression (Bhattacharyya et al. 2006). Although the direction of ARE-mediated translational regulation may vary and the mechanism itself is not yet fully understood, these reports suggest that ARE is a mediator of mRNA degradation and translation. Interestingly, not only ARE-RNAs are localized in P-bodies but also miRNAs and miRNA-regulatory factors (Liu et al. 2005; Eulalio et al. 2007). ARE-RNA may affect gene expression in various ways in specific cytoplasmic locations.

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

RHAU (alias: DHX36) is a putative RNA helicase identified by RNA-affinity chromatography using ARE of uPA mRNA and human HeLa nuclear extracts (Tran et al. 2004). It was termed RHAU for “RNA helicase-associated with AU-rich elements” since it contains DExH-conserved motifs giving rise to the putative RNA helicase activity.

1.04.01. RHAU as a destabilizing factor of ARE-RNA.

As RHAU has a specific affinity for the ARE sequence of mRNA, the effect of RHAU on AMD was studied first. In HeLa cells, overexpression of RHAU caused destabilization of reporter ARE (β-globin mRNA harbouring uPA-ARE) as well as endogenous uPA mRNA. Examination of the *in vitro* mRNA decay system also showed that recombinant RHAU protein accelerates deadenylation and decay of β-globin-ARE<sub>uPA</sub>. In contrast, downregulation of RHAU by siRNA in HeLa cells stabilized the reporter ARE. It was concluded that RHAU is a factor promoting degradation of ARE-containing mRNAs. RHAU requires ATPase activity, since the mutant E335A, which is unable to hydrolyze ATP, has no effect on the decay of ARE<sub>uPA</sub> either *in vivo* or *in vitro*. In this study, a destabilizing effect of RHAU was found for uPA-ARE but not other types of mRNA, e.g. for the uPA receptor,
which contains a different class of ARE. This suggests that RHAU has a specific role in AMD (Tran et al. 2004).

1.04.02. RHAU as a G4 DNA resolvase.

In 2005, Akman's group isolated RHAU as the major source of guanine quadruplex (G4) DNA-resolving activity in HeLa cell lysates (Vaughn et al. 2005). G4 DNA is a highly stable DNA structure composed of several layers of a guanine tetrad in which four guanine residues from the same or different strands are linked by Hogsteen-type hydrogen bonding (Maizels 2006). G4 structures are expected to occur in guanine-rich regions such as telomeres, ribosomal DNA, and immunoglobulin class switch regions, as well as in the promoter regions of several proto-oncogenes such as c-myc and c-kit, the transcriptional activity of which is repressed by this structure (Siddiqui-Jain et al. 2002; Maizels 2006; Shirude et al. 2007). Therefore, G4-resolving activity is expected to activate the transcription of genes containing G4 in the promoters. However, the biological functions of G4-DNA and G4-resolving enzymes (G4-resolvase) \textit{in vivo} are largely unknown and the physiological significance of RHAU G4 resolvase activity has also not been defined.

1.04.03. Affinity to other molecules - protein and RNA

Several RNA-related proteins have been found to interact with RHAU either via RNA or not. Examples of mRNA degradation factors include the exosome components, PM/Scl100 and hRrp40p, and poly(A) ribonuclease PARN, which interact with RHAU even in the absence of RNA (Tran et al. 2004). It was suggested, therefore, that RHAU first promotes deadenylation of ARE-RNA, and then recruits it into the exosome for rapid degradation. RHAU also interacts with further AUBPs, namely NF90 and HuR, that were co-precipitated in the initial RNA-affinity chromatography by which RHAU was isolated. These AUBPs both interact with RHAU in a manner dependent on RNA. In the case of NF90, strong interaction was observed in the presence of uPA-ARE but not IL2-ARE or mutated uPA-ARE. Downregulation of NF90 stabilized uPA-ARE. Thus, it is likely that both proteins have very specific roles in promoting decay of uPA-mRNA (Akimitsu, unpublished data).

RHAU was first isolated from HeLa nuclei with uPA-ARE oligonucleotides (Tran et al. 2004). However, \textit{in vitro} RNA electrophoretic mobility shift assays (REMSA) using recombinant RHAU protein and ARE-uPA showed that RHAU by itself has little interaction with ARE. The interaction was observed more clearly when protein and RNA were crosslinked, suggesting that RHAU interacts with RNA only transiently (Akimitsu and Lattmann, unpublished data). It seems that additional proteins are required for a more stable association of RHAU with RNA. One such protein is NF90. The intensity of the interaction of RHAU with uPA-
ARE was increased 2.7-fold by addition of recombinant NF90. Unlike RHAU, NF90 itself has strong affinity for uPA-ARE and, thus, NF90 may promote the interaction between RHAU and ARE that is required to stimulate mRNA degradation (Akimitsu, unpublished data).

1.04.04. Evolutional conservation and expression pattern.

According to sequence alignment data, RHAU is found in every clade in the Metazoa except for the phylum Nematode. The yeast DEAH-box protein YLR419w shows similarity to five human DExH proteins, including RHAU. Thus YLR419w is probably a common ancestor of these proteins (see Figure 2). YLR419w is dispensable in yeast and has not been characterized so far (Colley et al. 2000). RHAU is highly conserved, especially in vertebrates, through the central helicase core motifs 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 (Lattmann, unpublished data).

In humans, RHAU is moderately expressed in most tissue and cell types but is especially highly expressed in lymphocytes such as T-, B-, and NK cells, as well as in their precursors (Human GeneAtlas GNF1H, http://symatlas.gnf.org/SymAtlas/). Northern blot analyses of various mouse tissues showed highest expression of RHAU in thymus, also suggesting a possible role for RHAU in the immune system (Akimitsu, unpublished data). Bone marrow and blood were not tested. Many mRNAs encoding cytokines in immune cells contain ARE sequences and their expression is tightly controlled by mRNA degradation. RHAU may be involved in such regulation in lymphocytes.

1.04.05. Intracellular localization.

RHAU was originally identified in HeLa nuclear extracts. Ectopically expressed HA-RHAU was predominantly localized in the nuclei of HeLa cells (Tran et al. 2004). This localization pattern raised the possibility that RHAU has other functions in the nucleus, since mature mRNA degradation occurs in the cytoplasm or in cytoplasmic bodies. This hypothesis will be discussed further.
1.05. RNA helicases

1.05.01. Structure.

RNA helicases are ATP-hydrolytic enzymes found in virus, bacteria, archaea and eukaryotes, where they are the largest protein family involved in RNA metabolism (Anantharaman et al. 2002). All currently known RNA helicases belong to the helicase superfamilies 1-4, which include both DNA and RNA helicases. They share a highly conserved helicase domain consisting of several motifs (Tanner and Linder 2001; Linder 2006). A few RNA helicases belong to helicase superfamily 1 (SF1), including Upf1, an enzyme required for nonsense-mediated decay (NMD), but most RNA helicases belong to helicase superfamily 2 (SF2). RNA helicases in SF2 are further classified into three groups, DEAD-box, DEAH-box and DExH-box, based on the amino acid sequence of motif II in the helicase domain. 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). RHAU belongs to the DExH-box protein family and, therefore, has the gene symbol of DHX36.

DExH/D proteins contain at least eight conserved motifs (I, Ia, Ib, and II-VI) in the helicase core domain. These motifs have been characterized by biochemical approaches as they have ATP-binding and hydrolytic activity (I, II, VI), bind to nucleic acids (Ia, Ib, IV), or coordinate polynucleotide binding and ATPase activity (III and V) (Tanner and Linder 2001). Furthermore, recent studies of protein structure have provided more information on the helicase domain structure. Up to now, structures of two DEAD-box proteins, eIF4AIII (Andersen et al. 2006; Bono et al. 2006) and Vasa (Sengoku et al. 2006), and one viral DExH-box protein, HCV NS3 (Kim et al. 1998; Mackintosh et al. 2006), have been determined in the presence of nucleic acids. The helicase domain is formed by two domains connected via a flexible linker region. Without ATP or nucleic acids, the two domains are relatively open, especially in DEAD-box proteins. ATP and/or nucleic acid binding bring the two domains into a more defined arrangement (Jankowsky and Fairman 2007). Thus, it is possible that binding to nucleic acid 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; Iost et al. 1999; Polach and Uhlenbeck 2002; Talavera and De La Cruz 2005). DExH proteins, in contrast, show ATP hydrolysis without RNA, although RNA can still stimulate ATP hydrolysis (Shuman 1992; Tanaka and Schwer 2005; Tanaka and Schwer 2006). The DExH protein HCV NS3 indeed shows less dramatic movements upon binding to ATP and nucleic acid (Kim et al. 1998; Jankowsky and Fairman 2007). Whether RNA-dependent or not, changes in conformation caused by binding to ATP seem to be an important feature of RNA helicases when acting as ATP-driven switches at specific points of RNA metabolism.
1.05.02. helicase and RNase.

DExH/D proteins are characterized as ATP-dependent RNA helicases since some of the proteins, but not all, exhibit unwinding activity on duplex RNA molecules in vitro. The unwinding activity of viral DExH protein HCV NS3 has been monitored at the single-molecule level using a 60-bp RNA hairpin, which supported the model that DExH proteins first load onto the single-strand region of RNA and then translocate along one of the strands in a unidirectional and progressive fashion (Dumont et al. 2006). In contrast to the viral DExH proteins that can unwind several dozen base pairs, DEAD-box proteins are normally unable to unwind long duplexes but only a few base pairs, in a manner different to that of DExH proteins (Jankowsky et al. 2000; Cordin et al. 2006). DEAD-box protein Ded1 directly loads onto the duplex region and spontaneously initiates strand dissociation from the loading region. Single-strand regions increase the efficiency of duplex unwinding but this facilitates the loading of enzymes rather than loading onto the single-strand region itself (Yang and Jankowsky 2006). The physiological importance of duplex RNA unwinding activity has been less characterized than the biochemical unwinding experiments. For some RNA helicases, helicase activity is clearly required for the biological role. Dbp4p dissociates duplexes of U14 snoRNA and pre-rRNA, and thus duplex-unwinding activity is required for release of U14 from pre-ribosomes, an essential step in the pre-rRNA processing pathway (Kos and Tollervey 2005). However, the actual substrates of many other RNA helicases are unknown and, thus, the relevance of their unwinding activity for their biological function has not been clarified, even though they unwind duplexes in vitro (Tanner and Linder 2001; Cordin et al. 2006; Linder 2006).

The results of recent studies suggested that the activity of RNA helicases is not restricted to rearrangement of RNA secondary structure, but that it includes the modification of protein-RNA interactions (Jankowsky and Bowers 2006). Two proteins, viral DExH NPH-II and yeast DEAD-box protein DED1, have been shown to dissociate proteins from RNA in an ATP-dependent fashion with four different substrate RNPs in vitro (Jankowsky et al. 2001; Fairman et al. 2004; Bowers et al. 2006). Although the dissociation rate constant depends on various helicases and substrates, both proteins are able to displace exon junction complex (EJC), a protein complex bound upstream of exon-exon junctions, from the single-stranded spliced mRNAs, indicating that unwinding activity is not required for this activity (Jankowsky et al. 2001; Fairman et al. 2004; Bowers et al. 2006). These authors have proposed a novel feature of RNA helicases as a remodeler of RNPs. Since RNAs are invariably complexed with various proteins, the rearrangement of RNA-protein interactions by an RNA helicase is likely to be a feature of every step of the RNA metabolic pathway.
1.05.03. Various functions of RNA helicases.

RNA helicases are involved in all aspects of RNA metabolism. 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 DEAD-box helicases and 14 DExH-box helicases have been identified so far (Abdelhaleem et al. 2003; Linder 2006) and functions have been assigned in various steps of RNA metabolism. Four DExH helicases are involved in pre-mRNA splicing and one in ribosomal RNA processing; all of these are essential in yeast. Although YLR490w, an ancestor of five human DExH proteins including RHAU, is not essential for yeast viability, DHX9, which is also known as RNA helicase A (RHA) or NDH II and shares the same yeast ancestor, is required for mouse embryonic development (Lee et al. 1998). This suggests that RHA has gained additional functions during evolution.

Whilst it looks as though a unique RNA helicase is engaged in each RNA metabolic step, many authors have reported that a single RNA helicase harbours multiple functions acting at different steps from transcription, splicing, and RNA export to mRNA stability (Fuller-Pace 2006). RHA is one such protein, playing many roles in the regulation of gene expression. In the nucleus, RHA 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 promoters of the p16INK4a and MDR1 genes (Myohanen and Baylin 2001; Zhong and Safa 2004) and activates their transcription. RHA is also involved in RNA export mediated by the constitutive transport element (CTE) (Tang et al. 1997; Tang et al. 1999), in RNA splicing by interacting with SMN (survival motor neuron complex), and in the translation of selected mRNAs (Hartman et al. 2006; Bolinger et al. 2007). Most recently, RHA has been identified also in the RNA-induced silencing complex (RISC) in HeLa cells, functioning as an siRNA-loading factor (Robb and Rana 2007).

In another case, DEAD box proteins p68 (DDX5) and p72 (DDX17) have been shown also to regulate transcription via interaction with various transcription regulators, such as β-catenin (Yang et al. 2006), MyoD (Caretti et al. 2006), Smads (Warner et al. 2004), HDAC1 (Wilson et al. 2004), and p53 (Bates et al. 2005). They, thus, play a role in epithelial mesenchymal transition, myogenesis, regulation of apoptosis, general transcriptional repression, and tumorigenesis, respectively. Furthermore, it has also been suggested that they are required for pre-mRNA splicing (Liu 2002; Lin et al. 2005) and alternative splicing (Guil et al. 2003) as well as the processing of rRNA and miRNAs (Fukuda et al. 2007). These observations indicate that a single RNA helicase plays many different roles, depending on interactions with various molecules in different cellular environments.
Figure 2. Human DExH proteins. Amino acid sequences of fourteen human DExH proteins were aligned by an online program, MAFFT ver.6 (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/). Graphic tree was made based on the sequence distances between each protein using Tree View 1.6.6. Essential (in red): lethal phenotype described in knockout organisms. Not essential (in blue): knockout organisms are not lethal. Lattmann, unpublished data.
1.06. Spatial controls of RNA and proteins involved in RNA metabolisms.

Compartmentalization is one of the most important features of cells. Eukaryotic cells are themselves surrounded by a plasma membrane that fulfills specific roles but they also include many different compartments, like organelles, each of which contains a characteristic set of enzymes and other molecules active in its specialized role. The nucleus, for example, stores compacted DNA and enzymes to synthesize mRNAs. The nuclear membrane separates the sites of RNA synthesis and protein synthesis, thus avoiding the translation of premature mRNAs to proteins. Further subdomains have been characterized inside organelles or in the cytosol. These cellular “bodies” are not surrounded by membranes but by a local accumulation of selected molecules that can be visualized by specific markers. The cytosol contains P-bodies as well as stress granules formed in response to stress. Both bodies contain mRNA and specific RNA-binding proteins and function as sites of mRNA degradation (P-body), mRNA storage (stress granule), and suppression of translation (both of these organelles) (Anderson and Kedersha 2002; Kedersha et al. 2005; Eulalio et al. 2007; Parker and Sheth 2007). The nucleus is more complex than the cytoplasm, fulfilling many different roles in a small space, such as storage of genomic DNA, synthesis, processing, and export of RNA, as well as the degradation of premature and incorrectly processed RNAs. Therefore, it has been suggested that the nucleus has a very precise layout that ensures the efficiency of assorted nuclear activities. Many nuclear bodies have been characterized, such as nuclear speckles involved in the storage, assembly, and modification of pre-mRNA splicing factors, PML bodies playing a role in transcriptional regulation of specific genes, Cajal bodies involved in snRNP and snoRNP biogenesis and posttranscriptional modification of newly assembled spliceosomal snRNAs, and polycomb bodies containing silencing proteins, etc. The numbers and sizes of these nuclear bodies vary depending on cell type and conditions, which suggest that they are involved in regulatory steps of cellular metabolism (Matera 1999; Lamond and Sleeman 2003; Matera and Shpargel 2006).
To understand biological role(s) of RHAU in mammalian cells.

To determine subcellular localization of RHAU and its regulations.

To determine RHAU target genes and mRNAs.

To determine the effect of RHAU depletion in mammalian cells.
Materials and Methods

2.01. Plasmids

Oligonucleotides used in this work are presented in Appendix I. Plasmid pTER was kindly provided by Hans Clevers (van de Wetering et al. 2003). To construct pTER-shRHAU1 and pTER-shRHAU2, annealed oligonucleotides of shRHAU1-s, shRHAU2-s and shRHAU1-as, shRHAU2 were inserted into BglII/HindIII sites of the pTER vector to target RHAU mRNA at the site 1344-1364 nt and 2570-2590 nt, respectively. pTER-shLuc was kindly provided by A. Hergovich and B.A. Hemmings (Hergovich et al. 2007).

To derive the N-terminal fusion plasmid pEGFP-RHAU, full-length RHAU was cut out from pcDNA3-HA-RHAU (Tran et al. 2004) using BamHI/XhoI and inserted into the BglII/SalI sites of pEGFP-C1 (Clontech laboratories, Inc., Mountain View, CA). To introduce the ATPase-deficient mutation, pEGFP-RHAU-E335A was made using site-directed mutagenesis with oligonucleotides (E335A-s and E335A-as) that mutate the Glu335 of RHAU to Ala. To derive RHAU truncated mutants with N-terminal EGFP-tags, truncated forms of RHAU cDNAs were amplified by PCR using specific primers containing restriction sites and inserted into the BglII/EcoRI sites of pEGFP-C1. To derive the C-terminal fusion plasmids pRHAU-EGFP and pRHAU-E335A-EGFP, full-length RHAU was amplified by PCR using the primers RHAU 2 fw BamHI and RHAU1008 rv EcoRI with the plasmids pcDNA3-HA-RHAU and EGFP-RHAU-E335A, respectively, and inserted into the BamHI/EcoRI sites of pEGFP-N1 (Clontech).

pcDNA3-Flag-RHAUsm was made by replacing the HA tag of pcDNA3-HA-RHAU in HindIII/BamHI sites with annealed oligonucleotides coding the Flag sequence. To introduce silent mutations in the RHAU expression vector at the shRHAU-targeting site, we did site-directed mutagenesis using oligonucleotides RHAUsm-s and RHAUsm-as to amplify, using PCR, a mutated vector that contained two point mutations, G1350A and A1353G. To derive pGEX-RHAU(1-200aa), truncated RHAU was amplified by PCR using the primers RHAU 2 fw BamHI and RHAU 200 rv EcoRI with the plasmid pcDNA3-HA-RHAU and inserted into BamHI/EcoRI sites of pGEX-2T (GE health care life sciences). To derive pcDNA3-HA-p68 and pcDNA3-HA-p72, full-length cDNA of p68 and p72 were amplified using the primers p68-fw/p68-rv and p72-fw/p72-rv, respectively, using cDNA derived by reverse transcription of purified HeLa total RNA, and inserted into BamHI/XhoI sites of the pcDNA3.1(+) vector. pcDNA3.1(+) was made by inserting the annealed oligonucleotide fragment coding the HA sequence into the HindIII/BamHI sites of the pcDNA3.1(+) vector (Invitrogen Corporation, Carlsbad, CA). pcDNA3.1-HDAC1FLAG and pcDNA3.1-HDAC3FLAG were kindly from P. Matthias (FMI). The sequences of all plasmids made by PCR-cloning were confirmed.

2.02. Cell culture and transfection

HeLa cells and COS7 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% CO₂. T-REx™-HeLa cells (Invitrogen) were maintained as above with the additional supplement of 3 μg/ml blasticidin (Invitrogen). T-REx™-HeLa cells were stably transfected with pTER-shRHAU or pTER-shLuc vectors using FuGENE6 (Roche Applied Science, Rotkreuz, Switzerland) and selected with zeocin (InvinoGen, San Diego, CA) at a final concentration of 450 μg/ml. Zeocin-resistant colonies were picked up as independent clones. The independent clones from same transfections were pooled in some experiments. To induce shRNA expression, cells were treated with doxycycline (Sigma-Aldrich Co.) at a final concentration of 1 μg/ml. HeLa-shRHAU1 cells, stably transfected with pTER-shRHAU1, were used in all experiments except Figure 22. They are indicated as HeLa-shRHAU cells in these figures. In Figure 22, two different cell lines targeting different region of RHAU mRNA, HeLa-shRHAU1 (same cell line as HeLa-shRHAU in the other figures) and HeLa-shRHAU2 that were stably transfected with pTER-shRHAU1 and pTER-shRHAU2, respectively, were used. Transient transfection of plasmid DNA using FuGENE6 was performed according to instructions provided by the manufacturer. We used 1 μg plasmid DNA and 3 μl FuGENE6 per 35-mm dish.

2.03. Antibodies

Mouse anti-RHAU monoclonal antibody was generated against a peptide sequence which corresponds to the C terminal of RHAU, aa991-1007, and which has previously been reported (Vaughn et al. 2005). Rabbit anti-H3-K9 trimethylation and rabbit anti-NDH II (RNA helicase A) antibodies were kindly provided by A.H. Peters (Peters et al. 2003) and S. Zhang (Zhang et al. 1995), respectively. Commercially obtained antibodies were: mouse anti-DRBP76 (for detecting NF90) and mouse anti-Cleaved PARP (Asp214) from BD Biosciences (San Jose, CA), mouse anti-TRF2 (4A794) from Novus Biologicals, Inc. (Littleton, CO), rabbit Cleaved Caspase-3 (Asp175) from Cell Signaling Technology, Inc. (Danvers, MA), mouse anti-HDAC1 (2E10) from Millipore Corporation (Billerica, MA), rabbit anti-Histone H3 from Abcam plc. (Cambridge, UK), mouse anti-Ku (p80) (Ab-2, Clone111) from Lab vision Corp. (Fremont, CA), rabbit anti-BTF antibody (BL2521) from Bethyl Laboratories, Inc. (Montgomery, TX), mouse anti-β-tubulin, mouse anti-SC35, and mouse anti-FLAG® M2 from Sigma-Aldrich Co. (St. Louis, MO), and mouse anti-GAPDH (6C5), goat anti-ERK1 (C-16)-G, rabbit anti-CRM1 (H-300), mouse anti-Oct1 (E-8), rabbit anti-hnRNP C1/C2 (H-105), rabbit anti-HA (Y-11), and mouse anti-GFP (B-2) from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Mouse antibodies were all monoclonal antibodies.

2.04. Protein extraction and Western blotting

To prepare total cell lysates, cells were lysed with NP40 buffer (50 mM Tris-HCl, pH 7.4, 120 mM NaCl, 1% NP-40, 1 mM EDTA, 5 mM Na3VO4, 5 mM NaF, 0.5 μg/ml aprotinin, 1 μg/ml leupeptin) on ice for 30 min and centrifuged at 11,000 × g for 5 min at 4°C to remove cell debris. Typically, 20 μg of the total cell lysate were loaded for Western blotting. Nuclear fractionation was followed by Fey’s
protocol as previously described (Fey et al. 1986). HeLa cells were collected using PBS and subsequently lysed with CSK buffer (10 mM PIPES, pH 6.8, 50 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5 % Triton-X100) with Complete® (EDTA-free) (Roche Applied Science) for 3 min on ice and centrifuged at 650 × g for 5 min. The pellet was dissolved in the nuclear extraction buffer (10 mM PIPES, pH 6.8, 300 mM sucrose, 3 mM MgCl₂, 1 mM EGTA, 0.5 % Triton-X100, 0.25 M (NH₄)₂SO₄) with Complete®, left for 5 min on ice and then centrifuged at 1000 × g to obtain soluble (supernatant) and chromatin-rich (pellet) fractions. Each fraction was mixed with SDS-PAGE loading buffer and sonicated briefly before loading into the SDS-PAGE for the Western blotting. To visualize the bands, we used either the direct infrared fluorescence detection method or the chemiluminescence method. For the fluorescent blots, IR dye 800CW-conjugated secondary antibodies were used at a dilution of 1:10,000 and quantified using an Odyssey infrared imager (LI-COR Biosciences UK Ltd., Cambridge, UK). For the chemiluminescent blots, we used horseradish peroxidase (HRP)-conjugated secondary antibodies at a dilution of 1:4,000 and ECL™ western blotting detection reagents (Amersham Biosciences, Piscataway, NJ). The membranes were exposed to Kodak X-Omat LS films.

2.05. Immunoprecipitation

8×10⁵ HeLa cells were seeded in 10 cm dishes and transfected the next day with total 6 μg of plasmids using 18 μl of FuGENE6. After 48 h of transfection, cells were collected and lysed with IP buffer (20 mM HEPES pH 7.5, 3 mM MgCl₂, 150 mM NaCl, 0.3% CHAPS) and briefly sonicated. To the lysates, 400U/ml RNasin (Promega Co., Madison, Wis.) or 10 μg/ml RNaseA plus 100 U/ml RNaseT1 was added. The lysate was suspended with anti-FLAG M2 affinity gel (Sigma-Aldrich Co.), rotated for 2 h at 4°C, and then washed with the IP buffer. The precipitates were redissolved and loaded into SDS-PAGE for the Western blotting using anti-FLAG and anti-HA antibodies.

2.06. in situ extraction

HeLa cells (8×10⁴ cells per well) were seeded in 12-well plates with coverslips and transfected the next day with 500 ng of plasmids using FuGENE6. After 48 h of transfection, cells were washed with ice-cold PBS once and added 0.1 % Triton-X100 in PBS for 5 min on ice to permeabilize plasma membranes. Permeabilized cells were further treated with DNase or RNase by adding 100 U/ml DNase I plus 400 U/ml RNasin in CSK buffer or 20 μg/ml RNase A plus 100 U/ml RNase T1 in CSK buffer, respectively, and incubated at room temperature for 20 min. Cells were washed twice with PBS and fixed with 3.8 % paraformaldehyde in PBS for 10 min at room temperature. Cells were further double stained using anti-BTF (transcription factor, (Haraguchi et al. 2004)) antibody to stain nucleus both in DNase- and RNase-treated cells as well as DAPI to confirm breakdown of DNA in DNase-treated cells. Images taken by confocal microscope were analyzed to obtain numbers of EGFP-positive cells out of about 200 cells visualized by the BTF-staining.
2.07. Immunocytochemistry and image processing

HeLa cells (8×10⁴ cells per well) were seeded in 12-well plates with coverslips and transfected the next day with 500 ng of plasmids using FuGENE6. For inhibition of transcription, Actinomycin D (ActD: AppliChem GmbH, Darmstadt, Germany) or dichlororibofuranosyl benzimidazole (DRB: Sigma-Aldrich Co) was added 2 h before fixation to a final concentration of 5 μg/ml or 25 μg/ml, respectively. Cells were fixed with 3.8% paraformaldehyde in PBS 48 h after transfection, permeabilized with 0.5% Triton-X100 in PBS and blocked with 5% horse serum in PHEM buffer (25 mM HEPES, 10 mM EGTA, 60 mM PIPES, 2 mM MgCl₂, pH 6.9). Cells were incubated with primary antibodies in the same buffer at 4°C over night. Mouse anti-SC35, rabbit anti-H3 K9 trimethylation, rabbit anti-HA, rabbit anti-BTF, and mouse anti-TRF2 antibodies were used at dilutions of 1:4,000, 1:500, 1:200, 1:400, and 1:500, respectively. We used Cy2™, Cy3™, or Cy5™ - conjugated donkey secondary antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) at a dilution of 1:2,000 with 2.5% horse serum in PHEM buffer at room temperature for 40 min. Cells were then incubated with 500 ng/ml DAPI (Santa Cruz Biotechnology, Inc.) to identify the nuclei. ProLong® Gold antifade reagent (Invitrogen) was used for mounting. Images were acquired on a confocal microscope (LSM 510 META, Carl Zeiss GmbH, Jena, Germany) with a Plan-NeoFluar ×40/1.3 oil DIC objective (optical section ~1 μm). To avoid cross-talk, DAPI, EGFP, Cy3, and Cy5 fluorescence was detected sequentially using the 405 nm laser in combination with the BP420-480 nm filter, the 488 nm laser with the BP505-550 nm filter, the 543 nm laser with the BP561-646 nm filter, and the 633 nm laser with the BP646-732 nm filter, respectively.

2.08. Luciferase reporter assay

HeLa-shLuc or HeLa-shRHAU cells, pool of four different clones, were seeded in 12-well plates (1×10⁵ cells per well) with or without doxycycline (1 μg/ml). 24 h later, cells were transfected with 250 ng of pGL2-promoter (Promega Co., Madison, WI), a firefly luciferase-expressing plasmid, together with 1 ng of pRL-TK (for renilla luciferase expression as internal control) using FuGENE6. 48 h after transfection, cell lysates were prepared and luciferase expression was measured according to the given protocol (Dual-Luciferase Reporter Assay System, Promega). Firefly luciferase activity was normalized by renilla luciferase activity. Fold activation was derived from the normalization with dox- samples.

2.09. GeneChip microarrays and the analysis of RNA half-lives

HeLa-shLuc and HeLa-shRHAU cells were treated with doxycycline (1 μg/ml) for 6 days. On the 4th day of dox-treatment, four clones of each cell line were pooled and a total of 2×10⁶ cells were reseeded in 10 cm dishes. For the starvation experiment, the medium was replaced with serum-free DMEM on the 5th day, 24 h before the collection of RNA. The ActD-chase experiment was done on the 6th day of doxycycline treatment. 5 μg/ml ActD was added to the medium, and total
RNA was collected at 0, 30, 60, 90, and 120 min after the addition of ActD. Samples for time 0, representing the total amount of RNA collected from cells cultured in FCS-containing or starvation conditions were analyzed in triplicate, whereas ActD-treated samples for the mRNA decay study were analyzed in duplicate. Total RNA was isolated using the RNeasy kit from QIAGEN (Hombrechtikon, Switzerland).

Total RNA (5 μg) from each replicate was reverse transcribed and labeled using the Affymetrix 1-cycle labeling kit according to manufacturer’s instructions. Biotinylated cRNA (20 μg) was fragmented by heating with magnesium (as per Affymetrix’s instructions) and 15 μg of this fragmented cRNA was hybridized to Human U133 plus 2.0 GeneChips™ (Affymetrix, Santa Clara, CA). GC-RMA expression values and detection P-values were estimated using Refiner 4.0 from Genedata AG (Basel, Switzerland). Data analysis was performed using Analyst 4.0 from Genedata AG. The chip distributions were standardized by quantile normalization and they were scaled to make the median expression value, of genes with a detection P-value < 0.04, equal to 500. For the analysis of steady-state RNA levels, genes were required to have a detection P-value of less than 0.04 (Affymetrix default) in at least two replicates of at least one condition. The objective was to exclude genes that are not expressed in any condition. They were then subjected to a student t-test (P<0.05) and have a median fold change of 1.5 or 2 greater between samples dox+ and dox- or with and without starvation. Multiple testing errors were dealt with using a Benjamini and Hochberg false discovery correction.

To obtain mRNA half-lives, we used expression levels from the Affymetrix expression arrays at the start of the experiment (three biological replicates, “time 0”) and at four successive timepoints (30, 60, 90, and 120 minutes, two biological replicates each). First, mRNAs with long half-lives were identified, defined by expression levels that decreased less than 13% (= 1 - 2^(−120/600)) in the course of 120 minutes, corresponding to half-lives of 600 minutes or more. Half-lives and the corresponding standard errors of the remaining mRNAs were estimated by fitting the time-course expression data to an exponential decay function N(t) = N₀ × 2^(−t / t¹/²), where N(t) and N₀ correspond to the expression levels at timepoints t and zero, respectively, and t¹/² to the half-life, using the nonlinear least squares method as implemented in the R statistical program (www.r-project.org). Differences in half-lives were identified based on estimated half-lives and standard errors using the method described by Payton et al. (Payton et al. 2003). Half-lives with non-overlapping 88% confidence intervals were considered significantly different at a P value of 0.05.

For the general data analysis, the significance of the overlap between two sets of genes was calculated using the hypergeometric distribution as implemented in the R statistical program, which was also used to generate density plots of half-lives.

To find ARE-containing mRNAs, we used the human AU-rich element-containing mRNA database ARED (http://rc.kfshrc.edu.sa/ARED/). For the analysis of genes containing G4 structure in the promoter, the human promoters (defined as a sequence of 1000 nucleotides upstream of annotated transcripts that overlap
Affymetrix probesets) were obtained from Ensembl (www.ensembl.org, release 44) using a Perl script and the Ensembl Perl API (Curwen et al. 2004). Sequences were then scanned for the presence of G4 sequence using quadparser (Huppert and Balasubramanian 2005) with standard parameters. The entire set of microarray data is in GEO (Gene Expression Omnibus) with accession number GSE8192. The list of RHAU-regulated probe sets at steady-state mRNA level, by mRNA stability, and the list of starvation-sensitive genes are shown in Appendix II, III, and IV, respectively.

2.10. Cell growth and viability

HeLa-shLuc or HeLa-shRHAU cells, pool of four different clones, were cultured with or without doxycycline for 5 days. For the growth analysis in FCS-containing condition, 1.5×10^5 cells per well were seeded in 12-well plates and viable cell numbers were obtained every 24 h using the automated cell counter ViCell™ (Beckman Coulter, Inc., Fullerton, CA). For the starvation experiment, 3×10^5 cells per well were seeded in 12-well plates. 24 h later, the medium was replaced with serum-free medium and viable cell numbers and cell viability values were obtained every 24 h using ViCell™. Microscopic images showing cell morphology were captured by a phase contrast microscope. To count apoptotic cells, AnnexinV-positive cells were detected by FACS using anti-AnnexinV·APC antibody, according to a manufacturer’s instruction (BD Biosciences Pharmingen). To detect apoptotic marker proteins, starved cells were collected for Western blotting and stained by anti-cleaved caspase3 and anti-cleaved PARP antibodies.

2.11. Nuclear run-on assay

HeLa-shRHAU cells (clone 25) were treated for 6 days with doxycycline and then nuclear fractions were prepared as described (Medcalf et al. 1988). In vitro transcription was performed in 200 µl of run-on reaction buffer (5 mM Tris-HCl, pH 8.0, 2.5 mM MgCl₂, 150 mM KCl) containing cold ATP, CTP, GTP (5 mM each) and [γ-32P] UTP (100 µCi) and incubated at 30ºC for 30 min. RNA was purified and hybridized with specific cDNA (1 µg) immobilized on a positively charged nylon membrane for 3 days at 42°C. Hybridization signals were visualized and quantified using a Phosphoimager, and analyzed using Molecular Dynamics (version 5.2).

2.12. Real time PCR

First-strand cDNA was synthesized from total RNA (1 µg) isolated from HeLa-shRHAU cells transfected with Flag-RHAUs72 or empty vector (pBR-322), using the QuantiTect Reverse Transcription kit (QIAGEN) according to the manufacturer’s instructions. 1 µl of ten-times diluted RT reaction mixture was used to perform PCR with specific primer pairs (shown in APPENDIX I) corresponding to a particular gene of interest. Real-time PCR was performed using the QuantiTect SYBR Green PCR kit (QIAGEN) and the ABI PRISM 7000 sequence detector (Applied Biosystems, Foster City, CA) according to the
manufacturers’ instructions. We used NDUFA12 (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 12) for the normalization since expression of this gene was not altered in any samples analyzed by the microarray.

2.13. Tumor generation in nude mice

HeLa-TR-shRHAU1 or HeLa-TR-shRHAU2 cells were cultured with 1 μg/ml doxycycline for two days. Viable cells were counted and suspended in PBS (2×10^7 cells /ml). CD1 female nude mice (Charles River Laboratories, Inc., Sulzfeld Germany) were fed with water containing or not containing doxycycline (1mg/ml) two days prior to injection. HeLa cells in PBS (two million cells per injection) were subcutaneously injected into the right and left flanks of the nude mice. Once a tumor was detected, transcutaneous caliper measurements were used to measure the long and short dimensions of the tumor. Tumor volume was calculated on the assumption of an ellipsoidal volume, which was observed to be the predominant shape of the tumors. The tumor volume in milliliters was calculated according to the following formula, \( V=\frac{4}{3}\pi ab^2 \), where \( V \) represents volume and \( a \) and \( b \) represent the semiaxial dimensions.

2.14. GST-RHAU pull-down assay

Plasmids, pGEX-RHAU(1-200aa) or pGEX-2T, were transformed into *Escherichia coli* BL21 cells. Glutathion S-transferase (GST) fusion proteins were produced in the transformed BL21 cells and purified using glutathione sepharose 4B beads (GE healthcare). About 40 μg of purified GST proteins were incubated with 4 mg of HeLa nuclear or cytoplasmic S100 extracts in Buffer D (10 mM Hepes-KOH, pH 7.9, 3 mM MgOAc, 10% glycerol, 0.1 mM EDTA, 0.1 mM PMSF and 0.5 mM DTT) for two hours at 4 °C. Unbound proteins were removed by washing with Buffer D. Beads were boiled for 5 min in SDS-PAGE sample buffer and eluted proteins were analyzed in 10 % SDS-PAGE. Gels were stained with Coomassie blue. Proteins specifically interacting with GST-RHAU N-ter were identified by LC-MSMS.
Section 3 - Results

3.01. Possible roles of RHAU in the nucleus.

3.01.01. RHAU is mainly localized in the nucleus and tightly associated with RNA.

Previous studies have shown that exogenously expressed HA-RHAU is predominantly localized in the nucleus in HeLa cells (Tran et al. 2004). To investigate endogenous RHAU distribution, we performed cellular fractionation followed by Western blotting. Cells were first treated with detergent (0.5% Triton-X100) in CSK buffer, which permeabilized the plasma membranes. This buffer extracts cytoplasmic proteins from the cell and the supernatant, thus, contains cytoplasmic proteins such as \( \beta \)-tubulin and GAPDH, and the pellet contains nuclear proteins such as hnRNPC1/C2 and histone 3, as well as the transcription factor Oct1 and the splicing factor SC35 (Fey et al. 1986). A small but significant portion of endogenous RHAU was present in the nuclear insoluble fractions, with a distribution pattern similar to NF90, a dsRNA and ARE-binding protein. In contrast, large amounts of nuclear histone deacetylase 1 (HDAC1), telomere-binding protein Ku80, and RHA were extracted with ammonium sulfate buffer (compare lanes 2, 3, and 4 in Figure 3). These results indicate that RHAU is a nuclear protein associated with nuclear structure with a similar or stronger affinity than other RNA-/DNA-binding proteins.
In parallel, a similar analysis was done using the exogenously expressed EGFP-tagged RHAU. While mouse monoclonal and rabbit polyclonal antibodies against RHAU used in this work efficiently detected RHAU protein by Western blotting, they were unsuitable for detecting endogenous RHAU by immunocytochemistry. Therefore, to further investigate the cellular localization of RHAU, EGFP-tagged RHAU expression vectors were prepared and transiently transfected into HeLa cells. If EGFP-RHAU is tightly associated with nuclear structures, it should remain there even when nuclear membrane is permeabilized. To see if this was the case, cells were treated with 0.1% Triton X-100, washed out, then fixed with paraformaldehyde. Soluble nuclear protein such as EGFP was completely released from the nucleus in this condition (Figure 4B, EGFP, compare lanes fix and Triton). In contrast, significant level of fluorescence was still detected in the cells transfected with EGFP-RHAU albeit that the intensity was decreased (Figure 4A). In greater than 200 cells counted, 37.2% of transfected cells showed detectable EGFP-signal after the Triton-permeabilization (17.7% cells out of 47.6% transfected cells). Further DNase treatment to the permeabilized cells did not change proportion of EGFP-RHAU-positive cells, whereas almost no cells showed the signal after the RNase treatment (Figure 4, compare Triton-permeabilized and DNase, RNase). The results of biochemical fractionation and the in situ extraction suggested that RHAU tightly associated with higher nuclear structures via RNA.

Figure 4. In situ extraction of EGFP-RHAU. (A) EGFP fluorescence images of cells transfected with EGFP-RHAU. Cells were transiently transfected with EGFP-RHAU and either fixed or permeabilized with 0.1% Triton-X100. The permeabilized cells were further treated with DNase I (100 U/ml) with RNAsin or mixture of RNase A (20 μg/ml) and RNase T1 (100 U/ml), then fixed. (B) Cells showing significant EGFP-fluorescence were counted in greater than 200 cells in images taken from microscope. Average of two independent experiments is shown. Error bar, SEM.
3.01.02. Nuclear speckles and nucleolar cap-localization induced by transcriptional arrest.

The distribution of nuclear RHAU was further examined using EGFP-RHAU constructs. To avoid an artifact caused by terminal attachment of EGFP to RHAU, EGFP was tagged at either the N-terminal (EGFP-RHAU) or the C-terminal (RHAU-EGFP). Although the expression levels of differently tagged RHAU was about threefold different (Western blotting, Figure 5A, compare lanes 3 and 4), both proteins showed very similar distribution patterns. They were localized mainly in the nucleus excluding the nucleoli, with much lower expression in the cytoplasm. We found that RHAU was concentrated in nuclear speckles enriched with splicing factors and mRNAs (marked with splicing factor SC35) (Lamond and Spector 2003; Hall et al. 2006) but less abundant in heterochromatin (marked with H3-K9 trimethylation), supporting the idea that RHAU is closely associated with RNA but not compacted DNA (Figure 5B-a, b).

Therefore, we have examined whether RNA synthesis is linked to the specific nuclear localization of RHAU. Cells were treated with ActD, which intercalates into DNA and inhibits transcription by all types of RNA polymerase. In this condition, RHAU was no longer enriched in the nuclear speckles but formed prominent structures around the nucleoli. This structure was located close to but completely excluded from heterochromatin, as visualized by the antibody against H3-K9 trimethylation (Figure 5B-c, d). Time-course analysis showed that RHAU-containing cap-like structures became visible 1 h after the addition of ActD and enlarged subsequently. After 5 h, they completely occupied the nucleoli, but by then most cells were undergoing apoptosis (Figure 6). Treatment with DRB, an RNA polymerase II-specific inhibitor, exhibited a similar but milder effect on RHAU localization than ActD treatment: less RHAU was found around nucleoli and a significant amount was still concentrated in nuclear speckles (see Figure 7).

An ATPase-deficient mutant was also examined to see whether RHAU ATPase activity influences its cellular localization. EGFP-RHAU-E335A has an amino acid replacement at motif II of the conserved helicase domain from DEIH to DAIH and this mutation has been shown to cause a complete loss of RHAU ATPase activity in vitro (Tran et al. 2004). The ATPase-deficient mutant was localized only in the cytoplasm and its distribution was not altered by inhibition of transcription, suggesting that ATPase activity is necessary for the nuclear localization of RHAU (Figure 5C). As a control, EGFP alone was expressed and was seen to be distributed uniformly in the nucleoplasm under normal conditions. EGFP did not form cap-like structures upon treatment with any transcriptional inhibitors (Figure 5D).
**Figure 5. RHAU is enriched in the nucleus.** (A) Western blotting showing EGFP-tagged RHAU. HeLa cells were transfected with EGFP-tagged RHAU (EGFP-RHAU or RHAU-EGFP), ATPase-deficient RHAU mutants (EGFP-E335A or E335A-EGFP) or EGFP. Total cell lysates were analyzed by Western blotting using the antibodies indicated. (B) Immunofluorescence images of EGFP-expressing cells. HeLa cells were transiently transfected with vectors expressing EGFP-tagged proteins. For ActD treatment, the drug (5 μg/ml) was added to the culture 2 h prior to fixation. Cells were multiply stained with anti-SC35 antibody (nuclear speckles; in red), anti-H3 K9-trimethylation antibody (heterochromatin; in blue), and DAPI (DNA; in white). The merging of three colors for EGFP, SC35, and H3-K9trimet is shown. For cells transfected with RHAU-EGFP and E335A-EGFP, only merged images are shown. Scale bar: 5 μm.
3.01.03 Transcription-dependent localization of RHAU in the nucleolar caps with DEAD-box helicases p68 and p72.

The ActD-induced structures of RHAU around nucleoli resembled previously reported nucleolar caps around nucleoli in mammalian cells upon transcriptional arrest (Shav-Tal et al. 2005). In this report, DEAD-box RNA helicase p68 was found to be localized in nucleolar caps when cells were treated with ActD. To test whether RHAU is co-localized with p68 and its dimmer partner, p72, in the nucleolar caps, HA-tagged p68 or p72 were co-transfected with EGFP-RHAU and cells were stained with anti-HA and anti-SC35 antibodies. Under normal culture conditions, HA-p68 and HA-p72 were both concentrated in nuclear speckles as reported previously (Saitoh et al. 2004; Enukashvily et al. 2005), where EGFP-RHAU was also present (Figure 7 a, d: Co-localization of the three proteins appears as white areas in merged pictures resulting from a mix of red, blue, and green.). In transcription-arrested cells, p68 formed nucleolar caps and this was more marked with DRB than ActD (Figure 7 b, c). In contrast, p72 was more sensitive to ActD, forming a ring structure around nucleoli. In this situation of p72 overexpression, the localization of RHAU changed to a ring structure within p72 and no nucleoplasmic distribution was observed (Figure 7 e, f). Since single transfection of EGFP-RHAU or co-transfection with HA-p68 did not show such a remarkable structure, it seems that p72 recruited RHAU into such a structure around the nucleoli. In either case, RHAU was co-localized with p68 and p72 at the periphery of nucleolar caps, indicating that the structure formed by EGFP-RHAU was a part of the previously characterized nucleolar caps.
Figure 7. **RHAU is co-localized with p68 and p72 in nucleolar caps upon transcriptional inhibition.** (A) Immunofluorescence images of EGFP-expressing cells. ActD (5 μg/ml) or DRB (25 μg/ml) was added to the culture 2 h prior to fixation. Cells were stained with anti-HA antibody (in red), anti-SC35 antibody (in blue), and DAPI (in white). The merging of three colors for EGFP, HA, and SC35 is shown. Scale bar: 5 μm.

### 3.01.04 Interaction of RHAU with regulators of transcription.

To see whether RHAU interacts with p68 and p72, we transiently transfected Flag-RHAU together with HA-p68 or HA-p72 into HeLa cells and performed immunoprecipitation using anti-Flag antibody. HA-p68 and HA-p72 were co-precipitated with Flag-RHAU, indicating that RHAU interacts with p68 and p72 in cells (Figure 8A). Since p68 and p72 have been characterized as regulators of transcription, interaction between RHAU and these proteins suggest possible involvement of RHAU in transcriptional regulation. To see whether RHAU interacts with other known transcription regulators, the immunoprecipitation was performed with HDAC1 and 3. Histone deacetylases (HDACs) are general
transcription repressors and it has been reported that p68 and p72 associates with HDAC1, showing that these helicases repress transcription in some contexts (Wilson et al. 2004). We could detect the co-precipitation of RHAU when HDAC1 and HDAC3 were immunoprecipitated, suggesting that RHAU could be a part of transcriptional repression complex (Figure 8B). Since the addition of RNases into lysates abolished any of these interaction, RHAU likely associates with them via RNA.

3.01.05 N-terminal domain of RHAU is responsible for the nuclear and nucleolar caps localization.

RHAU consists of a central helicase core region and N-terminal and C-terminal extended domains (Figure 9A). The beginning of the N-terminus contains a glycine-rich motif and the end of the helicase core region possesses a putative nuclear export signal that is enriched with leucine. We made truncated mutants of RHAU in each domain to characterize essential domains of RHAU for the nuclear location. Deletion mutants of RHAU tagged with EGFP showed that the
200 N-terminal amino acids were required for the nuclear localization because the deletion of those residues showed complete cytoplasmic localization of RHAU (Figure 9B-d, e, f). In contrast, the N-terminal domain was sufficient for localization in the nucleus, and it was concentrated in the nuclear speckles (Figure 9B-a). Upon inhibition of transcription by ActD, all truncated mutants which would normally go to the nucleus showed nucleolar caps (Figure 9B-g, h, i), indicating that import to the nucleus is sufficient for the formation of these caps.

Figure 9. N-terminal domain of RHAU is responsible for the nuclear and nucleolar caps localization. Differently truncated forms of RHAU were fused with EGFP at their N-termini. Each construct was transfected into HeLa cells. ActD (5 μg/ml) was added 2 h prior to fixation. EGFP-fluorescence was captured and is shown in white. (A) A schematic representation of various EGFP-RHAU constructs with the summary of results of their cellular localization. (B) Fluorescent images of cells transfected with the above-shown EGFP-RHAU constructs before and after ActD treatment. Scale bar: 5 μm.
3.02. Microarray analysis using RHAU knockdown cells.

The intracellular localization studies have shown that: [1] RHAU is much more abundant in the nucleus than in cytoplasm; [2] in the nucleus, RHAU is concentrated in RNA-rich nuclear speckles; [3] RNA-synthesis influences the distribution pattern of RHAU in the nucleus. These results prompted us to consider the possibility that RHAU influences some RNA metabolic pathways in the nucleus and, therefore, it may affect global gene expression either directly or indirectly. To test this hypothesis and to identify the possible genes affected, we performed Affymetrix microarray analysis using RHAU-depleted cells.

3.02.01. Inducible RHAU-knockdown HeLa cell-line.

To establish HeLa-T-REx-derived cell lines in which RHAU-specific hairpin RNA (shRNA) could be induced by doxycycline (van de Wetering et al. 2003), plasmids expressing shRNAs to target RHAU (shRHAU) and luciferase (shLuc) as a control under the control of the Tet repressor were stably transfected and zeocin-resistant clones were picked. To reduce clone-specific effects, we pooled four independent clones from each shRNA-transfection. Such a pool was then treated as one experimental replicate in the analysis. Western blots showed that RHAU was reduced in all the clones down to 30% of that found in the controls after 6 days of doxycycline treatment (Figure 10A). In time-course experiments, maximal downregulation was observed after a 6-day doxycycline treatment and, therefore, a 6-day treatment was used in all subsequent experiments. Expression of shLuc in control cells was confirmed from the downregulation of luciferase activity by transient transfection assays (Figure 10B).

Figure 10. RHAU knockdown in HeLa cells by RHAU-specific shRNA expression. (A) Four independent clones derived from HeLa-shLuc or HeLa-shRHAU cells were pooled and treated or not treated with doxycycline (1 μg/ml) to induce shRNA for 6 days. RHAU knockdown in pooled and four individual HeLa-shRHAU clones was assessed by Western blotting using anti-RHAU antibody. Pool, pool of four individual clones; shRHAU clones, individual clones (B) HeLa-shLuc or HeLa-shRHAU cells were transiently transfected with firefly luciferase-expressing vector (pGL2-promoter) with or without co-treatment with doxycycline (1 μg/ml). Cells were collected 48 h after transfection. Firefly luciferase activity was normalized by renilla luciferase that was cotransfected as an internal control. Error bar, SEM of three replicates.
3.02.02. DNA microarray to measure steady-state mRNA and mRNA half-life.

RHAU was originally characterized as a destabilizer of uPA mRNA. As the steady-state level of mRNA reflects both its de novo synthesis and its degradation, we examined both steady-state levels of mRNAs and their half-lives by the microarray using RHAU knockdown cell lines. After complete knockdown of RHAU, cellular transcription was inhibited by ActD and total RNAs were collected every 30 min up to 120 min to determine the half-lives (see experimental procedure in Figure 11). The data from our microarrays showed that the expression of RHAU itself was successfully reduced to about 20% in doxycycline-treated (dox+) cells (Figure 11A). Acceleration of RHAU mRNA decay by shRNA-mediated mRNA degradation was also confirmed with a significant change in its half-life (Figure 11B). Thus, it was shown that microarray experiments could successfully detect changes in mRNA stability.

Figure 11. Microarray to measure both steady-state mRNA level and mRNA half-life. The scheme of microarray experiment is described. (A, B) Expression data of RHAU (Affymetrix name 223138_s_at) obtained from microarray analysis was plotted. Error bar, SEM of three or two replicates from microarray. (A) Steady-state mRNA level. (B) Decay of RHAU mRNA, which was accelerated by shRNA-mediated mRNA degradation.
Half-lives and the corresponding standard errors of every mRNA was estimated by fitting the time-course expression data of five time points to an exponential decay function using the nonlinear least squares method (See detail analysis in Material and Methods). We could confirm that ActD suppressed general transcription in our experiment since global decrease of mRNA levels in the ActD chase was detected by the microarray (Figure 12).

**Figure 12. Box plots of mRNA levels at five time points.** For each time point, a box plot of the observed expression levels is shown. The black line indicates the median, lower and upper box limits the first and third quartiles, respectively, and the whiskers show the total spread. Values further away than 1.5 times the interquartile range from the box were omitted as outliers. The difference of medians is significantly different at $P \leq 0.05$ if the notches around the medians of two box plots do not overlap.
3.02.03. Differences in steady-state levels after RHAU knockdown are not correlated to their half-lives.

To analyze microarray data, we first picked mRNAs showing significant differences in steady-state levels (time 0 of ActD treatment) in HeLa-shRHAU cells between doxycycline non-treated (dox-) and treated (dox+) conditions. As summarized in Table 1, 125 mRNAs showed more than a two-fold up- or down-regulation in RHAU-knockdown cells (dox+) compared with dox- cells. In contrast, only two mRNAs showed a twofold difference in expression after doxycycline treatment in control HeLa-shLuc cells, indicating little effect of doxycycline on global gene expression (See gene lists in Appendix II).

| Table 1. Comparison of probe sets with significantly altered steady-state levels in HeLa-shRHAU cells and other specific probe sets |
|---|---|---|---|---|
| **Steady-state** | **ARE** | **G4** | **mRNA half-life** |
| A | B | C | | |
| >1.5 fold up | 428 | 109 | 142 | 73 |
| down | 474 | 52 | 191 | 6 |
| > two-folds up | 58 | 13 | 22 | 13 |
| down | 67 | 5 | 28 | 2 |

Number of common probe sets between the sets that significantly increase or decrease their expression values in HeLa-shRHAU cells between dox- and dox+ (defined by \( P<0.05 \) and a minimal fold change, see Materials and Methods) and sets defined by additional characteristics are shown. The significance of the overlap is indicated as n.s.; not significant, *, \( P<0.01 \), **, \( P<0.001 \), ***, \( P<2\times10^{-16} \), calculated using the hypergeometric distribution, with \( n \) corresponding to the total number of genes in the column-set and \( N \) indicating the total number of genes analyzed in both column- and row-sets. (A) Fold difference of total mRNA amount in HeLa-shRHAU cells between dox- and dox+ (\( P<0.05 \)). \( N=30,599 \). Up, up-regulated in dox+; down, down-regulated in dox+. (B) Number of ARE-containing probe sets found in ARED (http://rc.kfshrc.edu.sa/ARED/). \( N=30,599, n=1,530 \); \( n \) was estimated under the assumption that the fraction of ARE-containing genes in the published screen (5%, (Bakheet et al. 2006)) is the same as the fraction of ARE-containing genes of all genes represented on the Affymetrix microarray. (C) Number of putative G4-sequence containing probe sets in the promoters (in 1 kb upstream from transcription starting sites) found by quadparser (Huppert and Balasubramanian 2005) with standard parameters. \( N=30,599, n=13,066 \); \( n \) was estimated under the assumption that the fraction of genes with G4-containing promoters in the published screen (42.7%, (Huppert and Balasubramanian 2005)) is the same as the fraction of genes with G4-containing promoters of all genes represented on the Affymetrix microarray. (D) Number of probe sets showing different half-lives (\( P<0.05 \)) between dox- and dox+ samples. Decrease or Increase, decreased or increased half-life in dox+. \( N=6,973, n_{\text{decrease}}=203, n_{\text{increase}}=99 \).
Correlation between the steady-state differences and their half-lives were then examined. Based on the half-life profiles, up- or down-regulated mRNAs in RHAU-knockdown were assigned to three groups (Figure 13). The major group (Group 1) includes those mRNAs whose steady-state levels were altered without changes in half-life, as represented by DAPK1 and CLUAP1 mRNAs (Figure 14). Groups 2 shows opposing correlation between steady-state level and mRNA decay, namely up-regulated steady-state levels with decreased half-lives or down-regulated steady-state levels with increased half-life (represented by SPRED1, Figure 14). Group 3 contains mRNAs whose steady-state levels were correlated with the mRNA decay rate as represented by CDKN1C, which showed decreased steady-state level and decreased mRNA half-life (Figure 14). Except for Group 3, involvement of the mRNA decay mechanism is unlikely, since changes in mRNA half-lives did not reflect steady-state mRNA levels. This is true for 99% of mRNAs whose steady state levels were affected by RHAU knockdown. These results suggest that the influence of RHAU on global gene expression is in general not through mRNA decay but rather through steps in the nucleus including transcription.

Among the mRNAs whose stability was up-regulated by RHAU knockdown, we collected a significant number of ARE-containing mRNAs that were found in the ARE-database, ARED (Bakheet et al. 2006) (Table 1B, see also Appendix II). However, the existence of ARE was not necessarily correlated to the misregulation of their half-lives, since the half-lives of the majority of ARE-containing mRNAs did not change in RHAU knockdown (5 out of 18 mRNAs showed changes of half-lives). This indicated that the effect of RHAU on steady-state mRNA level was not restricted to the ARE-mediated mRNA decay. We also analyzed whether those RHAU-regulated genes contained one or more G4 DNA structures in their promoters. According to bioinformatics analysis, about 40% of human promoters contain putative G4 sequences (Huppert and Balasubramanian 2005). In the down-regulated genes in RHAU knockdown, but not in the up-regulated mRNAs, we saw a slight enrichment of G4 sequences (53.5%) in their promoters, which was statistically significant (P<0.001) (Table 1C). Thus, they are candidate target genes of the G4-resolvase RHAU, as RHAU resolves G4 DNA in their promoters, which activates transcription. However, the effect of RHAU knockdown on the abundance of G4 DNA in promoters was mild, and in fact no significant enrichment of G4 sequences was seen in 2-fold down-regulated mRNAs, suggesting that there could be another mechanism affecting mRNA steady-state levels in RHAU knockdown cells.
### Results

<table>
<thead>
<tr>
<th>Group</th>
<th>Steady-state mRNA vs mRNA decay</th>
<th>Influence of mRNA decay rate on steady-state level</th>
<th>Change in transcription</th>
<th>Steady-state mRNA</th>
<th>t1/2</th>
</tr>
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<tbody>
<tr>
<td>Group 1</td>
<td>No change in mRNA decay</td>
<td>No</td>
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<td>up or down</td>
<td>no change</td>
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<td>Group 2</td>
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<tr>
<td>Group 3</td>
<td>Correlation</td>
<td>Yes</td>
<td>Yes or No</td>
<td>down</td>
<td>decrease</td>
</tr>
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</table>

![Figure 13. The relationship of steady-state difference and mRNA half-lives in RHAU-knock down cells.](image)

Three groups of steady-state different mRNAs and percentage of those mRNAs in up- or down- regulation is shown.
Figure 14. mRNAs regulated by RHAU. Expression values were obtained from microarray analysis. mRNAs whose expression levels were significantly affected after RHAU knockdown induced by doxycycline were selected and are represented here by six different mRNAs: DAPK1 (Affymetrix probe name: 203139_at), SPRED1 (226837_at), CLUAP1 (204576_s_at), CDKN1C (213348_at). The left panel shows steady-state levels of mRNAs normalized over the sample of dox- in cells. The right panel shows the mRNA decay curve. mRNA expression was normalized over time 0 as 100%.
3.02.04. Influence of RHAU on mRNA half-life.

In the analysis of the mRNA decay rate, we found 99 mRNAs were significantly stabilized and 200 mRNAs were destabilized in knockdown although, surprisingly, the steady-state levels of the majority of these mRNAs were not significantly affected (Table 2, see also gene lists in Appendix III). The density plot of their half-lives is shown in Figure 15A. The y-axis shows the density of genes in each half-life shown on the x-axis. In contrast to control shLuc cells which showed almost no difference between dox- and dox+, shRHAU cells showed a greater difference as overall distribution was shifted towards longer half-life in dox+, suggesting that RHAU tends to destabilize mRNAs as it was previously characterized. However, when we looked individual mRNAs, there were significantly more numbers of mRNAs showing decreased half-life in RHAU knockdown, suggesting that RHAU also stabilizes other mRNAs (Table 2). When we look at half-lives of mRNAs showing significant differenced on steady-state levels, mRNAs whose steady-state levels were down-regulated did not show any significant half-life differences following RHAU knockdown. In contrast, half-lives in up-regulated mRNAs became significantly shorter after RHAU knockdown (in red, compare dox+ and dox-). Although this tendency indicates that up-regulation of those mRNAs was attributable not to the increase in their half-lives but rather to enhanced transcription, it suggests that regulation of mRNA stability also exists at the same time, perhaps to compensate the increased transcription rate.

<table>
<thead>
<tr>
<th>Table 2. Number of probe sets with significantly altered mRNA half-life in HeLa-shRHAU or HeLa-shLuc cells treated with dox.</th>
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<td></td>
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<tr>
<td></td>
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<tr>
<td>shRHAU$^{a}$</td>
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<td>shLuc$^{b}$</td>
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(A and B) Number of probe sets showing statistically significant ($P<0.02$ or $P<0.05$) decrease or increase on their half-life between dox- and dox+ in HeLa-shRHAU or HeLa-shLuc cells. (C) Number of common probe sets between the sets of half-life differences ($P<0.05$, the number showed by B) and the sets that show significantly different steady-state level between dox- and dox+ (defined by $P<0.05$ and a minimal two-fold difference). 

$^{a}$, $n=5613$; $^{b}$, $n=17221$.  

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Figure 15. Distribution of RHAU-regulated mRNA half-lives. (A) Density plot of mRNA half-lives in shLuc cells (n=17221) or in shRHAU cells (n=5613). (B) Density plot of mRNA half-lives for gene sets whose steady-state levels were upregulated (red, n=58) or down-regulated (green, n=67) by RHAU knockdown in shRHAU cells.
3.02.05. Change in mRNA level in RHAU-knockdown cells can be rescued by exogenous RHAU expression.

To validate the microarray data, expression of several mRNAs was assessed by real-time PCR. We also examined whether the effect of RHAU knockdown can be reversed by exogenous expression of Flag-tagged RHAUsm. This expression vector harbored a silent mutation at the target site of shRNA (Flag-RHAUsm) allowing it to escape siRNA-mediated downregulation. Western blot analysis showed that exogenous Flag-RHAUsm was expressed at a level similar to endogenous RHAU, indicating that Flag-RHAUsm expression can restore RHAU in doxycycline-treated cells (Figure 16A). As shown in Figure 16B, real-time PCR is in agreement with the microarray analysis (compare dox- and dox+; lanes 1 and 2) and expression of Flag-RHAUsm rescued the effect of RHAU knockdown on many target mRNAs (dox+ with exogenous expression, in lane 3). Failure to reverse the effect of RHAU knockdown on some mRNAs suggests that these mRNAs are not direct targets of RHAU.

Figure 16. Rescue expression of RHAU by transient transfection of the silent RHAU mutant. The empty vector (pBR322) or Flag-RHAUsm (silent mutation) was transiently transfected in HeLa-shRHAU cells pre-treated with doxycycline (1 μg/ml) for 5 days. 24 h after transfection, the cell lysate and total RNA were prepared. (A) Western blot of total cell lysate of transfected cells. Endogenous or exogenous RHAU was detected using the anti-RHAU monoclonal antibody. (B) Real-time PCR using the collected RNAs (1-3) with various primer sets as indicated. 1: dox- with empty vector, 2: dox+ with empty vector, 3: dox+ with the expression of Flag-RHAUsm.
Finally, transcriptional activity was tested for RHAU candidate targets picked from the microarray. Nuclear run-on assay using isolated nuclei from HeLa-shRHAU cells showed an increase in transcriptional activity of one of the upregulated genes (DAPK1) in RHAU knockdown cells (Figure 17). Taken together, these results indicate that RHAU influences gene expression at different steps including transcription and mRNA decay, depending on the type of the gene.

**Figure 17. Nuclear run-on assay using HeLa-shRHAU cells.** Nuclei were isolated from doxycycline-treated or non-treated cells, and cell-free transcription was performed as described in the Materials and Methods section using radio-labeled UTP. Isolated radio-labeled RNA was hybridized with the indicated DNA probes cross-linked on the nylon membrane. The signal intensity of each probe was normalized by GAPDH.
3.03. RHAU on stress-response.

3.03.01. Influence of RHAU depletion on cell growth.

We have observed mild growth retardation of RHAU knockdown cells in normal culture condition, although they could continue proliferation for more than two weeks (Figure 18A). In contrast to the normal culturing condition, RHAU knockdown caused severer growth defect as a result of decreased viability when cells were serum-starved, despite doxycycline non-treated HeLa-shRHAU cells and control HeLa-shLuc cells (with or without doxycycline pre-treatment) were able to grow in starved medium (Figure 18B, C). To support it, more numbers of apoptotic annexinV-positive cells were detected in RHAU knockdown cells than controls in starvation and when treated with other apoptosis inducer, staurosporine (Figure 18D). Furthermore, Western blottings detecting apoptotic markers such as cleaved-caspase3 and cleaved-PARP showed that RHAU knockdown cells underwent apoptosis significantly faster than control cells in starvation medium (Figure 18E). These observations suggest that RHAU have anti-apoptotic effect in stressed culture conditions.

Figure 18. RHAU-knockdown cells undergo apoptosis upon serum-starvation. HeLa-shLuc or HeLa-shRHAU cells were treated or not treated with doxycycline for 5 days. Then cells were seeded in 12-well plates at 1.5×10^5 cells/well (A) or 3.0×10^5 cells/well (B) with or without doxycycline. (A) Viable cells were counted every 24 h by ViCell. (B) Culture medium was replaced with serum-free medium 24 h after cell seeding. Viable cell numbers and viability of cells were counted every 24 h using ViCell. Error bar, SD of four replicates. (C) Phase-contrast images taken for 24hr-starved HeLa-shRHAU cells either cultured with or without doxycycline. (D) HeLa-shRHAU cells cultured with serum-free medium for indicated hours or treated with 1 μM staurosporine (STS), protein kinase inhibitor that induces apoptosis, for 4 hours were collected to be analyzed by FACS to detect annexinV-positive cells. (E) HeLa-shRHAU cells were cultured in serum-free medium for indicated hours and collected for Western blotting. Same amount of whole cell lysates were loaded and analyzed using the antibodies indicated.
3.03.02. Microarray analysis using cells under serum-starvation.

We, therefore, attempted to identify target mRNAs of RHAU under stressed condition. HeLa-shRHAU cells were first treated with doxycycline for 5 days and then the medium was replaced with a serum-minus medium for 24 h prior to microarray analysis. In this analysis, we could first pick up as starvation-sensitive genes which showed different expression levels in starved conditions compared to serum-containing conditions irrespective of doxycycline pre-treatment (See gene lists in Appendix IV). The starvation-sensitive genes common in both HeLa-shLuc and HeLa-shRHAU cell line (in dox-) included the MAP-kinase inhibitor DUSP6 (14-fold up-regulation), the apoptosis-inducer TRAIL (13-fold up-regulation), and transgelin (20-fold down-regulation) which is involved in cytoskeleton reorganization; these may collectively contribute to the observed decrease in cell growth. We found that RHAU-regulated mRNAs were significantly enriched with these starvation-sensitive genes (Table 3B), indicating a close relationship between cellular responses induced by RHAU knockdown and serum starvation. In fact, most mRNAs that were up- or down-regulated in RHAU knockdown in the serum-containing condition showed the same changes under the serum-starvation condition (Table 3C). In addition, the effect of RHAU knockdown during serum-starvation was more pronounced for the down-regulated mRNAs, suggesting that RHAU is involved in the activation of gene expression in serum-starved conditions.

<table>
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<tr>
<th>TABLE 3. Comparison of probe sets with significantly altered steady-state levels in HeLa-shRHAU cells and starvation-sensitive genes.</th>
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<tbody>
<tr>
<td><strong>Steady-state</strong></td>
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<tr>
<td>&gt;1.5 fold up</td>
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<tr>
<td>&gt;1.5 fold down</td>
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<tr>
<td>&gt; two-folds up</td>
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<td>&gt; two-folds down</td>
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Number of common probe sets between the sets that significantly increase or decrease their expression values in HeLa-shRHAU cells between dox- and dox+ (defined by P<0.05 and a minimal fold change, see Materials and Methods) and sets defined by additional characteristics are shown. The significance of the overlap is indicated as n.s.; not significant, *, P<0.01; **, P<0.001; ***, P < 2e-16, calculated using the hypergeometric distribution, with n corresponding to the total number of genes in the column-set and N indicating the total number of genes analyzed in both column- and row-sets. (A) Fold difference of total mRNA amount in HeLa-shRHAU cells between dox- and dox+ (P<0.05). N=30,599. Up, up-regulated in dox+; down, down-regulated in dox+. (B) Starvation-sensitive genes. Probe sets showing more than 2-fold difference (P<0.05) in both HeLa-shLuc and HeLa-shRHAU cells between FCS-containing culture and serum-starved condition. N=30,599, n=593. (C) Probe sets showing 1.5 fold or 2-fold difference in starved HeLa-shRHAU cells between dox+ and dox-. N=30,599, n1.5fold-up=153, n1.5fold-down=1,685, n2fold-up=38, n2fold-down=256.
Section 4 - Discussions

4.01. RHAU is a nuclear-enriched protein.

Previous studies and the current work have shown that RHAU is localized predominantly in the nucleus, but with significant expression in the cytoplasm. Cell fractionation has also shown RHAU abundance in the nucleus and, furthermore, in the nuclear-insoluble fraction, suggesting that RHAU is tightly associated with the nuclear structure where it has a role. Since RHAU is a large nuclear protein with a molecular weight of 110 kDa, it is likely to be transported through the nuclear pore complex by an active transport mechanism requiring a nuclear localization signal (NLS) (Stewart 2007). Deletion experiments have shown that the N-terminal region of RHAU, containing a glycine-rich motif, is required for its nuclear localization. In addition, an EGFP-tagged N-terminal domain (of about 50 kDa) resulted in perfect nuclear localization and higher accumulation than with the full-length RHAU, suggesting that this region contains the NLS.

This was supported by observations in GST pull-down experiments where the N-terminal domain of RHAU was co-precipitated with the general nuclear import factors importin-α and importin-β (Figure 21). The nuclear-cytoplasmic transportation of RHAU may also be regulated by an active nuclear export mechanism involving a putative leucine-rich nuclear export signal (NES) located at the end of the helicase domain (see Figure 9). We observed that RHAU and a cytoplasmic isoform, RHAUΔ14, that lacks 14 amino acids in the helicase domain but still possesses the putative NES, were totally confined to the nucleus when cells were treated with leptomycin B, an inhibitor of the nuclear export factor CRM1 (Kudo et al. 1998). This suggests that RHAU is a nuclear shuttling protein (Figure 19). However, it is currently unclear whether RHAU is carried directly by CRM1 or in association with other molecules, including mRNA and mRNA-binding proteins. It seems that RHAU tightly associates with RNA in cells: [1] In situ nuclear extraction experiments showed that RHAU was completely released from the nucleus by treatment of cells with RNase but not DNase; [2] During

![Figure 19. Leptomycin B inhibits nuclear export of RHAU and RHAU isoform. COS7 cells were transfected with EGFP-RHAU or EGFP-RHAUΔ14. Leptomycin B (10 ng/ml) or its solvent (ethanol) was added to the medium 8 h prior to fixation. Cellular localization of EGFP-proteins was observed in greater than 200 cells under microscope. N: Nucleus, C: Cytoplasm.](image)
Discussion

immunoprecipitation, RHAU is much more accessible to antibody in lysates treated with RNases than without (Figure 8A, compare lanes 2 and 3 or 5 and 6 in IP; also observed in the previous report (Tran et al. 2004)); [3] RHAU interacts with various proteins including RNA-binding proteins only in the presence of RNA. Messenger RNAs are always associated with RNA-binding proteins from sites of transcription in the nucleus to the cytoplasm (Dreyfuss et al. 2002).

Although the major mRNA export pathway in mammalian cells is that via the TAP/p15 complex, which is a CRM1-independent pathway, it has been reported that CRM1 is specifically involved in nuclear export of ARE-containing mRNAs (Gallouzi and Steitz 2001). It is, therefore, reasonable to assume that RHAU is exported as a part of RNP complexes containing AREs via CRM1. Further investigation is required to determine which factors and mechanisms are involved in the nuclear export of RHAU.

The regulation of cellular traffic is important for RHAU function, since RHAU probably has different roles in different cellular compartments. Therefore, it is interesting to understand when and how its transport is regulated. To determine whether RHAU cellular transport is regulated in response to extracellular signals, we subjected cells to various stimuli. There was a significant difference in EGFP-RHAU localization when cells were serum starved (Figure 20) but not after treatment with insulin, EGF, or hydrogen peroxide (data not shown). RHAU tended to localize in the cytoplasm rather than the nucleus in serum-starved COS7 cells. Since this effect was not observed in HeLa cells, it may be cell- or cellular condition-specific but it is an interesting observation since we have shown that RHAU plays an anti-apoptotic role in cells cultured in serum-free medium. In this scenario, RHAU may have a cytoplasmic role, such as regulating mRNA decay during stress. It will be interesting to know whether RHAU localization varies with cell type. If the function of RHAU is correlated with its cellular localization, different localization patterns may be observed depending on cell function.

Figure 20. Serum-starvation causes cytoplasmic localization of EGFP-RHAU. COS7 cells were transfected with EGFP-RHAU. Cells were cultured in serum-free medium for 24 hours prior to fixation. Cellular localization of EGFP-proteins was observed in greater than 200 cells under microscope. N: Nucleus, C:cytoplasm.
4.02. ATPase activity and localization.

The regulation of the nuclear-cytoplasmic shuttling of RHAU is more complex than just a simple balance between nuclear import and export, since its ATPase activity and ongoing RNA synthesis are also involved. We observed that the ATPase-deficient mutant RHAU-E335A is completely excluded from the nucleus. Therefore, it is possible that movement of RHAU is tightly linked to its enzymatic activity; for example, its interactions with other proteins or RNAs important for nuclear import and/or export may be regulated by RHAU ATPase activity. The cytoplasmic localization of the RHAU mutant may be explained either by the acceleration of nuclear export or the inhibition of nuclear import. It is possible that RHAU nuclear activity depending on ATPase activity retains RHAU in the nucleus. However, RHAU hydrolyzes ATP more rapidly than other RNA helicases ($V_{\text{max}}: 77\text{nmol/min/\mu g}$, Akimitsu unpublished) and, therefore, it is likely that the turnover of RHAU association with other proteins is also rapid if the interaction requires ATPase activity. RHAU ATPase activity destabilizes ARE-mRNA (Tran et al. 2004). Thus, if ATP-hydrolysis is required for the release of ARE-mRNAs from the degradation complex containing RHAU, an ATPase-deficient RHAU mutant would be unable to dissociate from RNA, which would inhibit further import of the protein into the nucleus. Alternatively, RHAU may have different conformations depending on whether it is bound by ATP or ADP, thus affecting its interaction with regulatory proteins that influence RHAU movement across the nuclear membrane. In accordance with this, several RNA helicases have been reported to exhibit different conformations upon ATP and/or nucleic acid binding (Jankowsky and Fairman 2007).

4.03. Transcriptional arrest-dependent localization of RHAU to nucleolar caps.

In the nucleus, RHAU was distributed throughout the nucleoplasm but especially concentrated in the nuclear speckles. Nuclear speckles are irregular forms of subnuclear structure located in interchromatin regions of the nucleoplasm in mammalian cells. A speckle is a site at which actively transcribed mRNAs and RNA-processing factors are concentrated and, therefore, where efficient recycling of RNA-metabolic proteins, such as splicing factors, transcription factors, and nuclear export factors, is managed (Lamond and Spector 2003), (Hall et al. 2006). According to a proteomic analysis of biochemically isolated speckles from mouse liver nuclei, the components of nuclear speckles include not only splicing factors, transcription factors, and nuclear export factors, but also various RNA-related proteins, such as transcription factors and mRNA transport factors. Several DEAD/DExH RNA helicases, which might play a role in modifying or maintaining RNA-RNA and RNA-protein interactions in the speckles, were also identified (Saitoh et al. 2004). Since RHAU itself was not detected in this analysis and the RHAU signal in the speckles was not as prominent as that of SC35 in our localization study, RHAU may not be confined to the speckles but rather shuttles between these structures and other parts of the nucleus.
Dynamic movement of RHAU was indeed observed in transcriptionally arrested cells. Unexpectedly, we found that RHAU is localized in the cap-like structures around nucleoli in ActD-treated cells, which were located close to heterochromatin and contained the RNA helicases p68 and p72. The nucleolus is a highly dynamic organelle, disassembling and reforming during the cell cycle (Shaw and Jordan 1995; Raska et al. 2006). In quiescent cells or cells subjected to transcriptional arrest, the nucleolus segregates and a cap-like structure, the nucleolar cap, appears on its surface (Malatesta et al. 2000; Shav-Tal et al. 2005). During this event, protein and RNA components of other nuclear bodies such as Cajal-, SMN-, and PML bodies, as well as some nucleoplasmic RNA-binding proteins including the RNA helicases p68 and p72, are sorted into the nucleolar caps, whereas conversely some nucleolar proteins are dispersed into the nucleoplasm (Shav-Tal et al. 2005). Similarly, proteomic analysis has shown that the protein composition of isolated nucleoli alters significantly upon inhibition of transcription by ActD (Andersen et al. 2002; Andersen et al. 2005). This indicates that nucleolar components can be dynamically reorganized in response to the metabolic state of the cell. Transcription is the major energy-consuming process in the active nucleus and is functionally and physically coupled to other RNA metabolism steps. Thus, once transcription is arrested by ActD, this orchestration of the RNA metabolic process is broken down and nuclear morphology is significantly altered. ActD blocks RNA polymerase II complexes in their elongation process, by which part of the transcriptional machinery may be left sitting on the DNA template. On the other hand, an excessive pool of free post-transcriptional factors released from the transcription machinery may be sorted into the segregated nucleolus where pre-rRNAs accumulate, because many of those proteins possess RNA-binding activity. Therefore, proteins sorted to nucleolar caps upon transcriptional arrest are considered to be proteins dynamically moving in the nucleus and associated with various RNA metabolic processes. The RNA helicase RHAU may be one such regulatory protein shuttling between splicing speckles and other compartments in the nucleus, with the consequence that it changes its nuclear localization when transcription is arrested.

4.04. Microarray to determine the RHAU target gene and RNA.

The finding that RHAU is actively and dynamically translocated in the nucleus prompted an investigation of its function there. A DNA microarray approach was taken using the ActD-chase method to identify genes or mRNAs misregulated by knockdown of RHAU. Based on the data collected at five time points after the addition of ActD, exponential decay of mRNAs was recorded and 5,613 mRNA half-lives were compared in cells expressing or not expressing RHAU. Although it has been reported that transfection of siRNAs as well as transient expression of shRNA expressed from both plasmid and lentiviral DNA vectors can trigger an
interferon response (Bridge et al. 2003; Sledz et al. 2003), activation of PKR that upregulates interferon-β in response to dsRNAs (Kumar et al. 1994) was not detected in our system (data not shown). The expression of shLuc affected the expression of only two genes, which also suggests that inducible expression of shRNA provides little non-specific up- or downregulation of cellular transcription. Furthermore, changes in expression of many RHAU targets was reversed when a silent-mutant of RHAU was exogenously expressed, indicating that the genes tested were indeed targets of RHAU. Taken together, intrinsic and inducible expression of shRNA using stably transfected cells is an efficient tool to downregulate mammalian mRNAs, causing fewer side effects than other methods using RNA interference.

4.05. RHAU changes gene expression through a mechanism not involved in mRNA degradation.

Of the 125 gene sets showing at least a twofold change in steady-state expression relative to RHAU-expressing cells, only two had correlating alterations in mRNA half-life and steady-state level, suggesting that RHAU influence on gene expression is mostly not through regulation of mRNA decay. It is possible that RHAU is involved in transcriptional regulation in the nucleus, as transcription of DAPK1 was seen to be altered by RHAU knockdown in nuclear run-on assays. We also showed that RHAU interacts with the transcriptional regulators p68, p72, and HDACs, and that RHAU, p68 and p72 are closely localized in nuclear speckles as well as around the nucleolar caps in transcription-arrested cells. Since these interactions were RNA-dependent, it is likely that RHAU resides in a part of the large transcription-related complex including RNA. RHAU is concentrated in nuclear speckles in normal culture conditions, where it may associate with pre-mRNA, splicing factors and transcription factors. Thus, we also do not rule out the possibility that RHAU influences other RNA metabolic steps in the nucleus, such as splicing and mRNA export. Further studies are required to test how many RHAU-influenced genes are regulated by transcription or by other steps of RNA metabolism.

In addition, RHAU may regulate gene expression via direct binding to DNA. One possibility related to the G4 resolvase activity of RHAU. Single point mutation analysis and compounds stabilizing the G4 structure indicate that the structure formed in the c-myc promoter acts as an inhibitory cis-element of its transcription (Siddiqui-Jain et al. 2002). At least in this example, the G4 structure functions as a transcriptional repressing element and, thus, resolving this structure is expected to activate transcription. Indeed, there was a slight enrichment of genes containing putative G4 sequences in their promoters amongst the genes downregulated after RHAU knockdown, indicating that these genes may be activated by RHAU through its resolvase activity. However, despite the high frequency of genes containing putative G4 sequences (42.7% of the total
human genome (Huppert and Balasubramanian 2007)), so far only the c-myc gene has been shown experimentally to be regulated by a G4-forming sequence in the promoter. Thus, we need to further investigate whether the existence of the G4 structure in RHAU target genes in fact affects their transcription. G4-forming sequences are also found in telomeric repeats, which are considered important for the maintenance of telomeric structures and, consequently, for genome stability. It is unlikely, however, that RHAU is engaged in the maintenance of telomeric structures, since we did not observe RHAU co-localization with TRF2, a telomere-binding protein, in the HeLa cell nucleus (Figure 21).

4.06. Involvement of RHAU in mRNA degradation.

In the analysis of mRNA decay rate, 99 mRNAs were significantly stabilized and 200 mRNAs were destabilized after RHAU knockdown, although surprisingly the steady-state levels of the majority of these mRNAs were not significantly affected. A similar phenomenon was, in fact, observed in our original study of RHAU (Tran et al. 2004), where it was shown that overexpression of RHAU accelerated the decay of uPA mRNA despite the absence of changes in its steady-state levels, suggesting that RHAU increased transcriptional activity of the same gene under these conditions. Since many mRNAs were not only stabilized but also destabilized after RHAU knockdown, including both ARE and non-ARE mRNAs, the effect of RHAU on mRNA decay may not necessarily be restricted to ARE-containing mRNAs. At the moment, however, we are unable to distinguish between direct and indirect targets of RHAU in this study. It is also possible that some of the alterations in mRNA decay were caused by changes in the expression
of other mRNAs. Thus, further approaches, such as *in vitro* mRNA decay assays, will be required to determine the direct mRNA targets of RHAU.

4.07. Relationship of regulations by transcription and mRNA stability.

Of the steady-state upregulated mRNAs, 78% showed no changes (Group1 in Figure 13) in half-life and the remaining 22% showed decreased half-lives (Group2), which was a statistically significant enrichment. mRNAs in Group2 with both increased steady-state levels and shorter half-lives after RHAU knockdown appear contradictory but this might be explained by homeostatic regulation between transcription and mRNA decay. When transcription is activated, the stabilities of mRNAs coded by a given gene might be regulated in the opposite direction in preparation for imminent suppression of transcription. The activation of both transcription and mRNA decay at the same time is an energy-consuming process, but this response will ensure rapid restoration of the initial conditions once the signal for transcriptional activation has declined. In support of this hypothesis, half-life differences were observed for mRNAs whose steady-state levels were upregulated but not those downregulated, possibly because change in mRNA stability to prepare approaching upregulation of transcription is not necessary in the latter case. This observation implies some type of communication between mechanisms regulating transcription and mRNA degradation. RHAU may be a protein that is involved in two different regulatory pathways and that transfers feedback about gene expression to both mechanisms. It would be interesting to know at the molecular level how the two processes are functionally coordinated by RHAU.

4.08. RHAU in tumor cell growth.

RHAU knockdown in HeLa cells partially reversed the characteristics of cancer cells, which ceased growth in serum-starved conditions as a consequence of decreased viability. RHAU-expressing cells divided further for a few days under the same conditions. Preliminary experiments in nude mice inoculating HeLa-shRHAU cells targeting two different sequences of RHAU mRNA showed that RHAU knockdown slowed the growth of HeLa cell-derived tumors, suggesting that RHAU has a role in tumor progression (Figure 22). Several genes encoding potential tumor-suppressors were indeed upregulated in RHAU knockdown cells. For example, in RHAU knockdown cells the steady-state levels of DAPK1 (death-associated protein kinase1: 2.1-fold), SPRED1 (Sprouty-related protein with EVH-1 domain: 2.3-fold), and an MAP-kinase inhibitor, DUSP6 (dual-specificity phosphatase 6: 2.8-fold) were all upregulated. DAPK1 is a positive mediator of apoptosis (Bialik and Kimchi 2006). Cancer development and metastasis stemming from Lewis carcinoma cells, which do not express DAPK1, are
suppressed when DAPK1 expression is restored (Inbal et al. 1997). It has been shown also that SPRED1 inactivates the Ras-dependent MAP kinase signalling pathway (Wakioka et al. 2001), and that its overexpression inhibits cancer cell motility, leading to the suppression of metastasis (Miyoshi et al. 2004). Furthermore, hypermethylation in the promoter regions and, consequently, transcriptional suppression of DUSP6 (Jeffrey et al. 2007) has been reported in pancreatic cancer cells (Xu et al. 2005) and also for DAPK1 in various cancer cells (Tang et al. 2000; Tozawa et al. 2004; Chan et al. 2005; Kuester et al. 2007). According to the tumor database Oncomine 3.0 (http://www.oncomine.org/main/index.jsp), expression of RHAU is significantly increased in hepatocellular carcinoma, squamous cell carcinoma, and pancreatic adenocarcinoma, suggesting a proto-oncogenic property of RHAU in these cancers. Thus, examination of the role of RHAU in tumor model systems will be of great interest.

**Figure 22. Knockdown of RHAU in HeLa cells retards tumor growth in nude mice.** HeLa-shRHAU1 or HeLa-shRHAU2 were subcutaneously injected into the right and left flanks of CD1 female nude mice (two million cells per injection). Mice were fed with or without 1mg/ml doxycycline to induce shRNA expression in the injected cells and tumor diameter was measured constantly to estimate tumor volume. N=6 (3 mice per condition).


Results from the current study infer that RHAU has at least two functions in mammalian cells, regulating gene expression through both mRNA degradation and transcription (or another gene expression regulatory pathway in the nucleus).
So far, several RNA helicases have been shown to be multi-functional proteins, many of them involved in transcriptional regulation and other RNA-metabolism pathways (Fuller-Pace 2006). Examples include DEAD-box proteins p68 and p72, which are involved in the transcription regulation of various genes as well as in pre-mRNA processing and in alternative splicing. It is suggested that these RNA helicases play different cellular roles through interactions with various proteins. For instance, p68 can act as both a transcription activator and a repressor depending on whether it binds to co-activators like p300/CBP (Rossow and Janknecht 2003) or co-repressors like HDAC1 (Wilson et al. 2004) in different cellular contexts. It is possible that RHAU fulfils different functions in a similar way, through different molecular associations. We found that RHAU interacts with p68, p72 and HDACs in a fashion dependent on RNA (Figure 8). In addition, GST-pull-down assays using the GST-tagged RHAU N-terminal domain has identified many other RNA helicases, hnRNPs and factors involved in DNA repair or DNA replication both in the nucleus and in cytoplasm (Figure 23). Thus, RHAU seems to preferentially interact with RNA-related rather than DNA-related proteins. These results suggest that RHAU functions as part of various RNP complexes and is involved in different processes of RNA metabolism through interactions with other molecules.

Figure 23. GST-RHAU Nter pull-down using HeLa nuclear and cytoplasmic extracts. Bacterial purified GST or GST-RHAU-Nterminus (1-200aa) were incubated with HeLa nuclear extract (N), Cytoplasmic S100 extract (C), or binding buffer (Buf). GST proteins were collected with Glutathione sepharose, loaded in 10% SDS-PAGE and stained with Coomassie Blue. Proteins specifically interacting with GST-RHAU N-ter were identified by LC-MSMS.
Overall, the results indicate that RHAU is a protein involved in various processes of RNA metabolism but probably in no case as a principal factor. In mRNA degradation, RHAU only weakly interacts with ARE-RNA compared with other AUBPs and affects only limited numbers of ARE-RNA. In fact, depletion of RHAU had only a minor effect on the degradation of many mRNAs. In transcriptional regulation, it is unlikely that RHAU acts as a transcription factor binding directly to DNA and changing promoter activities since it seems to have a higher affinity for RNA than DNA. Furthermore, the threshold of impact on steady-state levels after depletion of RHAU was limited to about twofold in most genes. So, the question remains of what is the actual role of RHAU in mammalian cells.

I view RHAU as a more general and basic factor in RNA-protein complexes. The basic function of RHAU could be the maintenance or rearrangement of secondary structures of RNA and RNA-protein complexes in a process with low specificity. RHAU has no RNA-binding domain apart from the NTP-binding motifs in the helicase core domain; thus, it is unlikely that RHAU finds its specific targets itself. DExH-box proteins generally do not show so much specificity for their substrates in duplex unwinding. Yeast DEAH-box splicing factor Prp22p unwinds not only RNA-RNA but also RNA-DNA duplexes of various length (Tanaka and Schwer 2005). Human RNA helicase A also unwinds both dsDNA and dsRNA (Zhang and Grosse 1994). These in vitro experiments suggest that DExH-box helicases recognize structures of nucleotides such as the length of single and double strands rather than specific sequences. Furthermore, RHAU ATP-hydrolysis activity was activated by a wide range of polynucleotides as well as dsRNA, dsDNA and ssDNA ((Tran et al. 2004) and unpublished data from Akimitsu), suggesting that RHAU has low specificity for stimulation of enzymatic activity.

RHAU is presumably a protein passively used by other molecules in different contexts, rather than having an active function by itself. It gains specificity only through interactions with other proteins that bind to specific RNAs. For RHAU, NF90 is required for the interaction with AREuPA and AREuPA is required for the interaction with NF90. RHAU affects the decay of AREuPA only in the context of these two molecules. Proportionally, only a small number of RHAU molecules (a few percent of total RHAU expression) are found in such a complex in whole cell lysates, suggesting that other populations of RHAU are involved in different cellular processes through interactions with different molecules. If RHAU has a broad capacity for molecular interactions, there must be a way to increase its specificity. One possibility is by cellular compartmentalization. RHAU may be
restricted to particular cell compartments where it selectively associates with other molecules. In mammalian cells, RHAU is detected both in the nucleus and the cytoplasm, particularly in places where RNA exists at high concentrations. RHAU may affect transcriptional activity of some genes only when located at sites enriched with other transcription-related factors with which it interacts.

Thus, to further understand this molecule, more detailed information on cellular transportation is required. One problem in measuring RHAU activity in whole cell extracts is that the activity may be diluted below detection since RHAU is involved in many processes at the same time. Where RHAU movement can be restricted to one cellular compartment, its single function should be more prominent. The isolation of RHAU mutants with different cellular localizations would facilitate such an approach. It is also important to investigate interactions between RHAU and other molecules in situ. For this thesis, I have carried out immunoprecipitation experiments using whole cell lysates treated with RNases to examine protein-protein interactions and their RNA dependency. However, this approach does not necessarily reflect the in vivo situation. It lacks information on cellular compartments and, thus, molecular interactions occurring only in a specific cellular compartment would be lost in the lysates. Furthermore, treatment with RNases would destroy the structures of proteins associating with RNAs, so RHAU probably loses even direct protein-protein interactions in which RHAU recognizes particular protein structures. Therefore, the visualization of protein-protein or protein-RNA interactions in situ will be advantageous for understanding molecular interactions by RHAU. Fluorescence resonance energy transfer (FRET) using fluorescent proteins could be one method to provide such information. Furthermore, live-cell imaging should also provide important information, such as the velocity of molecular movements in different cellular conditions or the relationship between enzymatic activity and RHAU movement.

More important, the definition of the basic functions of RHAU is necessary in order to fully understand this molecule. Since RHAU activity exhibits low specificity, it is probably difficult to determine its RNA or protein substrates in crude whole cell extracts. An important issue then is how RHAU affects RNA-protein complexes, which could be answered by in vitro experiments, rather than to determine in which pathway RHAU is involved. Does it use the unwinding activity of duplex RNAs or RNPase activity to dissociate proteins from RNAs, or has it other as yet unknown activities? Answers to these basic questions will reveal the general function of RHAU and, thus, its involvement in various cellular processes.
Acknowledgements

First of all, I would like to express my deep gratitude to my supervisor, Dr. Yoshikuni Nagamine. I thank him for giving me the opportunity to pursue my studies in such a great environment. His educational philosophy “let students think by themselves” gave me many precious experiences in challenging experiments and, more important, valuable time to think deeply about my work. It has been really worthwhile training for me to learn how to pursue studies with my own ideas. I am certain that all the experiences I have encountered during my PhD work have provided me with wealth for my further life.

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And finally, I would like to thank Yutaka Matsuda for his advice and directions to many scientific works, as well as beyond.
### APPENDIX - I: Oligonucleotides used in this study

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<th>Primers used for cDNA cloning</th>
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<td>Flag-s</td>
<td>AGCTTGCAGCCCATGGAGACGATACCAAGACGAGGCAAGG</td>
</tr>
<tr>
<td>Flag-as</td>
<td>GATCCCAGGAACTGCGAAGAAGGTAATTCAGAGATACCTTCTTCGG</td>
</tr>
<tr>
<td>HA-s</td>
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</tr>
<tr>
<td>HA-as</td>
<td>GATCCCGCTGTATCTGTGCTGGTGTTCAGAGAGAAAACCAGCAGAAG</td>
</tr>
<tr>
<td>p68-fw-BamHI</td>
<td>AT GGATCC ATTCGCGGTTATTCGAGT</td>
</tr>
<tr>
<td>p68-rv-XhoI</td>
<td>C CTGGAG TATTTGGAAATATCCCTGGT</td>
</tr>
<tr>
<td>p72-fw-BamHI</td>
<td>AT GGATCC ATTCGCGGTTATTCGAGT</td>
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<tr>
<td>p72-rv-XhoI</td>
<td>C CTGGAG TATTTGGAAATATCCCTGGT</td>
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Primers used for real-time PCR  5' - 3'

<table>
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<th>Primer</th>
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<tr>
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<td>NDUFA12-rv</td>
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<td>MARK1-fw</td>
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<td>MARK1-rv</td>
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<td>TMF1-fw</td>
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<td>TMF1-rv</td>
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<td>DAPK1</td>
<td>QuantiTect Primer Assays (QIAGEN), Hs_DAPK1_SG_1</td>
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</table>

Oligonucleotides used in this thesis are shown.
Underline: sequence generating RHAU-specific shRNA (shRHAU1-s and –as, shRHAU2-s and -as) and mutation sites (RHAUsm-s and -as, E335A-s and -as). Italic: restriction sites.

Primers for real-time PCR were designed using Primer Express™ (Applied Biosystems, version 2.0.0). Primers’ Tm values are set about 60 °C.
### PROBE SETS SHOWING UP-REGULATION OF STEADY-STATE mRNA IN shRHAU dox+ CELLS

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold change dox+/-</th>
<th>Fold in SV</th>
<th>Fold change dox+/-</th>
<th>Fold in SV</th>
<th>t1/2 SE</th>
<th>t1/2 SE</th>
<th>dec inc</th>
<th>sig-change</th>
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<tbody>
<tr>
<td>707937_s_at</td>
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<td>1.10 1.43 1.60 1.49</td>
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</tbody>
</table>

**Up- and down-regulated probe sets in shRHAU cells or shLuc cells after doxycycline-treatment (dox+). ARE, clusters of ARE set in the ARED (http://rc.kfshrc.edu.sa/ARED/) are shown if gene contains an ARE sequence; Fold change, fold change between dox- and dox+ derived by expression value in dox+ divided by dox-; t1/2, half-life in each sample; SE, standard error of half-life; sig-change, whether there is a significant changes of half-life between dox- and dox+ (P<0.05); dec, decreased half-life in dox+; inc, increased half-life in dox+; NA, not available.
## Appendix - II

Probe sets showing down-regulation of steady-state mRNA in shRHAU dox+ cells.

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene Symbol</th>
<th>ARE</th>
<th>fold change</th>
<th>fold in STV</th>
<th>decay-cshc-in</th>
<th>decay-cshc-shHAU</th>
<th>decay-shc-in</th>
<th>decay-shc-shHAU</th>
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</thead>
<tbody>
<tr>
<td>223140_at</td>
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<td>0.89</td>
<td>0.94</td>
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Probe sets showing up-regulation of steady-state mRNA in shLuc dox+ cells.

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene Symbol</th>
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<th>decay-shc-in</th>
<th>decay-shc-shHAU</th>
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Probe sets showing up-regulation of steady-state mRNA in shLuc dox+ cells.

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Gene Symbol</th>
<th>ARE</th>
<th>fold change</th>
<th>fold in STV</th>
<th>decay-cshc-in</th>
<th>decay-cshc-shHAU</th>
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<th>decay-shc-shHAU</th>
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</tbody>
</table>
Appendix - III: RHAU-target mRNAs (on mRNA stability)

Probe sets showing significantly decreased half-life in shRHAU dox+ cells.

| Probe Set | Description | mRNA Stability
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<tr>
<th></th>
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<tbody>
<tr>
<td>259198_s_at</td>
<td>F75E, RhmF, and IH domain containing 4</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>266516_s_at</td>
<td>SET domain containing 2 (lymphocyte repressor candidate 5)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>259249_s_at</td>
<td>Atch 1c</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>259217_s_at</td>
<td>RHAU-target mRNA stability</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>267902_s_at</td>
<td>ATM (Serine/threonine kinase)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>259204_s_at</td>
<td>opositional element binding protein 3</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>259218_s_at</td>
<td>ESR1 (Estrogen receptor)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>265034_s_at</td>
<td>MTA2 (Multicopy transposable element)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>265035_s_at</td>
<td>OSR1 (Osteoblast-specific transcription factor)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
<tr>
<td>265036_s_at</td>
<td>OSR1 (Osteoblast-specific transcription factor)</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
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<td>271741_s_at</td>
<td>opositional element binding protein 3</td>
<td>Dox: Decreased half-life in shRHAU dox+ cells</td>
</tr>
</tbody>
</table>

Note: The table above lists probe sets showing significantly decreased half-life in shRHAU dox+ cells. Each probe set is associated with mRNA stability, indicating the impact of dox treatment on mRNA half-life.

**Legend:**
- **Dox:** Dox treatment
- **Steady state:** Steady-state mRNA levels
- **Half-life:** Half-life of mRNA in shRHAU dox+ cells compared to control

**References:**
- F75E, RhmF, and IH domain containing 4
- SET domain containing 2 (lymphocyte repressor candidate 5)
- Atch 1c
- RHAU-target mRNA stability
- ATM (Serine/threonine kinase)
- opositional element binding protein 3
- ESR1 (Estrogen receptor)
- MTA2 (Multicopy transposable element)
- OSR1 (Osteoblast-specific transcription factor)
Probe sets showing significantly increased half-life in shRHAU dox+ cells.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>MAF name</th>
<th>Genotypes</th>
<th>Counts</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>shRNAU</td>
<td>probe set 1</td>
<td>genotype A</td>
<td>count A</td>
<td>p-value A</td>
</tr>
<tr>
<td>shRNAU</td>
<td>probe set 2</td>
<td>genotype B</td>
<td>count B</td>
<td>p-value B</td>
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<td>probe set 3</td>
<td>genotype C</td>
<td>count C</td>
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</table>

Appendix - iii

- Probe sets showing significantly increased half-life in shRHAU dox+ cells.

<table>
<thead>
<tr>
<th>Probe set</th>
<th>MAF name</th>
<th>Genotypes</th>
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<th>p-value</th>
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<tbody>
<tr>
<td>shRNAU</td>
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</table>

Appendix - iii

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<thead>
<tr>
<th>Probe set</th>
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# Appendix - IV: Starvation-sensitive genes

Probe sets showing up-regulation in starving culture condition.

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Fold change</th>
<th>P-value</th>
<th>q-value</th>
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<tbody>
<tr>
<td>ENSMUSG00000003137</td>
<td>2.42</td>
<td>0.001</td>
<td>0.0001</td>
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<tr>
<td>ENSMUSG00000003138</td>
<td>2.36</td>
<td>0.005</td>
<td>0.0005</td>
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<tr>
<td>ENSMUSG00000003139</td>
<td>2.24</td>
<td>0.010</td>
<td>0.0010</td>
</tr>
<tr>
<td>ENSMUSG00000003140</td>
<td>2.18</td>
<td>0.015</td>
<td>0.0015</td>
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<tr>
<td>ENSMUSG00000003141</td>
<td>2.13</td>
<td>0.020</td>
<td>0.0020</td>
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<tr>
<td>ENSMUSG00000003142</td>
<td>2.08</td>
<td>0.025</td>
<td>0.0025</td>
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<tr>
<td>ENSMUSG00000003143</td>
<td>2.03</td>
<td>0.030</td>
<td>0.0030</td>
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<tr>
<td>ENSMUSG00000003144</td>
<td>1.98</td>
<td>0.035</td>
<td>0.0035</td>
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<tr>
<td>ENSMUSG00000003145</td>
<td>1.93</td>
<td>0.040</td>
<td>0.0040</td>
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<tr>
<td>ENSMUSG00000003146</td>
<td>1.88</td>
<td>0.045</td>
<td>0.0045</td>
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<tr>
<td>ENSMUSG00000003147</td>
<td>1.83</td>
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<td>0.0050</td>
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<tr>
<td>ENSMUSG00000003148</td>
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<td>ENSMUSG00000003149</td>
<td>1.73</td>
<td>0.060</td>
<td>0.0060</td>
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<td>ENSMUSG00000003150</td>
<td>1.68</td>
<td>0.065</td>
<td>0.0065</td>
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<tr>
<td>ENSMUSG00000003151</td>
<td>1.63</td>
<td>0.070</td>
<td>0.0070</td>
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<td>ENSMUSG00000003152</td>
<td>1.58</td>
<td>0.075</td>
<td>0.0075</td>
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<td>ENSMUSG00000003153</td>
<td>1.53</td>
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<td>ENSMUSG00000003154</td>
<td>1.48</td>
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<td>ENSMUSG00000003155</td>
<td>1.43</td>
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<td>ENSMUSG00000003156</td>
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<td>0.0095</td>
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<td>ENSMUSG00000003157</td>
<td>1.33</td>
<td>0.100</td>
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Note: The table above lists the genes that show significant up-regulation in starving culture conditions with their fold change values, p-values, and q-values.
Probe sets showing down-regulation in starving culture condition.

<table>
<thead>
<tr>
<th>Affymetrix ID</th>
<th>Description</th>
<th>Gene Symbol</th>
<th>fold change</th>
<th>SE</th>
<th>ShLuc</th>
<th>shRHAU</th>
<th>SE_shLuc</th>
<th>SE_shRHAU</th>
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<tbody>
<tr>
<td>1305724_s_at</td>
<td>transglutaminase</td>
<td>TALGL</td>
<td>0.067</td>
<td>0.042</td>
<td>0.006</td>
<td>0.006</td>
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<tr>
<td>209547_s_at</td>
<td>calpain</td>
<td>AAGT</td>
<td>0.963</td>
<td>0.034</td>
<td>0.005</td>
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<tr>
<td>209132_s_at</td>
<td>clathrin, clathrin/mannose-6-phosphate receptor</td>
<td>VPS54</td>
<td>1.004</td>
<td>0.074</td>
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<td>0.009</td>
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<tr>
<td>237825_s_at</td>
<td>interactin 7 receptor, interactin 7 receptor</td>
<td>I7R7</td>
<td>0.270</td>
<td>0.096</td>
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<tr>
<td>250232_s_at</td>
<td>aryl hydrocarbon receptor</td>
<td>ARNT</td>
<td>0.006</td>
<td>0.006</td>
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<tr>
<td>202160_s_at</td>
<td>mitochondrial complex 2</td>
<td>HSPD1</td>
<td>0.140</td>
<td>0.164</td>
<td>0.012</td>
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<td>202915_s_at</td>
<td>mitochondrial complex 2</td>
<td>HSPD1</td>
<td>0.100</td>
<td>0.066</td>
<td>0.016</td>
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<tr>
<td>240869_s_at</td>
<td>bcl2-like growth factor, bcl2-like growth factor</td>
<td>BCL2L1</td>
<td>0.176</td>
<td>0.116</td>
<td>0.033</td>
<td>0.033</td>
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<tr>
<td>228081_s_at</td>
<td>bcl2-like growth factor, bcl2-like growth factor</td>
<td>BCL2L1</td>
<td>0.212</td>
<td>0.152</td>
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<td>250219_s_at</td>
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<td>219065_s_at</td>
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<td>215177_s_at</td>
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<td>0.164</td>
<td>0.046</td>
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</table>

Up- and down-regulated probe sets in both HeLa-shLuc and HeLa-shRHAU cells (dox-) in serum-starved (STV) condition compared to not-starved condition. Fold change, between not-starved (cells cultured with FCS) and starved cells derived by expression value in FCS- divided by FCS+; SE, standard error of the fold-differences.
References


Bono, F., Ebert, J., Lorentzen, E., and Conti, E. 2006. The crystal structure of the exon junction complex reveals how it maintains a stable grip on mRNA. *Cell* 126(4): 713-725.


References


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**CURRICULUM VITAE**

**FUMIKO IWAMOTO**

Date and Place of birth: 14.02.1979, Fukuoka, Japan  
Nationality: Japanese  
Private Address: Gartengasse25 4125 Riehen, Switzerland  
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**EDUCATIONAL QUALIFICATION**

04. 2003 to 12. 2007 Friedrich Miescher Institute for Biomedical Research, University of Basel, Switzerland  
International Ph.D. Program  
Thesis: Biological roles of DExH RNA helicase, RHAU  
Supervisor: Dr. Yoshikuni Nagamine

04. 2001 to 03. 2003 Kyushu University, Fukuoka, Japan  
Graduate School of Sciences, Department of Biology  
Master of Science  
Thesis: Regulation of nuclear transport and protein degradation of PPARα and γ (mark A)  
Supervisor: Prof. Yukio Fujiki

07. 1998 to 08. 1998 University of Winnipeg, Canada  
English language course

04. 1997 to 03. 2001 Kyushu University, Fukuoka, Japan  
School of Sciences, Department of Biology  
Bachelor of Science (mark A)

**GRANTS AND FELLOWSHIPS**

09. 2006 EC Marie Curie scholarship for traveling grant for apoptosis meeting.  
01. 2005 to 12. 2005 Research Grant from Swiss cancer league.  
04. 2001 to 03. 2003 Scholarship from the Japan Scholarship Foundation.
## Research Experiences

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<tr>
<th>Ph.D study  04. 2003  to 12. 2007</th>
<th>Biological roles of DExH RNA helicase, RHAU</th>
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<tr>
<td><strong>Publication</strong></td>
<td>Transcription-dependent nucleolar cap localization and possible nuclear function of DExH RNA helicase RHAU</td>
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<tr>
<td><strong>Technical expertise</strong></td>
<td>RNA and DNA</td>
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<tr>
<td></td>
<td>Northern blot, RNA degradation assay, Nuclear Run-on assay, in vitro ATPase assay, in vitro transcription, synthesis and transfection of dsRNA, siRNA, Chromatin immunoprecipitation (ChIP), cloning of cDNA, site-directed mutagenesis, methylation-specific PCR, DNA microarray (statistical analysis using Expressionist, Excel, Pathway, and R)</td>
</tr>
<tr>
<td><strong>Protein</strong></td>
<td>Purification of recombinant proteins (from E.Coli and insect cells), Pull down experiment using GST proteins, Immunoprecipitation, Western blot, Purification of antibodies and antigens (recombinant His-protein)</td>
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<tr>
<td><strong>Cell and Animal model</strong></td>
<td>Immunofluorescence, Confocal microscope (ZEISS LSM meta), Luciferase reporter assay, apoptosis assay using FACS, cell proliferation assay, Tumor development experiments in nude mice</td>
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<tr>
<th>Bachelor and Master’s study  04. 2001  to 03. 2003</th>
<th>Regulation of nuclear transport and protein degradation of PPARα and γ</th>
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<tr>
<td><strong>Summary</strong></td>
<td>PPARs are ligand-induced transcription factors that have important roles on lipid metabolism and adipocyte differentiation. I have identified 50 amino acid-nuclear localization signals (NLS) of PPARs that were necessary and sufficient for their nuclear translocation. I found that ligands induce their nuclear translocation by live-cell imaging using confocal microscope. In addition to that, I discover ubiquitination of the NLS that were necessary for the protein degradation, thus I could show the important relationship between protein degradation and cellular compartmentalization.</td>
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## Technical Diploma

09.2003 Swiss animal course (module 1)

## Conferences Attended

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<tr>
<td>09.2006</td>
<td>14th Euroconference on Apoptosis, Chia, Italy</td>
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<td>10.2005</td>
<td>Novartis – FMI joint meeting on Epigenetics, Switzerland</td>
</tr>
<tr>
<td>07.2005</td>
<td>International helicase meeting: Helicases and NTP driven nucleic acid machines, Arolla, Switzerland (selected as an oral presenter)</td>
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<tr>
<td>10.2004</td>
<td>Novartis Corporate Research Conference, Boston, USA</td>
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<tr>
<td>10.2003</td>
<td>Novartis – FMI joint meeting on Epigenetics, Emmetten, Switzerland</td>
</tr>
<tr>
<td>06.2003</td>
<td>First International meeting on ARE’s and AUBP’s in mRNA stability and translational control, Florence, Italy</td>
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<tr>
<td>2003 –2007</td>
<td>Annual meetings of Friedrich Miescher Institute, Switzerland.</td>
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<tr>
<td>10.2002</td>
<td>75th Annual meeting of Japanese Biochemical Society, Kyoto, Japan</td>
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<td>12.2001</td>
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