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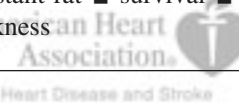
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# Epistatic Genetic Determinants of Blood Pressure and Mortality in a Salt-Sensitive Hypertension Model

George T. Cicila, Eric E. Morgan, Soon Jin Lee, Phyllis Farms, Shane Yerga-Woolwine, Edward J. Toland, Ramona S. Ramdath, Kathirvel Gopalakrishnan, Keith Bohman, Andrea L. Nestor-Kalinoski, Sadik A. Khuder, Bina Joe

**Abstract**—Although genetic determinants protecting against the development of elevated blood pressure (BP) are well investigated, less is known regarding their impact on longevity. We concomitantly assessed genomic regions of rat chromosomes 3 and 7 (RNO3 and RNO7) carrying genetic determinants of BP without known epistasis, for their independent and combinatorial effects on BP and the presence of genetic determinants of survival using Dahl salt-sensitive (S) strains carrying congenic segments from Dahl salt-resistant (R) rats. Although congenic and bicongenic S.R strains carried independent BP quantitative trait loci within the RNO3 and RNO7 congenic regions, only the RNO3 allele(s) independently affected survival. The bicongenic S.R strain showed epistasis between R-rat RNO3 and RNO7 alleles for BP under salt-loading conditions, with less-than-additive effects observed on a 2% NaCl diet and greater-than-additive effects observed after prolonged feeding on a 4% NaCl diet. These RNO3 and RNO7 congenic region alleles had more-than-additive effects on survival. Increased survival of bicongenic compared with RNO3 congenic rats was attributable, in part, to maintaining lower BP despite chronic exposure to an increased dietary salt (4% NaCl) intake, with both strains showing delays in reaching highest BP. R-rat RNO3 alleles were also associated with superior systolic function, with the S.R bicongenic strain showing epistasis between R-rat RNO3 and RNO7 alleles leading to compensatory hypertrophy. Whether these alleles affect survival by additional actions within other BP-regulating tissues/organs remains unexplored. This is the first report of simultaneous detection of independent and epistatic loci dictating, in part, longevity in a hypertensive rat strain. (*Hypertension*. 2009;53:00-00.)

**Key Words:** genetic hypertension ■ Dahl salt-sensitive rat ■ Dahl salt-resistant rat ■ survival ■ longevity ■ compensatory hypertrophy ■ relative wall thickness



Most human morbidity and mortality stem from complex diseases and disorders, of which phenotypes result from interactions of multiple genes with environmental factors. Hypertension is such a disorder, an independent predisposing factor in the development of several diseases responsible for adult morbidity and mortality, including atherosclerosis, coronary heart disease, peripheral artery disease, heart failure, renal failure, and stroke.<sup>1</sup> Little is known regarding the relationships between genetic determinants of blood pressure (BP) with genetic determinants of these diseases or overall mortality. We hypothesized that genetic factors contribute to the extended survival of some hypertensive subjects but not others. The obvious difficulty of using death as an end point in studying life span in human hypertensive subjects suggests that hypertension-survival relationships are better studied using animal models.

Inbred Dahl salt-sensitive (SS/Jr or S) and Dahl salt-resistant (SR/Jr or R) rat strains<sup>2</sup> are contrasting models of high and relatively normal BP, respectively, selectively bred from

outbred Sprague-Dawley rats under salt-loading conditions.<sup>3</sup> Supplemental dietary NaCl increases BP in S rats, with little or no effect on BP in R rats. Segregating populations and congenic strains derived from these inbred strains have been used to screen for and confirm chromosomal locations responsible for heritable BP strain differences (ie, BP quantitative trait loci [QTLs]).<sup>4,5</sup> S.R congenic strains, and substrains derived from them, were used extensively to identify 11 $\beta$ -hydroxylase (*Cyp11b1*) as a genetic determinant of BP on rat chromosome (RNO) 7<sup>6–8</sup> and to define limits of genomic segments containing BP genetic determinants on other chromosomes.<sup>4,9</sup> However, relationships between alleles within BP QTL-containing congenic intervals on different chromosomes have been little studied,<sup>10,11</sup> except when epistatic BP QTL interactions were first identified in genome scans. The effects of epistasis between BP QTLs on mortality have not been addressed in previous substitution mapping studies.

In the present study, we assessed rat genomic regions containing BP genetic determinants lacking known epista-

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sis<sup>6–8,12</sup> for their independent and combinatorial effects on BP and genetic determinants of survival. These BP QTL-containing intervals showed differing epistatic effects on BP, depending on the duration and concentration of the high-salt diet, and more-than-additive effects on survival, when chronically fed an even higher salt (4% NaCl) diet. Increased survival of RNO3+RNO7 bicongenic, compared with RNO3 congenic, rats was attributable, at least in part, to their maintaining lower BP despite prolonged exposure to a higher dietary NaCl intake. R-rat RNO3 congenic region alleles were also associated with measures of superior systolic function, with epistasis between R-rat RNO3 and RNO7 alleles leading to increased compensatory hypertrophy, as evidenced by increased end-diastolic relative wall thickness (RWT). These data are consistent with our hypothesis that interactions between alleles in different BP QTL-containing regions influence both BP and survival under salt-loading conditions and are traceable using S.R congenic strains as genetic tools.

## Methods

### Inbred and Congenic Rat Strains

Inbred Dahl S and R rat strains were developed<sup>2</sup> from outbred stock originally obtained from Dahl.<sup>3,13</sup> Development and characterization of rat chromosome 3 and 7 congenic substrains, S.R-(*D3Arb14-D3Mco36*) and S.R-(*D7Mco19-Exon2-Cyp11b1*); Figure S1, available in the online Data Supplement at <http://www.hypertensionaha.org>, were described previously.<sup>6,12</sup> Inbred and congenic rat strains were from our colony at the University of Toledo Health Science Campus and will be referred to throughout this manuscript as S, R, RNO3, and RNO7, respectively. Two backcross F<sub>1</sub>(S×R)×S populations (n=150 rats) were used to examine epistasis between RNO3 and RNO7 loci. Breeding and phenotyping of these populations were described previously<sup>14,15</sup> and are summarized in the online Data Supplement.

RNO3 and RNO7 congenic intervals containing R-rat low BP QTL alleles were introgressed into an S-rat genetic background resulting in the S.R-[(*D3Arb14-D3Mco36* and *D7Mco19-Exon2-Cyp11b1*)] rats, hereafter referred to as the RNO3+RNO7 bicongenic strain. Breeding was as follows: F<sub>1</sub> rats, bred by crossing RNO3 and RNO7 congenic rats, were backcrossed to RNO3 congenic rats. Progeny heterozygous for the RNO7 and homozygous for the RNO3 congenic intervals were crossed. Resulting progeny homozygous for both congenic intervals were bred to establish the bicongenic strain. All of the breeding and experimental protocols were approved by the institutional animal care and use committee of the University of Toledo Health Science Campus.

### Experiment 1

Age- and weight-matched rats (group 1: S, n=21; RNO3, n=15; RNO3+RNO7, n=20; and RNO7, n=19) were bred, housed, and studied concomitantly. Rats were weaned at 30 days of age onto a low-salt diet (0.3% NaCl, diet 7034; Harlan-Teklad). Two rats of different strains were randomly assigned to each cage. At 40 to 42 days of age, all of the rats were transferred to a 2% NaCl diet (diet 94217, Harlan-Teklad) for 28 days.

Systolic BP was measured using tail-cuff plethysmography for a subset of group 1 rats (S, n=15; RNO3, n=15; RNO3+RNO7, n=16; and RNO7, n=14) that were conscious, restrained, and warmed to 28°C<sup>16</sup> by operators unaware of the rats identity. BP of each rat was measured on consecutive days during weeks 3 (days 17 and 18) and 4 (days 26 and 27) of the 2% NaCl diet. Daily BP values were the mean of 3 to 4 consistent readings. Final BP values were the mean of the daily BP values. After BP measurement (day 28 after initiation of 2% NaCl diet), rats were transferred to a high-salt diet (4% NaCl, diet 83033, Harlan-Teklad) until they died or became obviously terminally ill, in which case they were euthanized by

carbon dioxide hypoxia. Rats were examined twice daily for signs of distress.

### Experiment 2

Two groups of rats were concomitantly bred to further characterize effects of R-rat RNO3 and RNO7 alleles on BP and survival: group 2A (S, n=8; RNO3, n=8; and RNO3+7, n=8) was used to measure BP by telemetry, and group 2B (S, n=23; RNO3, n=10; RNO3+7, n=10; and RNO7, n=10) was used to assess cardiac function by echocardiography and for terminal experiments. Both groups (2A and 2B) received the dietary NaCl regimen described in experiment 1, with renal function assessed by 24-hour urinary protein excretion (UPE). Group 2A rats were fed a 4% NaCl diet until they died or became terminally ill. Group 2B rats were fed a 4% NaCl diet for up to 46 to 48 days, when surviving rats were used in terminal experiments.

### Radiotelemetric BP Measurement

Transmitters were surgically implanted into rats 20 to 24 days after initiating the 2% NaCl diet, as described previously.<sup>17,18</sup> Rats recovered for a week before BP data was collected. Five sets of BP measurements (systolic and diastolic) were recorded at 5-minute intervals for 24- to 48-hour periods over 12 weeks. For each rat, a series of 6 moving averages (each over a 4-hour period) was calculated over the first 24-hours measured for each time point. An overall mean BP was calculated as the mean of 6 consecutive, 4-hour moving averages, for each of 5 time points measured in a rat.

### Urinary Protein Excretion

UPE was determined as described previously.<sup>19</sup> Urine was collected 3 times: (1) on a 0.3% NaCl diet, just before administering the 2% NaCl diet; (2) after 27 days on a 2% NaCl diet; and (3) after 38 days on a 4% NaCl diet (a total of 65 days on a higher-salt [2% or 4% NaCl] diet).

### Echocardiography

Left ventricular (LV) function was evaluated by echocardiography as described previously.<sup>20</sup> Briefly, rats were anesthetized with 1.5% to 2.0% isoflurane by O<sub>2</sub> inhalation, the chest shaved, and situated in the supine position on a warming pad. 2D guided M-mode studies were performed from parasternal long window using a 15-MHz linear array transducer. Study duration was typically 15 to 20 minutes per rat.

### Terminal Experiments and Histology

Group 2B rats surviving 46 days on the 4% NaCl diet (S, n=4; RNO3, n=6, RNO3+RNO7, n=6; and RNO7, n=3) were euthanized by pentobarbital overdose, plasma collected, and body weights measured. After blood collection, hearts were removed, blotted, and weighed. Kidneys were removed, decapsulated, blotted, and weighed separately. Left kidneys and portions of the heart were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks for subsequent sectioning. Plasma glucose, creatinine, and urea nitrogen concentrations were determined by the University of Toledo Medical Center Department of Pathology. Additional details regarding the phenotyping of experiment 2 rats are in the Supplemental Methods available online at <http://hyper.ahajournals.org>.

### Statistical Analysis

Normally distributed data were analyzed by 1-way ANOVA to determine overall significance followed by Tukey honestly significant differences or Games-Howell posthoc tests. Nonparametric data were analyzed by Kruskal-Wallis tests, followed by Mann-Whitney U pairwise comparison tests if significant differences were observed.  $P < 0.05$  was the criterion for statistical significance. Data are presented as the means ± SEMs.

Equality of the survival functions of the strains was evaluated by Kaplan-Meier and log-rank tests. Survival functions were compared pairwise, with the statistical significance criterion adjusted for multiple comparisons (Bonferroni correction). The effects of R-rat alleles within

**Table 1. Strain Differences in BP, BW, and Survival Among S and Congenic Rat Strains**

Strain	BP, Week 3, mm Hg	BP, Week 4, mm Hg	BW, Initial, g	Days Survived on 4% NaCl Diet
S	173.5±4.0 (15)	181.9±4.1 (15)	161.0±2.1 (20)	64.8±6.4 (21)
RNO3	154.7±1.7 (15)	160.5±2.3 (15)	145.1±3.6 (15)	96.9±7.1 (15)
RNO3+RNO7	152.7±2.6 (16)	157.8±3.2 (16)	150.2±3.4 (20)	126.4±8.5 (20)
RNO7	160.5±3.0 (14)	170.8±3.3 (14)	150.2±2.5 (19)	72.5±6.8 (19)
<i>P</i> (overall)	<0.0001	<0.0001	<0.0001	0.002
<i>P</i> (S vs RNO3)	0.002	0.001	0.002	0.002
<i>P</i> (S vs RNO3+RNO7)	0.001	0.0004	0.015	<0.0001
<i>P</i> (S vs RNO7)	NS, 0.067	0.052	0.004	NS
<i>P</i> (RNO3 vs RNO3+RNO7)	NS	NS	NS	0.016
<i>P</i> (RNO3 vs RNO7)	NS	0.018	NS	0.009
<i>P</i> (RNO7 vs RNO3+RNO7)	NS	0.013	NS	<0.0001

BW was measured on 40- to 42-day-old experiment 1 rats. Tail-cuff BP was measured during the third (days 17 and 18) and fourth (days 26 and 27) weeks on a 2% NaCl diet. After BP measurement, rats were maintained on a 4% NaCl diet until they died or became moribund. Numbers of rats per strain measured for each trait are in parentheses. NS indicates not significant ( $P>0.05$ ).

the RNO3 (R3) and RNO7 (R7) congenic regions on survival in experiment 1 and measures of cardiac and renal function in experiment 2 were examined using general linear models. Additional details are in Supplemental Methods available in the online Data Supplement.

## Results

To investigate the relationship between RNO3 and RNO7 BP QTLs, we analyzed the relationship between *Cyp11b* and *Edn3* (near these BP QTL peaks) with BP and body weight (BW)–adjusted heart weight a previously studied  $F_1(S \times R) \times S$  backcross population.<sup>7,14,15</sup> No interactions were observed between these loci for either trait (Table S1). A bicongenic strain (RNO3+RNO7) was bred to confirm additive actions of R-rat alleles in these congenic regions on BP and to examine effects of these alleles on survival in the context of an excessive dietary NaCl intake.

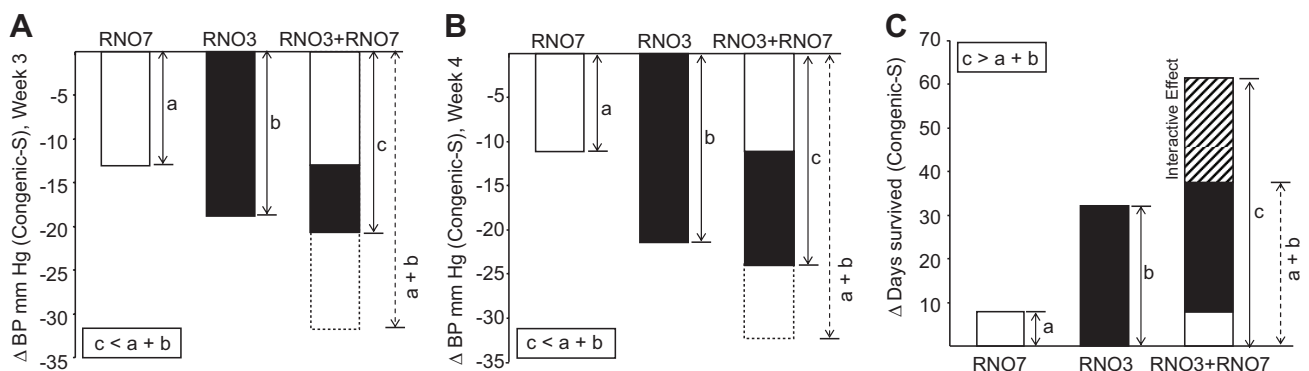
### Less-Than-Additive Effects of RNO3 and RNO7 QTLs on BP

Systolic BP was measured by tail-cuff plethysmography in concomitantly raised male S, RNO3 and RNO7 congenic, and RNO3+RNO7 bicongenic rats during the third and fourth

weeks of a 2% NaCl diet (Table 1 and Figure 1A and 1B). These BP measurement sets were strongly correlated ( $r=0.660$ ;  $P<0.0001$ ; Figure S2). Compared with the parental S strain, lower BP was observed for all 3 of the congenic strains at both time points (Table 1 and Figure 1A and 1B). Lower BP was observed in RNO3 compared with RNO7 congenic rats (160.5±2.3 versus 170.8±3.3 mm Hg, respectively;  $P=0.018$ ) and in RNO3+RNO7 bicongenic compared with RNO7 congenic rats in week 4 (157.8±3.2 versus 170.8±3.3 mm Hg, respectively;  $P=0.013$ ) but not between RNO3+RNO7 bicongenic and RNO3 congenic rats (Table 1). Indeed, the differential BP ( $\Delta$  BP; ie, mean BP of a congenic strain–mean BP of S) for RNO3+RNO7 bicongenic rats in both weeks 3 and 4 were lower than expected if the RNO3 and RNO7 congenic region low BP QTL alleles had additive effects (Figure 1A and 1B).

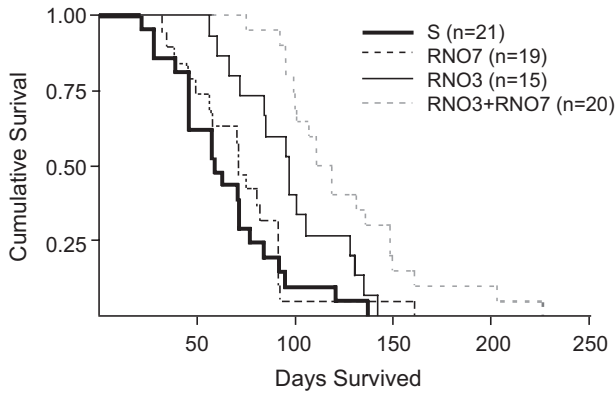
### Greater-Than-Additive Effects of RNO3 and RNO7 QTLs on Survival Under Salt-Loading Conditions

Because nonadditive BP effects were observed between R-rat alleles within these congenic intervals after 4 weeks on a 2%



**Figure 1.** Epistasis between genetic determinants of BP and survival in RNO3 and RNO7 congenic regions. Bars in Panels A and B depict decreased BP ( $\Delta$  BP) in congenic strains compared to S rats on a 2% NaCl diet. Bars in Panel C depict increased survival ( $\Delta$  survival) in congenic strains vs S rats that were subsequently fed a 4% NaCl diet. Increments labeled “a” and “b” depict effects of R-rat RNO3 and RNO7 congenic interval alleles, respectively, on  $\Delta$  BP and  $\Delta$  survival. The increment labeled “a+b” depicts the expected combined effects of R-rat alleles in both congenic intervals on  $\Delta$  BP and  $\Delta$  survival, if additive effects were exerted. The increment labeled “c” depicts the observed effects of R-rat alleles in both congenic intervals on  $\Delta$  BP and  $\Delta$  survival. Rats used were those described in experiment 1.





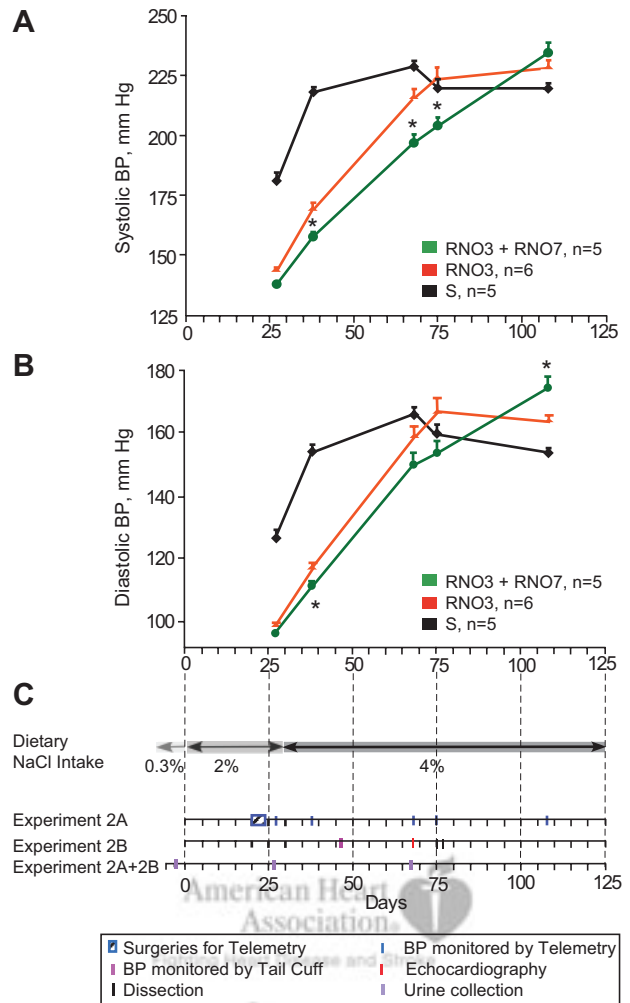
**Figure 2.** Cumulative survival curves for S, congenic, and bicongenic rats. Cumulative survival curves, with days survived on the 4% NaCl diet as the time variable, are shown for male S, RNO3, RNO3+RNO7, and RNO7 rats (experiment 1).

NaCl diet, our primary phenotypic measurement, we examined their effects on longevity after chronic salt loading. All of the rats (including some whose BP were not measured) were fed a higher-salt (4% NaCl) diet. RNO3 and RNO3+RNO7 strains survived significantly longer ( $96.9 \pm 7.1$  and  $126.4 \pm 8.5$  days;  $P=0.002$  and  $P<0.0001$ , respectively) compared with the parental S strain ( $64.8 \pm 6.4$  days; Table 1). Survival differences were not observed between RNO7 and S rats, although RNO7, compared with RNO3, rats survived significantly fewer days (Table 1). Surprisingly, differential survival of RNO3+RNO7 ( $\Delta$  survival; ie, mean survival of a congenic strain—mean survival of S rats, days on 4% NaCl diet) was much greater compared with the sum of the  $\Delta$  survivals for RNO3 and RNO7 congenic rats, indicating a strong interactive effect (Figure 1C). Survival functions of these 4 rat strains were significantly different ( $P<0.0001$ ; Figure 2), with all of the pairwise survival function comparisons significantly different (after Bonferroni correction), except for those of RNO7 with RNO3 or S rats.

Effects of RNO3 and RNO7 congenic interval alleles on survival were examined using a general linear model, with BP (measured during week 4 of the 2% NaCl diet) as a covariate. Both RNO3 congenic interval alleles (R3;  $P=0.037$ ) and main effects interactions ( $R3 \times R7$ ;  $P=0.030$ ; Figure S2), but not RNO7 congenic interval alleles (R7;  $P=0.11$ ), were associated with BP-adjusted survival. Increased BP-adjusted survival was observed for RNO3+RNO7 rats ( $115.3$  days) compared with S ( $84.5$  days;  $P=0.020$ ), RNO3 ( $86.9$  days;  $P=0.009$ ), or RNO7 ( $78.9$  days;  $P=0.002$ ) rats (Figure S3).

**Longer Exposure to Elevated Dietary NaCl Significantly Reduced BP in Bicongenic Compared With RNO3 Congenic Rats**

We next sought to identify factors responsible for increased survival of the RNO3+RNO7 bicongenic rats. Despite BP additivity not being observed in congenic rats fed a 2% NaCl diet (Figure 1 and Table 1), we hypothesized that RNO3 and RNO7 BP QTL allelic products might interact in rats maintained longer on a higher-salt (4% NaCl) diet, causing lower BP in bicongenic compared with RNO3 congenic rats. Experiment 2 was conducted to test this hypothesis. Similar



**Figure 3.** Longitudinal study of BP strain differences among S and congenic rats under an increasing dietary NaCl intake. A and B, Systolic and diastolic BP measurements by radiotelemetry, in male S, RNO3 congenic, and RNO3+RNO7 bicongenic rats (group 2A) chronically exposed to an elevated dietary NaCl intake. \*Significant BP differences between RNO3 congenic and RNO3+RNO7 bicongenic rats. Numbers of rats per strain measured and statistical analyses of systolic and diastolic BP are in Table S2. C, Experiment 2 design. Experimental manipulations and measurements are shown separately for each rat group above a timeline showing days after 2% NaCl diet initiation.

to our earlier results (Table 1), S rats had higher BP (systolic and diastolic) compared with both RNO3 and RNO3+RNO7 rats after 27 days on a 2% NaCl diet, with no significant difference observed between RNO3 and RNO3+RNO7 rats (Table S2). However, BP strain differences were observed between RNO3 and RNO3+RNO7 after additional time on a diet with an even higher salt (4% NaCl) content. Lower systolic BP was observed for bicongenic rats compared with RNO3 congenic rats after 38 days ( $157.7 \pm 2.3$  versus  $169.6 \pm 2.1$  mm Hg;  $P=0.0004$ ), 68 days ( $196.6 \pm 3.6$  versus  $215.6 \pm 4.1$  mm Hg;  $P=0.039$ ), and 75 days ( $203.8 \pm 3.7$  versus  $223.7 \pm 4.6$  mm Hg;  $P=0.0003$ ) of salt loading (Figure 3A and Table S2). Similarly, lower diastolic BP was also observed for RNO3+RNO7 compared with RNO3 rats after 38 days of salt loading ( $110.2 \pm 2.0$  versus  $115.7 \pm 1.8$  mm Hg;  $P=0.039$ ; Figure 3B and Table S2). Interestingly, the BP of

**Table 2. Strain Differences in Echocardiographic Parameters Among S and Congenic Rat Strains Maintained on Elevated Dietary NaCl**

Strain (n)	LVdD, cm	LVsD, cm	FS, %	Vcf, 1/s	RWT
S (6)	0.74±0.04	0.42±0.04	44±4	5.48±0.59	0.67±0.01*
RNO3 (7)	0.73±0.04	0.34±0.04	54±3	7.94±0.60	0.67±0.09
RNO3+RNO7 (7)	0.69±0.02	0.30±0.02	57±3	8.48±0.50	0.85±0.03†
RNO7 (4)	0.86±0.04	0.52±0.05	40±3	5.30±0.78	0.50±0.05
<i>P</i> (overall)	0.036	0.004	0.005	0.002	0.023
<i>P</i> (S vs RNO3)	NS	NS	NS	0.031	NS
<i>P</i> (S vs RNO3+RNO7)	NS	NS	0.039	0.007	0.010
<i>P</i> (S vs RNO7)	NS	NS	NS	NS	0.041
<i>P</i> (RNO3 vs RNO3+RNO7)	NS	NS	NS	NS	NS, 0.08
<i>P</i> (RNO3 vs RNO7)	NS	0.017	0.035	0.043	NS
<i>P</i> (RNO7 vs RNO3+RNO7)	0.024	0.004	0.013	0.012	0.010
<i>P</i> (R3)	0.019	0.001	0.0005	0.0002	0.026
<i>P</i> (R7)	NS	NS	NS	NS	NS
<i>P</i> (R3×R7)	0.042	NS, 0.08	NS	NS	0.025

Echocardiography was performed on surviving group 2B rats exposed to a 4% NaCl diet for 40 to 41 days (ie, 69 to 70 days on higher-NaCl diets). Numbers of rats per strain studied for each trait are in parentheses unless indicated otherwise. NS indicates not significant ( $P>0.05$ ); R3, allelic content within the RNO3 congenic interval; R7, allelic content within the RNO7 congenic interval; LVdD, LV end-diastolic diameter; LVsD, LV end-systolic diameter.

\*4 S rats.

†6 RNO3+RNO7 rats.

S and RNO3 congenic (but not bicongenic) rats plateaued, with S rats reaching this level first (Figure 3 and Table S2).

In addition to the above telemetry experiment, we assessed the BP of group 2B rats surviving for 17 to 18 days on a 4% NaCl diet (69 to 70 days on a higher dietary NaCl intake) by tail-cuff plethysmography. The timeline of all of the experiments is given in Figure 3C. RNO3+RNO7 rats had lower BPs compared with S and RNO7 rats ( $P=0.001$  and  $P=0.015$ , respectively; Table S3) but not RNO3 rats, which approached significance ( $P=0.08$ ). Both RNO3 (R3;  $P=0.002$ ) and RNO7 (R7;  $P=0.026$ ) congenic interval alleles, but not the main effects interaction (R3×R7;  $P$  value not significant), were associated with significant differences in tail-cuff BP.

### R-Rat RNO3 Alleles Are Associated With Superior Cardiac Function After Prolonged Exposure to Excessive Dietary NaCl

The cardiac function of group 2B rats surviving 40 to 41 days on a 4% NaCl diet was assessed by echocardiography, with representative M-mode images shown in Figure S4. Overall, inbred and congenic rat strains in this study could be ranked for echocardiographic parameters, from best to worst, as follows: RNO3+RNO7≥RNO3≥S≥RNO7 (Table 2). Measures of systolic function (LV fractional shortening [FS]) and mean velocity of circumferential fiber shortening (Vcf) were similarly improved in RNO3 and RNO3+RNO7 rats compared with S and RNO7 rats (Table 2). Bicongenic rats showed the most cardiac hypertrophy (as determined by RWT and LV RWT) compared with the other tested strains (Table 2). RNO3 congenic interval alleles were associated with significant differences in the following parameters: FS ( $P=0.0005$ ), Vcf ( $P=0.0002$ ), RWT ( $P=0.026$ ), LV end-diastolic diameter ( $P=0.019$ ), and LV end-systolic diameter

( $P=0.001$ ; Table 2). Interestingly, RNO3 and RNO7 congenic interval alleles were epistatic for LV end-diastolic diameter ( $P=0.042$ ) and RWT ( $P=0.025$ ; Table 2).

### Effects of RNO3 and RNO7 Congenic Interval Alleles on Renal Function Were Highly Dependent on Dietary NaCl Intake

Male S, RNO3 and RNO7 congenic, and RNO3+RNO7 bicongenic (groups 2A and 2B) rats were assessed for renal function on 3 dietary NaCl regimens by measuring 24-hour UPE. Because significant strain differences in BW were observed at each urine collection, 24-hour UPE/BW was analyzed. UPE/BW (24-hour) was first measured in rats maintained on a low-salt (0.3% NaCl) diet, with higher 24-hour UPE/BW observed for S and RNO3 rats compared with RNO3+RNO7 and RNO7 rats ( $P\leq 0.001$ ; Table 3), with RNO7 congenic interval alleles associated with differences in 24-hour UPE/BW ( $P<0.0001$ ; Table 3). However, after 28 days on a higher-salt (2% NaCl) diet, S and RNO7 rats had higher 24-hour UPE/BW compared with RNO3 and RNO3+RNO7 rats ( $P<0.01$ ; Table 3), with RNO3 congenic interval alleles associated with 24-hour UPE/BW differences ( $P<0.0001$ ). After 38 days on a 4% NaCl diet, RNO3 rats had lower 24-hour UPE/BW compared with RNO3+RNO7 rats ( $P=0.012$ ; Table 3) with RNO7 congenic interval alleles associated with 24-hour UPE/BW differences ( $P=0.042$ ).

### Terminal Morphometric and Biochemical Assessment of Inbred and Congenic Rats

No significant strain differences in body, kidney, or heart weights were observed (Table S4). There were also no significant strain differences in circulating creatinine, glucose, or urea nitrogen values (Table S4). However, mean

**Table 3. Longitudinal Study of UPE Among S and Congenic Rat Strains Under an Increasing Dietary NaCl Intake**

Dietary Conditions	Collection 1	Collection 2	Collection 3
Dietary salt content	0.3% NaCl	2.0% NaCl	4.0% NaCl
Days on higher-salt diet (2% and/or 4% NaCl)	0	28	67 (38 d on 4% NaCl diet)
Strain	24-h UPE/BW, g/kg		
S	83.4±5.8 (18)	907±59 (18)	1621±169 (9)
RNO3	81.9±7.2 (18)	467±26 (18)	1042±153 (13)
RNO3+RNO7	33.1±4.8 (18)	565±41 (17)	1672±135 (15)
RNO7	37.1±11.0 (10)	814±57 (10)	1716±143 (5)
<i>P</i> (overall)	<0.0001	<0.0001	0.009
<i>P</i> (S vs RNO3)	NS	<0.0001	NS, 0.06
<i>P</i> (S vs RNO3+RNO7)	<0.0001	<0.0001	NS
<i>P</i> (S vs RNO7)	0.0003	NS	NS
<i>P</i> (RNO3 vs RNO3+RNO7)	<0.0001	NS	0.012
<i>P</i> (RNO3 vs RNO7)	0.001	<0.0001	NS, 0.08
<i>P</i> (RNO7 vs RNO3+RNO7)	NS	0.007	NS
<i>P</i> (R3)	NS	<0.0001	NS, 0.08
<i>P</i> (R7)	<0.0001	NS	0.042
<i>P</i> (R3×R7)	NS	0.052	NS

Numbers of rats per strain measured for each trait are in parentheses. R3 indicates allelic content within the RNO3 congenic interval; R7, allelic content within the RNO7 congenic interval; NS, not significant ( $P>0.05$ ).

circulating creatinine values for all 4 of the strains were higher than the rat reference range,<sup>21</sup> as were mean circulating urea nitrogen values for all but bicongenic rats.

S, RNO3 and RNO7 congenic, and RNO3+RNO7 bicongenic rat kidney sections showed similar, extensive renal vascular changes, consistent with the presence of malignant hypertension (data not shown). Similarly, heart sections from these inbred and congenic strains were evaluated for arterial stenosis, hypertrophic myocytes, and interstitial fibrosis. No strain differences were observed among these 4 strains for these 3 phenotypes (Table S5).

### Discussion

Over the past 2 decades, hundreds of QTLs for BP and related traits have been identified in rodent models and humans,<sup>4,5,22–24</sup> although few were characterized with respect to either interaction with other BP QTLs or effects on mortality. Two QTLs for survival in the context of an excessive dietary NaCl intake were identified in the present study. RNO3 congenic rats carried a newly identified survival QTL, whereas R-rat RNO7 congenic interval alleles did not independently affect survival. The latter contrasts with our previous results, where in males, R-rat RNO7 alleles within a much larger congenic region (Figure S1) significantly increased survival compared with S rats.<sup>14</sup> However, in the present study, R-rat RNO7 alleles were associated with increased survival under salt-loading conditions in RNO3+RNO7 bicongenic rats, where their products could interact with those of R-rat RNO3 alleles.

### Dietary NaCl, Epistasis, and BP

Surprisingly, low BP QTL alleles within the RNO3 and RNO7 congenic intervals of bicongenic rats showed BP epistasis highly

dependent on the content and/or duration of exposure to a high dietary NaCl intake (Tables 1 and S2 and Figure 1 and 3). In these RNO3+RNO7 bicongenic rats, less-than-additive effects were observed after a 2% NaCl diet compared with the greater-than-additive effects observed with additional, prolonged exposure to a higher-salt (4% NaCl) diet. Also, when BP was measured under our standard conditions (ie, after 4 weeks on a 2% NaCl diet), low BP QTL alleles in RNO3+RNO7 rats showed less-than-additive effects in contrast with the greater-than-additive effects observed previously in another bicongenic rat strain.<sup>10,25</sup> The differing interactive effects of low BP QTL alleles on different chromosomes observed in these 2 bicongenic strains further reflects the intricate gene-environment relationships in complex traits like BP.

R-rat RNO7 congenic interval alleles demonstrated modest BP effects in this study compared with the much larger BP and survival affects of the R-rat RNO7 alleles in S.R-Cyp11b,<sup>14</sup> from which it was derived. This suggests that S.R-Cyp11b rats carried additional R-rat RNO7 BP QTL and survival QTL allele(s). Substitution mapping<sup>8</sup> suggests that the additional RNO7 low BP QTL allele(s) in S.R-Cyp11b congenic rats<sup>14</sup> do not act independently of those of the RNO7 congenic substrain<sup>6</sup> used in this study.

Overall, it is clear that BP alone does not completely explain the observed extended survival of the bicongenic rats. There may be factors within and/or outside the cardiovascular and renal systems that dictate the extended survival of the bicongenic rats. In this report, we chose to test the hypothesis that differential functionality of the heart and/or kidney may contribute to differences in survival.

### Cardiac Function and Survival

Echocardiographic evaluation of cardiac function suggested that RNO3 congenic region alleles were associated with



preservation of systolic function under salt-loading conditions (Table 2). RNO3+RNO7 rats displayed superior systolic function (significantly higher FS and Vcf) compared with S rats. However, no FS and Vcf differences were observed between RNO3+RNO7 and RNO3 rats, suggesting that these strains exhibited similar increases in systolic function compared with S rats (Table 2). Furthermore, no epistasis between RNO3 and RNO7 congenic interval alleles was observed for either measure of systolic function (Table 2). In contrast, RNO7 congenic rats did not demonstrate improved systolic function compared with S rats. Together, these data indicate that, under salt-loading conditions, RNO3 congenic region alleles are primarily responsible for the observed increased systolic function of RNO3+RNO7 bicongenic and RNO3 congenic rats compared with S rats.

However, the above systolic function differences do not explain the longer survival of RNO3+RNO7 compared with RNO3 rats. Echocardiographic evaluation found bicongenic rats to have the highest cardiac hypertrophy (as determined by RWT) among the tested strains. This observation, combined with epistasis between RNO3 and RNO7 congenic interval alleles for RWT (Table 2), suggested that greater RWT contributes to the increased longevity of bicongenic rats (Table 2), consistent with previous studies of pressure overload-induced heart failure, where increased survival was observed for rats with greater RWT.<sup>26</sup>

### Renal Function and Survival

Clearly, the inbred and congenic strains used in this study showed heritable differences in renal function. R-rat RNO7 alleles (in bicongenic and RNO7 congenic rats) were associated with decreased UPE/BW (Table 3), compared with strains (S and RNO3 congenic) lacking these alleles, on a low-salt (0.3% NaCl) diet. After exposure to the 2% NaCl diet, this effect disappeared, and, instead, R-rat RNO3 alleles were associated with decreased UPE/BW (Table 3), possibly reflecting the lower BP of RNO3 and bicongenic rats compared with S and RNO7 rats (Figure 3 and Table 1).

Although these differences in measures of renal function may be related to initial BP strain differences, they are unlikely responsible for increased survival of RNO3+RNO7 rats. Treatment with an even higher, 4% NaCl, diet paradoxically led to lower UPE/BW for RNO3 rats compared with RNO3+RNO7 (as well as S and RNO7) rats. Indeed, histological examination of their renal sections after such treatment found similar, substantial renal vascular changes, consistent with malignant hypertension, that were also reflected in high circulating creatinine and urea nitrogen levels in these 4 rat strains (Table S5).

### Mortality as a BP QTL Study Criterion

Although genomic regions containing alleles protecting from morbidity and simultaneously increasing longevity are clearly advantageous, few BP QTLs have been tested for effects on survival.<sup>14</sup> In this context, whereas transgenic Sprague-Dawley rats overexpressing *Npy*<sup>27</sup> and transgenic Dahl S rats expressing the R-rat *Atp1a1* allele<sup>28</sup> showed decreased BP and increased survival, other studies did not associate decreased BP with increased survival.<sup>29–33</sup> The dual beneficial

effects of decreasing morbidity (by lowering BP) and increasing survival suggest that the RNO3 and RNO7 BP QTLs can be viewed as priorities for further genetic dissection.

### Perspectives

The genetic contribution to human life span is estimated to be ≈20%.<sup>34</sup> However, study designs to identify such genes in humans and to determine whether they remain operational in a morbid human condition, such as hypertension, are limited.<sup>35</sup> These newly identified survival QTLs, one acting independently (on RNO3) and the other epistatically (on RNO7), illustrate how available congenic strains can be exploited for dissecting genes underlying life-span differences among hypertensive subjects and facilitate further positional cloning of causative genes. In addition, our study in rat models demonstrates how heritable elements dictating small BP changes in hypertensive subjects can lead to differential mortality through epistatic mechanisms. Because our analysis of the effects of these alleles on cardiac and renal function was limited, it remains to be investigated whether these RNO3 and RNO7 alleles might also exert their effects through actions occurring in other tissues/organ systems involved in the maintenance of BP homeostasis.

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### Disclosures

None.

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# Hypertension

JOURNAL OF THE AMERICAN HEART ASSOCIATION

## Online Supplement

# Epistatic Genetic Determinants of Blood Pressure and Mortality in a Salt-Sensitive Hypertension Model

*Short Title: Epistatic Blood Pressure QTL Effects on Mortality*

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## Supplemental Methods:

Breeding the  $F_1$ (SxR) Backcross Populations. Briefly,  $F_1$  females were crossed to S males. Backcross progeny, both male and female, were weaned at 30 days and transferred to a high salt (8% NaCl) diet (Teklad diet TD82050, Harlan-Teklad; Madison, WI) at 35 days of age. Tail-cuff BP was measured after 4.5 weeks on the 8% NaCl diet. Following BP measurement, rats were euthanized by pentobarbital overdose, and their BW and HW measured. Linear regression was used to eliminate the strong influence ( $r = 0.75$ ) of BW from heart weight. BP was normalized for population and sex differences and BW-adjusted HW was normalized for sex differences. Both the breeding and phenotyping of these two segregating populations have been previously described in detail<sup>1,2</sup>.

24 Hour Urine Collection. Rats from both study groups (Groups 2A and 2B) in Experiment 2 were weaned onto a low salt (0.3% NaCl) diet and maintained on this diet until transferred to a 2% NaCl diet. After 28 days on this 2% NaCl diet, rats were then maintained on a 4% NaCl diet until they died or became moribund. Initially, only 18 S rats were included in this metabolic study, with 8 additional rats used to replace those that died.

Urinary Protein Excretion (UPE). The pyrogallol red-based QuanTtest Red Total Protein Assay (Quantimetrix, Redondo Beach, CA) was used to estimate the protein concentration in the urine samples in a 96-well format. The absorbance of each sample at 600 nm was measured using a VERSAmax tunable microplate reader from Molecular Devices (Sunnyvale, CA) and compared to those of the standard curve. A standard curve was calculated by measuring the absorbance of human protein standard dilutions (QuanTtest, 25-200 mg/dL). Linear regression of this standard curve was used to calculate the protein concentrations (mg/dL) in the urine samples.

Echocardiography. LV end-diastolic diameter (LVDd), LV end-systolic diameter (LVSD), septal wall thickness (SWT), and posterior wall thickness (PWT) were determined from M-mode images acquired from the long axis view at the midpapillary level. LV fractional shortening (FS) was calculated using this equation:  $FS = [(LVDd-LVSD)/LVDd] \times 100$  and relative wall thickness (RWT) was calculated as  $(SWT+PWT)/LVDd$ . Ejection time (ET) was determined from color-flow directed Doppler pulsed-wave traces of mitral and aortic flow measured at the level of the LV outflow tract, and the velocity of circumferential shortening (Vcf) was calculated as  $FS/ET$ . Data was later analyzed offline with software resident on the ultrasound system.

Terminal Measurement of Organ Weights and Circulating Factors: Terminal experiments on Group 2B rats were planned 38 days after the start of the 4% NaCl diet. At this time, only 50% of RNO7 and 30% of S rats survived, whereas 78% of RNO3 and 94% of RNO3+RNO7 rats survived and thus sufficient RNO3 and RNO3+RNO7 rats would be available for assessing cardiac function and dissection (Group 2B rats), as well as, a final renal function assessment (Groups 2A and 2B rats).

Histology: Kidneys and hearts were bisected and immersed in 10% neutral buffered formalin for 24 h and processed in an automated tissue processor for histology and microscopy. Tissues were subsequently embedded in paraffin and four-micrometer-thick paraffin sections were prepared for histological examination. Sections stained with hematoxylin and eosin or Masson's trichrome were evaluated in a blinded fashion for pathological changes. Several slides were also stained with Prussian blue for the presence of hemosiderin (*i.e.*, iron), a hemoglobin breakdown product.



Cardiac Pathology: Heart sections were evaluated for pathology including arterial stenosis, hypertrophic myocytes, acute and/or chronic ischemia, hemorrhage and fibrosis. The mean percentage of arterial stenosis was determined by comparing luminal diameters with arterial wall thickness, and was calculated as the quotient of arterial wall thickness divided by the arterial diameter. Total wall thicknesses and arterial diameters were summed and the quotient of the total wall thicknesses divided by the total arterial diameters was taken as the average percent stenosis. This was to minimize the effects differences in relative vessel size would have on this measure. Myocardial hypertrophy was categorized into degrees of prominence based upon the relative presence of hypertrophic myocytes in the heart sections. The categories were divided into grades ranging from 1 to 3 with increasing presence of hypertrophic myocytes (grade 1, rare; grade 2, occasional; and grade 3: frequent). The degree of fibrosis was determined utilizing both hematoxylin and eosin-stained and trichrome-stained heart samples, with the degree of fibrosis categorized into grades of increasing prominence ranging from 0 to 2 (grade 0, no fibrosis; grade 1, focal fibrosis; and grade 2, minimal fibrosis).

Renal Pathology: Kidney sections were evaluated for pathology including arterial changes relating to hypertension as well pathology of the glomeruli, renal tubules, collecting ducts, small renal veins and the interstitium of the renal parenchyma. The presence of fibrosis was also evaluated utilizing both hematoxylin and eosin-stained and trichrome-stain sections from each rat kidney.

Statistical Analysis: SPSS (SPSS, Chicago, IL) and SAS (SAS Institute; Cary, NC) software was used for statistical analyses. Normally distributed data was analyzed by one-way ANOVA to determine overall significance. Data with significant differences was further analyzed using Tukey HSD or Games-Howell post-hoc tests to determine inter-strain significance depending on whether or not homogeneity of

variance was observed. Extreme outliers were removed from non-parametric data using the box-plot method, followed by data re-analysis using the above-described procedures. Data still non-parametric was analyzed with Kruskal-Wallis tests to assess overall significance, followed by Mann-Whitney U pair-wise comparison tests if significant differences were observed.  $P < 0.05$  was the criterion for statistical significance. Data is presented as the mean  $\pm$  the standard error of the mean (SEM).

The Kaplan-Meier method and the logrank test were used to evaluate equality of the survival functions of the strains. Pair-wise comparisons of survival functions were performed, with the criterion for statistical significance adjusted for multiple comparisons (Bonferroni correction). A general linear model (GLM) was used to examine the effects of R-rat alleles within the RNO3 (R3) and RNO7 (R7) congenic regions on the survival of Experiment 1 rats on the 4% NaCl diet, with BP (measured during week 4 of the 2% NaCl diet) included as a covariate. A Shapiro-Wilk test was used to assess whether the residuals were normally distributed. Days survived on the 4% NaCl diet were adjusted for BP (measured during week 4 of the 2% NaCl diet), with results expressed as the least squares mean for each strain. GLMs were used to examine effects of RNO3 and RNO7 congenic region alleles on measures of cardiac and renal function in Experiment 2.

## References – Supplemental Methods and Data

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**Table S1. Systolic Blood Pressure and Body Weight-Adjusted Heart Weight by Genotype Combination for Selected Loci in an F<sub>1</sub>(S x R) x S Rat Population.**

Genotype		n	Quantitative Trait	
<i>Cyp11b</i>	<i>Edn3</i>		Blood Pressure, mm Hg	Body Wt.-Adjusted Heart Wt., mg
SS	SS	34	193.4 ± 4.1	1196 ± 20
SS	SR	35	184.3 ± 5.1	1137 ± 20
SR	SS	39	184.1 ± 3.6	1134 ± 15
SR	SR	42	171.1 ± 3.6	1083 ± 17
P ( <i>Cyp11b</i> )			0.007	0.002
P ( <i>Edn3</i> )			0.008	0.003
P ( <i>Cyp11b</i> x <i>Edn3</i> )			NS	NS

Tail-cuff BP was measured in male and female F<sub>1</sub>(S x R) x S rats after 4.5 weeks on an 8% NaCl diet. Rats were then euthanized by pentobarbital overdose and their body and heart weights measured. Linear regression was used to eliminate the strong influence ( $r = 0.75$ ) of BW from heart weight (HW). BP was normalized for population and gender differences and BW-adjusted HW was normalized for gender differences. Systolic BP and BW-adjusted HW (mean ± SEM) for each genotype combination are given. S = S-rat allele, R = R-rat allele.



**Table S2. Longitudinal Study of Strain-Differences in Blood Pressure among S and Congenic Rat Strains Under an Increasing Dietary NaCl Intake.**

**Systolic Blood Pressure, mm Hg**

Days after 2% NaCl diet started	S	RNO3	RNO3 + RNO7	P (overall)	P (S vs. RNO3)	P (S vs. RNO3 + RNO7)	P (RNO3 vs. RNO3 + RNO7)
27 (2% NaCl)	181.0 ± 3.6 [5]	143.3 ± 1.5 [6]	137.8 ± 1.6 [5]	<0.0001	<0.0001	<0.0001	NS, 0.08
38 (4% NaCl)	217.9 ± 2.1 [5]	169.6 ± 2.1 [6]	157.7 ± 2.3 [5]	<0.0001	<0.0001	<0.0001	0.0004
68 (4% NaCl)	228.2 ± 2.6 [3]	215.6 ± 4.1 [4]	196.6 ± 3.6 [4]	<0.0001	0.034	<0.0001	0.039
75 (4% NaCl)	219.3 ± 4.2 [3]	223.7 ± 4.6 [4]	203.8 ± 3.7 [5]	0.0003	NS	0.005	0.0003
108 (4% NaCl)	219.7 ± 1.7 [1]	228.2 ± 3.0 [2]	234.4 ± 4.1 [2]	NS	NA	NA	NA

**Diastolic Blood Pressure, mm Hg**

Days after 2% NaCl diet started	S	RNO3	RNO3 + RNO7	P (overall)	P (S vs. RNO3)	P (S vs. RNO3 + RNO7)	P (RNO3 vs. RNO3 + RNO7)
27 (2% NaCl)	125.2 ± 3.0 [5]	97.9 ± 1.4 [6]	95.1 ± 1.6 [5]	<0.0001	<0.0001	<0.0001	NS, 0.08
38 (4% NaCl)	153.0 ± 1.6 [4]	115.7 ± 1.8 [6]	110.2 ± 2.0 [5]	<0.0001	<0.0001	<0.0001	0.039
68 (4% NaCl)	164.9 ± 2.3 [3]	157.5 ± 3.3 [4]	148.8 ± 4.1 [4]	0.012	NS	0.004	NS, 0.08
75 (4% NaCl)	158.2 ± 3.9 [3]	165.3 ± 4.6 [4]	152.2 ± 4.2 [5]	NS	NA	NA	NA
108 (4% NaCl)	152.5 ± 1.7 [1]	162.3 ± 2.2 [2]	172.9 ± 3.5 [2]	0.0005	NS	0.0005	0.025

Systolic BP and diastolic BP measurements were collected by radio-telemetry from Group 2A rats maintained on a 2% NaCl diet for 28 days and then transferred to a 4% NaCl diet until they died or became moribund. The number of days on an elevated salt diet (2% NaCl and/or 4% NaCl) and the diet rats were fed (in parentheses) are provided for each time point BP data was collected. Numbers of rats per strain measured for each trait, at each time point, are in brackets. NS, not significant (P>0.05). NA, not applicable because P(overall) >0.05.

**Table S3. Comparison of Tail-Cuff Blood Pressure among S and Congenic Rat Strains Chronically Maintained on an Excessive Dietary NaCl Intake.**

Strain	n	BP, mm Hg	$\Delta$ BP (congenic - S), mm Hg
S	19	257.2 $\pm$ 5.1	
RNO3	10	242.6 $\pm$ 6.2	-14.6
RNO3 + RNO7	9	214.9 $\pm$ 8.9	-42.3
RNO7	10	250.5 $\pm$ 9.8	-6.7
P (overall)		<0.0001	
P (S vs. RNO3)		NS	
P (S vs. RNO3 + RNO7)		0.001	
P (S vs. RNO7)		NS	
P (RNO3 vs. RNO3 + RNO7)		NS, 0.08	
P (RNO3 vs. RNO7)		NS	
P (RNO7 vs. RNO3 + RNO7)		0.015	
P (R3)		0.002	
P (R7)		0.026	
P (R3 x R7)		NS	

The BP of surviving Group 2B rats, maintained on a 4% NaCl diet for 17-18 days (69-70 days on an elevated dietary NaCl intake), was assessed using tail-cuff plethysmography.  $\Delta$  BP, difference in mean systolic BP of congenic - mean systolic BP of S. R3, allelic content within the RNO3 congenic interval. R7, allelic content within the RNO7 congenic interval. NS, not significant ( $P > 0.05$ ).

**Table S4. Comparison of Anatomic and Metabolic Parameters Among S and Congenic Rat Strains Chronically Maintained on an Excessive Dietary NaCl Intake.**

Parameter	S	RNO3	RNO3 + RNO7	RNO7
Body Wt., g	357.0 ± 26.0 [4]	367.5 ± 4.7 [6]	384.2 ± 7.3 [6]	360.3 ± 15.3 [3]
Heart Wt., g	1.67 ± 0.07 [4]	1.67 ± 0.05 [6]	1.78 ± 0.05 [6]	1.82 ± 0.07 [3]
Heart Wt./Body Wt., g/kg	4.76 ± 0.46 [4]	4.56 ± 0.16 [6]	4.63 ± 0.12 [6]	5.09 ± 0.40 [3]
Kidney Wt., g	4.17 ± 0.14 [4]	3.90 ± 0.04 [5]	4.01 ± 0.11 [6]	3.79 ± 0.14 [3]
Kidney Wt./Body Wt., g/kg	11.87 ± 0.92 [4]	10.60 ± 0.13 [5]	10.45 ± 0.21 [6]	10.53 ± 0.18 [3]
Glucose, Plasma, mmol/L	7.9 ± 1.1 [4]	6.9 ± 0.2 [6]	7.9 ± 0.4 [6]	6.7 ± 0.6 [3]
Urea Nitrogen, Plasma, mmol/L	9.9 ± 2.1 [4]	9.3 ± 0.7 [6]	8.0 ± 0.6 [6]	11.6 ± 0.6 [3]
Creatinine, Plasma, mmol/L	57.5 ± 10.5 [4]	60.4 ± 4.8 [6]	60.4 ± 2.7 [6]	73.7 ± 5.9 [3]

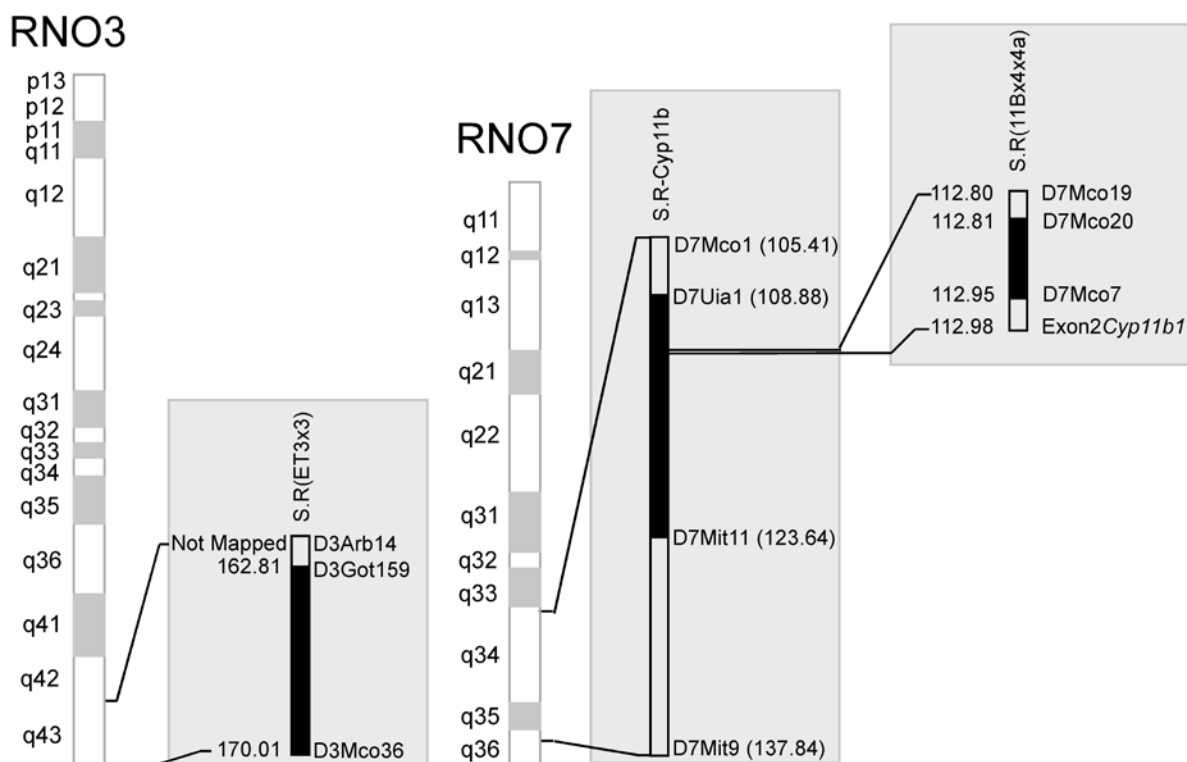
Group 2B rats were maintained on a 2% NaCl diet for 28 days and then transferred to a 4% NaCl diet until they died or became moribund. Surviving rats, maintained on a 4% NaCl diet for 46-48 days (*i.e.*, 75-77 days on an elevated dietary NaCl intake) were used in these terminal experiments. Numbers of rats per strain measured for each trait are in brackets.

**Table S5. Comparison of Cardiac Pathology Parameters among S and Congenic Rat Strains Chronically Maintained on an Excessive Dietary NaCl Intake.**

<b>Parameter</b>	<b>S</b>	<b>RNO3</b>	<b>RNO3 + RNO7</b>	<b>RNO7</b>
n	3	6	6	3
Cardiac arterial stenosis, %	54.0 ± 7.0	55.0 ± 3.4	57.3 ± 3.9	53.3 ± 1.9
Cardiac interstitial fibrosis	1.00 ± 0.58	1.83 ± 0.17	1.83 ± 0.17	2.00 ± 0.00
<u>Cardiac hypertrophy</u>	<u>1.67 ± 0.33</u>	<u>2.00 ± 0.26</u>	<u>2.00 ± 0.26</u>	<u>2.00 ± 0.57</u>

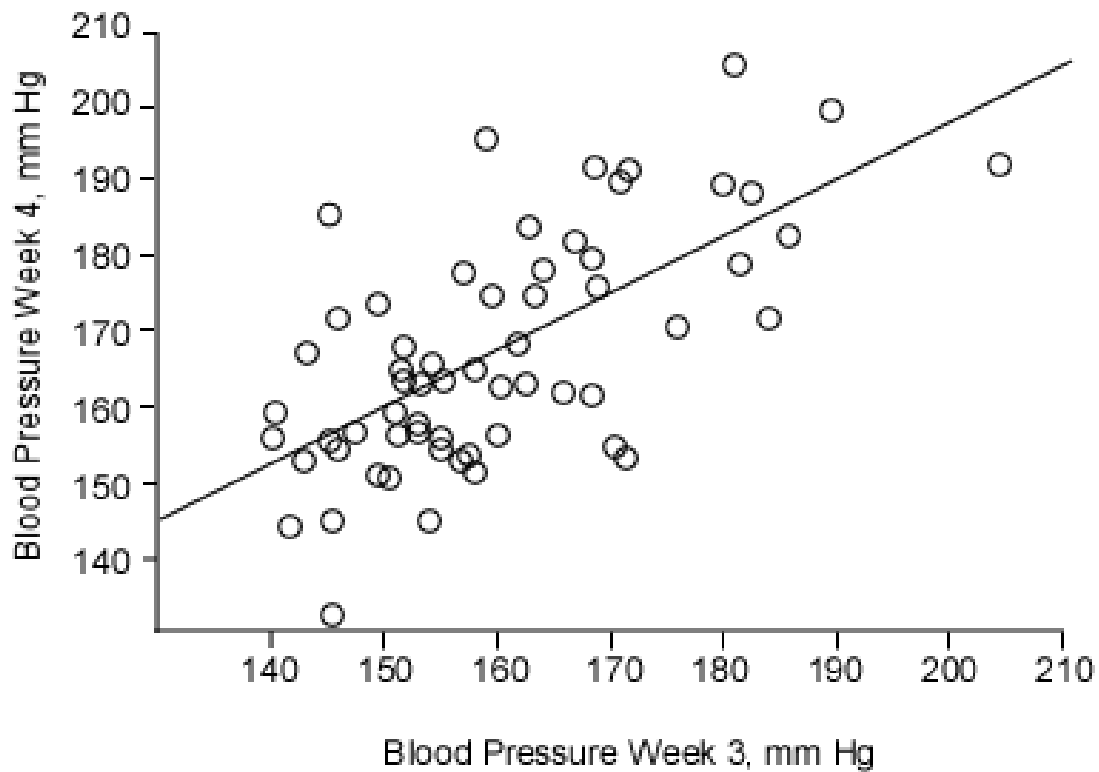
Rats (Group 2B) were maintained on a 2% NaCl diet for 28 days and then transferred onto a 4% NaCl diet until they died or became moribund. Surviving rats were maintained on a 4% NaCl diet for 46-48 days (*i.e.*, 75-77 days on an elevated dietary NaCl intake) before being euthanized. Stained sections prepared from these rat hearts were evaluated by a pathologist. Sections were evaluated for the presence of cardiac interstitial fibrosis using the following criteria: 0, none; 1, focal; and 2, minimal. Sections were evaluated for the presence of hypertrophic myocytes using the following criteria: 1, rare; 2, occasional, and 3, frequent.





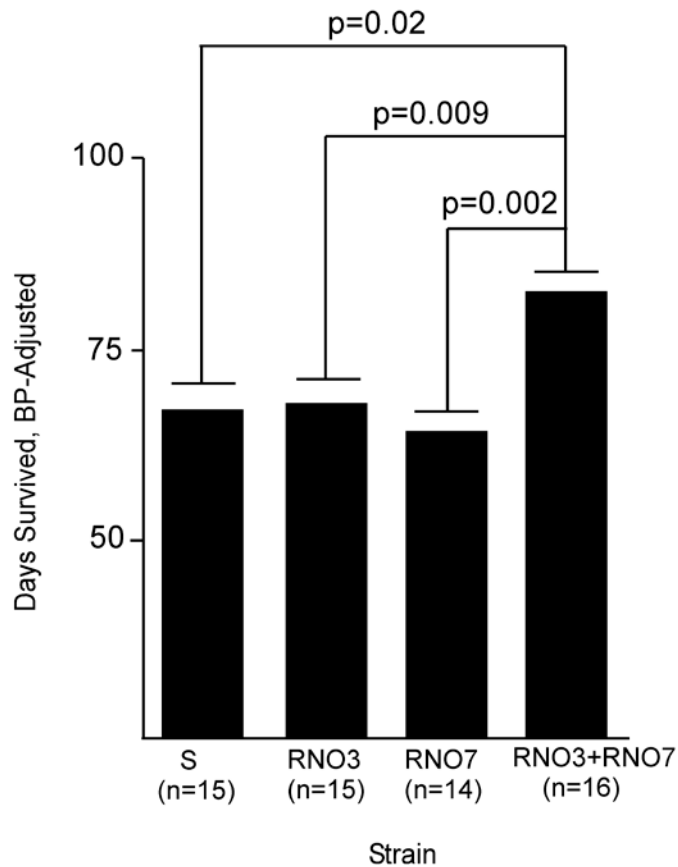
**Figure S1. Physical Maps of the RNO3 and RNO7 Congenic Intervals.** R-rat RNO3 and RNO7 regions introgressed into congenic and bicongenic strains are shown compared with cytogenetic maps of RNO3 and RNO7, respectively. Extents of congenic regions are depicted as bars, with filled portions indicating intervals containing donor strain (R-rat) alleles and unfilled portions of bars indicating intervals containing recombinant endpoints of introgressed R-rat chromosome. The introgressed region of R-rat derived RNO7 in the S.R-(*D7Mco19*–*D7Mco7*) substrain<sup>3</sup> (extreme right) is shown compared with that of S.R-Cyp11b<sup>1</sup>, the congenic strain from which it was derived.

**Figure S2**



**Figure S2: Correlation Between Blood Pressure Measurements During Week 3 and Week 4 On a 2% NaCl Diet between S and Congenic Strains.** Tail-cuff BP measured during week 3 was plotted against tail-cuff BP measured during week 4 for each rat (Experiment 1) and a significant correlation was observed between these two BP measurements ( $P < 0.0001$ ). The formula for the regression line was  $BP \text{ week } 4 = 47.62 + 0.749 (\text{BP week } 3)$  and the correlation coefficient ( $r$ ) was 0.66.

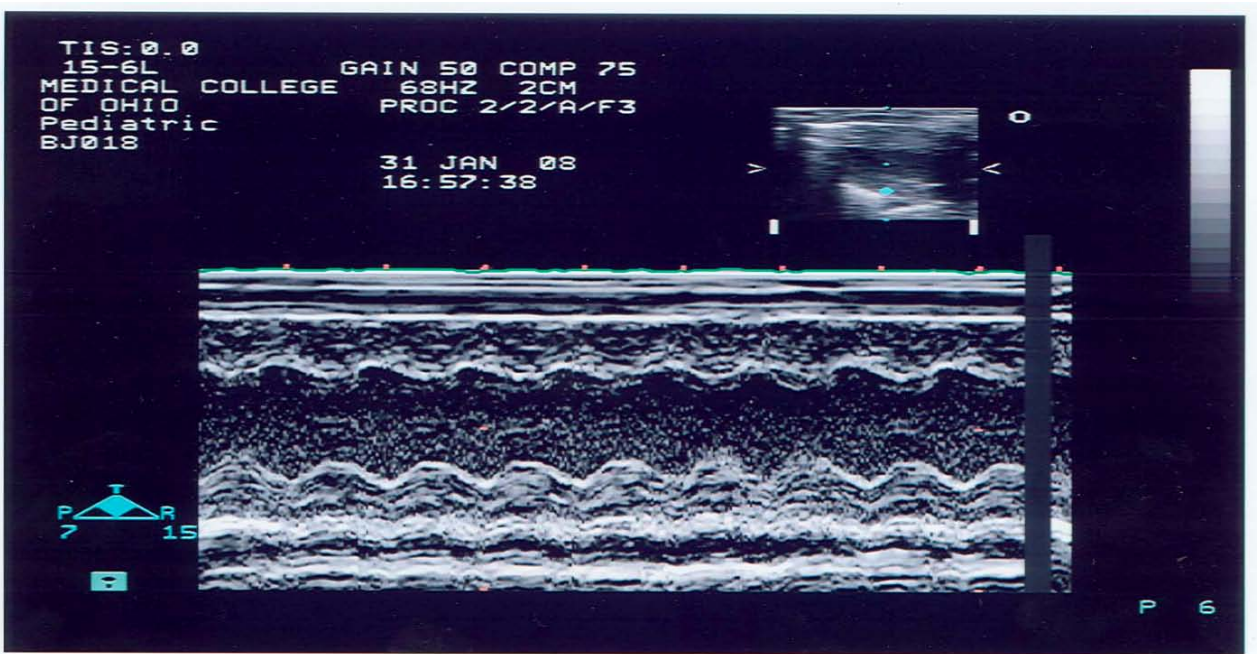
Figure S3



**Figure S3: Increased BP Adjusted-Survival of RNO3+RNO7, Compared to S and RNO3 and RNO7 Congenic Rats Is Observed.** Effects of R-rat alleles within the RNO3 (R3) and RNO7 (R7) congenic intervals on the survival of S and congenic rats in the context of an elevated dietary salt intake were assessed. BP (during week 4 of the 2% NaCl diet) was used as a covariate to adjust days survived on the high (4% NaCl) diet for each strain, with the results presented as the least squares mean.

Figure S4

A)



B)

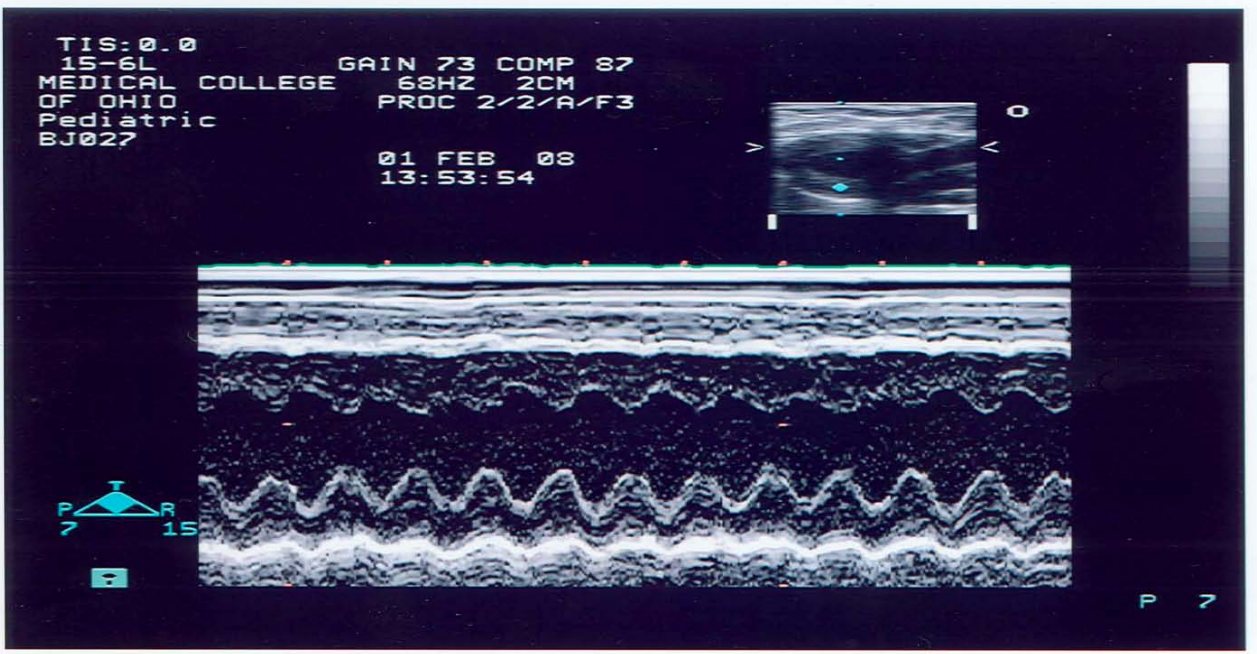


Figure S4. Representative M-Mode Images. (A) S rat and (B) RNO3+RNO7 bicongenic rat after 4% sodium diet. Visualized depth is 2 cm in both Panels.