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# The novel heart-specific RING finger protein 207 is involved in energy metabolism in cardiomyocytes

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ABSTRACT

A failing heart shows severe energy insufficiency, and it is presumed that this energy shortage plays a critical role in the development of cardiac dysfunction. However, little is known about the mechanisms that cause energy metabolic alterations in the failing heart. Here, we show that the novel RING-finger protein 207 (RNF207), which is specifically expressed in the heart, plays a role in cardiac energy metabolism. Depletion of RNF207 in neonatal rat cardiomyocytes (NRCs) leads to a reduced cellular concentration of adenosine triphosphate (ATP) and mitochondrial dysfunction. Consistent with this result, we observed here that the expression of RNF207 was significantly reduced in mice with common cardiac diseases including heart failure. Intriguingly, proteomic approaches revealed that RNF207 interacts with the voltage-dependent anion channel (VDAC), which is considered to be a key regulator of mitochondria function, as an RNF207-interacting protein. Our findings indicate that RNF207 is involved in ATP production by cardiomyocytes, suggesting that RNF207 plays an important role in the development of heart failure.



## 1. Introduction

The number of people suffering from heart failure (HF) has been increasing worldwide, especially in countries with markedly aging populations [1]. Although pharmacological and device-based therapies for HF have made dramatic progress over the past few decades, the one-year mortality rate for HF is still high at 10% [2]. The heart beats approximately 100,000 times per day, and the oxygen consumption of the heart accounts for 10% of the body's total oxygen consumption [3]. To meet this tremendous energy demand, the body's cardiac metabolism is strictly regulated under normal conditions [4–6].

The cardiac metabolism in a failing heart is different from that in a normal heart. The metabolic changes observed in HF are defined by reduced cardiac energy production that may arise from progressive disorder in substrate utilization and mitochondrial function [3,7]. Since the heart demands large amounts of energy to maintain contractile function, a reduced production of adenosine triphosphate (ATP) may directly lead to contractile dysfunction [8]. It is thus rational to assume that the cardiac metabolism plays an important role in the pathophysiology of HF.

Two of the most common cardiac pathologies that eventually lead to HF are (1) cardiac hypertrophy induced by pressure overload and (2) ischemia/reperfusion injury, and it has also been reported that cardiac metabolic changes occur in a condition called the 'compensated state' [7,9]. Although the clarification of metabolic changes in HF has progressed, little is known about the mechanisms that cause energy metabolic alterations in HF. Elucidation of the mechanisms through which cardiac metabolic changes occur in HF could thus uncover new therapeutic strategies for HF.

The really interesting new gene (RING)-finger domain contains eight metal-binding residues that coordinate two zinc atoms for protein folding and biological function [10]. >200 proteins containing a RING-finger domain (RNF) are encoded in the human genome. It has been reported that several RNF proteins play essential roles in many cellular processes including signal transduction [11], apoptosis [12] and lipid metabolism [13]. It was also recently demonstrated that mutations of some RNF proteins are involved in colorectal cancer [14], RIDDLE syndrome [15] and Moyamoya disease [16]. The RNF proteins RNF41 and RNF146 were reported to be associated with the development of cardiac dysfunction [17,18]. It has been shown that some of the RNF proteins

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are tissue-specifically expressed [19] and that the mRNA of RNF207 is specifically expressed in the heart [20,21]. However, the detailed function of RNF207 in the heart has not been elucidated.

The VDAC was first characterized as a mitochondrial outer membrane protein and it has three isoforms in humans: VDAC1, VDAC2 and VDAC3 [22]. The VDAC was initially considered to be a channel through which ions and metabolites such as ATP, adenosine diphosphate (ADP) and nicotinamide adenine dinucleotide (NADH) pass between the cytosol and mitochondria, but recent studies have shown that VDACs play an essential role in energy homeostasis, selective mitochondrial autophagy (mitophagy) and calcium homeostasis [23–25].

In the present study, we found that RNF207 protein is specifically expressed in the heart. A depletion of RNF207 in neonatal rat cardiomyocytes (NRCs) induced a reduction of ATP concentration and mitochondrial dysfunction. RNF207 interacts with the outer mitochondrial membrane protein VDAC1. The expression of RNF207 was significantly reduced in mouse models of common heart diseases including cardiac hypertrophy, ischemic-reperfusion injury and HF. Taken together, these findings suggest that RNF207 is involved in cardiac energy metabolism and the pathogenesis of common heart diseases.

## 2. Materials and methods

### 2.1. Cloning and plasmid construction

We amplified mouse *Rnf207* cDNAs from a mouse heart cDNA library by polymerase chain reaction (PCR) with BlendTaq (Takara, Tokyo) using the following primers: 5′-TAAATGTCTGGAGCAATTTTTGCG-3′ (RNF207 forward), 5′-TTGTCAGGCTTGGTAGCAAGCCCC-3′ (RNF207 reverse). We amplified mouse *Vdac1* cDNAs from cDNAs of mouse myoblast C2C12 cells by PCR with BlendTaq (Takara) using the following primers: 5′-ATGGCCGTGCCTCCCACATA-3′ (VDAC1 forward) and 5′-TTATGCTTGAAATTCCAGTCCTAGG-3′ (VDAC1 reverse). We subcloned the amplified fragments into pBluescript II SK(+) (Stratagene, La Jolla, CA) and verified the sequences. The resulting cDNA fragments were subcloned into the vectors pCGN-HA, p3 × FLAG, p3 × FLAG-CMV7.1, pcDNA3.1/myc-His and pMX-puro. *Rnf207* cDNAs including deletion mutants and point mutations and *Vdac1* cDNAs including deletion mutants were amplified by PCR and subcloned into p3 × FLAG and p3 × FLAG-CMV7.1.

### 2.2. Cell culture

Human embryonic kidney 293 T (HEK293T) cells, mouse myoblast C2C12 cells (ATCC, Manassas, VA) and their derivatives were cultured under an atmosphere of 5% $CO_2$ at 37 °C in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich, St. Louis, MO) supplemented with 10% (v/v) fetal bovine serum (Invitrogen, Carlsbad, CA), 55 μM β-mercaptoethanol (GIBCO, Grand Island, NY), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acid, penicillin (10 U $ml^{-1}$) and streptomycin (0.1 mg $ml^{-1}$) (Sigma). Cells stably overexpressing 3 × Flag-RNF207 were established by a retroviral expression system using pMX-puro with puromycin (8 μg $ml^{-1}$, Sigma) selection as described previously [26].

### 2.3. Primary culture of rat neonatal ventricular myocytes

We prepared ventricular cardiomyocytes from 2 to 3-day-old Crl:(WI) BR-Wistar rats (Sankyou Labo Service, Tokyo). A cardiomyocyte-rich fraction was obtained by centrifugation through a discontinuous Percoll gradient as described previously [27].

### 2.4. Mice

C57BL/6 J mice were purchased from CLEA Japan (Tokyo). The mice were used for experiments at 10–12 weeks of age (weight, 23–27 g). The mice were bred in a pathogen-free environment and kept under a constant 12-h light–dark cycle at a temperature of 23–25 °C. Standard chow and water were provided. All of the animal protocols were reviewed and approved by the Animal Welfare Committee of Hokkaido University. The work presented in this study is covered by the Animal Protocol No.APN-13-0074. All of the research staff who performed the procedures using live animals were pre-approved by the Animal Welfare Committee of Hokkaido University based on their completion of required animal use and care training and experience in animal experiments.

### 2.5. Mass spectrometry analysis

Immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with silver staining. The silver-stained bands were excised from the gel. The proteins therein were subjected to in-gel reduction, S-carboxyamidomethylation and digestion with sequence-grade trypsin (Promega, Fitchburg, WI) as described previously [28]. The resultant peptides were analyzed by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC ESI-MS/MS) (LCQ DECA and LTQ XL; Thermo Fisher Scientific, Waltham, MA). The data were analyzed using Mascot software (Matrix Science, Boston, MA).

### 2.6. Transfection, immunoprecipitation and Western blot analysis

Cells were transfected by Fugene HD reagent (Roche, Branchburg, NJ) and lysed in a solution containing 50 mM Tris-HCl (pH 7.6), l50 mM NaCl, 0.5% Triton X-100, leupeptin (10 μg $ml^{-1}$), 1 mM phenylmethylsulfonyl fluoride, 400 μM $Na_3VO_4$, 400 μM EDTA, 10 mM NaF, and 10 mM sodium pyrophosphate. The cell lysates were centrifuged at 16,000 *g* for 15 min at 4 °C, and the resulting supernatant was collected. Protein concentrations were measured (Pierce BCA, Rockford, IL, USA) and lysates that had the same amounts of protein were incubated with anti-FLAG M2 agarose (Sigma) or anti-HA-agarose (Sigma) for 1 h at 4 °C.

The beads was separated by centrifugation and washed five times with ice-cold lysis buffer, and the protein complexes were then eluted by 3 × FLAG peptide (Sigma) or 3 × HA peptide (Sigma). Immune complexes were detected with primary antibodies, horseradish peroxidase–conjugated antibodies to mouse or rabbit IgG (GE Healthcare Bioscience, Princeton, NJ) and an enhanced chemiluminescence system (GE Healthcare).

### 2.7. Antibody generation

Glutathione *S*-transferase (GST)-fusion proteins including 117–141 amino acid residues of mouse RNF207 were expressed in XL-10 cells using the pGEX4T-2 plasmid vector (GE Healthcare) and then purified by reduced glutathione-Sepharose beads (GE Healthcare). The recombinant protein was used for immunization in rabbits. A rabbit polyclonal anti-RNF207 antibody was affinity-purified using a recombinant RNF207-conjugated Sepharose 4B column.

### 2.8. Recombinant protein and in vitro binding assay

GST-fused protein of mouse VDAC1 was expressed in XL-10 cells using the pGEX6P-1 plasmid vector (GE Healthcare) and then purified by reduced glutathione-sepharose beads (GE Healthcare). $His_6$-Flag-tagged mouse RNF207 proteins including deletion mutants were expressed in Rosetta blue cells with the use of the pET30 plasmid vector (Novagen, Madison, WI) and then purified with the use of ProBond metal affinity beads (Invitrogen).

For the production of recombinant proteins in Sf9 cells, we subcloned full-length mouse *Rnf207* cDNAs into pFastBac HTc with epitope tags and expressed epitope-tagged full-length mouse *Rnf207* with the BAC-to-BAC system (Clontech Laboratories, Mountain View, CA).