Title

Cardiac CRFR1 Expression Is Elevated in Human Heart Failure and Modulated by Genetic Variation and Alternative Splicing

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Myocardial expression of Corticotropin-Releasing Factor Receptor 1 (CRFR1) is elevated in human heart failure and is modulated by genetic variation and a novel CRFR1 splice variant.

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Abstract

Corticotropin-releasing factor (CRF) and the CRF-related peptides, urocortin (Ucn)1, Ucn2 and Ucn3 signal through receptors CRFR1 and CRFR2 to restore homeostasis in response to stress. The Ucns exert potent cardioprotective effects and may have clinical utility in heart failure. To explore the activity of this system in the heart, we measured levels of myocardial gene expression of the CRF/Ucn family of ligands/receptors and investigated genetic variation and alternative splicing of CRFR1 in 110 heart failure patients and 108 heart donors. Using quantitative real-time PCR, we detected *CRFR1*, *CRFR2*, *CRF*, *Ucn1*, *Ucn2* and *Ucn3* in all samples. *CRFR2α* was the most abundant receptor and *Ucn3* the most abundant ligand, both in patients and donors. Compared with donors, cardiac expression of *CRFR1*, *CRF* and *Ucn3* was higher ($p<0.001$) and *CRFR2α* lower ($p=0.012$) in patients. In patients and donors, genetic variation within *CRFR1*, represented by the chromosome 17q21.31 inversion polymorphism, was associated with markedly higher *CRFR1* expression ($p<0.001$), making *CRFR1* and *CRFR2α* expression almost equivalent in some patients. A novel, truncated splice variant of CRFR1, designated CRFR1j, was identified and shown to exert a dominant-negative effect on CRFR1 signaling *in vitro*. The novel variant was expressed in a greater proportion of patients (60%) than donors (3%, $p<0.001$). In summary, cardiac expression of *CRFR1*, *CRF* and *Ucn3* genes is elevated in heart failure and may contribute to activation of the CRF/Ucn system in these patients. A common variant within the *CRFR1* gene and a novel *CRFR1* splice variant may modulate CRFR1 expression and signaling.

Introduction

The corticotropin releasing factor (CRF) family of ligands and receptors are key regulators of stress and metabolism and exert a wide range of systemic and local effects through endocrine, paracrine and autocrine signalling (1). The CRF-related peptides, urocortin (Ucn)1, Ucn2 and Ucn3 have emerged as an important group of cardiac regulators that have considerable therapeutic potential in the treatment of heart failure and ischemic heart disease (2). The Ucns exert multiple beneficial effects on the heart and vasculature including increased cardiac contractility and cardiac output, improved hemodynamic, neurohormonal and renal function and protection from ischemia/reperfusion injury (3). In patients with heart failure, plasma levels of Ucn1 are elevated and correlate with markers of disease severity and cardiac dysfunction (4). Antagonism of endogenous Ucns in experimental heart failure promotes adverse hemodynamic, neurohormone and renal effects (5), suggesting that the Ucns form part of the beneficial compensatory response to this condition and may have prognostic utility in heart failure patients.

The actions of CRF and the Ucns are mediated via two families of class B1 G-protein coupled receptors, CRFR1 and CRFR2, which exist in numerous alternatively-spliced forms and exhibit distinct patterns of expression in the central nervous system and periphery that reflect their diverse physiological functions (1). CRF has high affinity for CRFR1, but not CRFR2(6); Ucn1 has high affinity for both receptors (6); Ucn2 and Ucn3 bind exclusively to CRFR2 (7,8). The CRFR2 receptor is abundantly expressed within the left ventricle and intra-myocardial vasculature of the heart (predominantly CRFR2α in humans, CRFR2β in rodents) at levels that are approximately equivalent to other well-characterized cardiovascular receptors such as the angiotensin II type 1 receptor (9,10). The genes encoding the Ucns are also expressed at high levels, particularly *Ucn1* and *Ucn3* (10-12), and Ucn1-like immunoreactivity in the left ventricle is elevated in heart failure patients (13,14). In contrast, expression of CRFR1 and CRF is thought to be weak or absent in the human heart (10). However, a comprehensive analysis of the

77 myocardial gene expression profile of all CRF family members in heart failure patients and controls has
78 not been performed to date.
79
80 To contribute to our understanding of how the CRF family of ligands and receptors may influence heart
81 function and pathology, we examined the relationship between levels of gene expression of the CRF
82 family members in myocardial tissue from heart failure patients and from heart donors with no previously
83 diagnosed heart disease, and investigated genetic variation and alternative splicing as potential regulatory
84 mechanisms of this system in the heart.
85
86 **Methods**
87 ***Collection of Human Heart Tissue Samples***
88 Heart tissue from the left ventricular free wall of the myocardium of organ donors (n=108) and heart
89 failure patients (n=110) was collected by the Cleveland Clinic Kaufman Center for Heart Failure human
90 heart tissue bank between 1993 – 2006, as previously described (15). The study was approved by the
91 Cleveland Clinic Internal Review Board (IRB 2378) and adhered to the principles outlined in the
92 Declaration of Helsinki. All procedures followed were in accordance with institutional guidelines and all
93 families and/or patients provided informed consent.
94
95 ***Quantitative Real-Time PCR (RT-qPCR)***
96 Total RNA was extracted from frozen tissue as previously described (15). RNA concentration and purity
97 were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, Montanin, DE). The
98 mean RNA concentration and 260:280 ratio (± standard deviation) in heart donors was 768 ± 282 ng/L
99 and 2.06 ± 0.03, and in heart failure patients was 902 ± 426 ng/L and 2.04 ± 0.03. Although donor tissues
100 were stored longer than heart failure tissues prior to RNA extraction (donor samples 6.1 ± 3.2 years, heart
101 failure patients 1.9 ± 1.0 years, $p<0.001$), assessment of the integrity of the RNA by gel electrophoresis
102 confirmed intact non-degraded RNA in all samples.

Quantitative real-time PCR (RT-qPCR) was performed in duplicate for *CRFR1*, *CRFR2*, *CRFR2α*, *CRFR2β*, *CRF*, *Ucn1*, *Ucn2* and *Ucn3* on a Lightcycler 480 Real-Time PCR system (Roche Diagnostics, Indianapolis, IN). Primer sequences are provided in Supplemental Table 1 and reaction conditions are provided in Supplemental Methods. Expression levels were normalized to signal recognition particle 14 (*SRP14*) and eukaryotic elongation factor 1A1 (*EEF1A1*), two reference genes previously validated for use in human myocardium from heart failure patients and donors (16).

Genotyping Chromosome 17q21.31 Inversion Polymorphism

Genomic DNA was extracted from frozen tissue as previously described (15). Heart donors and heart failure patients were genotyped for a 900kb inversion polymorphism on chromosome 17q21.31 (chr17q21.31) by amplifying a 238 bp insertion within the 17q21.31 locus that is representative of the H1 haplotype, as published previously (17). This polymorphism segregates with much of the genetic variation within *CRFR1* and was used as a marker for genetic variation at this locus. Amplimers were sequenced on a 3100-Avant Genetic Analyser (Applied Biosystems) to confirm specific amplification. Genotypes were validated by re-genotyping 38% of samples selected at random, which gave 100% concordance with original genotypes. Hardy-Weinberg statistics were calculated using the online calculator www.oege.org/software/hwe-mr-calc.shtml (18).

Semi-Quantitative Reverse Transcription PCR (RT-PCR)

Nested primer sets were used to identify alternatively spliced transcripts of *CRFR1* as previously described (19). Alternatively spliced forms of CRFR1 were characterized by sequencing amplimers of different sizes. Nested primer sets specific for the previously unreported, novel *CRFR1* splice variant identified in this study were designed using Primer3 (20). The novel variant was recorded as 'present' in samples where the predicted 121bp band was detected in both replicates. For samples where amplification was detected in one replicate (17%), samples were re-tested in triplicate and the novel variant recorded as

'present' if amplification was detected in at least two replicates. Primer sequences are provided in Supplemental Table 2 and reaction conditions for nested PCR assays are provided in Supplemental Methods.

Plasmid Constructs

An expression plasmid encoding the novel *CRFR1* splice variant, *CRFR1j*, was subcloned into the pcDNA3.1+ (Invitrogen) expression vector using nested PCR primers that incorporated EcoR1 or Not1 restriction sites (for primer sequences and reaction conditions see Supplemental Methods), and transformed into One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen) according to manufacturer's instructions. Vector DNA was purified with Plasmid Mini kits (Qiagen) and sequenced on a 3100-Avant Genetic Analyser (Applied Biosystems) to confirm the presence of the expected cloning insert. Expression plasmids for human CRFR1α were generated as previously described (21).

Transient Transfections and Luciferase Reporter Assays

HEK293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Sigma) and 2mM glutamine (Invitrogen). Cells ($1.8x10^6$) were seeded in 10cm tissue culture dishes in 10mL of medium, incubated overnight, washed in Optimem (Invitrogen) without serum and transfected for 6 hours with a mix of Lipofectamine 2000 (Invitrogen), CRE-luciferase reporter plasmid, β-galactosidase plasmid and varying amounts of the expression plasmids encoding the novel *CRFR1* splice variant, *CRFR1* or empty vector diluted in Optimem (Invitrogen), as indicated. Cells were then incubated with complete media (containing serum) overnight, trypsinized and seeded at a density of $1x10^5$ cells/well in a poly-D-lysine-coated 48-well plate in 300μL complete media. After 24 hours cells were washed in DMEM+0.1% Bovine Serum Albumin (BSA) and treated for 4 hours with varying concentrations of the CRFR1 agonist, sauvagine (0-100nM) or vehicle, in triplicate, in 300μL serum-free media. The cells were harvested in lysis buffer (1% Triton X-100, 25mM glyclglycine, pH 7.8, 15mM

$MgSO_4$, 4mM EGTA and 1mM DTT). Luciferase reporter activity was measured using D-luciferin luciferase substrate on a Modulus Microplate Reader (Turner Biosystems) and normalized to β-galactosidase (measured using ONPG) to control for transfection efficiency. Reported data correspond to the luciferase activity of each plasmid (or combination of plasmids) relative to the no-treatment control.

Cell Surface Expression Assays

COSM6 cells, cultured in DMEM supplemented with 3% fetal bovine serum (Sigma), were transiently transfected as described above, with a mix of Lipofectamine 2000 (Invitrogen) and varying amounts of the expression plasmids encoding myc-tagged *CRFR1*, the novel *CRFR1* splice variant, soluble mouse *CRFR2α* (*sCRFR2α*) (22) or empty vector diluted in Optimem (Invitrogen) as indicated. After 24 hours, cells were seeded across a poly-D-lysine-coated 24-well plate ($1x10^6$ cells/well, in triplicate), incubated overnight in 500μL complete media, washed in HDB+0.1% BSA and incubated for 2 hours with mouse anti-myc antibody (Sigma, diluted 1:200) in 500μL HDB+0.1% BSA. The cells were washed, incubated for 1 hour with a HRP-conjugated anti-mouse antibody (Sigma) diluted 1:1000 in 250μL HDB+0.1% BSA, washed again and incubated with TMB substrate until a blue color change was observed. The reaction was stopped with 0.18M sulphuric acid and absorbance at 450nm measured on a Modulus Microplate Reader. Reported data correspond to the level of myc-tag detected on the cell surface for each plasmid (or combination of plasmids) relative to the vector control.

Statistical Analysis

Clinical characteristics and sample storage time were compared between heart donors and patients using Chi-square and ANOVA analyses. Gene expression data displayed consistently skewed distributions and were log-transformed before analysis and geometric means and 95% confidence intervals have been reported. All gene expression analyses were adjusted for potential confounding factors (age, gender, ethnicity and sample storage time). Gene expression levels of *CRFR1*, *CRFR2α*, *CRF*, *Ucn1*, *Ucn2* and

Ucn3 were compared between heart donors and patients using linear regression. The relationship between expression levels of each gene was analyzed using Pearson correlation. Correlation matrices were plotted with R software (http://www.R-project.org) and the Corrplot package version 0.30 (23,24). The association between the chromosome 17q21.31 inversion polymorphism and levels of *CRFR1* and *CRFR2α* gene expression was analyzed assuming an additive genetic model with linear regression. Associations between the presence of the predicted novel *CRFR1* splice variant and clinical characteristics were performed using Chi-square and ANOVA tests. Functional assays were analyzed with linear regression. All statistical analyses were performed with SPSS Statistics version 21.0 (SPSS Inc., Chicago, IL) and a P-value <0.05 taken to indicate statistical significance.

Results

Heart Donor and Heart Failure Patient Characteristics

The clinical characteristics of heart failure patients are shown in Table 1. Clinical characteristics of heart donors were described previously (15). Compared with heart donors, heart failure patients were, on average, 7.4 years older ($p<0.001$) and a greater proportion were male ($p<0.001$) and of non-European ancestry ($p=0.003$).

Myocardial Gene Expression of the CRF Family of Ligands and Receptors

Expression of *CRFR1*, *CRFR2α*, *CRF*, *Ucn1*, *Ucn2* and *Ucn3* was detected within the left ventricle of the myocardium of heart donors and heart failure patients (Figure 1). In heart donors, *CRFR2α* was the most abundantly expressed receptor. On average, expression levels of *CRFR1* and *CRFR2β* were 22-fold and 142-fold lower than *CRFR2α*, respectively, with *CRFR2β* only reaching detectable levels in 39% of samples. The most abundantly expressed ligand was *Ucn3*, closely followed by *Ucn1*. Expression levels of several family members were correlated (Figure 2a). The strongest relationships were between *CRFR2α* and *Ucn2* ($r=0.817$, $p<0.001$), *CRF* and *Ucn1* ($r=0.771$, $p<0.001$), and *Ucn1* and *Ucn2* ($r=0.641$,

p<0.001).

In heart failure patients, expression levels of *CRFR1*, *CRF* and *Ucn3* were higher than heart donors (*CRFR1*: 3.0-fold, p<0.001; *CRF*: 2.5-fold, p<0.001; *Ucn3*: 2.3-fold p<0.001, Figure 1) and levels of *CRFR2α* were lower than heart donors (1.5-fold, p=0.012, Figure 1). Although *CRFR2α* remained the most abundant receptor subtype, the relative expression of *CRFR2α* to *CRFR1* decreased from 22:1 in heart donors to 4:1 in heart failure patients. Expression levels of *CRF* and the *Ucn* genes were even more strongly correlated in patients than in donors (Figure 2b). The strongest relationships were between *CRF* and *Ucn3* (r=0.948, p<0.001), *CRFR2α* and *Ucn2* (r=0.898, p<0.001), and *CRFR1* and *Ucn3* (r=-0.792, p<0.001).

Chr17q21.31 Haplotype and CRFR1 Gene Expression

Much of the genetic variation within the *CRFR1* gene segregates with a large ~900kb inversion polymorphism at chromosome 17q21.31, which occurs as two distinct haplotypes H1 and H2. The H1/H2 haplotype frequency in heart donors was 52% H1H1, 47% H1H2, 1% H2H2 and in heart failure patients was 75% H1H1, 23% H1H2, 2% H2H2. The haplotype frequency was in Hardy-Weinberg equilibrium in patients (p=0.956), but not in donors (p=0.004).

In both heart donors and heart failure patients, individuals carrying one or more copies of the rarer H2 allele had higher levels of *CRFR1* compared with other individuals (heart donors: 3.0-fold, p<0.001; heart failure patients: 3.8-fold, p<0.001, Figure 3a). These individuals also tended to have lower levels of *CRFR2α*, although this was only significant in heart donors (heart donors: 1.5-fold, p=0.016; heart failure patients: 1.1-fold, p=0.677, Figure 3b). Consequently, the ratio of *CRFR2α*:*CRFR1* was markedly reduced in individuals with the H2 allele, with *CRFR2α* and *CRFR1* expression nearly equivalent in heart failure

patients carrying the H2 allele (heart donors: H1H1vs H2 carriers, 30:1 vs 6:1 p<0.001; heart failure patients: H1H1vs H2 carriers, 6:1 vs 1.6:1, p<0.001, Figure 3c).

Characterizing a novel CRFR1 splice variant, CRFR1j

A previously unreported splice variant of *CRFR1*, designated *Homo sapiens* corticotropin releasing hormone receptor variant 1j, was discovered in a subset of heart donors and heart failure patients, in which exons 4 and 6 of the *CRFR1* gene were spliced out (Figure 4a and 4b). The deletion causes a frameshift and a premature 'stop' signal in exon 9 of the *CRFR1* gene. Analysis of the Ambion FirstChoice total RNA survey panel showed that CRFR1j is expressed in brain, thyroid, thymus, esophagus, trachea, adipose, small intestine, colon, bladder, prostate, testes, ovary, cervix and placenta, and, to a lesser extent, lung, heart, skeletal muscle and kidney (Figure 4c). CRFR1j gene expression was not detected in liver or spleen. The full-length functional CRFR1 isoform (CRFR1α) was present in all tissues expressing CRFR1j, except for esophagus and skeletal muscle (Figure 4c). The *CRFR1j* mRNA transcript was detected in a greater proportion of heart failure patients than heart donors (62% of patients vs 3% of donors, p<0.001). In heart failure patients the presence of *CRFR1j* mRNA was associated with higher myocardial expression of *CRFR1α* (3.0-fold, p<0.001). Patients in whom *CRFR1j* could be detected were more likely to carry the chr17q21.31 H2 allele (34% vs 11%, p=0.011).

Functional assays demonstrated that HEK293T cells transfected with CRFR1α and treated with increasing doses of sauvagine (a CRFR1 agonist) showed expected proportional increases in activation of the cAMP response element in a luciferase reporter assay (Figure 5a). In contrast, cells transfected with the CRFR1j construct failed to show a response, indicating that CRFR1j was unable to activate the cAMP signaling pathway (Figure 5b). Co-expression of CRFR1α with increasing doses of CRFR1j proportionally reduced activation of the cAMP response element by CRFR1α, suggesting that CRFR1j may exert a dominant negative effect on CRFR1α (p<0.001 for 0.1 and 1 nmol/L doses of sauvagine, Figure 6a). Cell surface assays indicated that CRFR1j may inhibit expression of CRFR1α at the cell surface (p=0.053, Figure 6b).

Co-expression of a control gene, sCRFR2α, with CRFR1α showed that the dominant negative effect on cell surface expression was specific to CRFR1j, rather than a consequence of non-specific competitive transcription/translation of another mRNA (Figure 6c).

Discussion

Our findings demonstrate that all members of the CRF family of ligands and receptors are expressed within the left ventricle of the human myocardium, including *CRFR1* and *CRF*. In heart failure, down-regulation of *CRFR2α* and up-regulation of *CRFR1* changed the relative abundance of these receptors from more than 20:1 in favor of *CRFR2α* in heart donors to only 4:1 in favor of *CRFR2α* in heart failure patients. This was associated with increased expression of *CRF* and *Ucn3*, suggesting that both CRFR1 and CRFR2 signaling are activated in this condition. Furthermore, our data suggest that *CRFR1* expression may be altered in association with genetic variation at the *CRFR1* gene locus and that CRFR1 signaling may be modulated by a novel, truncated splice variant of CRFR1, designated CRFR1j. This novel variant was detected in a wide range of human tissues and has a dominant-negative effect on CRFR1 signaling *in vitro*. CRFR1j was rarely detected in donor heart tissue, but was detected in >60% of left ventricle tissues from heart failure patients, where it was associated with higher expression of *CRFR1*. This suggests that expression of the novel variant may be induced when *CRFR1* expression is high.

Previous studies have shown that *CRFR2α* and the *Ucns* are abundantly expressed in the left ventricle of normal heart (10,12). Our study confirms these findings and demonstrates robust expression of *CRFR1* and *CRF* in the left ventricle in normal and diseased states. This suggests that the distribution of CRF receptors in humans is considerably more heterogeneous than in rodents and that peripheral tissues, including the heart, express both receptor subtypes (1). In heart donors, expression of *CRFR2* greatly predominated over *CRFR1* and there was weak correlation between expression of the CRF family members. In contrast, in heart failure, the difference in the relative abundance of the receptors was

markedly reduced and expression of all ligands was strongly correlated. These changes may form part of the beneficial compensatory response to poor cardiac output in these patients.

Plasma levels of Ucn1 are elevated in human heart failure and may be associated with disease severity(4). Although two studies have reported increased Ucn1-like immunoreactivity in cardiac tissue from heart failure patients compared with controls (13,14), the source of circulating Ucn1 in heart failure patients was unknown. In an ovine model of experimental heart failure, Charles *et al* recorded increases in plasma Ucn1 across the kidney, liver/gastrointestinal tract and head, but not the heart (25). Consistent with this finding, we found that although *Ucn1* gene expression was relatively abundant in the myocardium, it did not differ between heart failure patients and donors. This suggests that elevated plasma Ucn1 in heart failure patients reflects production from non-cardiac sources.

In contrast to *Ucn1*, we observed a marked increase in expression of *CRF* and *Ucn3* in heart failure patients compared with donors. Moreover, *Ucn3* expression was positively correlated with LVEF, an echocardiographic marker of cardiac function, suggesting it may have greater potential as a prognostic marker and/or therapeutic agent in heart failure than Ucn1 or Ucn2. It is currently unknown whether levels of cardiac gene expression of *Ucn3* correlate with plasma concentrations.

Genetic variants within the genes encoding the CRF family of ligands and receptors have been associated with a range of predominantly anxiety- and stress-related conditions including depression (26), panic disorder (27) and suicide (28). Our data suggest that genetic variation at the *CRFR1* gene locus is strongly associated with levels of *CRFR1* gene expression. *CRFR1* is located at chromosome 17q21.31, one of the most structurally complex and evolutionarily dynamic regions of the human genome (29). The region occurs as two distinct haplotypes, H1 and H2, which span ~1.5 Mb and includes a ~900 kb inversion that encompasses several genes including *CRFR1* (30). Much of the genetic variation within *CRFR1* segregates with this polymorphism. Among European populations the H1 haplotype has been associated

with increased risk of a group of neurodegenerative diseases including Alzheimer's disease, which are characterized by accumulation of fibrillar aggregates of microtubule-associated protein tau (MAPT) in the brain cortex (31). The *MAPT* gene is located within the 17q21.31 inversion region, in close proximity to *CRFR1*. *MAPT* is expressed at elevated levels within the cerebral cortex of H1 carriers and this most likely represents the functional mechanism underlying the association between the inversion polymorphism and increased risk of neurodegenerative disease (31). Our data provide a second example of altered expression of a gene located within this region in association with the inversion haplotype, with individuals with the H2 haplotype having higher levels of *CRFR1* compared with H1 homozygotes, regardless of disease status. This relationship may help explain the association between genetic variation at the *CRFR1* locus and increased risk of anxiety and stress related conditions.

In contrast with other classes of GPCRs, the exon-intron organization of class B1 GPCRs, of which CRFR1 and CRFR2 are members, permits extensive alternative splicing enabling expression of numerous splice variants that differ in their ligand-specificity, binding and G-protein coupling (1). Although the physiological relevance of many of the CRFR1 and CRFR2 splice variants is uncertain, their complex pattern of expression suggests they may not be functionally redundant(19,32-35). We have identified a novel, truncated form of CRFR1, designated CRFR1j, that was observed to down-regulate CRFR1 signaling by reducing expression of the full-length CRFR1 receptor at the cell surface. This splice variant lacks exons that encode part of the receptor's ligand-binding and transmembrane domains and the predicted protein sequence shares homology with only the first 3 exons of the full-length CRFR1 receptor. CRFR1j expression was predominantly detected in heart failure patients and was associated with higher CRFR1 levels, leading us to speculate that it may be induced in disease or when *CRFR1* expression is high. However, several questions regarding the physiological and clinical relevance of CRFR1j in the heart remain, including its subcellular localisation, whether endogenous levels influence cell surface expression and signalling of endogenous CRFR1, and whether its presence influences clinical outcome in heart failure.

In summary, we have demonstrated robust expression of genes encoding all members of the CRF family of ligands and receptors within the left ventricle of the human myocardium. Our data suggest that both CRFR1 and CRFR2 contribute to the coordinated compensatory response to heart failure and that genetic variation at the *CRFR1* locus and alternative splicing may modulate CRFR1 activity in the heart.

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Table 1. Baseline Characteristics of Heart Failure Patients and Heart Donors

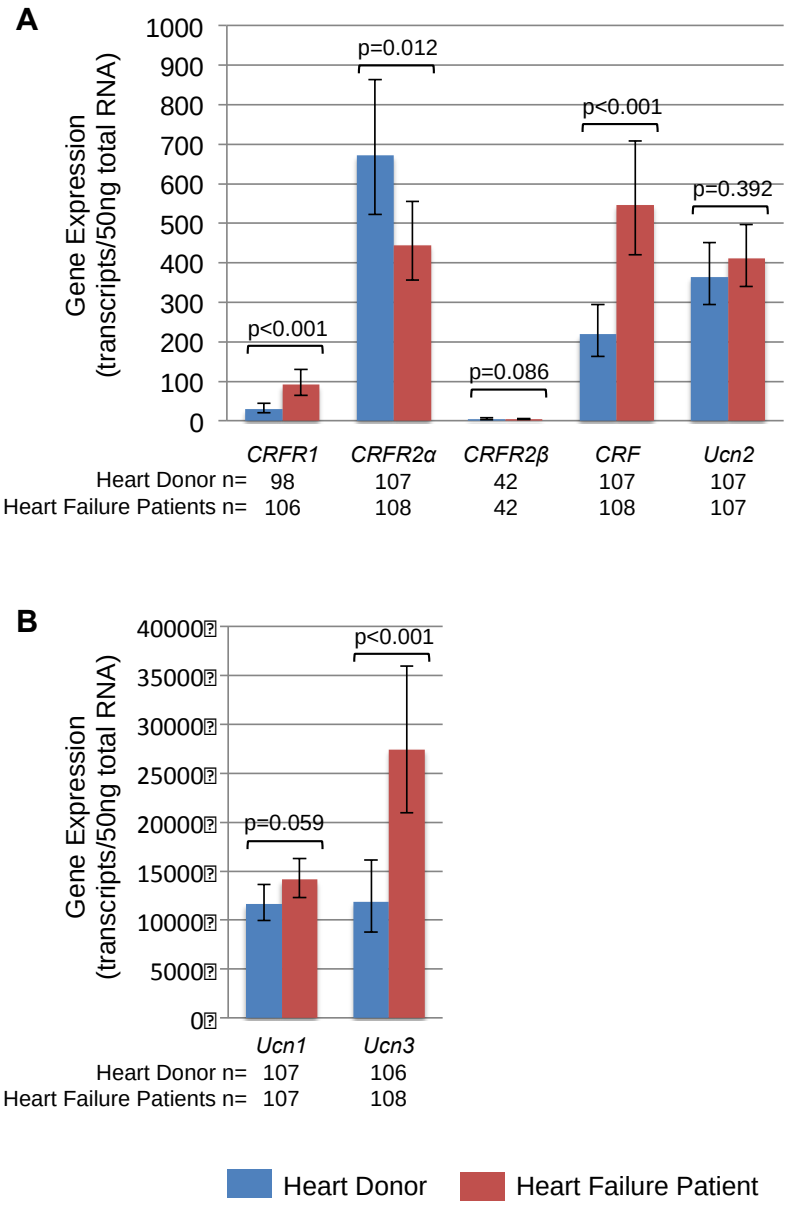
Baseline Characteristics	**n**	**Heart Failure Patients**	**n**	**Donors**
Age* (years)	110	55.3 ± 12.2	107	48.0 ± 12.5
Gender (male/female)	110	89/21 (81% male)	107	55/52 (51% male)
Ethnicity	110	82% European	106	96% European
		15% African American		4% African American
		3% Hispanic		
Cigarette smoking (past/present)	47	38/2 (81%/4%)	-	-
Body mass index* (kg/m^2)	64	27.0 ± 5.1	-	-
Diastolic blood pressure* (mm Hg)	78	63.9 ± 8.7	-	-
Systolic blood pressure* (mm Hg)	78	97.2 ± 12.4	-	-
Total cholesterol† (mmol/L)	87	3.7 (3.0 – 4.5)	-	-
LDL† (mmol/L)	87	1.9 (1.5 – 2.5)	-	-
HDL* (mmol/L)	87	1.1 ± 0.4	-	-
Triglycerides† (mmol/L)	87	1.2 (0.9 – 1.6)	-	-
Glucose† (mmol/L)	94	5.7 (4.9 – 6.8)	-	-
Brain natriuretic peptide† (pmol/L)	82	174 (95 – 351)	-	-

LVEF* (%)	94	15.7 ± 5.5	61	52% ± 15.3
Creatinine† (μmol/L)	91	106 (88 – 132)	-	-
Duration of heart failure (years) †	70	6.1 (2.6 – 10.1)	-	-
Family history of heart disease	92	16 (17.4%)	-	-
Previously diagnosed hypertension	100	47 (47.0%)	-	-
Type 2 diabetes	100	24 (24.0%)	-	-
Myocardial infarction	100	48 (48.0%)	-	-
ACE inhibitor treatment	110	58 (52.7%)	-	-
β-blocker treatment	110	61 (55.5%)	-	-
Loop diuretic treatment	100	69 (69.0%)	-	-
Spironolactone treatment	100	52 (52.0%)	-	-

* mean and standard deviation

† median and inter-quartile range

Figure 1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Gene Expression
(transcripts/50ng total RNA)
1000
900
800
700
600
500
400
300
200
100
0
p=0.012
p<0.001
p=0.086
p<0.001
p=0.392
CRFR1
CRFR2α
CRFR2β
CRF
Ucn2
Heart Donor n= 98 107 42 107 107
Heart Failure Patients n= 106 108 42 108 107
B
Gene Expression
(transcripts/50ng total RNA)
40000
35000
30000
25000
20000
15000
10000
5000
0
p<0.001
p=0.059
Ucn1
Ucn3
Heart Donor n= 107 106
Heart Failure Patients n= 107 108
Heart Donor
Heart Failure Patient

Figure 2

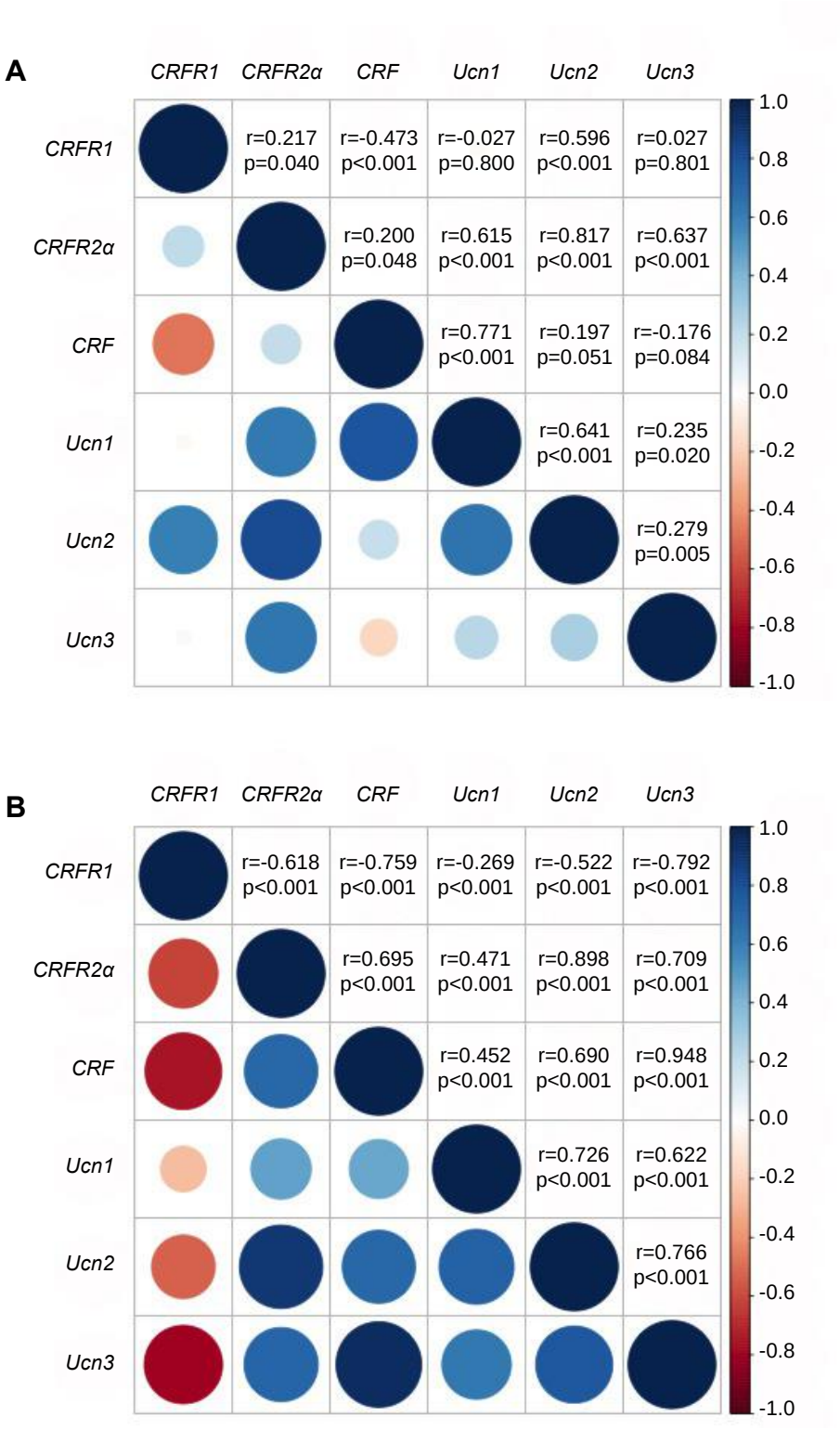
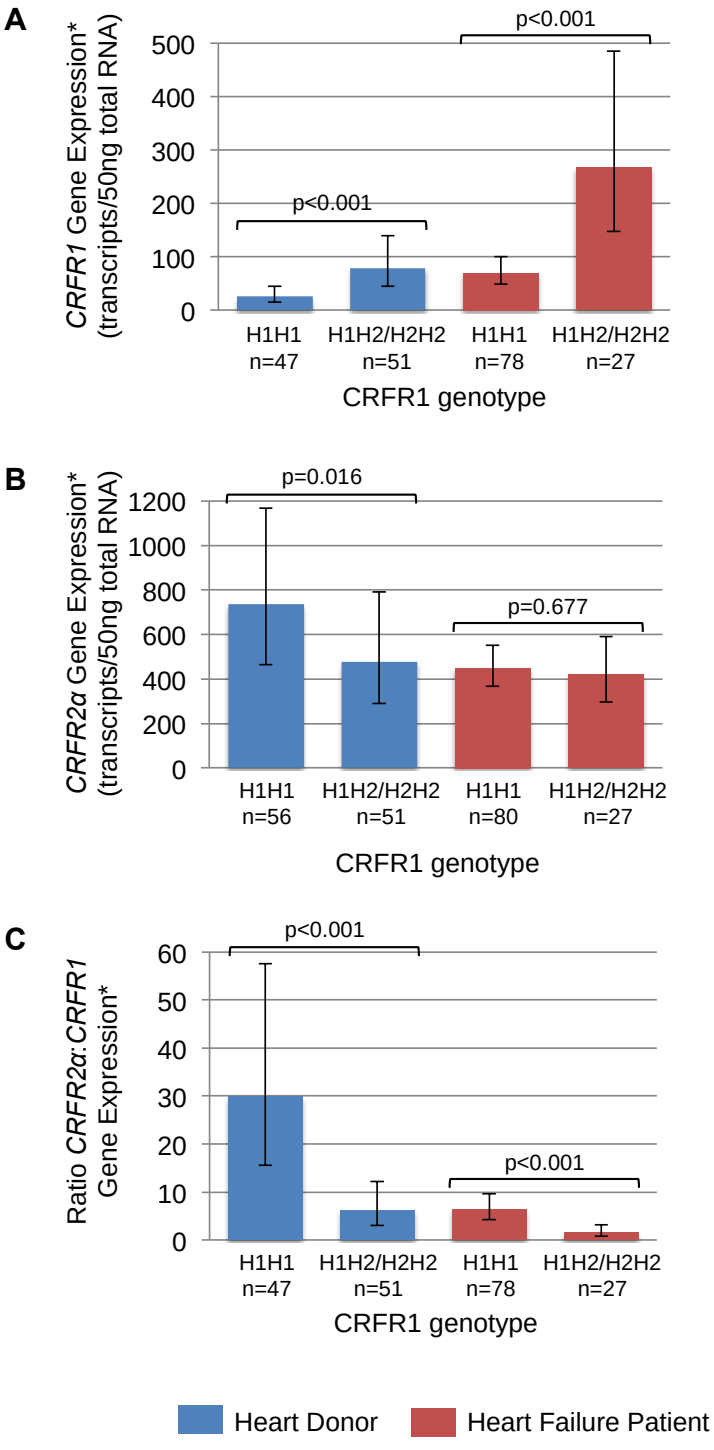

A
CRFR1
CRFR2α
CRF
Ucn1
Ucn2
Ucn3
r=0.217 p=0.040
r=-0.473 p<0.001
r=-0.027 p=0.800
r=0.596 p<0.001
r=0.027 p=0.801
r=0.200 p=0.048
r=0.615 p<0.001
r=0.817 p<0.001
r=0.637 p<0.001
r=0.771 p<0.001
r=0.197 p=0.051
r=-0.176 p=0.084
r=0.641 p<0.001
r=0.235 p=0.020
r=0.279 p=0.005
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
B
CRFR1
CRFR2α
CRF
Ucn1
Ucn2
Ucn3
r=-0.618 p<0.001
r=-0.759 p<0.001
r=-0.269 p<0.001
r=-0.522 p<0.001
r=-0.792 p<0.001
r=0.695 p<0.001
r=0.471 p<0.001
r=0.898 p<0.001
r=0.709 p<0.001
r=0.452 p<0.001
r=0.690 p<0.001
r=0.948 p<0.001
r=0.726 p<0.001
r=0.622 p<0.001
r=0.766 p<0.001
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0

Figure 3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CRFR1 Gene Expression* (transcripts/50ng total RNA)
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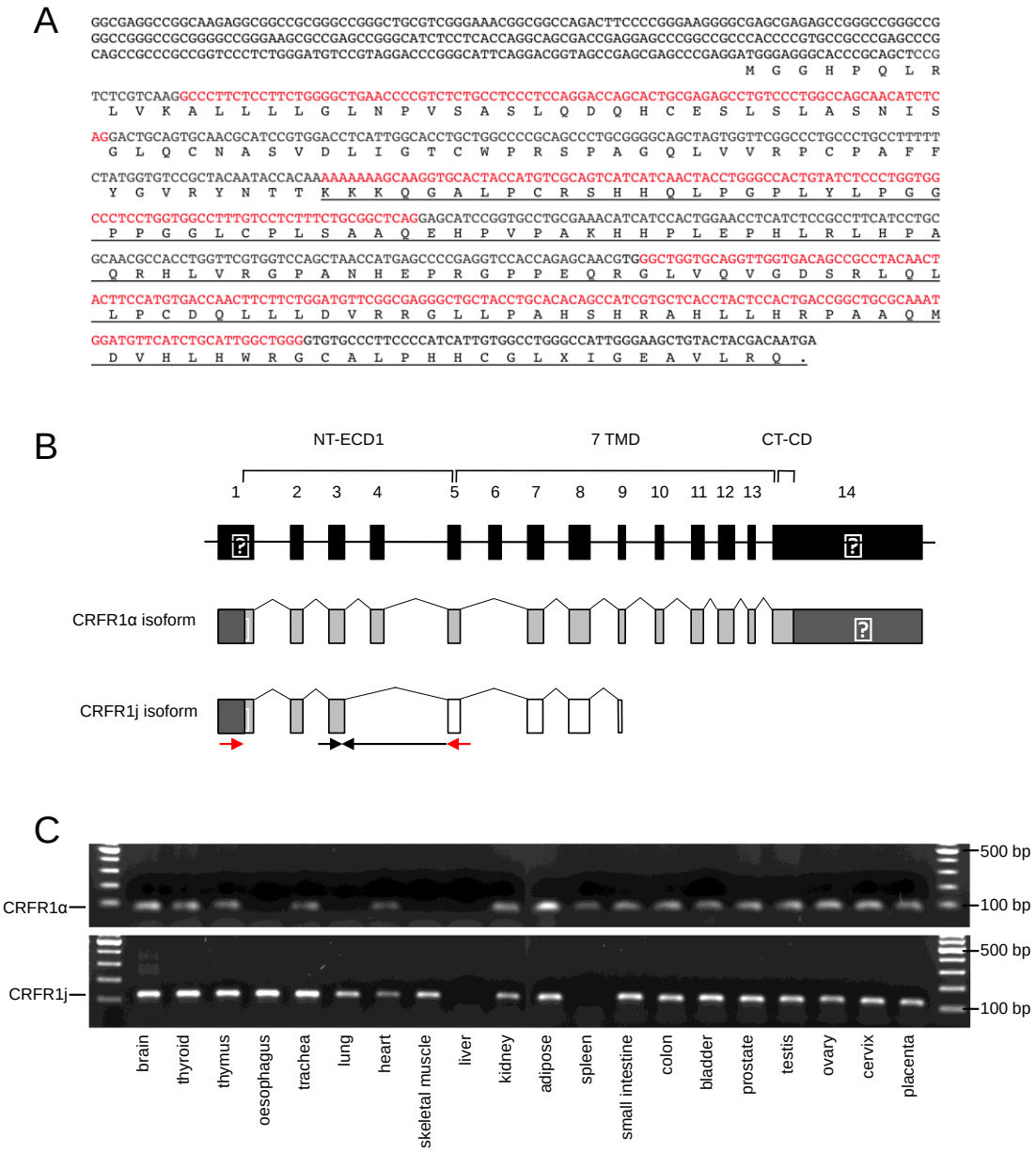
p<0.001
500
400
300
200
100
0
H1H1 n=47
H1H2/H2H2 n=51
H1H1 n=78
H1H2/H2H2 n=27
CRFR1 genotype
B
CRFR2α Gene Expression* (transcripts/50ng total RNA)
p=0.016
p=0.677
1200
1000
800
600
400
200
0
H1H1 n=56
H1H2/H2H2 n=51
H1H1 n=80
H1H2/H2H2 n=27
CRFR1 genotype
C
Ratio CRFR2α:CRFR1 Gene Expression*
p<0.001
p<0.001
60
50
40
30
20
10
0
H1H1 n=47
H1H2/H2H2 n=51
H1H1 n=78
H1H2/H2H2 n=27
CRFR1 genotype
Heart Donor
Heart Failure Patient

Figure 4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CAGCCGCCCGCCGGTCCCTCTGGGATGTCCGTAGGACCCGGGCATTCAGGACGGTAGCCGAGCGAGCCCGAGGATGGGAGGGCACCCGCAGCTCCG
M G G H P Q L R
TCTCGTCAAGGCCCTTCTCCTTCTGGGGCTGAACCCCGTCTCTGCCTCCCTCCAGGACCAGCACTGCGAGAGCCTGTCCCTGGCCAGCAACATCTC
L V K A L L L L G L N P V S A S L Q D Q H C E S L S L A S N I S
AGGACTGCAGTGCAACGCATCCGTGGACCTCATTGGCACCTGCTGGCCCCGCAGCCCTGCGGGGCAGCTAGTGGTTCGGCCCTGCCCTGCCTTTTT
G L Q C N A S V D L I G T C W P R S P A G Q L V V R P C P A F F
CTATGGTGTCCGCTACAATACCACAAAAAAAAAGCAAGGTGCACTACCATGTCGCAGTCATCATCAACTACCTGGGCCACTGTATCTCCCTGGTGG
Y G V R Y N T T K K K Q G A L P C R S H H Q L P G P L Y L P G G
CCCTCCTGGTGGCCTTTGTCCTCTTTCTGCGGCTCAGGAGCATCCGGTGCCTGCGAAACATCATCCACTGGAACCTCATCTCCGCCTTCATCCTGC
P P G G L C P L S A A Q E H P V P A K H H P L E P H L R L H P A
GCAACGCCACCTGGTTCGTGGTCCAGCTAACCATGAGCCCCGAGGTCCACCAGAGCAACGTGGGCTGGTGCAGGTTGGTGACAGCCGCCTACAACT
Q R H L V R G P A N H E P R G P P E Q R G L V Q V G D S R L Q L
ACTTCCATGTGACCAACTTCTTCTGGATGTTCGGCGAGGGCTGCTACCTGCACACAGCCATCGTGCTCACCTACTCCACTGACCGGCTGCGCAAAT
L P C D Q L L L D V R R G L L P A H S H R A H L L H R P A A Q M
GGATGTTCATCTGCATTGGCTGGGGTGTGCCCTTCCCCATCATTGTGGCCTGGGCCATTGGGAAGCTGTACTACGACAATGA
D V H L H W R G C A L P H H C G L X I G E A V L R Q .
B
NT-ECD1
7 TMD
CT-CD
1 2 3 4 5 6 7 8 9 10 11 12 13 14
CRFR1α isoform
CRFR1j isoform
C
CRFR1α
CRFR1j
500 bp
100 bp
500 bp
100 bp
brain
thyroid
thymus
oesophagus
trachea
lung
heart
skeletal muscle
liver
kidney
adipose
spleen
small intestine
colon
bladder
prostate
testis
ovary
cervix
placenta

Figure 5

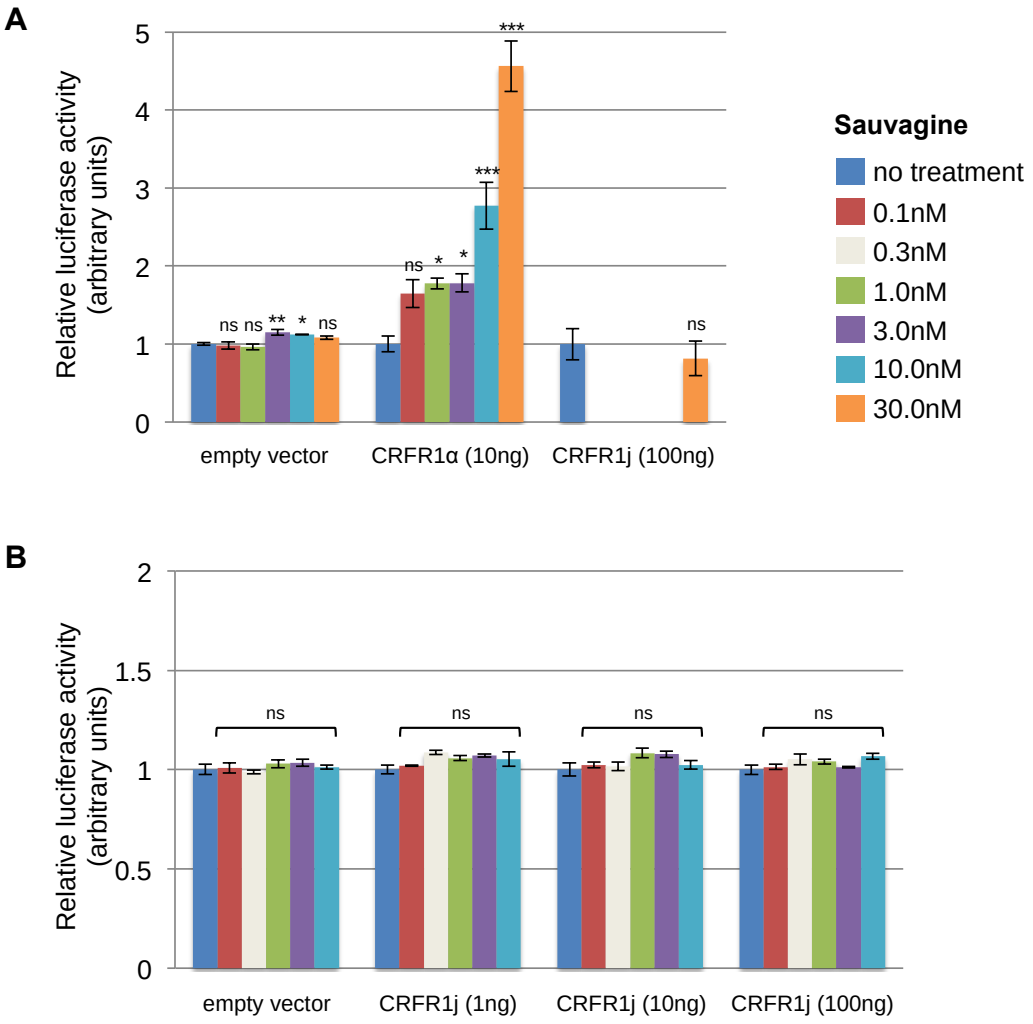
A
Relative luciferase activity (arbitrary units)
0
1
2
3
4
5
ns ns ** * ns
ns * * *** ***
ns
empty vector
CRFR1α (10ng)
CRFR1j (100ng)
Sauvagine
no treatment
0.1nM
0.3nM
1.0nM
3.0nM
10.0nM
30.0nM
B
Relative luciferase activity (arbitrary units)
0
0.5
1
1.5
2
ns
ns
ns
ns
empty vector
CRFR1j (1ng)
CRFR1j (10ng)
CRFR1j (100ng)

Figure 6

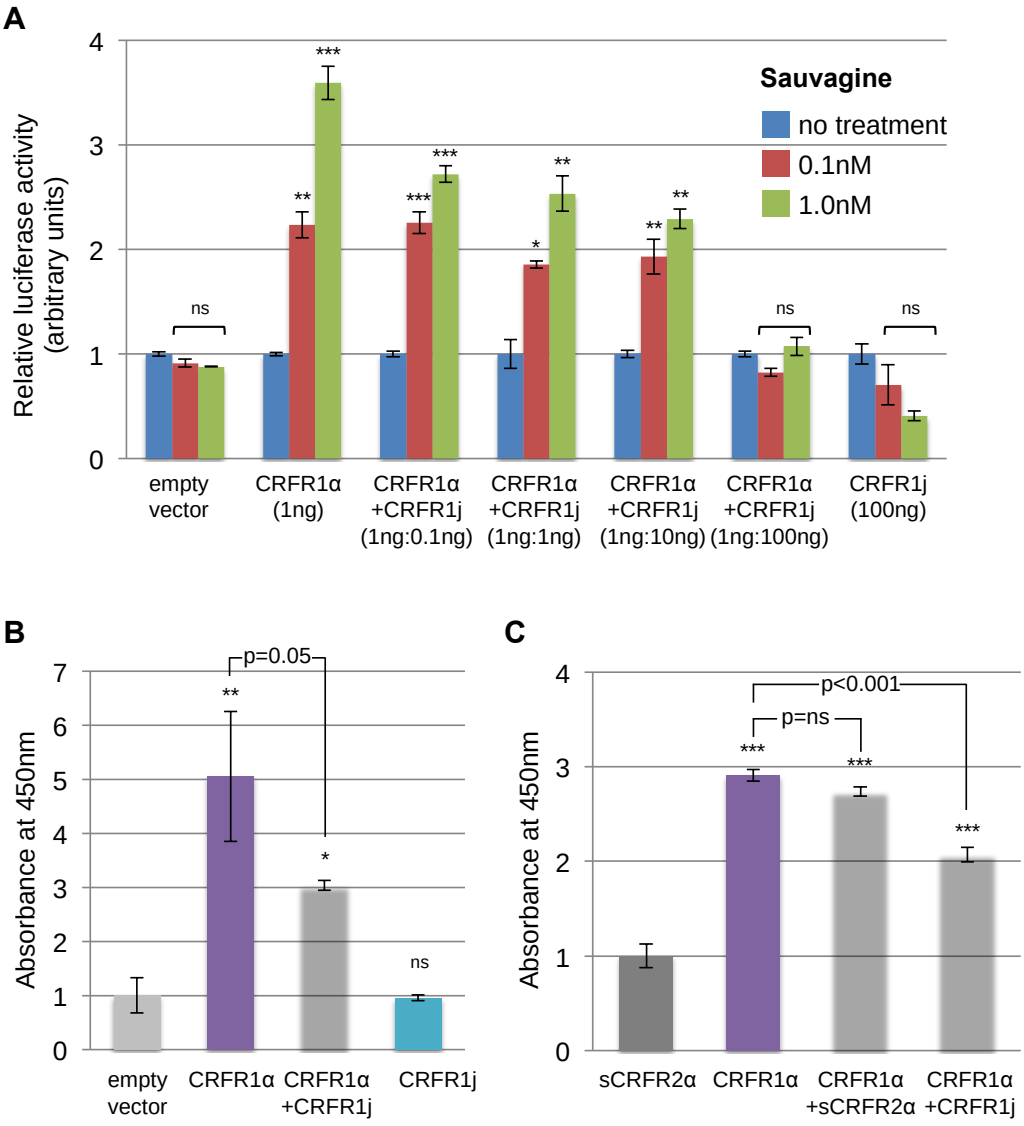
A
Relative luciferase activity (arbitrary units)
Sauvagine
no treatment
0.1nM
1.0nM
empty vector
CRFR1α (1ng)
CRFR1α +CRFR1j (1ng:0.1ng)
CRFR1α +CRFR1j (1ng:1ng)
CRFR1α +CRFR1j (1ng:10ng)
CRFR1α +CRFR1j (1ng:100ng)
CRFR1j (100ng)
ns
B
Absorbance at 450nm
p=0.05
empty vector
CRFR1α
CRFR1α +CRFR1j
CRFR1j
C
Absorbance at 450nm
p<0.001
p=ns
sCRFR2α
CRFR1α
CRFR1α +sCRFR2α
CRFR1α +CRFR1j

Figure Legends

Figure 1. Myocardial gene expression of the CRF family of ligands and receptors in heart donors (blue) and heart failure patients (red). (A) Expression levels of the CRF receptors, *CRF* and *Ucn2*, and (B) *Ucn1* and *Ucn3* in the left ventricle of the human heart adjusted for age, gender, ethnicity and sample storage time. In patients, expression levels of *CRFR1*, *CRF* and *Ucn3* were higher and *CRFR2α* were lower compared with donors. Data represent geometric means and 95% confidence intervals.

Figure 2. Correlation matrix of gene expression levels of the CRF family of ligands and receptors in the left ventricle of the human heart, in (A) heart donors and (B) heart failure patients. Gene expression levels were adjusted for age, gender, ethnicity and sample storage time. The upper half of each figure displays the Pearson correlation coefficients and p-value for each association. The lower half of each figure gives a pictorial representation of these values: the Pearson correlation coefficient is represented by the colour of each circle (positive correlations are blue, negative correlations are red), while the p-value is indicated by the size of the circle (larger circle sizes indicate highly statistically significant relationships).

Figure 3. Associations between a large inversion polymorphism on chromosome 17q21.31 (which spans the *CRFR1* gene) and *CRFR1* and *CRFR2α* expression in heart donors (blue) and heart failure patients (red). (A) Levels of *CRFR1* expression were higher in donors and patients with the H2 haplotype. (B) Levels of *CRFR2α* expression were lower in donors with the H2 haplotype. (C) Consequently, the ratio of *CRFR2α*:*CRFR1* expression was markedly lower in donors and patients with the H2 haplotype, making *CRFR1* expression almost equivalent to *CRFR2α* expression in patients with the H2 haplotype. Gene expression data were adjusted for age, gender, ethnicity and sample storage time. Data represent geometric means and 95% confidence intervals

Figure 4. Sequence and structural representation of the novel CRFR1 splice variant j. (A) DNA and protein sequence of CRFR1j, illustrating the predicted protein sequence that results from loss of exons 4

and 6, which leads to a shift in reading frame and a new stop codon in exon 9 (underlined sequence). Alternate exons are indicated by black and red text. (B) Schematic illustration of the structure of the CRFR1j and the full length, functional CRFR1 isoform (CRFR1α), showing that only the first three exons of CRFR1j are predicted to be identical to the functional receptor. The stop codon in exon 9 of CRFR1j results in a truncated protein compared with CRFR1α. Black boxes indicate exons of the *CRFR1* gene sequence. Grey boxes indicate exons translated in the correct reading frame to generate functional CRFR1α and white boxes indicate exons predicted to be translated in an alternative reading frame. Arrows indicate the location of nested primers used for semi-quantitative PCR of novel CRFR1j splice variant. The reverse primer for the second PCR reaction spans the unique CRFR1j splice junction between exons 3 and 5. NT-ECD1=amino-terminal extracellular domain 1, 7 TM=7 transmembrane domains, CT-CD=carboxy-terminal cytoplasmic domain. (C) RT-PCR indicating the presence or absence of CRFR1α and CRFR1j mRNA in range normal human tissues (expected band size for CRFR1α=69 bp and for CRFR1j=121 bp).

Figure 5. The novel CRFR1j splice variant is unable to activate the intracellular cAMP signaling pathway. (A) Activation of a cAMP-response element luciferase reporter in HEK293T cells transfected with CRFR1α (full-length, functional isoform) or CRFR1j, following treatment with the CRFR1 agonist, sauvagine (0-30nM). Cells transfected with CRFR1α showed increasing activity with increasing sauvagine concentration. In contrast, cells transfected with 10x the amount of CRFR1j showed no activity at the highest dose of sauvagine. (B) Activation of a cAMP-response element luciferase reporter by sauvagine (0-10nM) in HEK293T cells transfected with varying amounts of the novel CRFR1j splice variant (1-100ng), indicating a complete lack of activation at all concentrations. Experiments were performed in triplicate. Data represent means and standard errors. Statistics compare sauvagine treatment with the no treatment group for each transfection; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns $p>0.05$.

Figure 6. Dominant-negative activity of the novel CRFR1 splice variant j on the full-length CRFR1α receptor. (A) Activation of a cAMP-response element luciferase reporter by sauvagine (0.1nM, 1nM) in HEK293T cells transfected with a fixed amount of CRFR1α and increasing amounts of CRFR1j. Luciferase activity decreased with increasing CRFR1j at both concentrations of sauvagine ($p<0.001$ for both analyses), indicating a potential dominant-negative effect of CRFR1j on CRFR1 signaling. (B) Cell surface expression of CRFR1α in COSM6 cells transfected with myc-tagged CRFR1α alone or in combination with CRFR1j, showing reduced cell surface expression in co-transfected cells. Experiments were performed in triplicate. Data represent means and standard errors. Statistics compare sauvagine treatment with the no treatment group for each transfection, unless otherwise indicated; * $p<0.05$; ** $p<0.01$; *** $p<0.001$; ns $p>0.05$.