Received: 16 March 2022 | Accepted: 21 April 2022

DOI: 10.1002/ame2.12251

REVIEW

To unwind the biological knots: The DNA/RNA G-quadruplex resolvase RHAU (DHX36) in development and disease

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Funding information
National Key Research and Development Program of China, Grant/Award Number: 2019YFA0801601; National Natural Science Foundation of China, Grant/Award Number: 31930029, 91854111 and 31571490

Abstract

The G-quadruplex (G4) sequences are short fragments of 4-interval triple guanine (G) with frequent and ubiquitous distribution in the genome and RNA transcripts. The G4 sequences are usually folded into secondary "knot" structure via Hoogsteen hydrogen bond to exert negative regulation on a variety of biological processes, including DNA replication and transcription, mRNA translation, and telomere maintenance. Recent structural biological and mouse genetics studies have demonstrated that RHAU (DHX36) can bind and unwind the G4 "knots" to modulate embryonic development and postnatal organ function. Deficiency of RHAU gives rise to embryonic lethality, impaired organogenesis, and organ dysfunction. These studies uncovered the pivotal G4 resolvase function of RHAU to release the G4 barrier, which plays fundamental roles in development and physiological homeostasis. This review discusses the latest advancements and findings in deciphering RHAU functions using animal models.

KEYWORDS
development and disease, DHX36, G-quadruplex, RHAU

1 | INTRODUCTION

G4 sequences are short fragments of 4-interval triple guanine (G) with frequent and ubiquitous distribution in the genome and RNA transcripts (Figure 1A). The G4 sequences are usually folded into secondary "knot" structure (G4 structure) via Hoogsteen hydrogen bond to exert negative regulation on a variety of biological processes, including DNA replication and transcription, mRNA translation, and telomere maintenance (Figure 1B). There are helicases to relieve the G4 structure barrier, and the known dual DNA/RNA G4 helicases are RHAU, DDX21, and DHX9.

The discovery of RHAU dates back to 2004 when Dr. Yoshikuni Nagamine's laboratory in the Friedrich Miescher Institute for Biomedical Research (a part of the Novartis Research Foundation) in Basel, Switzerland, identified a new AU-rich element (ARE)-binding protein that was associated with the ARE fragment of the urokinase plasminogen activator mRNA.[1] This new protein was named RNA helicase associated with AU-rich elements (RHAU) and was found to promote the degradation of ARE-containing mRNAs.[1] Protein sequence analysis of RHAU demonstrated the identical amino acid composition with the ATP-dependent DEAD/DEAH-box helicase 36 (DDX36 or DHX36).[2] One year later, it was found that RHAU possessed the DNA G4 resolving activity to bind and unwind DNA G4 structure, and was hence called G4 resolvase 1 (G4R1).[3] By then, 3 names had been given to this protein: RHAU, DHX36, and G4R1. Afterwards, the G4 resolvase activity of RHAU was extended to RNA molecule through in vitro biochemical analysis.[4] These early pioneering studies have uncovered the primary property of RHAU as DNA/RNA G4 resolvase/helicase to unwind the G4 secondary structure and laid a solid foundation for elucidating the biological function of RHAU in mouse models. Later on, Dr. Nagamine's group generated the global *Rhau* knockout mice that were embryonic lethal at around embryonic day 7.5 (E7.5), indicating the essential role of RHAU in mouse development.[5] A following study of hematopoietic-specific *Rhau* deletion mice revealed

(A) CGGGUGCGCGGGCGGGCGGCGGGCACCAUG
CGGGGAGGCUGUCCCCAGGGGUGGGCAGCA

(B)

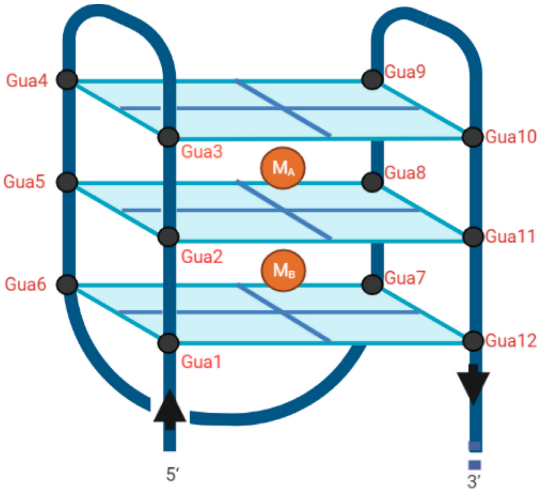

FIGURE 1 Nucleic acid sequence and secondary structure of G-quadruplex. (A) The G-quadruplex's nucleic acid sequence in the 5′-UTR region of mouse Nkx2-5 mRNA. (B) Schematic diagram of the secondary G-quadruplex structure (vertically stacked G-tetrad).

the indispensable function of RHAU in erythropoiesis and detected the potential G4-forming sequences in the promoter region of several genes in regulating red blood cell development.[5] This study implied that RHAU might regulate gene expression through its DNA G4 resolvase activity, which was verified by investigating the germ-cell-specific *Rhau* knockout mice. RHAU is most abundant in the testis, and deficiency of RHAU in the spermatogonia caused male infertility that was probably a consequence of impaired expression of *c-kit*, a gene containing G4 sequence in its promoter region.[5] Our group inactivated RHAU in the cardiac progenitor cells of mouse embryo and in the postnatal cardiomyocytes in mice and revealed its pivotal function in heart development and function through coupled regulation on RNA translation and stability.[7,8] These in vivo experiments not only confirmed the DNA/RNA G4 resolving activity of RHAU, but also comprehensively explored the distinct molecular mechanisms and a variety of biological functions of RHAU, which will be discussed in detail in this review.

2 | STRUCTURAL BIOLOGY OF RHAU AND ITS ASSOCIATION WITH SUBSTRATES

RHAU belongs to the DEAH-box helicase family, and it is therefore of great interest to explore the structural alteration of RHAU association with its substrates in order to understand the enzymatic regulatory mechanisms. Structural biological studies have uncovered that RHAU protein preserves the common structural characteristics of the DEAH-box helicase family. DEAH-box RNA helicases have a core component consisting of 2 RecA-like domains that are flanked by the N-terminal domain and the C-terminal domain[9,10] (Figure 2A). RecA is an *E. coli* protein that functions in DNA recombination, and the RecA-like structural domain possesses ATPase activity to move macromolecules or move along macromolecules (peptides or nucleic acids).[11] Thus, the proteins carrying the RecA-like domains are called motor ATPase. The C-terminal domain of RHAU protein comprises 4 subdomains of degenerate-winged-helix (WH), ratchet-like (RL), oligonucleotide and oligosaccharide-binding-fold-like (OB), and constitutive transport element (CTE). At the N-terminus of RHAU protein, the 2 RecA-like structural domains are strengthened through a glycine-rich element (Gly) followed by an RHAU-specific motif (RSM).[12] RSM contains 2 α-helixes and is essential for the association of RHAU with G4[13] (Figure 2B). As RHAU binds to the G4 sequence, the RecA1, RecA2, and C-terminal domains create a conformation as a trefoil (Figure 2B). The N-terminal RSM domains are connected to the RecA1 domain of the trefoil through a random sequence, and its α1-helix contacts the 5′ (top) face of the bound G4.[9] There is a channel apparent in the "trefoil" structure, which facilitates the pass-through of the 3′-end single strand of G4.[9] RHAU unwinds the G4 structure by pulling nucleotide residues one by one towards the 3′ to 5′ direction through the above channel, which is achieved by the RecA-like domain 2 (RecA2) via catalyzing the hydrolysis of ATP to provide energy.[9,14]

Although the RSM motif is critical in binding the G4, experimental studies have confirmed the participation of the OB domain in the process,[9] and computational modeling suggests the involvement of the RecA2 in the association of RHAU with the G4.[15]

3 | RHAU FUNCTIONS PRIMARILY AS DNA/RNA G4 RESOLVASE

3.1 | RHAU regulates gene expression via its DNA G4 resolvase activity

Shortly after its discovery, RHAU was uncovered as possessing DNA G4 resolving activity to bind and unwind DNA G4 structures, and was hence termed G4 resolvase 1 (G4R1) (Figure 3).

The protein of Yin Yang 1 (YY1) possesses multifaceted function and may modulate tumorigenesis.[16–19] It was found that there were G4 structures in the promoter of *YY1* gene and in the 5′-UTR (untranslated region) of its mRNA, and these G4 structures imposed effects on the expression of luciferase reporter. Further study indicated that RHAU bound to the G4 sequence of the *YY1* promoter in vitro, and overexpression of RHAU increased endogenous YY1 levels.[20] Although this was an in vitro study, it shed a light on the regulation of gene expression by RHAU through functioning on the G4 structures in the promoter regions.

Another in vitro cellular study reported that RHAU could interact with HDAC1/4 to monitor the expression of tissue-nonspecific alkaline phosphatase (TNAP), an important gene involved in bone mineralization and bone regeneration.[21,22] It was found that RHAU bound to a specific element in the *Tnap* promoter and deletion of *Rhau* significantly reduced the *Tnap* transcription level.[23] However, whether this type of regulation is G4 dependent is unknown and needs further confirmation in *Rhau* mutant mice.

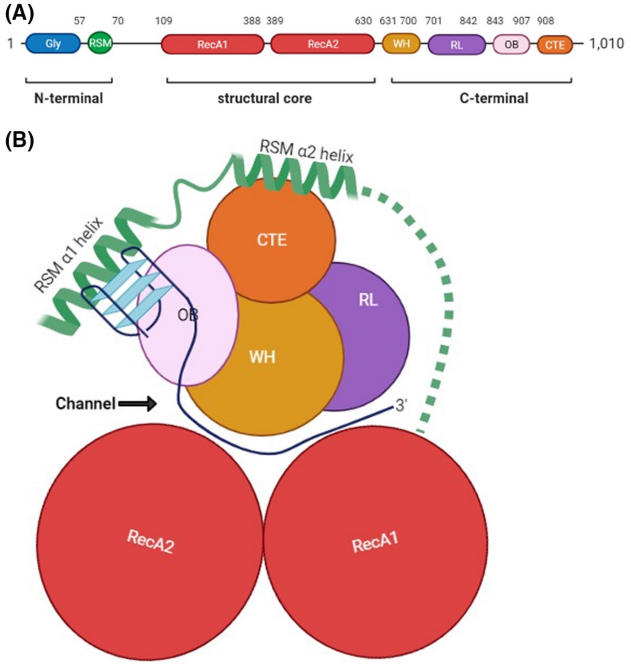

FIGURE 2 Schematic diagram showing the domains and structural regions of RHAU protein. (A) The protein domains of RHAU: the N-terminal domain, structural core, and the C-terminal domain. (B) Geometric representation of the complex of RHAU bound to ssDNA. CTE, constitutive transport element; OB, oligosaccharide-binding-fold-like; RL, ratchet-like; RSM, RHAU-specific motif; WH, degenerate-winged-helix.

3.2 | RHAU exerts post-transcriptional regulation of mRNA through RNA G4 resolvase activity

The first demonstration of the RNA G4 unwinding ability of RHAU was reported in 2008, and the study was performed in HeLa cell lysates[4] (Figure 3). A recent analysis examined the global regulatory mRNA targets of RHAU and revealed more than 4500 mRNAs with G4-forming and G-rich sequences that were preferentially interacting with RHAU in human cell lines.[24,25] Deletion of *Rhau* caused significantly enhanced levels of the target mRNA with reduced protein output, suggesting that RHAU directed mRNA degradation and translation.[26] mRNAs with G4s were enriched in stress granules (SG), and *Rhau* knockout (KO) resulted in increased stress granule formation and protein kinase R (PKR/EIF2AK2, cell stress response marker) phosphorylation. Meanwhile, *Rhau*-KO cells also displayed cellular stress, such as decreased cell proliferation and morphological changes. Thus, it was speculated that RHAU was involved in the alleviation of cell stress induced by G4.[27]

RHAU also plays an important role in the process of mRNA processing. Eukaryotic mRNAs need to obtain a poly (A) tail before maturation. This process is called pre-mRNA 3′-end processing. In the cellular DNA damage response, pre-mRNA 3′-end processing is usually inhibited and subsequently impairs protein production.[28] RHAU specifically binds and unwinds the G4 in the *p53* mRNA precursor, which is necessary to maintain 3′-end processing of the *p53* mRNA precursor after UV-induced DNA damage. Mutation of the G4 in the *p53* mRNA precursor suppressed the ability of RHAU to maintain pre-mRNA 3′-end processing. Therefore, it was believed that RHAU functions to achieve this type of compensation mechanism, safeguarding *p53* mRNA precursor processing and subsequent translation after UV damage.[29]

3.3 | RHAU associates with noncoding RNA to regulate biological processes

TERC is a 451-nt-long noncoding RNA (lncRNA), a core component of telomerase. RHAU bound to the G4 structure of the 5′-region of TERC, and associated with the telomerase holo-enzyme.[30] The P1 helix on TERC was a critical element that defined the boundary of the reverse transcription template. However, the 5′-region of TERC contained multiple G4 structures, which hindered the formation of P1 helix. RHAU could resolve these obstructive G4s and promoted the untwisted chain to form a stable P1 helix. Knockout of RHAU resulted in a decrease in average telomere length.[30]

It was reported that the precursor-miRNA-134 (pre-miR-134) accumulated in the dendrites of hippocampal neurons and synapses[31] and RHAU mediated the dendritic localization of pre-miR-134 by directly associating with the terminal loop of pre-miR-134 (Figure 3). This kind of binding was essential for miR-134-dependent inhibition of target gene expression and controlled the dendritic spine size.[32] This finding was supported by another study where knockdown of *Rhau* in nerve cells disrupted nuclear localization of pre-miR-134 and inhibited BDNF-dependent dendritic development.[33]

The mammalian genomes encode a large amount of long noncoding RNAs (lncRNAs) with a length of more than 200 nucleotides.[34,35] Unlike small noncoding RNAs (such as miRNAs), lncRNAs function through a variety of distinct mechanisms.[36,37] lncRNAs are often dysregulated in diverse human cancers.[36,37] For instance, lncRNA FLJ39051, also known as GSEC (G-quadruplex-forming sequence containing lncRNA), was one of the lncRNAs upregulated in colorectal cancer and could bind RHAU through its G4 forming sequence to suppress the resolvase activity of RHAU. This study suggests that inhibition of RHAU activity by GSEC through its G4 structure facilitates the migration of colon cancer cells.[38]

On the other hand, RHAU binding to RNA is not entirely G4-dependent. Recent studies have found that the noncoding RNA BC200 (BCYRN1) is particularly enriched in RHAU immunoprecipitation.[39,40] Although BC200 neither contains a G4 structure nor binds to the G4 interaction motif of RHAU, it had a direct affinity for RHAU on in vitro analysis. It was found that the adenosine-rich region at the 3′-end of BC200 binds to the C-terminal region of RHAU, while the cytosine-rich region at the 3′-end of BC200 is necessary for association with unwound G4. This study provides a possible regulatory manner by which RHAU governs BC200 to modulate G4-containing RNA or DNA sequences.[41]

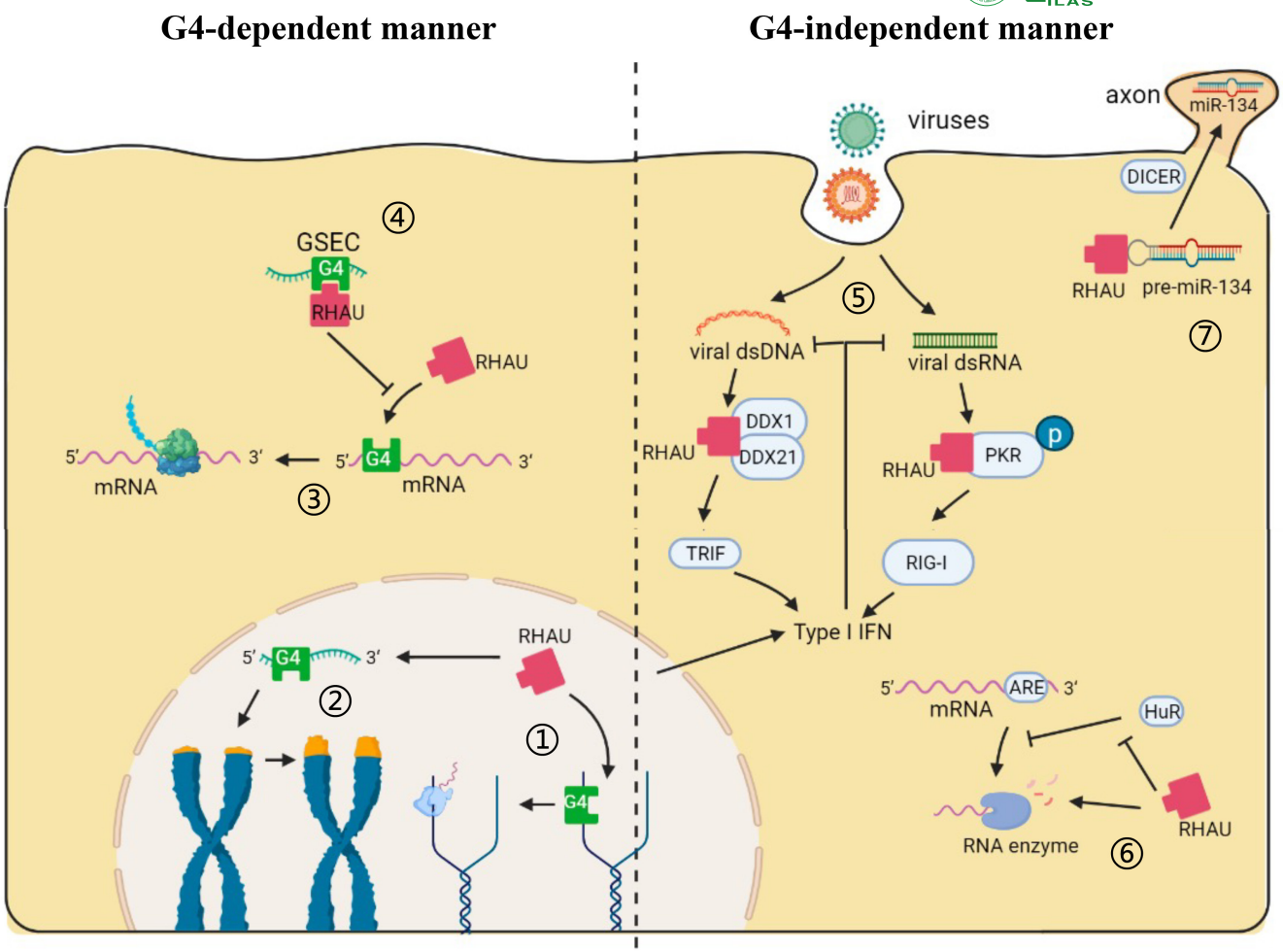

FIGURE 3 RHAU works primarily in G4-dependent manner. ① RHAU regulates gene expression via its DNA G4 resolvase activity. ② RHAU promotes telomere lengthening. ③ RHAU exerts post-transcriptional regulation of mRNA through RNA G4 resolvase activity. ④ and ⑦ RHAU associates with noncoding RNA to regulate biological processes. ⑤ RHAU senses viral nucleic acid and plays a role in host defense. ⑥ RHAU promotes mRNA degradation by binding to the ARE.

3.4 | RHAU senses viral nucleic acid to defend against pathogens

Sensing microbial DNA helps detect microbial infection, which in turn activates the innate immune system. The RNA helicases DDX1, DDX21, and RHAU could assemble into a viral sensor to detect the invasion of viruses in myeloid dendritic cells (Figure 3). Subsequently, the adaptor molecule TRIF triggered the type I IFN and cytokine responses to suppress the viruses.[42,43] Another study demonstrated that these RNA helicases participated in antiviral immune response in fish. In the kidney of common fish with viral infection, the upregulation of these RNA helicases occurs before the upregulation of type I IFN genes.[44] Furthermore, it was found that RHAU formed a complex with dsRNA-dependent protein kinase (PKR) to sense dsRNA in the cytoplasm for inhibition of RNA virus invasion. In the complex, RHAU assisted dsRNA binding and PKR phosphorylation that activated RIG-I to trigger antiviral IFN responses. Knockout of RHAU reduced the secretion of IFN and abolished the ability to resist RNA virus.[27]

Taken together, RHAU works primarily in G4-dependent manner to regulate multiple biological processes in cells. RHAU interacts with a variety of nucleic acid molecules and is involved in the regulation of gene transcription and translation, mRNA degradation, and more complex and diverse interactions with noncoding RNAs.

4 | GENETIC STUDY OF *RHAU*-DEFICIENT MICE

4.1 | RHAU plays an essential role in early embryonic development

Immediately after identification of RHAU, Dr. Nagamine's group started to generate *Rhau* mutant mice for understanding its physiological functions. First, they produced the global (whole body) *Rhau* knockout mice and revealed an early embryonic lethality of the mice ($Rhau^{-/-}$) at around embryonic day 7.5 (E7.5), while at E6.5, the $Rhau^{-/-}$ mice appeared normal[5] (Figure 4). These findings

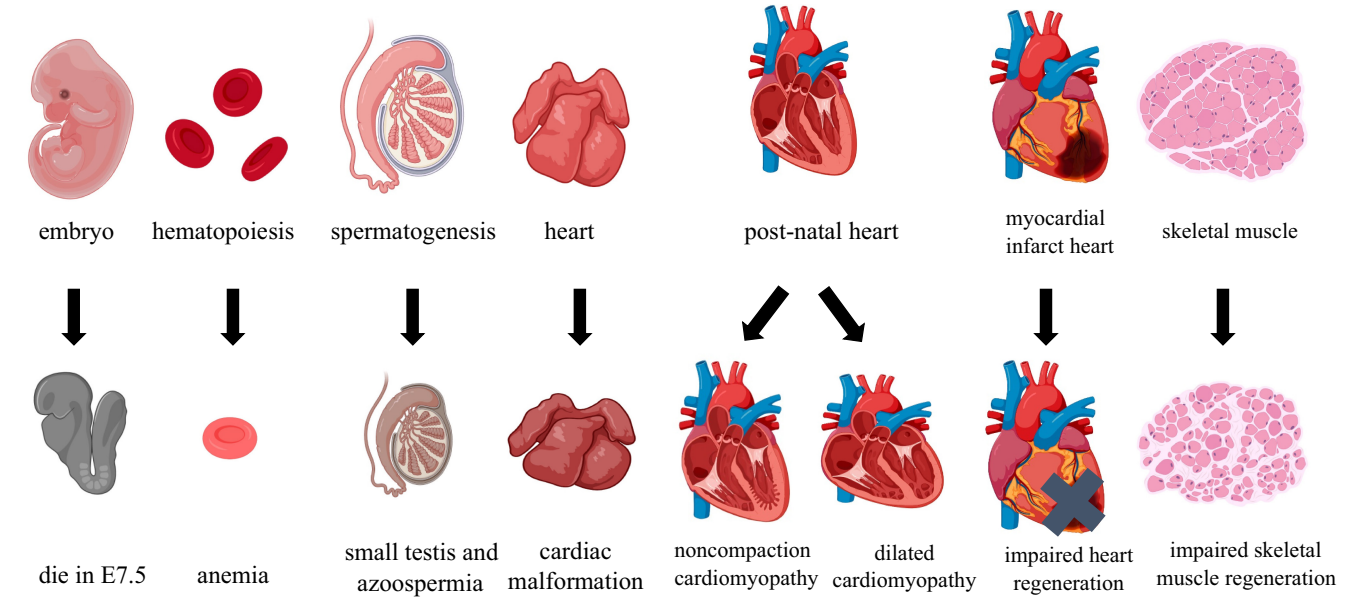

FIGURE 4 RHAU functions in early embryogenesis, organ/tissue development, and regeneration.

demonstrated the essential role of RHAU in early embryogenesis (gastrulation). To overcome the hindrance of embryonic lethality of conventional *Rhau* knockout mice, Dr. Nagamine's group thereafter designed conditional alleles of *Rhau* in mice for tissue-specific deletion of *Rhau*.

4.2 | RHAU in hematopoiesis and spermatogenesis

In a collaborative study, Dr. Nagamine and Dr. Matthias' groups inactivated RHAU in the hematopoietic stem cells using *Vav1*-mediated Cre excision. They uncovered anemia in the hematopoietic-specific *Rhau* deletion mice as a result of erythropoietic differentiation defects (Figure 4). Bioinformatic analysis of the dysregulated genes in *Rhau* mutant mice identified a number of G4-containing sequences in the promoter of genes involved in regulating cell proliferation. From these results, they hypothesized that RHAU might monitor gene expression through resolving the G4 in promoter regions.[5]

These initial studies of *Rhau* global and tissue-specific deficiency mice have built a strong foundation to decipher the functions of RHAU in organogenesis and tissue formation. To explore the role of RHAU in sperm development, the germ-cell-specific *Rhau* deletion mice were produced, and these mice showed small testis together with missing mature sperm (azoospermia). RHAU deficiency in germ cells resulted in an increase of the G4 structures in the genome, leading to a decrease in spermatogonial differentiation.[6] Further studies have shown that RHAU could bind to the G4 motifs in the *c-kit* promoter to facilitate its transcription, which in turn gave rise to c-kit protein to regulate spermatogonia differentiation.[6] This work provides the first evidence that RHAU controls gene expression via G4 resolvase activity in mice.

4.3 | RHAU in heart development and postnatal heart function

During early mouse embryonic development, gastrulation is completed by E7.5 and the 3 germ layers of ectoderm, mesoderm, and endoderm are present.[45] The cardiac mesoderm derives immediately from the nascent mesoderm following accomplishment of gastrulation to specify into cardiac tissues for heart development.[46] Therefore, the first organ to form and function is heart for establishing the circulatory system. As stated above, the global *Rhau* knockout mice are embryonic lethal at around E7.5, and it is rational to hypothesize that RHAU may play a critical role in heart development. We thus tested this hypothesis and deleted *Rhau* in the nascent mesoderm through *Mesp1*-Cre mediated excision and in the cardiomyocytes via *Nkx2-5*-Cre directed elimination. These cardiac-specific *Rhau* deletion mice displayed severe cardiac malformations along with reduced cardiomyocyte proliferation, leading to embryonic lethality at E12.5[7] (Figure 4). Further study revealed that *Rhau* deletion resulted in a substantial increase of *Nkx2-5* mRNA level. However, the protein level of NKX2-5 was profoundly reduced in the *Rhau*-deficient heart compared with control. Biochemical and molecular studies uncovered 2 conserved regulatory elements located in the 5′- and 3′-UTR in monitoring *Nkx2-5* mRNA translation and stability. While the G4 structure in the 5′-UTR suppressed *Nkx2-5* mRNA translation, the AU-rich element (ARE) in the 3′-UTR advanced *Nkx2-5* mRNA decay. RHAU associated with the G4 structure in the *Nkx2-5* mRNA to facilitate translation but promoted mRNA degradation by binding to the ARE. In the absence of RHAU, the *Nkx2-5* mRNA molecules accumulated because the degradation machinery was disrupted. However, these *Nkx2-5* mRNA could not translate into protein owing to the G4 barrier, resulting in elevated mRNA level and reduced protein level. Thus, we disclosed

a novel mechanism of coordinated *Nkx2-5* mRNA translation and degradation, which is regulated by RHAU and is involved in heart development.[7]

Our findings demonstrated an essential role of RHAU in embryonic heart development. We then asked whether RHAU regulated postnatal heart function. To address this question, we generated mouse models of RHAU deficiency in the cardiomyocytes of postnatal mice through the *Myh6-Cre*-mediated RHAU inactivation. These *Rhau* deletion mice developed progressive dilated cardiomyopathy (DCM) leading to heart failure and were all lost within 6 months[8] (Figure 4). Mechanistic study discovered G4 in 2 other mRNA molecules, *Hexim1* (*Hexamethylene bisacetamide inducible 1*) and *YAP1* (*Yes1-associated transcriptional regulator*), that are important for both embryonic heart development and postnatal heart function. In a regulatory manner similar to that of *Nkx2-5* mRNA, RHAU bound to *Hexim1* and *YAP1* mRNA to control mRNA translation and stability.[8]

To expand the study of RHAU in late cardiac development, especially in the formation of ventricular chambers, we deleted *Rhau* in embryonic cardiomyocytes using *cTNT*-Cre and identified the noncompaction cardiomyopathy (NCC) phenotype[47] (Figure 4). In this study, we found that RHAU modulated gene expression level of several genes, including *Myh7*, *Nkx2-5*, and *Hey2*, that are involved in ventricular trabeculation and compaction. We further revealed that RHAU regulated *Myh7* transcription by unwinding G4 structures in the promoter region of *Myh7*, leading to reduced MYH7 but enhanced MYH6 protein level. This study uncovered the critical function in modulating ventricular trabeculation and compaction, and implies that misregulation of RHAU function might be involved in the pathogenesis of NCC.[47]

Collectively, these studies declared the pivotal function of RHAU in regulating embryonic heart development and postnatal heart contractility, and RHAU deficiency caused heart defects and pathological heart remodeling, leading to heart failure and mortality.

4.4 | RHAU in heart and skeletal muscle regeneration

Our study of RHAU in postnatal heart function identified YAP1 as a RHAU-regulatory downstream target. YAP1 has been extensively explored in organ/tissue regeneration, and a conclusion was drawn from numerous studies that activation of YAP1 boosts organ/tissue regeneration.[48] Therefore, we investigated whether RHAU played a role in neonatal heart regeneration. Using a myocardial infarct model, we demonstrated that RHAU inactivation impaired heart regeneration in neonatal mice, indicating of a critical function of RHAU in heart repair and regeneration.

Recently, Dr. Huating Wang's group reported that RHAU was also involved in skeletal muscle regeneration. The skeletal muscle repair depends profoundly on satellite cell (SC) activation to form new fibers, which induced the expression of *Rhau*. Deletion of *Rhau* in the adult SCs impeded cell proliferation and muscle regeneration (Figure 4). Mechanistically, it was found that RHAU facilitated the translation of *Gnail* mRNA through associating with and resolving the G4 structures in the 5′-UTR to activate SC expansion.[49]

Together, these 2 works have demonstrated the crucial function of RHAU in muscle regeneration and repair. Manipulation of RHAU protein level may provide therapeutic application for tissue/organ regeneration and repair in the future.

5 | CONCLUSION

In this review, we focused on *RHAU* knockout animal models and their phenotypes to understand the physiological function together with the regulatory mechanisms of RHAU. The accumulating evidences obtained from tissue/organ-specific *Rhau* deletion mice supported the conclusion that RHAU employed DNA and RNA G4 resolvase activity to unwind the G4 structures in the genome and mRNA molecules to regulate gene expression, mRNA translation, and stability. The G4 resolvase activity of RHAU played an essential role in early embryogenesis, organ/tissue development, and regeneration. These findings imply that dysregulated RHAU function may cause pathological consequences, such as heart failure and muscle injury, and manipulation of RHAU G4 resolvase activity may have therapeutic application in the clinic.[50]

Likewise, numerous G4 structures found in the human genome and mRNA indicate that G4 and its major resolvase, RHAU, played important roles in regulating gene transcription and translation.[51,52] Previous studies have found that RHAU has an inhibitory effect on the tumorigenesis of lung cancer and breast cancer.[53–55] However, the role of RHAU under these disease conditions remains largely unknown. Furthermore, the regulatory relationship between RHAU and cancer-related gene activation should be explored in cancers. A comprehensive understanding of the G4-dependent and G4-independent functions of RHAU will provide new insights into the mechanisms of developmental defects and tumorigenesis.

ACKNOWLEDGMENTS

We thank Dr. Yingchao Shi, Wenli Fan, Xiaodong Wang, and Tianyang Zhao for their help in preparation of this review.

FUNDING INFORMATION

This work was supported by grants from the National Key Research and Development Program of China (grant no. 2019YFA0801601) and grants from the National Natural Science Foundation of China (grant nos. 31571490, 31930029, and 91854111) (to Z.Y.)

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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How to cite this article: Yang C, Yao J, Yi H, Huang X, Zhao W, Yang Z. To unwind the biological knots: The DNA/RNA G-quadruplex resolvase RHAU (DHX36) in development and disease. *Anim Models Exp Med*. 2022;5:542-549. doi: 10.1002/ame2.12251