Published in final edited form as:
Hypertension. 2011 April ; 57(4): 764–771. doi:10.1161/HYPERTENSIONAHA.110.165803.

AUGMENTED RIFIFYLIN IS A RISK FACTOR LINKED TO ABERRANT CARDIOMYOCYTE FUNCTION, SHORT QT-INTERVAL AND HYPERTENSION

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Abstract

Using congenic strains of the Dahl salt-sensitive S rat introgressed with genomic segments from the normotensive Lewis rat, a blood pressure quantitative trait locus was mapped within 104kilobases on chromosome 10. The goal of the current study was to conduct extensive phenotypic studies and further fine-map this locus. At 14 weeks of age, the blood pressure of the congenic rats fed a low salt diet, was significantly higher by 47mm Hg ($p<0.001$) compared to that of the S. A time-course study showed that the blood pressure effect was significant from very young ages of 50–52 days (13mm Hg, $p<0.01$). The congenic strain implanted with electrocardiography transmitters demonstrated shorter QT-intervals and increased heart rate compared with S ($p<0.01$). The average survival of the congenic strain was shorter (134 days) compared with S (175 days, $p<0.0007$). The critical region was narrowed to <42.5kilobases containing 171 variants and a single gene, Rififylin. Both the messengerRNA and protein levels of rififylin were significantly higher in the hearts of the congenic strain. Overexpression of rififylin is known to delay endocytic recycling. Endocytic recycling of fluorescently labeled holotransferrin from cardiomyocytes of the congenic strain was slower than that of S ($p< 0.01$). Frequency of cardiomyocyte beats in the congenic strain (62±9 beats/min) was significantly higher than that of the S (24 ± 6 beats/min, $p<0.001$). Taken together, our study provides evidence to suggest that early perturbations in endocytic recycling caused by the overexpression of Rffl is a novel physiological mechanism potentially underlying the development of hypertension.

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Disclosures
None

Keywords

Cardiovascular diseases; blood pressure; heart rate; genetic; arrhythmia

Introduction

Previously, a blood pressure (BP) quantitative trait locus (QTL) was located within 104kb on rat chromosome 10 using congenic strains developed by introgressing alleles from the normotensive LEW (Lewis) rat onto the genome of the hypertensive salt-sensitive (S) rat [1]. The BP QTL alleles within the 104kb region from the normotensive LEW rat are pro-hypertensive because they further increase the BP of the hypertensive S rat [1]. The purpose of the current study was to (1) further narrow the BP QTL region, (2) expand the phenotyping and (3) study the function of the candidate gene/s.

The importance of the study is that the QTL was further fine-mapped within a shorter segment of <42.5kb containing a single gene, Rififylin or RING Finger and FYVE-like domain-containing protein (*Rffl*). By overexpressing Rififylin in HeLa cells, Coumailleau [2] *et al* have demonstrated that Rififylin delays recycling of membrane components from the endocytic recycling compartment to the plasma membrane. Rififylin is also an endosome-associated ubiquitin ligase 3 [3]. The current study presents data to suggest that Rififylin is a novel gene linked to increased heart rate, BP and mortality in the congenic strain compared with S.

Methods

Animals

All animal experiments were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and as per approved protocols by the Institutional animal care and use review committee of the University of Toledo College of Medicine. The primary congenic strain described in this study is S.LEW(10)×12×2×3×5[1].

Blood Pressure Measurements

Rats were weaned at 30 days of age and administered low salt diet (0.3% NaCl, Harlan Teklad) for 30 days. Systolic blood pressure (BP) was measured using the tail-cuff method as described previously[1]. The day after the last BP measurements, rats were euthanized and whole heart weights were recorded. Blood pressure was also confirmed by two independent radiotelemetry experiments using the equipment, probes and software from Data Sciences International (DSI), as described previously [1]. Experiment 1) S (n=10) and congenic (n=10) rats on a low salt diet at the age of 75–78 days were surgically implanted with C40 blood pressure transmitters; Experiment 2) C10 blood pressure transmitters were implanted into 30 day old rats.

Echocardiography

Left ventricular (LV) function of S and congenic rats were evaluated by echocardiography as described previously [4,5].

Statistical Analyses

All phenotypic data obtained from the two groups (congenic and S rats) were statistically analyzed by Student’s t-test except the time course analyses of blood pressure by telemetry, which was assessed by two way ANOVA followed by Tukey’s post hoc test. A p-value of <0.05 was considered statistically significant. Statistical analyses of the microarray data

were performed with RMA (Robust Multiarray Averaging) and BH (Benjamini and Hochberg) adjustment using the R statistical package (version 2.8.1).

Genomic and cDNA Analyses

Tail DNA from adult rats and mRNA from hearts of neonates and 53 days old rats were used for genomic and cDNA analyses, respectively, as described under the expanded online methods section (Please see http://hyper.ahajournals.org). Transcript expression of *Rffl* was analyzed by Real Time PCR (BioRad) and expression levels relative to *Gapdh* were calculated by the $2^{-\Delta\Delta CT}$ method [6].

Immunoblot Analyses

Western blotting experiments and the sources of antibodies are detailed in the online methods section (Please see http://hyper.ahajournals.org).

Whole-genome Transcriptional Profiling

RNA was isolated from the hearts of concomitantly raised, male, 53 day old S and congenic rats (n=6/group) using TRIzol and purified by RNeasy kit (Qiagen). RNA from two animals was pooled. Three such RNA samples from S and congenic rats were hybridized to Affymetrix Rat Expression Arrays 230 2.0. Statistical analyses of the microarray data were performed using R statistical package (version 2.8.1). The microarray data are in compliance with the Minimum Information About Microarray Experiments (MIAME) and are available at the Gene Expression Omnibus (GEO) database (GSE23643).

Cardiomyocyte Preparation and Culture

Neonatal ventricular myocytes were isolated and cultured from S (n=9) and congenic (n=10) rats as described previously [7,8].

Transferrin Recycling

Transferrin recycling was studied as described previously [2] and details are provided in the online methods section (Please see http://hyper.ahajournals.org). Mean fluorescent intensity was measured in Image J at individual time points of the acquired images.

Cardiomyocyte Beats

Cardiomyocytes from S and congenic rats were grown on glass cover slips for 4–7 days and placed on a stage-mounted microscope. Cardiomyocytes were chosen at random and cell beats were visually counted [9] and data analyzed by a two-tailed, unpaired *t*-test.

Biotelemetry-ECG

The electrocardiogram (ECG) was monitored on a continuous basis with a biotelemetry system (DSI). The transmitter devices were surgically implanted in 32 day old rats under general anesthesia. The devices were inserted into the peritoneal cavity and electrodes from the transmitter were arranged in Lead II configuration. ECG data were collected at 5 minute intervals for 24 hours and analyzed using Ponemah v.4.90 software (DSI). Normalization of QT interval was conducted with the Bazett's formula specifically for rats. T-test (available within the Ponemah software) was used for statistical analysis of the ECG data.

Results

Screening for recombinants within the congenic strain S.LEW×12×2×3×5

To fine-map the BP increasing locus the congenic strain S.LEW×12×2×3×5, this strain was backcrossed to S. F1 rats were intercrossed to obtain 1995 F2 rats. None of these F2 animals were recombinants.

Fine-mapping the BP QTL to <42.5kb

Using newly identified single nucleotide polymorphisms between S and LEW (Fig S1, Table S1, please see http://hyper.ahajournals.org) the previously located 104kb QTL region was resolved to <42.5kb between the base pairs 71028112 and 71070581. Sequencing of this <42.5kb interval from S and S.LEW×12×2×3×5 resulted in the identification of 171 variants (Table S2, please see http://hyper.ahajournals.org). The critical <42.5kb region contained a single predicted gene, Rififylin (Fig 1). The homologous region of this segment in humans also contains Rififylin as the single gene (Fig 1, www.ncbi.nlm.nih.gov).

Extended phenotyping

The experimental design for all the phenotyping studies are schematically represented in Fig S2 (Please see http://hyper.ahajournals.org).

Experimental Group 1—The BP increasing effect of LEW alleles of the BP QTL was previously reported after feeding high salt (2% NaCl) to the rats [1]. To test whether the hypertension increasing property conferred by this highly resolved genomic segment is independent of dietary salt S and S.LEW×12×2×3×5 congenic rats were fed with a low salt (0.3% NaCl) diet. BP measured by tail-cuff of the congenic strain (225 ± 3 mm Hg, n=10) was higher by 46 mm Hg than that of the S (178 ± 4 mm Hg, $p<0.0001$) (Fig 2a). Relative heart weight of the congenic strain (4.69± 0.10) was also significantly higher than that of the S (3.91±0.11) ($p<0.0001$). At 93 and 110 days, the hearts of both congenic and S rats were assessed by echocardiography. The detailed results of the comparisons are presented in Table 1. Representative two-dimensional and M mode images in Figure 2b showed that the congenic strain exhibited concentric cardiac hypertrophy as evidenced by increased wall thickness and reduced LV volume. At this time point, cardiac output of the congenic rats was also higher than that of the S (Table 1).

Experimental Group 2—A time course study with radiotelemetry corroborated this result and demonstrated that both systolic and diastolic BP were higher in the congenic rats (Fig 2c, Table S3, please see http://hyper.ahajournals.org).

Experimental Group 3—In order to determine whether the changes in BP precede the alterations in cardiac function, both systolic and diastolic BP of young rats (43 days) were monitored by radiotelemetry (Fig 2d, Table S4, please see http://hyper.ahajournals.org). At young ages of 50–52 days of age, both systolic and diastolic BP of the congenic rats were significantly higher than that of the S ($P<0.01$, Figure 2d, Table S4, please see http://hyper.ahajournals.org). However, there was no change in cardiac function at this time point of 50–52 days (Table S5, please see http://hyper.ahajournals.org). The difference in systolic BP between S and the congenic strain was further amplified with time, reaching a significant difference of >25 mm of Hg when the rats were 109–110 days old (Fig 2d). The increased BP of the congenic strain was associated with significantly shorter survival compared with the hypertensive S (Fig 2e). The median survival of the congenic strain was 134 days whereas that of the S was 175 days ($p<0.0007$).

Experimental Group 4—The interval between the start of the QRS complex and end of the T wave called as the QT-interval of a heart beat was recently reported in a genome-wide study to be associated with single nucleotide polymorphisms around the human *RFFL* gene [10]. This association was also confirmed in a second genome-wide association study [10,11]. To assess whether the functional alterations caused by *Rffl* in our model are similarly associated with QT-interval, we surgically implanted electrocardiogram transmitters into 45 day old S and congenic rats and monitored their ECG. As shown in Figures 3a and 3b, the QT interval of the congenic strain was significantly shorter than that of the S. While the QT interval which is a useful index of the ventricular action potential duration, the action potential duration varies directly with heart rate (an increase in heart rate shortens the action potential and vice versa). Thus, QT interval also varies with heart rate and could be corrected for this variable using the Bazett's correction. QTcb, which applies the Bazett's correction for QT interval, was also significantly decreased in the congenic strain compared with S (Fig 3c).

Overexpression of Rififylin in the hearts of congenic compared with S

To assess the candidacy of the single predicted gene, Rififylin, we sequenced all exons of *Rffl*, five of which were located within the <42.5kb region. There were no exonic variants. We then explored for differential transcription of *Rffl* as the next possibility. A microarray experiment with small sample numbers of n=3 rats/group suggested a trend for increased expression of *Rffl* in the hearts of congenic rats compared with S (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zjonleysiyeqing&acc=GSE23643). cDNA products of Rffl obtained by RT-PCR (Fig 4a) were further confirmed by Realtime PCR (n=6 rats/group) (Fig 4b). The mRNA expression of *Rffl* was significantly higher in the congenic strain compared with S (Fig 4b). Accordingly, Rffl protein was also higher in the hearts of congenic strain compared with S (Fig 4c).

Higher levels of polyubiquitinated proteins in the hearts of congenic

As shown in Figure 4d, the total polyubiquitinated proteins from the hearts of the congenic strain were significantly higher than that of the S ($p<0.01$). Microarray data of these hearts showed that only six transcripts were significantly differentially expressed (Table 2). Among these, two transcripts were prominently downregulated in the congenic strain. These were methyl CpG binding protein 2 (*Mbd2*), which is a suppressor of transcription [12] and c18orf54, a transcript of unknown function. Because the ubiquitin-proteasome system influences transcription[13–15], we reasoned that the downregulation of *Mbd2* in the heart is perhaps a consequence of the increased polyubiquitination of cardiac proteins in the congenic strain.

Functional analysis of rififylin overexpression in cardiomyocytes

Rififylin was overexpressed in the hearts of neonatal congenic rats (Fig 5a). Overexpression of Rififylin is described to cause delayed endocytic recycling [2]. Therefore we quantitated endocytic recycling in isolated cardiomyocytes by the transferrin recycling assay. Recycling was significantly slower in the congenic compared with S (Fig 5b, c). After 30 minutes of incubation, % fluorescence retained in cardiomyocytes from congenic rats (29%) was higher than that in the S (12%, $p <0.01$) (Fig 5b and c). The frequency of cardiomyocyte beats from the congenic strain (62 ± 9 beats/min) was significantly faster than that of the S (24 ± 6 beats/min, $p<0.001$) (Fig 5d). Heart rate of the congenic rat (432 ± 17) was significantly higher compared with S (399 ± 10) ($p<0.05$).

Discussion

The data presented in our study suggest that a BP QTL is further fine-mapped within <42.5kb containing a single candidate protein coding gene, Rififylin and 171 candidate quantitative trait nucleotides. The congenic strain containing LEW alleles at this QTL region has elevated BP, develops cardiac hypertrophy and has a shorter QT interval. These phenotypes are associated with an elevated mRNA and protein expression of rififylin for unknown reasons. There are two known functions of overexpression of both the transcript and protein product of the gene, Rififylin: 1) delayed recycling of endosomes [2] and 2) increased polyubiquitination of proteins [3]. Both of these functions were differential between the congenic strain and S. The delay in recycling of endosomes was demonstrated in neonatal cardiomyocytes of the congenic strain. These physiological differences were associated with alterations in beats/min of neonatal cardiomyocytes from the congenic animals which also had shorter QT intervals and increased heart rates. These early changes in cardiac function are known predictors of hypertension [16,17] and observed to occur before significant differences in blood pressure are detected between the congenic strain and S. Taken together, the data suggests that overexpression of *Rffl* represents one of the mechanistic factors likely to be contributing to the observed increase in blood pressure of the congenic strain. Cardiac hypertrophy observed later in the study is likely influenced by the higher blood pressure, but the concentric nature of the hypertrophy remains intriguing.

The contractile function of cardiomyocytes is primarily dependent on the surface expression of ion channels [18]. While mutations in many ion channels *per se* are discovered to cause alterations in myocardial repolarization [19–21], it is possible that functional alterations in proteins involved in the intracellular transport of the channels to and from the plasma membrane could also affect membrane repolarization by regulating the dynamic control of their endocytosis and exocytosis from intracellular storage compartments. One such storage compartment is the endocytic recycling compartment (ERC), a peri-nuclear collection of tubular organelles that mature from sorting endosomes [22]. An independent study has demonstrated that the recycling of endosomes is delayed in the presence of an excess of rififylin [2]. We have demonstrated that this function of rififylin is recapitulated in cardiomyocytes from the congenic strain which overexpress rififylin. This observation lead us to suspect alterations in myocardial repolarization, which was further supported by the shorter QT-intervals observed in the congenic strain because QT-intervals reflect myocardial repolarization as measured by the electrocardiogram[23].

Rififylin is also reported to be an endosome-associated ubiquitin ligase 3 [3]. Therefore we assessed the polyubiquitination status of cardiac proteins. Higher levels of polyubiquitinated proteins observed in the congenic strain compared with the S is suggestive of increased availability of endosome-associated ubiquitin ligase 3 activity as a result of increased expression of *Rffl*. Because the ubiquitin-proteasome system influences transcription [13–15], we reasoned that the observed downregulation of *Mbd2* is perhaps a consequence of the increased polyubiquitination of cardiac proteins in the congenic strain compared with S.

An obvious question that arises from our current study is, ‘why is *Rffl* overexpressed in the congenic strain compared with S?’ The various possibilities include the following:(1) exonic or intronic variants within the *Rffl* gene, (2) variants within the promoter of *Rffl*, (3) variants upstream and downstream of *Rffl* that are binding sites for transcription enhancers/ suppressors, (4) variants in non-coding RNAs, (5) variants within yet undefined regulatory elements of *Rffl* and (6) Trans-elements on the remainder of the S rat genome that interact with LEW alleles within the <42.5kb QTL region to regulate the expression of *Rffl*. Sequencing has revealed that while there are no exonic variants of *Rffl*, there are 171 candidate variants between S and LEW within the <42.5kb region. To begin addressing the

question of why *Rffl* is overexpressed in the congenic strain, a detailed follow-up study of each of these candidate variants is required, but this is beyond the scope of the current study which was primarily focused on extended phenotyping. Nevertheless, the importance of the current study is that it provides evidence for aberrant endocytic recycling in cardiomyocytes to be further considered as a novel physiological mechanism potentially underlying blood pressure regulation.

Perspectives

Several mapping studies point to candidate genes on human chromosome 17 as plausible for blood pressure control in humans [24]. Two independent genome-wide association studies (GWAS) [10,11] also point to single nucleotide polymorphisms (SNPs) on human chromosome 17 that are associated with systolic BP. Further, one of these groups conducted a large meta-analysis of three GWAS in 13,685 individuals of European ancestry from the Framingham Heart Study, the Rotterdam Study and the Cardiovascular Health Study, as part of the QTGEN consortium and identified association of multiple minor alleles near the *RFFL* locus in humans with shorter QT-intervals [10]. This association was also confirmed in a second study [11]. Our finding of a genomic segment containing rififylin similarly linked to QT-intervals in rats serves as a functional validation of these GWAS [10,11]. Further, our observation in rats that early changes in QT-interval contributed to the development of hypertension suggests that these individuals could be at risk for developing hypertension. Interestingly, over 29% of the cohorts screened by GWAS [10] were hypertensive, thus lending support to our interpretation. Overall, our studies provide the impetus to target the endosomal recycling machinery via *Rffl* as a novel mechanism for prevention of aberrant heart function leading to hypertension in individuals with abnormal heart rhythms.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding Sources

This work was conducted through funds provided by the NIH to BJ (HL020176, HL076709). EEM acknowledges a postdoctoral fellowship award from the American Heart Association.

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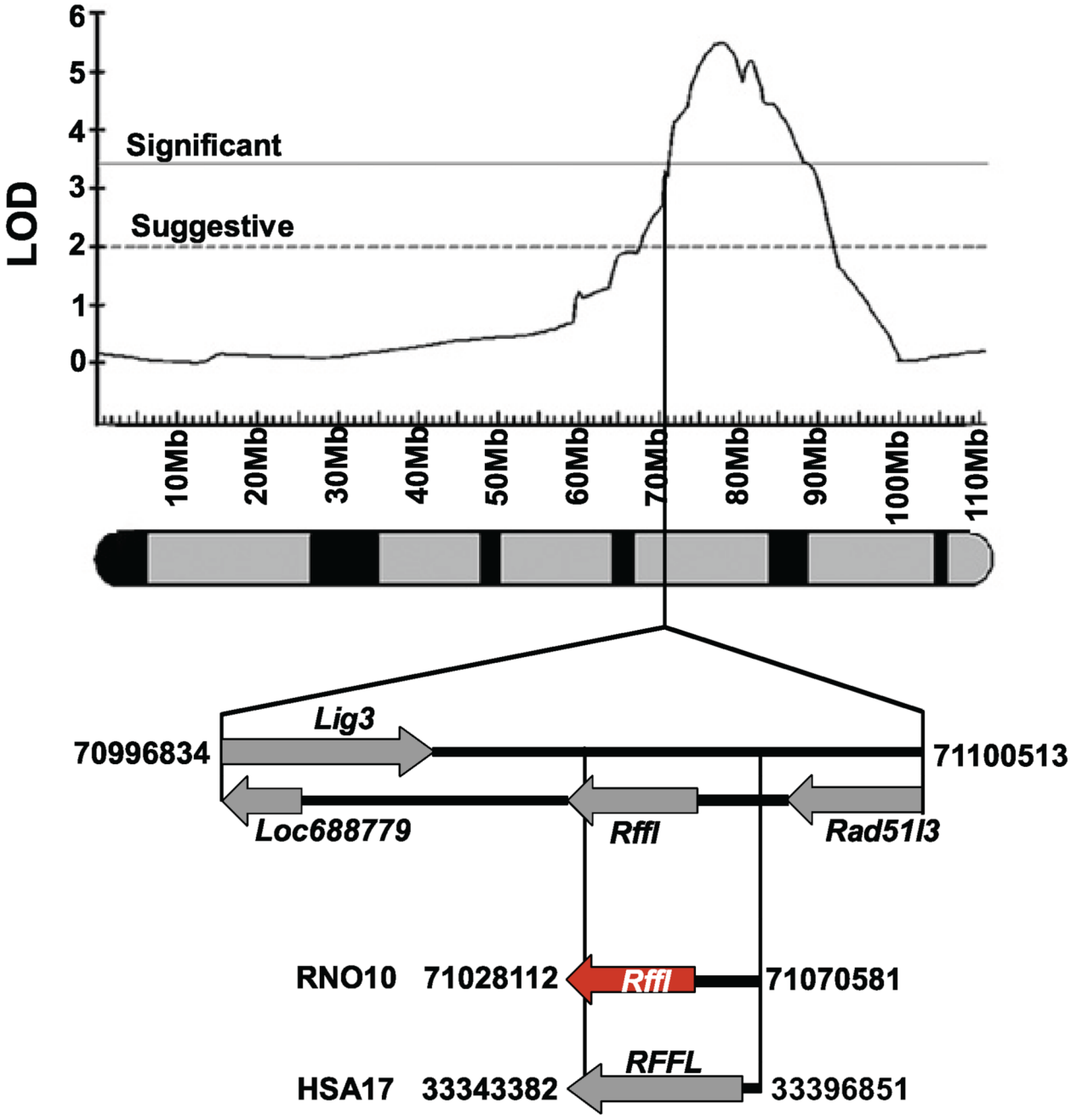

Figure 1. Mapping the blood pressure locus on rat chromosome 10

(a) The LOD plot for BP using the F2 (S × LEW) population on rat chromosome 10 (RNO10) is followed by the previously published BP QTL containing the four genes shown by the arrows marked as *Lig3*, LOC688779, *Rffl* and *Rad51l3*. The region containing *Rffl* in red is the current mapped location. The homologous segment on human chromosome 17 (HSA17) is also shown. Numbers alongside RNO10 and HSA17 represent base pair locations.

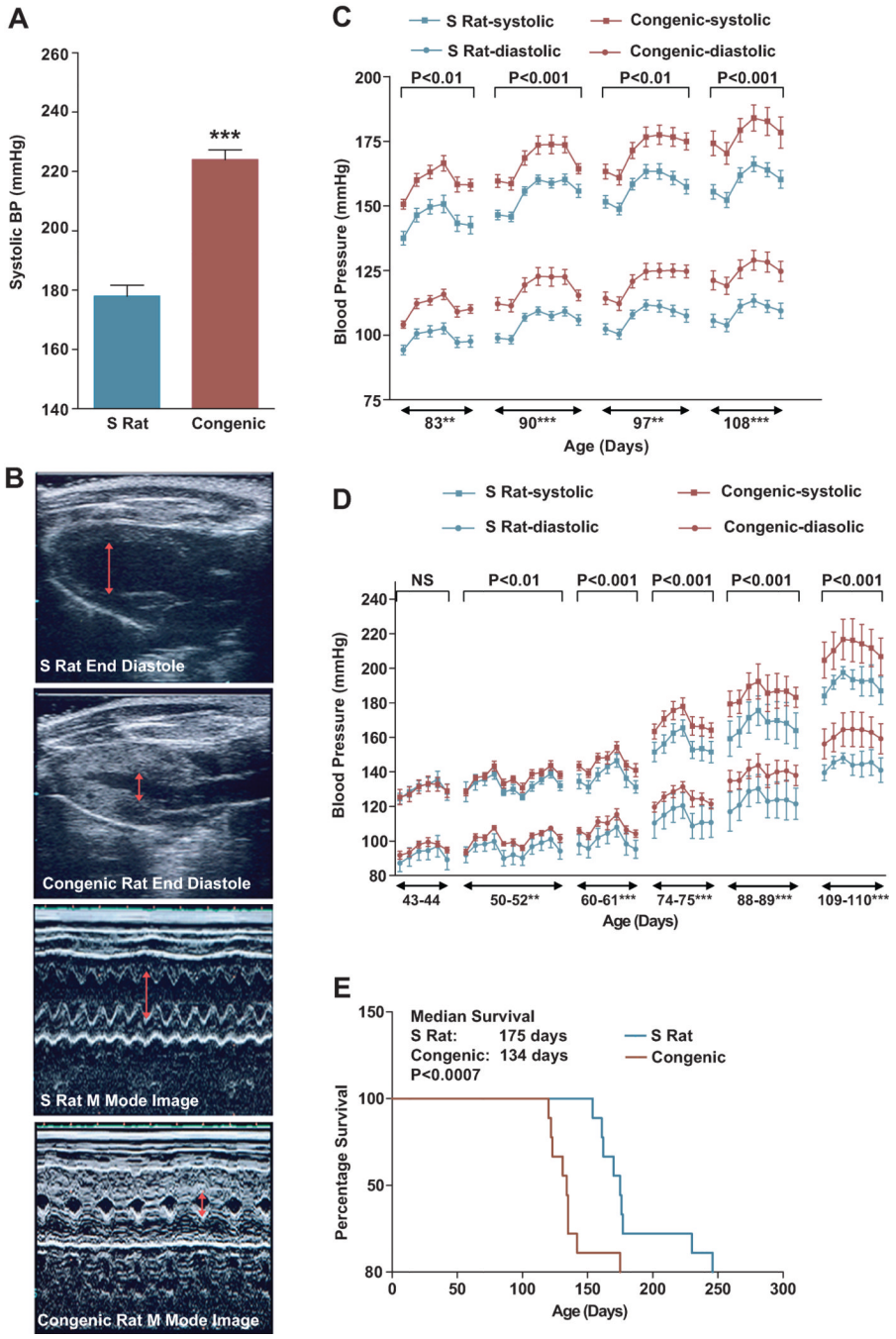

Figure 2. Time course assessment of cardiac function and blood pressure

(a) Mean systolic BP effect ± SEM by the tail-cuff method of 110 day old rats; S (n=10) and S.LEW×12×2×3×5 congenic (n=10) rats. (b) Echocardiographic measurements were obtained on 110 day old S and congenic rats. Two dimensional images represent the end diastole from S rat and congenic rat. M-mode images from S rat and S.LEW×12×2×3×5 congenic rat. (c) BP data corroborated by radiotelemetry using C40 transmitters. S (n=13) and S.LEW×12×2×3×5 congenic (n=13) rats. Data plotted is the recording obtained once every 5 min continuously for 24hrs and averaged for 4 hr intervals. P values indicated are obtained by two-way ANOVA followed by Tukey's test (d) Time course assessment of BP by radiotelemetry. The data shown are from 5 rats/group on a low (0.3% NaCl) diet,

implanted with the C10 transmitters. Data plotted was obtained by telemetry recording once every 5 min continuously for 24–72 hrs and averaged for 4 hr intervals. Levels of statistical significance by two-way ANOVA followed by Tukey's test are indicated on the x-axis as follows: ***$p<0.001$, **$p<0.01$, *$p<0.05$. (e) Kaplan-Meier survival curves. Animals (S and S.LEW×12×2×3×5 congenic rats, n=12/group) were fed with 0.3% dietary salt (NaCl) for 116 days and then fed with 2% NaCl until their natural death ($p<0.0007$).

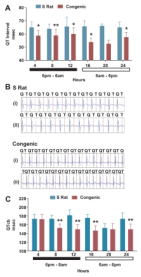

Figure 3. Short QT intervals in the Congenic strain compared with S

Bars ± SEM represent ECG recording once every 5 min continuously for 24 hrs and averaged for 4 hr intervals. (a) Uncorrected short QT interval was observed in the congenic. (b) Representative Electrocardiogram recordings from individual S (n=3) and S.LEW×12×2×3×5 Congenic (n=3) rats. (c) Corrected QT interval (QTcb) ± SEM (by Bazett method). ** $p<0.01$, *$p<0.05$

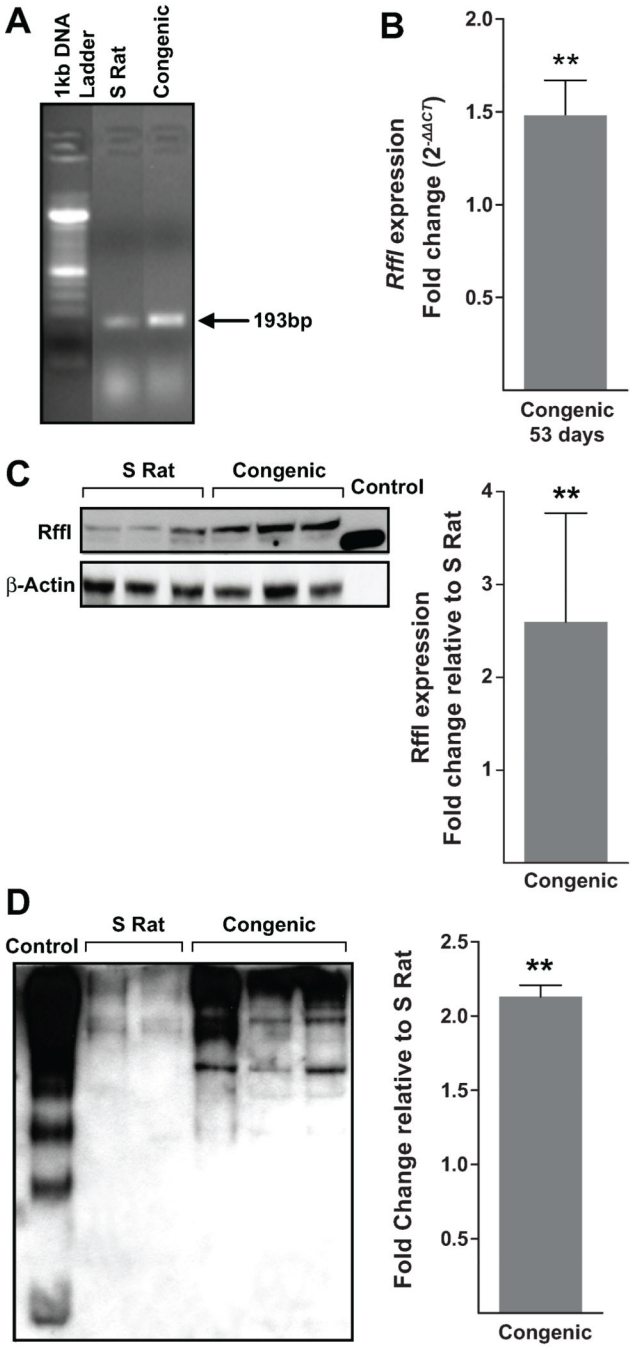

Figure 4. Expression analysis of Rififylin and Levels of polyubiquitinated proteins

(a) Expression of *Rffl* transcript at 53 days of age as detected by RT-PCR. (b) Quantification of *Rffl* transcripts by Real time PCR (n=6 animals/group). (c) Immunoblot of Rffl in whole-cell lysates from S (n=3) and congenic (n=3) rat hearts at 53 days of age. 36.41kDa, RFFL (NP_0010717368, 2aa–99aa) partial recombinant protein was used as positive control and β-Actin was the loading control. Quantification of Rffl protein± SEM is shown on the right. **p<0.01. (d) Immunoblot for polyubiquitinated proteins in whole-cell lysates from S (n=2) and congenic (n=3) rat hearts at 110 days of age. Control: provided by the Pierce Ubiquitination enrichment kit. Quantification of these proteins ± SEM is shown in the right. **p<0.01.

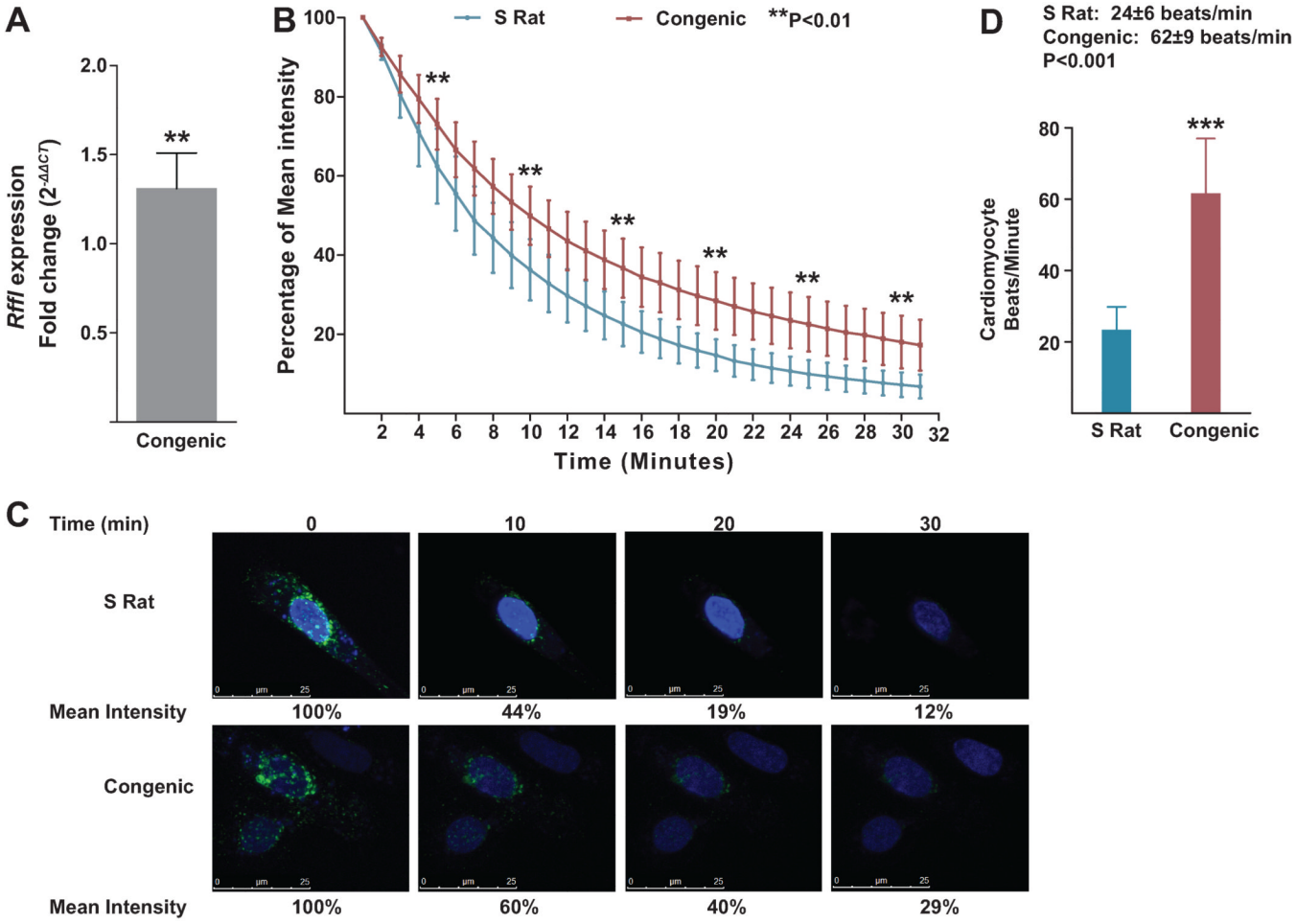

Figure 5. Cardiac functional analysis of Rififylin

(a) Differential expression of *Rffl* gene transcripts from neonatal S.LEW×12×2×3×5 congenic rat hearts (n= 6/group) by Real-time PCR. (b) Defective transferrin recycling in the congenic cardiomyocytes. The disappearance of fluorescently labeled transferrin was plotted using the initial mean intensity of labeled internalized transferrin (± SEM) by the cardiomyocytes taken as 100% in three independent experiments conducted in duplicates. (c) Representative images of data presented in panel (b) from cardiomyocytes recycling transferrin. Green, Alexa^{488}-Transferrin; Blue, DRAQ5. (d) Differential rates of beats/min of neonatal cardiomyocytes. Experiments were conducted twice and beats counted three times for each experiment. *** p<0.001, ** p<0.01

Table 1

Echocardiographic measurements in S and Congenic rats

Parameter	93 Days			110 Days		
	S(n=12)	Congenic (n=10)	P	S(n=12)	Congenic (n=7)	P
SV (ml)	0.32±0.02	0.45±0.06	P<0.02	0.36±0.04	0.58±0.06	P<0.005
C.O. (ml/min)	139±9	194±24	P<0.03	165±19	249±33	P<0.03
C.I. (ml/min/kg)	376±24	588±65	P< 0.003	422±47	665±77	P<0.01
ESA (cm2)	0.35±0.04	0.36±0.04	ns	0.42±0.02	0.34±0.07	P>0.06
Ao OFT (cm)	0.33±0.01	0.35±0.01	ns	0.33±0.02	0.39±0.01	P<0.03
RWT	0.56±0.08	0.58±0.01	ns	0.55±0.02	0.91±0.30	P>0.09

* Abbreviations: SV: stroke volume; C.O: cardiac output; CI: cardiac Index; ESA: end systolic area; Ao OFT; Aortic outflow tract, RWT: relative wall thickness; ns: not significant

Table 2

Cardiac transcriptome analysis of congenic rats compared with S

Probe ID	Fold change	p Value	Gene Symbol	Gene Name
1379740_at	3.594	0.001	*LOC361346*	Similar to chromosome 18 open reading frame 54
1380293_at	2.905	0.0006	*LOC361346*	similar to chromosome 18 open reading frame 54
1388774_at	2.647	0.0004	*Mbd2*	methyl-CpG binding domain protein 2
1377563_at	−1.565	0.0374	*Lmod3*	leiomodin 3 (fetal)
1377112_at	−1.629	0.0374	*Cda*	cytidine deaminase
1369983_at	−1.635	0.0374	*Ccl5*	chemokine (C-C motif) ligand 5
1378848_at	−1.665	0.0374	*LOC361187*	similar to ankyrin repeat and SOCs box-containing protein 5

*Statistical analyses of the microarray data were performed with RMA (Robust Multiarray Averaging) and BH (Benjamini and Hochberg) adjustment using the R statistical package (version 2.8.1).

†The complete microarray data is available to the reviewers at the following link: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=zjonleysiyeqing&acc=GSE23643