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Altered enhancer and promoter usage leads to differential gene expression in the normal and failed human heart

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Abstract

Background: The failing heart is characterized by changes in gene expression. However, the regulatory regions of the genome that drive these gene expression changes have not been well defined in human hearts.

Methods: To define genomewide enhancer and promoter use in heart failure, Cap Analysis of Gene Expression (CAGE sequencing) was applied to three healthy and four failed human hearts to identify promoter and enhancer regions used in left ventricles. Healthy hearts were derived from donors unused for transplantation and failed hearts were obtained as discarded tissue after transplantation.

Results: CAGE sequencing identified a combined potential for ~23,000 promoters and ~5000 enhancers active in human left ventricles. Of these, 17,000 promoters and 1800 enhancers had additional support for their regulatory function. Comparing promoter usage between healthy and failed hearts highlighted promoter shifts which altered amino-terminal protein sequences. Enhancer usage between healthy and failed hearts identified a majority of differentially utilized heart failure enhancers were intronic and primarily localized within the first intron, revealing this position as a common feature associated with tissue-specific gene expression changes in the heart.

Conclusions: This dataset defines the dynamic genomic regulatory landscape underlying heart failure and serves as an important resource for understanding genetic contributions to cardiac dysfunction. Additionally, regulatory changes contributing to heart failure are attractive therapeutic targets for controlling ventricular remodeling and clinical progression.

Keywords

heart failure; cardiomyopathy; gene expression; promoter; enhancer; regulatory sequences

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Heart failure with reduced ejection fraction is characterized by altered metabolism and function, each which contribute to an inability to meet demands for normal activity. Heart failure is associated with global changes in gene expression, and some of these gene expression changes directly drive pathological and adaptive remodeling.¹ For example, the failed heart shifts its metabolism towards glycolysis, driven in part by gene expression changes.² Within the failed human heart, a distinct form of myosin heavy chain is expressed,^{3, 4} as are alternatively spliced forms of *TNNT2* and *TTN*, encoding troponin T and titin, respectively, and these changes directly modify contractility and compliance.⁵⁻⁷ In addition, mutations in many of these genes directly lead to cardiomyopathy and heart failure.^{8, 9}

Gene expression is regulated by promoters, which locate immediately upstream from the target gene, and enhancers, which can reside much further away from the target gene. Transcription factors are proteins that bind promoter and enhancer sequences, which interact in three-dimensional space to regulate gene expression. The genomic regions responsible for regulating normal and pathological gene expression in the heart are incompletely understood. A few specific genetic regulatory regions have been characterized,^{10, 11} but comparatively few genomewide analyses have been conducted. Surveys of the cardiac epigenome have been carried out using developing mouse hearts and embryonic stem cell derived-cardiomyocytes.^{12, 13} One study of mouse hearts subjected to pressure overload, examined chromatin conformation to indicate potential active regulatory regions.¹⁴ However, the mature human hearts has its own gene expression program that differs from mouse hearts and stem cell-derived cardiomyocytes.

Estimates of number of active heart enhancers vary from several thousand to tens of thousands depending on the approach used.^{15, 16} An assessment of active cardiac enhancers relied on detecting p300/CBP binding sites from one human fetal and one adult failed heart.¹⁵ This analysis identified ~5,000 active enhancers in fetal tissue and ~2,000 active enhancers in adult tissue, with approximately half of adult heart enhancers also active in fetal heart.¹⁵ A similar approach used normal human and mouse hearts integrating p300/CBP binding sites with active histone (H3K27ac) marks.¹⁶ This combined analysis suggested more than 80,000 potential heart enhancers.¹⁶ Although these studies provide valuable datasets, the genomic alterations seen in human heart failure require better definition.

Genes can be expressed from more than one promoter. Alternative promoters are estimated to affect 30-50% of human genes.¹⁷ Alternative promoters may alter the 5' untranslated (UTR) of transcripts or may also affect the amino terminus of the encoded protein. Both of these alterations can have functional consequences. Additionally, alternative promoters can also influence the effect of genetic variants on protein function.¹⁸ Despite the potential of broad proteome differences due to alternative promoter usage, a genomewide view of promoter shifts in human heart failure is lacking.

Cap-Analysis of Gene Expression (CAGE) determines RNA transcriptional start sites at high resolution.¹⁹ Enhancer RNAs (eRNAs) are short RNAs produced from enhancer sites. CAGE sequencing detects eRNAs from enhancers, seen as short bidirectional transcripts, as well as the unidirectional sequences from the capped ends of longer transcripts, reflecting

promoters.^{20, 21} To define alternative promoter and enhancer use in heart failure, we generated CAGE sequence data from healthy and failed human left ventricles. We relied on a no-amplification non-tagging CAGE sequencing protocol, to permit more robust and less biased detection of transcriptional start sites.¹⁹ We combined CAGE seq with RNA sequencing from the same hearts to better compare healthy and failed hearts. In aggregate, we identified >23,000 unidirectional signals and >5,500 bidirectional signals in the heart. The promoters used by highly-expressed, tissue specific genes, like those encoding the sarcomere proteins, had distinct molecular signatures. We found enhancers in tissue specific genes often mapped to first introns, and the features of these enhancers suggest they are highly critical for driving cardiomyocyte-specific gene expression. We compared regulatory regions between failed and healthy hearts and identified 129 genes with differential promoter usage in heart failure. These alternative promoters have the potential to encode proteins with unique amino-termini, highlighting potential protein composition shifts in the failed heart which may contribute to heart failure.

METHODS

For detailed methods, see Expanded Methods.

Approvals.

Subjects provided informed consent under IRB approval from the University of Chicago or Northwestern University.

Transparency and Data Availability.

All scripts/code used in this analysis are available upon request. Raw and processed sequence data is publicly available at NCBI-GEO under accession number GSE147236.

RNA-Extraction, Library Preparation, and Sequencing.

Healthy and failed left ventricle samples were obtained from failed transplants or as discarded tissue, respectively. Living subjects provided consent. Healthy left ventricular samples were obtained from hearts provided by the Gift of Hope of Illinois and were found to be unsuitable for transplant due to age or prior cardiac surgeries. All patients were declared to be brain dead as the result of cerebral hemorrhage and familial consent was obtained for organ use in research. Custom nAnt-iCAGE-seq (no-Amplification-no-Tagging Illumina Cap Analysis of Gene Expression libraries) libraries were prepared by DNAFORM (Japan) following a previously-described protocol.¹⁹ Approximately 50pM of pooled libraries was sequenced using a NextSeq 500 (Illumina) to yield ~400 million total 75bp single end reads (Supplemental Table 1). RNA-seq libraries were prepared using the TruSeq mRNA-seq library preparation kit (Illumina). Libraries were pooled in equimolar amounts and loaded on the HiSeq 4000 (Illumina) to generate ~40 million 150bp paired-end reads/sample. CAGE-Seq analysis was conducted using adapted methods.^{22–29} Epigenetic datasets of interest were downloaded from their respective locations (Supplemental Table 2) and compared to CAGE sequence clusters using HOMER.³⁰

RESULTS

Identifying genetic regulatory regions of the left ventricle.

RNA sequencing assays gene expression by looking at the entire length of RNA transcripts. CAGE sequencing identifies RNAs originating from promoters and enhancer regions by targeting just the 5' capped ends of transcripts. Since gene expression patterns differ between normal and failing hearts, we generated CAGE sequence datasets from left ventricle (LV) from three healthy and four failed hearts. Healthy LV samples were those acquired but not used for transplant due to age or other incompatibility. Failed hearts were obtained at the time of transplant from patients with a range of ages (Table 1). The small sample size did not allow the consideration of age and primary gene mutation in the analysis. Each library was sequenced to high depth with comparable alignment rates (Supplemental Table 1). To generate a comprehensive list of all potential promoters and enhancers, the initial analysis combined the results from healthy and failed hearts. CAGE sequence analysis identified 23,676 promoter regions, defined as unidirectional sequence clusters, and 5,647 enhancers, defined as bidirectional sequence clusters.

The promoter sequences, seen as unidirectional CAGE sequence clusters, were mapped relative to known genes (example shown in Supplemental Figure 1 and pipeline shown in Supplemental Figure 2). 70.1% of these promoter sequences mapped near transcriptional start sites (± 100 bp), as would be expected for promoter function (Figure 1A). An additional 8.1% of these unidirectional clusters, mapped between 100 and 1000bp upstream of transcriptional start sites. The remaining 21.8% of sequence clusters mapped to untranslated regions, coding and noncoding exons, introns or intergenic regions.

We next analyzed the promoter clusters for the presence of transcription factor binding motifs. The 70.1% of clusters mapping within 100bp of transcriptional start sites were highly enriched for GFY-Staf, Sp1, and Elk/ETS binding motifs, which are transcription factors known to bind promoters.³¹ Clusters mapping into other regions showed minimal enrichment of these motifs (Figure 1B). To provide additional support for the promoter-enriched sequence clusters, ATAC sequencing and H3K4me3 ChIP-seq datasets from human left ventricles were evaluated since these data indicate open and active chromatin; Supplemental Table 2 provides the source information. The promoter clusters overlapped considerably with ATAC-seq and H3K4me3 ChIP-seq signals, providing strong support for these CAGE seq clusters as identifying promoters (Figure 1C). These clusters also showed high CTCF and Pol2A binding, as well as a reduction of H3K4me1 histone modifications, which is consistent with these regions being promoters and not enhancers (Supplemental Figure 3). Taken together, the unidirectional CAGE sequence clusters bear the genomic signatures of active promoters.

Bidirectional eRNA clusters indicate likely enhancer regions. We similarly annotated bidirectional CAGE clusters for their position relative to genes. Only 44.5% of bidirectional clusters mapped ± 100 bp within transcriptional start sites. In contrast to unidirectional clusters, 24.3% of clusters mapped to gene introns and 7.6% were intergenic (Figure 1D). These intergenic and intronic enhancer clusters showed enrichment of GATA, GRE, and MEF2 transcription factor binding motifs. Each of these transcription factors are essential

for cardiomyocyte specification and maintenance (Figure 1E).³² Intergenic bidirectional clusters showed enrichment of open chromatin signals (ATAC-seq), H3K4me1, and H3K27Ac histone modifications in human left ventricles. Intronic clusters also showed a similar pattern, but with lower magnitude (Figure 1F). The intergenic and intronic bidirectional clusters showed enrichment of CTCF and Pol2A binding as well as reduced H3K4me3 modifications (Supplemental Figure 3). These patterns are highly consistent with the role of intronic and intergenic bidirectional CAGE sequence clusters as being enhancers, rather than promoters. Furthermore, these patterns represent multiple, independently-derived sources of evidence that bidirectional eRNA transcription signify functional enhancers.

Two types of promoters.

Mammalian promoters can initiate transcription across broad or narrow genomic regions, and these promoter shapes, broad or narrow (sharp), correlate with distinct transcriptional regulatory mechanisms.³³ We evaluated cardiac promoters predicted from CAGE clusters for these two major types of promoters by calculating the interquartile range (IQR) of promoter CAGE clusters by determining the base pair distance between 10% and 90% of a promoter's total signal. We observed the expected two distinct populations, defined as sharp (IQR < 10bp) and broad promoters (IQR ≥ 10bp) (Figure 2A). Broad promoters were those associated many different cellular functions, including housekeeping functions. In contrast, genes with sharp (narrow) promoters were those encoding proteins critical for tissue specific functions seen by the presence myofilament, muscle tissue and muscle contractions genes (Figure 2B). Thus, tissue specific genes important for left ventricular specification and function were more likely to have sharp promoters.

Sharp and broad promoters also displayed differential enrichment of upstream sequence DNA-binding motifs. Sharp promoters had TATA motifs at positions 30–33 upstream of the predominant transcriptional start site, representing canonical TATA boxes (Figure 2C). Broad promoters were devoid of TATA motifs, and instead showed enrichment of GC nucleotides consistent with CpG-islands.³⁴ Sharp, tissue-specific promoters were also more highly expressed compared to broad promoters, and this observation was driven by a smaller population of very highly expressed sharp promoters, for example those encoding sarcomere genes (Figure 2D). We compared promoter shape between healthy and failed hearts and found a small but significant genomewide increase in promoter IQR in failed hearts, consistent with a slight widening of transcriptional start sites in heart failure (Figure 2E).

Predicted enhancers map within the first Intron.

A large proportion of the predicted enhancers mapped to introns. We observed that the majority (69%) of intronic enhancers in this dataset mapped to the first intron (Figure 3A). First introns are more conserved than other introns and correlate with higher levels of gene expression.³⁵ In the LV CAGE sequence data, these first intron enhancers generated more eRNA than enhancers in other introns, but were not wider and did not differ in their balance of bidirectionality (Figure 3B). Notably, these intronic enhancers mapped within genes enriched for cardiac and muscle gene ontology terms (Figure 3C). This finding identifies that highly expressed, tissue specific genes, like those encoding myofilament proteins, often have enhancers in their first introns.

Correlation of CAGE-sequence and RNA-sequence data.

RNA sequencing, which evaluates the transcripts along their entire length, was carried out on the same LV samples and was compared to CAGE sequencing. CAGE sequencing, which assays just the 5' end of capped transcripts can assay gene expression, but is more sensitive to RNAs derived from promoters and enhancer regions. Since the assays were performed on the same tissue, a tight correlation between expression values were observed. Consistent with this, there was a tight correlation between CAGE sequencing and RNA sequencing results for any given gene (Figure 4A). Additionally, we assessed correlations between pairs of samples. In general, healthy hearts correlated best with other healthy hearts, and failed hearts compared best with failed hearts. The RNA sequence and CAGE sequence expression estimates were most correlated for matched samples except for Failed Heart 4, which likely reflects the lower CAGE sequence read depth in this sample (Figure 4B). We next compared gene expression differences between failed and nonfailed hearts using both CAGE and RNA sequence datasets. Overall, RNA sequencing was more sensitive and therefore identified more upregulated and downregulated genes; approximately half of the genes identified by CAGE sequencing were also identified by RNA sequencing (Figure 4C). The types of genes differentially expressed were similar in both sequence datasets. Genes associated with developmental pathways and extracellular matrix organization were upregulated in heart failure while genes associated with catabolism were downregulated in heart failure (Figure 4D), consistent with prior gene expression profiling of failed hearts.^{1, 2}

CAGE sequencing-defined enhancer regions are validated by other enhancer datasets.

Of the ~1,800 candidate enhancer regions identified by CAGE sequencing, data was available from 45 of these in the Vista Enhancer Browser, a list of enhancers tested in an *in vivo* reporter assay using transgenic mouse embryos.³⁶ Of the 45 present in Vista, 31 (70%) demonstrated enhancer activity in the developing mouse heart (Supplemental Figure 4A). We also compared CAGE sequence-predicted enhancers to those predicted by H3K27Ac and p300 ChIP sequencing from developing and adult human and mouse tissues.¹⁶ CAGE-sequence-defined enhancers showed significantly higher overlap to H3K27Ac/p300 ChIP regions compared to length-matched scrambled control regions (Supplemental Figure 4B). One study with H3K27Ac ChIP-seq data from healthy and failed human hearts was similarly compared and showed significant overlap (Supplemental Figure 4C).³⁷ Finally, we compared CAGE sequence-defined enhancer predictions from the FANTOM consortium, which used CAGE sequencing across many non-diseased tissues to define enhancers.³⁸ We observed significant overlap with FANTOM predictions (Supplemental Figure 4D), but we found many additional enhancers beyond the FANTOM predictions because we used a higher depth of sequencing (Supplemental Table 1). The intersection of these multiple datasets corroborates the cardiac enhancers now identified by CAGE sequence in both healthy hearts and failed hearts.

Alternative promoter usage in heart failure.

In the LV, 3,032 (23%) expressed genes had evidence for more than one promoter (Figure 5A). For these multi-promoter genes, we compared the average percent-usage of each promoter in healthy and failed hearts and found 609 promoters in 325 genes with a shift

10% (Figure 5B). Of these, 149 promoters in 124 genes occurred after the exon containing the start codon, indicating the potential to alter the amino-terminal amino acid sequence of the resulting protein (Figure 5C). Of the 124 genes identified as having alternative promoters that occur after start codons in heart failure, many are associated with sarcomere regulation or muscle structure development, including *TNNT*, *MYOT*, and *SPEG*. This indicates the heart failure can result in alternative proteins due to promoter shifts. We annotated a significant promoter switch in *PRKAG2*, a gene linked to hypertrophic cardiomyopathy and critical to heart metabolism.³⁹ Three major *PRKAG2* promoters were identified, encoding three different isoforms- γ 2a, γ 2-3b, and γ 2b (Figure 5D). In healthy hearts, the relative expression of these three transcripts is 53% γ 2b, 28% γ 2-3b, and 17% γ 2a. In heart failure, these percentages significantly shift with 29% γ 2b, 59% γ 2-3b, and 10% γ 2a isoform (Figure 5E). Notably, the γ 2-3b isoform encodes a unique 32 amino acid sequence at the amino-terminus (Figure 5F). Total expression of *PRKAG2* was not different between healthy and failed hearts. We interrogated the 30kb upstream of the γ 2b and γ 2-3b isoforms for transcription binding motifs and found an enrichment of Smad and GRE motifs upstream of the γ 2-3b isoform, suggesting a role for these transcription factors (Supplemental Table 3).

Enhancer usage shifts in heart failure.

The CAGE sequence analysis identified ~1,800 LV enhancer regions in intergenic and intronic regions (Figure 1A). Clustering analysis of the normalized expression levels showed an overall similar profile of enhancer usage across the healthy LVs, but disparate enhancer usage across the four failed LVs (Figure 6A). Comparing enhancer usage across healthy and failed LV revealed 264 enhancers that changed significantly in heart failure (raw p-value 0.05). To assess whether differential enhancer transcription was associated with differential transcription factor binding site profiles, we compared transcription factor motif instances across enhancers in healthy and failed LVs. We found SMAD2, NFIX, NFAT, TCF7L2, ZNF740, and AR binding motifs enriched in enhancers that changed in heart failure. SMAD2, NFIX, TCF7L2, and AR binding motifs were found more often in downregulated enhancers. While NFAT and ZNF740 binding motifs were found more often in upregulated enhancers. RNA-sequencing demonstrated that *SMAD2* and *NFAT5* were significantly upregulated in heart failure (Figure 6C).

Figure 6D illustrates alternative enhancer use within the first intron of *TRPM7*, which encodes the transient receptor potential cation channel subfamily M member, a gene implicated in ischemic cardiomyopathy and cardiac rhythm.^{40, 41} This intronic enhancer showed significantly lower eRNA expression in heart failure (Figure 6E), concomitant with a significantly lower expression of *TRPM7* in failed hearts (Figure 6F). We also identified an enhancer cluster upstream of the *NPPA/NPPB* gene loci (Figure 7). *NPPA/NPPB* encode natriuretic peptides, which serve as important clinical biomarkers of volume overload in heart failure. One enhancer of this cluster was detected in all samples and showed higher expression of eRNA in heart failure (Figure 7C), consistent with the regulatory region responsible for the upregulation of natriuretic proteins in heart failure.

DISCUSSION

Defining the promoterome of the human LV in health and disease.

Heart failure is known to be accompanied by shifts in gene expression, including the re-expression of developmentally expressed genes. We now describe genomewide promoter usage in left ventricle, and further highlight how promoter usage shifts in the failed LV. In total, we report ~17,000 high likelihood promoters active in the adult human heart. We observed two major promoter types, the sharp TATA-box-associated and the broad CpG island-associated.³⁴ Sharp promoters had single or a few transcriptional start sites and were linked to highly-expressed, tissue-specific genes like *MYH7*, *TTN*, and *MYL2*. Broad promoters had a wider distribution of transcriptional start sites and included both housekeeping and some tissue specific genes. The slight but significant increase in genomewide promoter width in failed left ventricles may suggest a loss of tight regulation of transcriptional start sites, which could derive from epigenetic modifications or transcriptional factor profile differences.

Alternative promoter usage in heart failure.

Approximately ~20% of genes active in the human left ventricle have more than one active promoter, similar to what has been reported for other cell types.^{42, 43} Promoter switches that alter the noncoding regions can affect translational efficiency, imparting developmental and tissue specificity.⁴⁴ Some promoter switches can directly alter the amino-terminus of the resulting protein. We provide as an example a failure-linked promoter shift in the *PRKAG2* gene. Mutations in *PRKAG2* cause hypertrophic cardiomyopathy and arrhythmias.³⁹ In healthy left ventricles, we found that ~55% of transcripts originated from the γ 2b promoter and ~35% originate from the γ 2–3b promoter. In failed left ventricles ~60% of the *PRKAG2* transcripts represent the γ 2b–3b isoform. The γ 2b–3b isoform includes a unique 32 amino-acids that may affect the ability of the AMPK complex to interact with troponin I and regulate contraction dynamics.⁴⁵ In the UK Biobank, a polymorphism, rs10224210, which is in the first intron of the γ 2–3b isoform, associates with cardiovascular disease. This specific sequence could alter γ 2b–3b isoform expression through a first intron enhancer, and this association provides additional evidence that the γ 2b–3b isoform may be an important mediator of heart failure. Upregulation of the γ 2b–3b isoform in failed hearts may also influence how mutations in *PRKAG2* are expressed. The γ 2b isoform is expressed in healthy left ventricle and in cultured fibroblasts, as seen in the GTEx dataset, indicating isoform shifts might affect multiple cell types in the heart.

Differential enhancer usage in heart failure.

Heart failure is associated with transcriptional changes.¹ We found that enhancer usage was more variable in failed ventricles, which may indicate genomewide dysregulation of gene expression. *SMAD2* binding motifs were enriched in differentially used heart failure enhancers, and this is highly consistent with the known upregulation of TGF- β signaling in failing hearts.^{46, 47} The enrichment of this motif in differential enhancers may reflect increased TGF- β /SMAD activation in cardiomyocytes and/or a larger proportion of cardiac fibroblasts in the failed left ventricle tissues. We highlighted a specific differential enhancer located within the first intron of the *TRPM7* gene. *TRPM7* encodes kinase domain-

containing cation channel. Deletion of *Trpm7* in mice disrupts cardiac automaticity and causes cardiac hypertrophy and fibrosis.⁴¹ In ischemic cardiomyopathy, *TRPM7* was significantly down regulated in the left atria and ventricle.⁴⁰ Together, these findings support a reduction in *TRPM7* in the setting of end stage heart failure. Sequences upstream of the *NPPA/NPPB* gene loci were also identified as differentially activated in heart failure. The comparable region in the mouse genome has been shown to regulate expression of these genes, validating its function *in vivo*.⁴⁸ As natriuretic peptide elevation serves as a biomarker for heart failure, this enhancer region may be an attractive target to modulate natriuretic factor expression in heart failure.

Conclusions and study limitations.

This study used CAGE sequencing to define a broad spectrum of predicted cardiac promoters and enhancers, with focus on their differential use in heart failure. We identified 129 genes that had alternative 5' sequences between failed and healthy hearts. We also identified 264 enhancers that were differentially used between the failed and healthy hearts. Due to the small cohort size, age, sex, and race could not be considered in the analysis. The majority of samples were of European descent, and controlling for age was not possible as age correlates with heart failure status. Therefore, this analysis was uncorrected for these covariates, and thus may limit the broader applicability of this data. The use of bidirectional eRNA transcription as a genomewide mark of enhancer function is relatively new and the exact role of eRNAs is unknown. To address this, we compared the CAGE sequencing findings to multiple independent approaches which have been used to predict enhancers. Despite this, these approaches may still over or underestimated the true number active enhancer regions. We observed variability in differential promoter and enhancer usage in failed heart, as the normal control hearts showed tighter correlations to each other. This variability may reflect the end stage process of heart failure. While a larger dataset may be more revealing, the diversity of response in the failed hearts mirrors what has been observed when RNA sequencing was used to define transcripts produced for *TTN*, a large gene that has been examined in multiple failed hearts.^{49, 50} The wide array of transcripts produced from even this single gene may underscore that a lack of uniform response itself could contribute to heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Non standard abbreviations:

ATAC-seq	Assay for Transposase-Accessible Chromatin sequencing
bp	base pair
CAGE-seq	Capped Analysis of Gene Expression sequencing
ChIP	chromatin immunoprecipitation
CTCF	CCTCT binding factor
eRNA	enhancer RNA
H3K4me1	histone H3, mono-methylated on lysine 4
H3K4me3	histone H3, tri-methylated on lysine 4
H3K27ac	histone H3, acetylated on lysine 27
IQR	interquantile range
LV	left ventricle
Pol2A	RNA polymerase II
PRKAG2	Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma
TPM	transcripts per million
TRPM7	transient receptor potential cation channel subfamily M member
UTR	untranslated region

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What is new?

- We identified genomic regulatory regions used in the human left ventricle from normal and failed hearts using CAGE sequencing.
- We found more than 120 genes using alternative promoters in heart failure where the resulting protein changed its amino terminus because of this promoter shift.
- The PRKAG2 gene shifts its promoter usage in failed hearts, which could change the action or location of the AMP activated protein kinase (AMPK) enzyme.

What are the clinical implications?

- The failed heart expresses different genes compared to the normal heart; some of these gene expression changes are adaptive and others are maladaptive.
- These gene expression changes are directed by distinct regulatory genomic regions.
- These regulatory regions are targets for genetic manipulation to potentially alter or treat heart failure.

Figure 1. The genomic position of promoters and enhancers used by human left ventricles (LV). **A.** Using CAGE sequencing of LVs, more than 23,000 promoters were found across the genome, and 78.2% mapped near the transcriptional start sites (TSS) of genes (orange and blue combined, 1-1000bp from TSS). **B.** The promoter sites were analyzed for transcription factor binding sites. Elk, Sp1, and GFY-Staf transcription factor binding sites were enriched in promoters near transcriptional start sites (orange bars). **C.** The promoters near transcriptional start sites showed epigenetic marks of open chromatin and active histone marks (orange lines in ATAC Seq and H3K4me3 ChIP Seq), indicating these regions are

open and active in the heart. **D.** More than 5,500 enhancer signals were found across the genome, and >30% of these enhancers mapped into intronic and intergenic regions (green and brown combined). **E.** Intronic and intergenic enhancers were enriched for cardiac transcription factor binding sites like GATA, GRE, and Mef2, which are transcription factors known to be important for heart function (green and brown bars). **F.** Intronic and intergenic enhancer sites also had signals of active enhancer function, seen as ATAC-seq and H3K4me1 and H3K27Ac histone marks (green and brown lines in each). Dashed lines in **C** and **F** represent negative controls. *TSS*, transcriptional start site.

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Figure 2. Cardiac promoters have sharp or broad transcriptional start sites.

There are two types of promoters, those that initiate transcription over a narrow region (<10bp, sharp promoter) or over a broad range (10-100bp). **A.** The >17,000 CAGE promoters signals near transcriptional start sites can be divided into these two groupings, sharp and broad. **B.** Genes with broad promoters (red bars) encoding proteins with housekeeping functions (cellular localization, gene expression, organelle organization, cellular metabolic processes). In contrast, genes encoding proteins of the muscle filament and muscle contraction had sharp promoters (blue bars). **C.** Sharp promoters have TATA regulatory motifs, while broad promoters, including those for tissue specific genes, are defined by CpG islands. **D.** Genes with sharp promoters, which include tissue specific genes, are expressed at higher levels than genes with broad promoters. The left panel shows that sharp promoters have higher normalized transcripts per million (TPM) levels (80.5 vs 73.5). The right panel indicates that sharp promoters span a shorter genomic distance (39bp vs 98bp). **E.** On average, failed hearts have slightly wider promoters compared to healthy hearts, which likely reflects differences in gene expression that occur during heart failure. Significance determined by two-tailed nonparametric Mann Whitney Test ($p = 0.05$ (*), 0.0005 (***)). *TSS*, transcriptional start site. *IQR*, interquantile range. *bp*, basepair.

Figure 3. The majority of intronic cardiac enhancers localize to the first intron.

A. Of the 1,371 enhancers that fell within introns, ~70% of intronic enhancers mapped to the first intron of a gene. **B.** First intron enhancers were expressed more highly than other intronic enhancers, while other features like the width and expression from both strands were similar between first intronic enhancers and other intronic enhancer. Thus, first intronic enhancers were similar to other enhancers except that they were expressed more highly. **C.** Intronic enhancers were more likely to be found in tissue specific genes, like those that encode components of the sarcomere. Significance determined by two-tailed nonparametric Mann Whitney Test ($p = 0.05(*)$).

Figure 4. Gene expression and eRNA expression correlate.

RNA sequencing (RNA seq) measures all RNAs while CAGE seq measures expression capped transcripts including the short enhancer RNAs (eRNAs). **A.** Overall, there was good correlation between a gene's expression level measured by RNA seq and measured by CAGE seq. Each dot represents a single gene, and genes with high expression by RNA sequencing also had high expression by CAGE sequence analysis. This pattern was seen in both healthy (teal) and failed (red) hearts. **B.** The correlation coefficient of genomewide gene expression levels showed that expression in healthy hearts correlated best with other

healthy hearts, and failed hearts compared best with failed hearts. **C.** Comparing gene expression between healthy and failed heart identified differentially expressed genes and eRNAs. RNA-seq identified more upregulated and downregulated genes, and approximately half of the genes identified by CAGE-seq were also identified by RNA-seq. **D.** Genes associated with developmental pathways and extracellular matrix organization were upregulated in heart failure while genes associated with mitochondrial transport and catabolism were downregulated in heart failure.

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Figure 5. Heart failure was associated with significant changes in promoter usage.

A. Approximately 20% of genes active in the heart use more than one promoter. **B.** For genes with more than one promoter, promoter usage is altered between failed and healthy. The x-axis represents the difference between a promoter's average percent usage in the healthy or failed LVs. Left-shifted promoters make up a greater percentage of gene expression in failed ventricles and right-shifted promoters are a greater percentage in healthy ventricles. **C.** ~25% of promoter shifts result in using a promoter 3' downstream of the start codon, potentially adding new sequences to the 5' end of the mRNA or amino terminus of

the protein. **D.** 124 genes were identified as potentially changing protein structure by using an alternative promoter in heart failure. An example is shown for the *PRKAG2* gene. Three known isoforms of *PRKAG2* are represented at the bottom ($\gamma 2a$, $\gamma 2-3b$, $\gamma 2b$). Above the promoter of each transcript, the CAGE-seq signal for healthy (blue) and failed (red) hearts is shown and the scales of each representation are indicated in black. **E.** The promoter percent usage indicates the $\gamma 2-3b$ form is upregulated and the $\gamma 2b$ form is downregulated in failed heart. Significance determined by a two-tailed Student's t-test. **F.** The predicted amino acid sequences translated from each *PRKAG2* isoform are shown. (p 0.05(*), 0.005(**)) *PRKAG2*, Protein Kinase AMP-Activated Non-Catalytic Subunit Gamma.

Figure 6. Differential enhancer usage in heart failure.

A. Enhancer expression clustered more tightly in the healthy hearts compared to failed hearts, which may reflect more dysregulated gene expression in heart failure. **B.** Some enhancers are increased while other are decreased in healthy versus failed hearts. **C.** Differentially used enhancers preferentially have SMAD2, NFIX, NFAT, TCF7L2, ZNF740 and AR transcription factor binding motifs. *SMAD2* and *NFAT*, were themselves differentially expressed in the healthy versus failed hearts (purple asterisks). **D.** An example of a differentially used enhancer is shown in the first intron of the *TRPM7* gene. A reduction

of eRNA signal is seen in the failed hearts (red) compared to the healthy hearts (blue). **E.** eRNA expression was significantly reduced from the *TRPM7* enhancer. **F.** Overall *TRPM7* gene expression was correspondingly reduced. Additional failed hearts were added to increase power. ($p = 0.05$ (*), 0.0005 (***)). *FC*, fold change. *TRPM7*, transient receptor potential cation channel subfamily M member 7.

Figure 7. An enhancer for the *NPPA/NPPB* genes carries the signature of activation in heart failure.

A. The *NPPA/NPPB* locus, which encodes ANP and BNP, and is known to be upregulated in heart failure. Three upstream enhancers are shown in purple, and these were identified by CAGE seq. These same regions overlap with marks of active and open chromatin (H3K27Ac in blue and ATAC-seq in red). **B.** The CAGE-seq signals of the enhancers shown in A demonstrate increased signal in failed (red) compared to healthy (blue) hearts. **C.** eRNA expression from enhancer 1 shows increased expression in failed compared to healthy hearts. (p < 0.005(**)).

TABLE 1.

Left Ventricle Tissue Source Demographics, Phenotypes, and Mutations.

Sample	Primary phenotype	Additional phenotypes	Sex	Race	Age	Primary gene mutation(s)
Healthy 1	Healthy	-	M	Caucasian	62	N/A
Healthy 2	Healthy	-	M	Caucasian	47	N/A
Healthy 3	Healthy	-	F	Caucasian	76	N/A
Heart Failure 1	Cardiomyopathy	Ventricular Tachycardia	M	Caucasian	20	<i>TPM1</i> D230N
Heart Failure 2	Cardiomyopathy	-	M	Hispanic	16	<i>TTN</i> c.42521-5 C>G, <i>TNNT2</i> K210del
Heart Failure 3	Cardiomyopathy	Becker Muscular Dystrophy	M	Caucasian	54	<i>DMD</i> IVS +1 G>T
Heart Failure 4	Cardiomyopathy	Limb Girdle Muscular Dystrophy	F	Caucasian	26	<i>LMNA</i> c.1142-1157+1del17