Heat shock proteins and small nucleolar RNAs are dysregulated in a Drosophila model for feline hypertrophic cardiomyopathy

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Abstract

In cats, mutations in myosin binding protein C (encoded by the MYBPC3 gene) have been associated with hypertrophic cardiomyopathy (HCM). However, the molecular mechanisms linking these mutations to HCM remain unknown. Here, we establish Drosophila melanogaster as a model to understand this connection by generating flies harboring MYBPC3 missense mutations (A31P and R820W) associated with feline HCM. The A31P and R820W flies displayed cardiovascular defects in their heart rates and exercise endurance. We used RNA-seq to determine which processes are misregulated in the presence of mutant MYBPC3 alleles. Transcriptome analysis revealed significant downregulation of genes encoding small nucleolar RNA (snoRNAs) in exercised female flies harboring the mutant alleles compared to flies that harbor the wild-type allele. Other processes that were affected included the unfolded protein response and immune/defense responses. These data show that mutant MYBPC3 proteins have widespread effects on the transcriptome of co-regulated genes. Transcriptionally differentially expressed genes are also candidate genes for future evaluation as genetic modifiers of HCM as well as candidate genes for genotype by exercise environment interaction effects on the manifestation of HCM; in cats as well as humans.

Keywords: MYBPC3; cMyBP-C; Drosophila melanogaster; feline HCM; hypertrophic cardiomyopathy

Introduction

Cardiovascular disease is the leading cause of death worldwide, accounting for 30% of all deaths. The American Heart Association estimates that over 85 million people in the United States have cardiovascular disease, with an associated health-care cost of $320 billion dollars. Cardiomyopathy refers to disease of the heart muscle and can be classified as either primary or secondary. Secondary cardiomyopathy results from extrinsic factors such as hypertension, ischemia, and metabolic disorders, while primary cardiomyopathy occurs in the absence of extrinsic factors. Hypertrophic cardiomyopathy (HCM) is a primary myocardial disease characterized by thickening of the left ventricle myocardium, impaired diastolic function, myocardial fibrosis, and altered calcium kinetics in the cell (Houston and Stevens 2014; Fraiche and Wang 2016; Kimura 2016). It affects over 1 in 500 people in the general population and ~700,000–725,000 are affected in the United States (Maron et al. 2016; Sen-Chowdhry et al. 2016). Patients diagnosed with HCM may experience one or more of the following symptoms: shortness of breath, chest pain during exercise, fainting, heart-palpitations, heart failure, or sudden cardiac death.

In most cases, HCM is caused by mutations in genes encoding proteins involved with sarcomere structure or function and is often inherited as a Mendelian autosomal dominant trait (Bonne et al. 1998). Seventy percent of HCM cases have been linked to mutations in 11 sarcomeric genes, leaving ~30% of cases with unidentified causes (Efthimiadis et al. 2014; Roma-Rodrigues and Fernandes 2014). Most mutations associated with HCM occur in the MYBPC3 gene that encodes myosin binding protein C (cMyBP-C). These are either frame-shift or splice-site mutations resulting in a truncated protein, or missense mutations in which a single DNA nucleotide change results in an amino acid substitution (ClinVar database; https://www.ncbi.nlm.nih.gov/clinvar/) (Landrum et al. 2016). As of January 27, 2020, the ClinVar database has curated 1594 MYBPC3 variants, of which 342 have been classified as pathogenic for HCM. Phenotypic heterogeneity is
prevalent within and between families, suggesting that
tumors of the sarcomere are not the sole determinant of the HCM
phenotype.

The human MYBPC3 gene encodes the 1274 amino-acid (140 kDa)
myosin-associated protein cMyBP-C that contributes to
the structural integrity of the sarcomere and regulates myocardial
contraction (Hartzell 1985; Gilbert et al. 1996; Weisberg and
Winegrad 1996). The cMyBP-C protein consists of 11 domains
(C0–C10)—8 immunoglobulin-like C2 domains and 3 fibronectin
type III domains (Sequeira et al. 2014). Five phosphorylation sites
have been identified, four are located in the cMyBP-C regulatory
region (M-domain) and one is in the proline-alanine rich domain
(Gruen et al. 1999; Kuster et al. 2013). Myocardial contraction is
achieved by the sliding movement of myosin-containing thick fil-
aments and the actin-containing thin filaments in the sarcomere.
Each cMyBP-C molecule is tethered to the myosin thick filament
through its C-terminus (C7–C10) and interacts with actin via its
N-terminal domains (C0–C2). The contraction and relaxation pro-
cesses are triggered by a structural change in the myosin head
domains while they are bound to actin and coupled with ATP hy-
drolysis (Inchingolo et al. 2019). In addition to actin and myosin,
the sarcomere contains multiple other proteins (including cal-
modulin, titin, and troponin) that serve as binding partners with
cMyBP-C to regulate cardiovascular health (Heling et al. 2020).

HCM is a leading cause of cardiovascular sudden death among
competitive US athletes (Maron et al. 2009, 2016). Between the
years 1980 and 2006, it was estimated that 251 athletes among
690 diagnosed with a primary cardiovascular disease died of sud-
cen cardiac death due to HCM (Maron et al. 2009). In 2015, the
first study to screen for sarcomeric gene mutations was con-
ducted on a cohort of 102 unrelated young Japanese athletes with
abnormal ECG findings (Kadota et al. 2015). Heterozygous
Arg106Trp and Thr1046Met mutations were identified in
MYBPC3 in four of 102 athletes, with two patients for each of the muta-
tions
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Drosophila models have been successfully developed for nu-
merous human diseases, including diabetes (Alfa and Kim 2016),
glucoma (Carbone et al. 2009), retinal degeneration (Coiley 2012),
Alzheimer’s disease (Fernandez-Funez et al. 2015), sleep disorders
(Donelson and Sanyal 2015), and obesity (Smith et al. 2014). Fly
models of cardiovascular disease emerged over 20 years ago with
the development of assays to measure heart function and develop-
ment (Bodmer 1993; Ocorr et al. 2014). Many cardiac related
genes are evolutionarily conserved between Drosophila and
humans including those involved in heart development (Bodmer and
Venkatesh 1998; Vogler and Bodmer 2015), cardiac function
(Medioni et al. 2009; Kooij et al. 2014), ion channels (Ocorr et al.
2007; Cavaliere and Hodge 2011), and contractile function
(Cammrato et al. 2011; Chakraborty et al. 2015). It is likely that
these conserved genes may be functioning in common physiolog-
ical pathways, enabling an ideal translational model for verte-
brate cardiac disease.

The insect heart consists of a hollow muscular tube that runs
from the posterior abdomen into the thorax. The hemolymph
(i.e. insect “blood”) flows freely within the body cavity where it makes direct contact with internal tissues and organs. Both the mammalian and insect hearts are divided into chambers that are separated by tiny valves through which fluid (blood or hemolymph) enters the heart. In insects, each of the four chambers contains six myocardial cells that facilitate the flow of hemolymph through the dorsal vessel. The aorta is the tube composed of myocardial cells that transports the hemolymph from the head into the body cavity (Ponzio et al. 2002; Bier and Bodmer 2004; Zhu et al. 2016). Since other invertebrate genetic models such as C. elegans lack a heart, Drosophila presents the best simple model organism for translational studies on cardiac disease.

Materials and methods
Drosophila culture
All flies were maintained on cornmeal-agar-molasses medium at 25°C and 70% humidity, and a 12:12 h light:dark cycle.

Transgenic lines expressing feline MYBPC3
The HCM MYBPC3 variants were cloned from the cDNA of feline heart tissue (generically donated by Dr. Kathryn Meurs, NC-State University) to the pUAST-attb donor expression vector [which contains the white+ (w+) marker] using standard techniques, and subsequently validated by Sanger sequencing (Sanger et al. 1977; Bischof et al. 2007). The WT MYBPC3 clone was used as the parental template for site-directed mutagenesis to obtain the pathogenic variants (A31P and R820W) using appropriate primer pairs (Supplementary Table S1). The variant clones were validated by Sanger sequencing to ensure that the desired mutagenesis had occurred (Supplementary Table S1 and Supplementary Figure S1). All MYBPC3-pUAST-attb constructs were submitted for PhiC31 transformation (Groth et al. 2004) to Model System Injections (Durham, NC, USA). Transgenic flies bearing the WT, A31P, and R820W MYBPC3-pUAST-attb constructs were generated at a common integration site using PhiC31 transformation (Groth et al. 2004) in the y, w[Pint, y-]; Chr2/Chr2, P(attP2, y+) isogenic genotype. Generation-0 (G0) flies were crossed to a white-eyed double-mutant line homozygous for a cardiac- and the heart rate was measured by counting the number of beats (i.e. pulses of the trachea) within a 15 s interval (Cooper et al. 2009). The movement of the trachea occurs due to pulling of the attachments from the heart (dorsal vessel). The heart rate measurement was repeated for four 15 s intervals per individual. Each heart rate within a 15 s interval is multiplied by 4 to calculate the beats per minute (BPM). Tukey and T-tests were performed using JMP Pro, Version 14 (SAS Institute Inc., Cary, NC, USA, 1989–2019).

Climbing and exercise assays
On the day before the assay, flies of the three genotypes expressing MYBPC3 in cardiac tissue and the control genotype were sorted by sex (50 flies/sex/lane/replicate) and allowed to recover overnight from exposure to CO2. Three biological replicates/sex/line were assayed. To measure their inherent climbing ability, we used the countercurrent distribution apparatus designed by Benzer (1967). Flies were placed in their start-tube, gently tapped to the bottom of the tube and allowed 15 s to climb vertically. The apparatus was shifted once to the right and gently tapped such
that any flies that climbed were collected into the neighboring tube. The flies were again allowed to climb vertically for 15 s after shifting the apparatus back to its start position. The process was repeated for a total of 7 climbing opportunities. At the end of the assay, the eight tubes containing flies were removed and each fly assigned a score from 1 (did not climb) to 8 (climbed seven times). The mean climbing score for each replicate was calculated as: \[ \frac{\sum (i \times N_i)}{\sum N_i} \] where \( N_i \) is the number of flies in the \( i \)th tube.

The flies were then returned to their original vial containing food and allowed to recover overnight. The following day, the flies were exposed to an intense exercise regime. The flies were transferred to empty vials and placed vertically in a rack secured to a shaker (VWR Signature High-Speed Microplate Shaker). The shaker was programmed to pulse for 10 s (600 rpm), causing the flies to fall to the bottom of the fly vial, followed by a 10 s rest period. During the 10 s rest period, the flies “exercise” by climbing upwards as they are innately negatively geotactic. The process was repeated for 1 h. At the end of the exercise regime, the flies were again scored for their climbing ability using the countercurrent distribution apparatus. Flies were then frozen at –80°C for RNA-sequencing. Standard least squares and Tukey test analyses were performed using JMP® Pro, Version 13 (SAS Institute Inc., Cary, NC, USA, 1989–2019) using the full model: \[ Y = \mu + G + S + T + G*S + G*T + S*T + G*S*T + Rep(G*S*T) + e, \] and the reduced model (by sex): \[ Y = \mu + G + T + G*T + e, \] where \( G, S, T, \) and \( Rep \) are the genotype (control, WT, A31P, and R820W), sex (M or F), treatment (exercised vs nonexercised), and replicate, respectively.

Genome wide expression profiling

Larvae and 1-week old adult flies from the three genotypes expressing MYBPC3 in cardiac tissue and the control genotype were collected over \( CO_2 \) and allowed to recover from exposure to \( CO_2 \) for 24 h prior to being flash-frozen for RNA-sequencing. In addition, 1-week old adult flies were also collected and flash frozen for RNA-sequencing after exposure to the exercise regimen described above. The samples were stored at –80°C until ready to process. Samples were processed for two biological replicates each of 10 pooled males and 10 pooled females from each genotype under normal rearing conditions and before and after 1 h of exercise, and for two biological replicates of 10 pooled larvae from each genotype, for a total of 40 samples.

Total RNA was extracted with QIAzol lysis reagent (Qiagen) and the Quick-RNA Miniprep Zymo Research Kit (Zymo Research). Ribosomal RNA (rRNA) was depleted from 5 μg of total RNA using the Ribo-ZeroTM Gold Kit (Illunina, Inc.). Depleted mRNA was fragmented and converted to first-strand cDNA using Superscript III reverse transcriptase (Invitrogen). Second strand cDNA was synthesized using dUTP instead of dTTP to label the second strand cDNA. cDNA from each sample was used to produce barcoded cDNA libraries using NEXTflexTM DNA barcodes (Bioo Scientific) with an Illumina TruSeq compatible protocol. Briefly, each sample was subjected to end-repair (Enzymatics), adenylation of 3‘-ends (Enzymatics), and ligation of indexed adapters (Enzymatics and Bioo Scientific). Each enzymatic reaction was purified using 1.8X Agencourt AMPure XP beads (Beckman-Coulter). Size selection of each library was performed using Agencourt AMPure XP beads (Beckman Coulter) to an approximate insert size of 130 bp and a total library size of ~250 bp. Second strand cDNA was digested with Uracil-DNA Glycosylase prior to PCR-enrichment to produce directional cDNA libraries.

PCR-enrichment of the purified barcoded DNA was carried out with KAPA HiFi Hot Start Mix (Kapa Biosystems) and NEXTflex Primer Mix (Bioo Scientific). Libraries were quantified using the Qubit dsDNA HS kit (Life Technologies) and their sizes determined with the 2100 Bioanalyzer (Agilent Technologies). Each sample was diluted to equal molarity, quantified, multiplexed, denatured, and diluted to 15 pM. Clonal clusters for each pooled library sample were generated on the Illumina cBot and then sequenced on the Illumina HiSeq2500 using 125 bp single-read v4 chemistry (illumina Inc.). We generated multiplexed libraries containing 12 samples each. Each multiplexed library loaded on one lane of the HiSeq2500.

We demultiplexed the RNA sequences using the Illumina bc12fastq program; assessed read quality using the FastQC program and pre-processed reads using Cutadapt (Martin 2011) to remove residual adapter sequences. Residual ribosomal RNA (rRNA) sequences were removed following alignment of sequencing reads to known rRNA sequences using TopHat (Trapnell et al. 2009). All remaining reads were processed using the CLC Genomics Workbench 11 (https://www.qiagenbioinformatics.com/) which is based on previously published methods (Mortazavi et al. 2008). Reads were aligned to the D. melanogaster reference genome release 6.13 (Flybase.org). Unaligned reads were mapped to the MYBPC3 transcript (NCBI Accession: NM_000256.3). Read counts and differential expression analyses were assessed using the CLC Genomics Workbench 11. Principal component analysis (PCA) was conducted in JMP® Pro, Version 14 (SAS Institute Inc., Cary, NC, USA, 1989–2019). Gene expression changes were investigated for male and female transgenic flies (attP2 control, WT, A31P, and R820W) under nonexercised and exercised conditions. PCA was performed using normalized read counts of the total number of expressed genes (~16,000 genes).

Differential expression analyses were conducted for multiple comparisons to uncover differences due to expression of mutant MYBPC3 under exercised and nonexercised conditions, for males, females, and larvae. We identified genes that were significantly differentially regulated at a false discovery rate (FDR) < 0.05 and with Log2FC > 0.5 or Log2FC < −0.5. Heat maps were generated using the online tool Heatmapper (http://www2.heatmapper.ca/expression/). To place the differentially expressed genes in their biological context, the following online bioinformatics tools for gene ontology, pathway analysis and identification of human orthologs were used: The Reactome Pathway Portal (www.reactome.org), Network Analyst STRING algorithm (www.networkanalyst.ca) (Szklarczyk et al. 2015), DAVID (https://david.ncifcrf.gov), PANTHER Pathway (www.pantherdb.org/pathway/), and FlyBase (www.flybase.org). These tools allowed us to place feline candidate genes in a functional context by assigning biological processes to candidate genes associated with HCM risk.

Knockdown of candidate genes using RNAi

We used RNA interference (RNAi) to knockdown the expression of candidate genes implicated by our differential expression analyses. Homozygous UAS-RNAi lines (Act57B, Act79B, Act88F, E71alpha101E, Hsp22, Mhc, Mhc2, and TyrR) were obtained from the Vienna Drosophila Resource Center (VDCR) (Dietzl et al. 2007). These flies were crossed to flies containing either the ubiquitin driver (Ubi156-Gal4) or the cardiac-specific Gal4 driver (TinC-Gal4) to disrupt their gene expression. The progenitor genotypes (VDCR lines 60100 and 60000) were also crossed to both drivers as a control for lines from the KK[Panc31] or GD[Pi-element] RNAi libraries, respectively. Larvae heart rates of the F1 progeny were measured as described above.
Data availability
Supplementary data have been uploaded to the GSA Figshare portal. The expression data are deposited in the Gene Expression Omnibus under the accession ID GSE141574. Supplementary Figure S1 shows the Sanger sequencing chromatograms of the A31P and R820W mutations generated by site-directed mutagenesis. Supplementary Figure S2 shows the RNA expression of feline MYBPC3 in our Drosophila model for feline HCM, using both RT-PCR and RNA-seq data. Supplementary Figure S3 shows the volcano plots of the differential expression analysis of genes in the MYBPC3 transgenic lines (WT, A31P, and R820W) compared to the control line (attP2). Supplementary Figure S4 shows the principal component analyses of the RNA-seq data. Supplementary Figure S5 shows the protein–protein interaction (PPI) networks that were generated for significantly differentially expressed genes. Supplementary Figure S6 shows larval heart-rates resulting from RNA interference knockdown. Supplementary Table S1 lists the primer sequences used for cloning, site-directed mutagenesis, RT-PCR and Sanger sequencing. Supplementary Table S2 shows the results of the statistical analysis for the exercise assays. Supplementary File S1 is the gene expression browser for the RNA-seq data. Supplementary File S2 is the results of the differential expression analysis. Supplementary File S3 provides the data to generate the heat maps. Supplementary File S4 lists the gene ontology-biological processes (GO-BP) for the significantly differentially expressed genes. Supplementary material is available at figshare DOI: https://doi.org/10.25387/g3.13228268.

Results
Expression of feline MYBPC3 alleles in Drosophila
HCM is the most common heart disease in cats. Two MYBPC3 gene mutations, A31P and R820W, were previously identified in the Maine Coon and Ragdoll breeds, respectively. To understand the disease etiology associated with these variants, we used the Gold4/UAS system to express feline MYBPC3 in the fly cardiovascular system. We cloned the WT and variants (A31P and R820W) of feline MYBPC3 to the pUAST-attp expression vector and generated Drosophila UAS-MYBPC3 transgenic lines throughout the text. The lines were validated for gene expression of feline MYBPC3 using RT-PCR and the normalized counts from the RNA-sequencing data for larvae, males, and females (Supplementary Figure S2). As expected, the three MYBPC3 genotypes (WT, A31P, and R820W) show feline MYBPC3 gene expression while the control line (attP2) does not, under both exercised and nonexercised conditions. We assayed these lines for their heart rates and exercise endurance and performed whole-transcriptome profiling using RNA-seq.

Effects of feline variants of MYBPC3 on heart rate and exercise endurance
We measured heart rates from larvae expressing feline WT and mutant MYBPC3 alleles in the Drosophila cardiovascular system using a heart-specific Gal4 driver. We observed a significant increase in heart rate of the A31P (P = 2.7 × 10^{-3}) and R820W (P = 2.5 × 10^{-4}) mutant alleles compared to the WT MYBPC3 WT allele (Figure 1). Importantly, there was no significant difference in heart rate between the control genotype and the MYBPC3 WT allele, indicating that expressing the WT feline MYBPC3 gene has no detectable effect on heart rate.

We assessed the cardiovascular endurance of the transgenic lines by performing a baseline climbing assay and a second climbing assay immediately following 1 h of intense exercise (Figure 2, Supplementary Table S2). Climbing ability was significantly reduced following exercise for all genotypes in both sexes, with the greatest reductions among the genotypes expressing feline MYBPC3. The climbing ability of the pre-exercised flies was only moderately significantly different between the four genotypes in either sex. However, following the exercise regime, the A31P and the R820W genotypes showed a significant decrease in climbing ability compared to the MYBPC3 WT genotype in both sexes, and all three MYBPC3 genotypes had reduced climbing ability compared to the control post-exercise. This decrease was much greater in males than females.

Gene expression changes due to expression of MYBPC3 alleles
We quantified genome-wide gene expression in flies that express the three MYBPC3 transgene alleles (WT, A31P, and R820W) in the heart and for the control genotype (attP2) using RNA sequencing. Although we expressed feline MYBPC3 specifically in the cardiovascular system of the fly, we measured genome-wide transcription of the whole organism. Therefore, any differential effects caused by expression of the MYBPC3 alleles may ultimately cause downstream effects of other genes, across different tissues.

We assessed gene expression in third instar larvae and in the 1-week-old males and females before and after exposure to an intense exercise regime (Supplementary File S1). We performed pairwise analyses of differential gene expression for each of the three MYBPC3 transgenic lines compared to the control, separately for larvae and for exercised and nonexercised males and females (Supplementary File S2, Figure S3). We deemed genes to be significantly differentially expressed if the FDR < 0.05 and the Log2 fold change (Log2FC) was >0.5 (upregulated) or <−0.5 (downregulated). Table 1 lists the number of significantly differentially expressed genes among the transgenic lines by developmental
stage, exercise treatment and sex. For each of the contrasts, many genes overlap between two or more MYBPC3 alleles (Figure 3). A PCA was performed on the normalized RNA-seq read counts corresponding to the set of ~16,000 expressed genes (Supplementary Figure S4). The first (60.2%) and second (13.2%) components represent most of the expression pattern. This analysis revealed a clear separation between males and females, by treatment (non-exercised vs exercised). Heat maps illustrating differential expression patterns of genes reveal clusters with differentially regulated patterns of expression between WT and the mutant lines (A31P and R820W) (Figure 4 and Supplementary File S3). To identify enrichment for genes with particular functions among those differentially expressed in response to MYBPC3, we tested the sets of significantly upregulated and downregulated genes identified from each transgenic line (by sex and condition) for GO-BP enrichment using Panther 14.1 (Supplementary File S4).

**Variants of feline MYBPC3 elicit transcriptional responses to unfolded proteins in larvae**

We identified 15,965 expressed genes among the WT, A31P, and R820W MYBPC3 transgenic lines relative to the attP2 control (Table 1) in larvae, with 365 (WT), 146 (A31P), and 139 (R820W) significantly differentially expressed genes. Among the significant genes, 88 were common to all three transgenic lines (Figures 3 and 4). The A31P and R820W larvae show significant enrichment of the following GO-BP categories compared to WT MYBPC3: response to heat (GO:0009408 and GO:0034605), response to unfolded proteins (GO:0006986 and GO:0034620), peptide metabolic process (GO:0006518), and chaperone-mediated protein folding (GO:0061077) (Supplementary File S4). These categories are represented by genes that include Hsp70Ab, Hsp70Bb, Gba1a, CG8773, and CG8774. Human HSC70 is the ortholog of Drosophila Hsp70Ab/Hsp70Bb and has recently been shown to serve as a chaperone for both WT and several mutant forms of MYBPC3 protein using co-immunoprecipitation/mass spectrometry (Glazier et al. 2018). Glucosylceramidase beta (GBA) is the human ortholog of Drosophila Gba1a. Mutations in GBA have been associated with Gaucher disease (GD) type III in patients presenting with cardiovascular calcifications (Beutler et al. 1995; Chabas et al. 1995; George et al. 2001). Drosophila models for GD have been established to study the effects of mutant Gba1 (Suzuki et al. 2015; Davis et al. 2016; Kinghorn et al. 2016; Cabasso et al. 2019). A Drosophila model for GD using two existing GBA1 mutant fly lines recapitulated hallmarks of GD including shortened lifespan, neuroinflammation, and activation of the unfolded protein response to unfolded proteins in larvae.

**Table 1** Number of differentially expressed genes

| SEX      | AGE | Condition | Total number of expressed genes |
|----------|-----|-----------|---------------------------------|
|          |     | Larvae    |       | Females 1-week | Males 1-week | Males 1-week |
|          |     | Not-exercised | 15,965 | Not-exercised | EXERCISED | Not-exercised | EXERCISED |
| **Wild-type (WT)** |       |                           |        |                   |           |               |           |
|          | Total |                           |        |           | 52        | 321          | 272        |
|          | Up-regulated |                           |        |           | 62        | 32           | 147        |
|          | Down-regulated |                           |        |           | 303       | 75           | 125        |
| **A31P** |       |                           |        |                   |           |               |           |
|          | Total |                           |        |           | 146       | 134          | 430        |
|          | Up-regulated |                           |        |           | 20        | 73           | 283        |
|          | Down-regulated |                           |        |           | 126       | 61           | 147        |
| **R820W** |       |                           |        |                   |           |               |           |
|          | Total |                           |        |           | 139       | 113          | 312        |
|          | Up-regulated |                           |        |           | 25        | 79           | 156        |
|          | Down-regulated |                           |        |           | 114       | 34           | 156        |

Pairwise comparisons of differentially expressed genes of wild type (WT, u[1118]/y; w; Chr2/+; UAS-WT-MYBPC3/TinC4-Gal4), A31P (u[1118]/y; w; Chr2/+; UAS-A31P-MYBPC3/TinC4-Gal4) and R820W (u[1118]/y; w; Chr2/+; UAS-R820W-MYBPC3/TinC4-Gal4) MYBPC3 alleles expressed in cardiac tissue compared to the attP2 control (u[1118]/y; w; Chr2/+; attP2/TinC4-Gal4). Differential expression analysis was performed using CLC Genomics Workbench 11 (Qiagen). Significantly differentially expressed genes were selected based on FDR P-value < 0.05 and Log2FC > 0.5 (up-regulated) or Log2FC < −0.5 (down-regulated).
Expression of feline MYBPC3 in female Drosophila reveal distinct patterns of gene regulation between exercised and nonexercised flies, including a cluster of differentially regulated snRNAs

We identified over 15,000 expressed genes when we performed RNA-sequencing on adult females of the transgenic and control genotypes and compared nonexercised flies to those that were exposed to a 1 h exercise regime: 15,290 expressed genes for non-exercised flies and 15,370 genes for exercised flies (Table 1). There were 107 (WT), 134 (A31P), and 113 (R820W) significantly differentially expressed genes for the exercised females, of which 16 were common to all three transgenic lines; and 52 (WT), 49 (A31P), and 126 (R820W) significantly differentially expressed genes for the nonexercised females, of which 16 were common to all three transgenic lines (Table 1, Figures 3 and 4). No gene ontology categories were significantly enriched for the WT or the R820W nonexercised females (Supplementary File S4). For the A31P non-exercised females, the following GO-BP categories were enriched: response to heat (GO:0009408 and GO:0034605), response to unfolded protein (GO:0006986, GO:0034620), chaperone-mediated protein folding (GO:0061077), and immune system process (GO:0002376). The protein-folding processes are represented mostly by genes encoding heat-shock proteins (Hsp22, Hsp70Bb, and Hsp70Bc). Heat-shock proteins are a family of evolutionary conserved proteins that function as molecular chaperones. These proteins recognize and form complexes with incorrectly folded or denatured proteins and assist with their correct folding or degradation (Jagla et al. 2018). It is conceivable that the MYBPC3 A31P variant is misfolded, resulting in the up-regulation of the Hsp proteins possibly eliciting an immune response (Bolhassani and Agi 2019). When females from the transgenic lines are exercised, the same GO-BP categories as the nonexercised females are enriched, for the WT and A31P lines (response to heat, response to unfolded protein and chaperone-
mediated protein folding); however, the only Hsp gene represented by these categories is Hsp70Bb. No GO-BP categories were enriched for the exercised R820W variant genotype.

Several clusters of differentially expressed genes were coordinately up- or down-regulated between the MYBPC3 genotypes under each exercise regime. For nonexercised females, clusters of downregulated genes in WT were upregulated in A31P and R820W. These clusters include GO-BP categories such as carbohydrate metabolic process (Mal-A1 and Mal-A6), transmembrane transport (CG17751, CG6484, CG16727, and Mdr50), oxidation-reduction process (Cyp6d21) and proteolysis (Jon25Bii and aoe). In exercised females, clusters of genes were downregulated for R820W, but upregulated for WT and A31P. These clusters include GO-BP categories such as muscle system process (Fhos), myofibril assembly (Act88F) and a cluster of 23 small nucleolar RNAs (including snoRNA-lola-a, snoRNA-lola-b, and snoRNA-Psi-28S) (Figures 4 and 5). The snoRNAs are a class of small noncoding RNAs that guide post-transcriptional modifications such as the conversion of uridines into pseudouridines. Other functions of snoRNAs include regulation of mRNA editing, alternative splicing, and gene silencing (Bachellerie et al. 2002; Bratko and Rogelj 2014; Deoghoria and Majumder 2018). A genome wide association study (GWAS) involving 5244 participants of the PROspective Study of Pravastatin in the Elderly at Risk (PROSPER) identified SNPs in the snoRNA cluster on chromosome 14q32 that were significantly associated with heart failure, and showed that snoRNA gene expression in this cluster is upregulated during cardiovascular disease (Hakansson et al. 2019). Altered expression of H/ACA snoRNAs has also been implicated in other human diseases including leukemia, prostate cancer and nonsmall cell lung cancer (for a review see, McMahon et al. 2015). It is conceivable that the snoRNAs identified by our study regulate the disease etiology of HCM.

**Exercise induces an enrichment of heat shock protein genes in male MYBPC3 transgenic lines**

We identified over 16,000 expressed genes in adult males for each exercise regime (Table 1). A total of 321 (WT), 376 (A31P), and 239 (R820W) genes were significantly differentially regulated for the nonexercised males and 272 (WT), 430 (A31P), and 312 (R820W) genes were significantly differentially regulated for the exercised males. Among the significantly differentially regulated genes, 95 (nonexercised males) and 71 (exercised males) were in common between the WT, A31P, and R820W transgenic lines (Table 1, Figures 3 and 4). The top GO-BP categories for all three transgenic lines in unexercised males include defense response to bacteria (GO:0019731, GO:0050829, and GO:0050830), immune response (GO:0006959 and GO:0006955), response to heat (GO:0034605, GO:0009408, and GO:0034605), and response to unfolded protein (GO:0006986, GO:0034620, and GO:0061077). A comparison of differentially expressed genes in exercised and nonexercised male flies revealed that genes involved in the response to unfolded protein or to protein refolding (Hsp70Aa, Hsp70Ab, Hsp70Ba, Hsp70Bb, Hsp70Bc, and Hsp70Bbb) were uniquely enriched in the exercised flies (Figure 6).

Several clusters of differentially expressed genes were coordinately regulated between the MYBPC3 genotypes under each exercise regime. A cluster of upregulated genes was induced by R820W expression in nonexercised males that were downregulated in WT and A31P, including genes involved in muscle
contraction (mcl1 and Tm1) and protein refolding (CG14207 and l(2)ef1) (Figure 4D and Supplementary File S4). In addition, another cluster of genes was downregulated in R820W and upregulated in WT and A31P, including genes involved in G-protein coupled receptor signaling (Msr1, Rh4, Rh5, Rh6, AkhR, and mthl3), axon guidance (tok, daw, and Lsp2), and oxidation-reduction process (Hpd, Cyp4ac1, and CG4335). In the exercised males, there was a large, downregulated cluster for WT that is upregulated in
the A31P line, but lowly-expressed in R820W (Figure 4E and Supplementary File S3). GO-BP processes within this cluster include proteolysis (CG31928, CG6508, CG31926, ndl, and stet), post-transcriptional gene silencing (CG9925 and Marf1), DNA replication (Rnl1, Mcm10, Orc2, and PCNA), and chorion-containing eggshell formation (Cp7Fa, Cp7Fb, Cp7Fc, Muc12Ea, and dec-1).

Transcriptional responses to proteolysis and oxidation-reduction processes of exercised flies compared to nonexercised WT flies

We compared the gene expression profiles of nonexercised flies that express the WT-MYBPC3 allele to exercised WT, A31P, and R820W MYBPC3 flies (Supplementary Files S2 and S3, Figures 3 and 4). The comparison of nonexercised WT to exercised WT flies revealed 26 genes in females and 179 genes in males that were exclusively significantly differentially regulated when the flies underwent the exercise regime (Figure 3B). A major portion of these genes have biological processes relating to oxidation-reduction (Cyp4ac2, Cyp6a8, Cyp6a2, and Cyp6a17), mannose metabolic process (LMani-LManVI), proteolysis (Nep9, Nep7, and Jon99Fii, Jon65Ai, Jon99Fii, Ser8), and transmembrane transport (Tret1-1, MF51, and MF51S). The top up-regulated genes include asRNA: CR45271, CG17672, CG43773, and hrcRNA: CR44080 while the top-down-regulated genes include Hsp70bb, Hsp70aa1, Impl1, and hrcRNA: CR44602 and phi. Heat map analysis (Figure 4B and Supplementary File S3) revealed 37 genes (162 genes) that were regulated when the WT flies were exercised. The following biological processes were significantly over-represented (FDR < 0.05) in this case; immune/defense response (FDR < 0.0036) and response to incorrect/unfolded protein (FDR < 0.008). These categories may indicate that exercise activates genes that reduce inflammation (i.e. immune response) and muscle damage (i.e. proteolysis) to assist with recovery post-exercise.

A31P exercised females showed 6 exclusively significantly up-regulated and 12 significantly down-regulated genes compared to the WT nonexercised females. In exercised A31P males, 50 were exclusively up-regulated and 220 were exclusively down-regulated compared to the nonexercised WT males. Cytochrome P450 genes were significantly down-regulated in both females (Cyp6a17 and Cyp6a2) and in males (Cyp6a19 and Cyp6a2). In females, two additional cytochrome P450 genes, Cyp4p2 and Cyp6a8, were up-regulated. Down-regulated genes in the exercised A31P males represented a variety of functions including: cell death (Drep-2, CG31928, and CG6508), defense response (Hsp27, CG9925, CBF, and Pehp1), protein processing (Bace, pcl, stg, CG18190, Cdk1, lok, Cdk2, and stet), proteolysis (CG32834 and CG31681), and oxidation-reduction process (Nox, CG12398, RnsR, Rnl1, Cyp6a19, Cyp6a2, PH4alphaP, and CG4009).

In the exercised female flies expressing R820W-MYBPC3, 17 genes were exclusively up-regulated and 72 were down-regulated compared to the WT-MYBPC3 nonexercised flies. The down-regulated genes are involved in a variety of processes including proteolysis (CG4998, gammaTry, and CG6607), metabolic processes (ldg2, Tps1, CG6295, Gpo-1, Hml, vre1, and CG463), and cell adhesion (Dscam4). Of the upregulated genes, 10 have unknown functions. Three genes (Cp7Fa, Cp96, and Cp18) are involved in multicellular organism development; two genes (CG4595 and CG4009) are involved in oxidation-reduction processes; the gene c(3)C is involved in synaptonemal complex assembly; and CG5966 is involved in lipid catabolic processes.

The exercised male flies expressing R820W-MYBPC3, 66 genes were exclusively up-regulated and 64 were down-regulated compared to the nonexercised WT-MYBPC3. Up-regulated genes included those involved in metabolic processes (tob1, Cht4, Ec3t, Amyryl, Mal-A7, and Mal-B2); locomotion/flight (CG31148 and TmpC4); oxidation-reduction process (mt: ND3, su(f)), Gapdh1, and CG6910), and proteolysis (Pha1). Down-regulated genes included those involved in proteolysis (CG31233, CG17633, CG7025, Jon99Fii, CG11911, CG5246, Jon99Fii, and Ser8), response to endoplasmic reticulum stress (bgm) and transmembrane transport (CG4562, CG42269, CG8654, CG30722, and CG4822).

Heat map analysis (Figure 4B) in females (males) revealed 2 (1) major clusters of genes that were down-regulated in nonexercised WT flies, but up-regulated among the exercised flies (WT, A31P and R820W). Included in these clusters are genes involved in muscle system processes (Jn, Neurochondrin, Mlct1, Mlct2, Mhc, Tm1, kon, and Fhos), defense response (Bom51, Bom52, Bom55, Dso2, and msopa), and muscle contraction (Act57B, Act79B, and Act88F). In males, several up-regulated genes in nonexercised and exercised WT flies were down-regulated only in exercised A31P and R820W flies. These include 32 protein-coding (CG) genes with unknown biological process, genes involved in microtubule-based movement (Sad2c, CG7276, CG8407, and CG10859), and oxidation-reduction processes (Cyp6a16, Gpo3, and Ttx1). Six genes involved in proteolysis (CG11034, CG11912, CG18179, CG18180, Jon25Bii, and Jon74E) were down-regulated in WT (nonexercised and exercised), but up-regulated in exercised A31P and R820W males.

Knockdown of candidate genes leads to arrhythmias

We used RNAi knockdown to functionally test whether reduced expression of candidate genes implicated by the differential expression analyses affect larval heart rates. We selected 8 candidate genes for RNAi mediated suppression of gene expression (Act79B, Act79F, Act88F, Eifalpha100E, Hsp22, Mhc, Mlct2, and TyrR) specifically in the cardiovascular system of the fly using the TnpC4-Gal4 driver line. Knockdown of five genes (Act79B, Act79F, Mhc, Mlct2, and TyrR) significantly decreased larval heart rate compared to the control line, while knockdown of two genes (Eifalpha100E and Hsp22) significantly increased the heart rates (Supplementary Figure S6). The Homophila database (http://homophila.sdsce.edu) reports that the Act genes (Act79B, Act79F, and Act88F) and Mlct2 are close matches to the human ACTC1 gene (NP_005150) and MYL2 (NP_000423) genes, respectively. Mutations in both these genes have been associated with familial HCM (van Wan ing et al. 2019; Manivannan et al. 2020). Next-generation sequencing of an Italian family with HCM revealed an Ala21Val mutation in ACTC1 4-Gal4 (NP_000423) genes, respectively. These patients presented with palpitations and ventricular tachycardia due to structural defects of the myofilaments resulting from the Ala21Val mutation. In a Lebanese family presenting with congenital heart defects and arrhythmias, a Met84Thr mutation in MYBPC3 was identified by Sanger sequencing (Augier et al. 2015). The structural consequences of this mutation were examined using 3D structural analysis and was found to reside in a region of the Actin filament in extremely tight apposition to the myosin head. Disease-causing ACTC1 mutations can disrupt
both electrostatic and hydrophobic contacts, resulting in perturbation of the interaction between Actin, Tropomyosin and Myosin (Augière et al. 2015). An RNAi screen in Drosophila primary cell culture of heart muscle disease genes associated with congenital myopathies and cardiomyopathies lead to abnormal muscle phenotypes in primary culture (Bai et al. 2008). For example, knockdown of Mhc and Mlc2 (both genes are involved in the regulation of Myosin function) resulted in the lack of striation in both Actin and Myosin filaments. Knockdown of Actin isoforms including Act57B in primary cell culture resulted in thinner or shorter myofibril structures, possibly resulting from an arrest in myofibril assembly from the lack of available Actin monomers (Bai et al. 2008). RNAi knockdown of Mhc in Drosophila cardiomyocytes with the heart-specific Hand-Gal4 driver line resulted in softening of the cardiomyocytes, due to reduction in myofibrillar density (Kaushik et al. 2011). In addition, the authors identified severe impairment of the heart tube with significant loss of rhythmic contractions. Results from our RNAi knockdown experiment together with the previous studies mentioned here demonstrate that these genes, when knocked-down or mutated, may result in myofibril structural defects resulting in cardiovascular defects.

**Discussion**

HCM affects 10–15% of the pet cat population (Payne et al. 2015; Freeman et al. 2017). Most affected cats remain free of clinical signs; however, a proportion experience serious complications, including congestive heart failure, arterial thromboembolism, and sudden cardiac death, similar to human HCM (Paige et al. 2009; Payne et al. 2015). Breeds including Maine Coon, Ragdoll, Persian, and Sphynx are predisposed to HCM, suggesting a genetic component to the disease (Kittleson et al. 2015). Two missense mutations in MYBPC3 were identified in the Maine Coon (A31P) and Ragdoll breeds (R820W) (Meurs et al. 2005, 2007). The molecular mechanism(s) by which mutations in MYBPC3 give rise to HCM remains poorly understood, particularly given the broad spectrum of clinical manifestations, from benign or asymptomatic to sudden death. Studies of the genetic factors and biological mechanisms that underlie this disease are as challenging in felines as they are in humans, as they maintain extensive variation due to differences in factors including genetics, disease, medication, and diet. Model organisms can control for these factors. A powerful strategy for studying the molecular effects of vertebrate disease variants is to create transgenic *D. melanogaster* bearing the disease gene/variants and express them in cardiac tissue using the versatile Gal4/UAS system (Brand and Perrimon 2013) and leukemia (Valleron et al. 2012). When deregulated, the snoRNAs identified by this study may be involved in the pathogenesis of HCM particularly under conditions of cardiac stress. The study of snoRNAs and their effect on human disease pathogenesis is an emerging field that could lead to the development of therapeutics that would modulate the expression of H/ACA snoRNA.

Here, we developed a transgenic fly model for feline HCM by expressing feline WT MYBPC3 and two MYBPC3 variants associated with disease (A31P and R820W) in the Drosophila cardiovascular system, and assessed the cardiovascular health and transcriptional consequences specific to each MYBPC3 variant. Transcriptionally co-regulated genes in the presence of MYBPC3 mutations relative to the MYBPC3 WT allele are potential modifiers affecting the variable effects of MYBPC3 mutations in different genetic backgrounds, and transcriptionally co-regulated genes that differ between exercised and nonexercised flies with the MYBPC3 mutations are potential candidate genes affecting genotype by environment interactions affecting manifestation of disease.

One potential caveat for this approach is that there is no experimental evidence for Drosophila orthologs of mammalian MYBPC3. The DRSC Integrative Ortholog Prediction Tool (DIOPT) (https://www.flyrnai.org/cgi-bin/DRSC_orthologs.pl) identifies four Drosophila proteins (Unc-89, Sla, Hbs, and Sns) as potential orthologs, however, the DIOPT score for orthology is low (1/15) for each of these. Our control for possible effects of global misregulation of an exogenously expressed gene is to compare the effects of the missense variants with the WT feline gene and fly control genotype.

We measured exercise endurance and larval heart rates as a proxy to assess the cardiovascular health of our MYBPC3 transgenic lines. Previously, RNAi knockdown of *Atg* genes in Drosophila muscle and heart led to impaired locomotor function and displayed an accelerated age-dependent loss of cardiac function (Xu et al. 2019). The flight ability and cardiac structure and function were also affected in a Drosophila model for HCM by expressing a mutation in human β-cardiac myosin heavy chain, known to cause HCM (Kronert et al. 2018). Several studies have shown that exercise training prevents age-related heart dysfunc- tion in Drosophila (Piazza et al. 2009; Sujkowski et al. 2015; Zheng et al. 2017; Sujkowski and Wessells 2018; Blice-Baum et al. 2019). These results indicate that measurements of locomotor function are valuable for examining heart health in fly models for HCM. We therefore assessed whether the MYBPC3 variants were able to mimic symptoms presented by patients diagnosed with HCM by assessing the cardiovascular health of the transgenic lines by quantifying heart rate and exercise endurance for each genotype.

We observed a significantly increased heart rate of both the A31P and R820W mutants compared to the WT (Figure 1). However, there was no significant difference in heart rate between the WT MYBPC3 allele and the control genotype, showing that expression of the WT feline MYBPC3 gene does not impair heart rate. Exercise assays for cardiovascular endurance showed that the A31P and R820W mutants had significantly reduced endurance post-exercise in both sexes (Figure 2).

Gene expression analyses using RNA-sequencing revealed significantly regulated genes that responded to the expression of feline MYBPC3 variants. These included a cluster of 23 snRNAs that were downregulated by R820W but upregulated by WT only in the exercised females. Fifteen of these snoRNAs are encoded by introns and belong to the box H/ACA class that guide pseudouridylation (conversion of uridine to pseudouridine) of the target 28S RNA (Yoshihama et al. 2013). Down-regulation of box H/ACA snoRNAs (such as SNORA15 and SNORA24) has been associated with human diseases including dyskeratosis congenita (Bellodi et al. 2013) and leukemia (Valleron et al. 2012). When deregulated, the snoRNAs identified by this study may be involved in the pathogenesis of HCM particularly under conditions of cardiac stress. The study of snoRNAs and their effect on human disease pathogenesis is an emerging field that could lead to the development of therapeutics that would modulate the expression of H/ACA snoRNA.

Genes encoding Heat shock proteins (Hsp and Hsc) were also de-regulated in this study. The Hsp70 gene family are molecular chaperones that promote the refolding of misfolded proteins or suppress protein aggregation (Kettern et al. 2011; Rosenzweig et al. 2019), thus protecting the cell from deleterious proteotoxic effects. Here, we observed down-regulation of five Hsp genes in the R820W larvae (Hsc70, Hsc70-2, Hsp60C, Hsp60D, and Hsp60B) with concomitant up-regulation in the WT larvae. In adult flies, the genes Hsp70Bbb and Hsp70Aa were significantly downregulated after WT flies experienced the exercise regime. Exercised A31P females and males show an overall up-regulation of several Hsp genes that are down-regulated in exercised R820W and WT transgenics. In a previous study (Glazier et al. 2018), co-
immunoprecipitation analyses in a cardiomyocyte MYBPC3 model for HCM showed that HSC70 is a chaperone for WT and mutant MYBPC3. HSC70 likely plays a role in maintaining cardiomyocyte proteostasis by assisting with the folding and turnover of MYBPC3 (Glazier et al. 2018); however, further studies on its precise role in HCM are needed. A Drosophila model for AF showed that overexpression of Drosophila Hsp23 was protective against contractile dysfunction and cardiac remodeling (Zhang et al. 2011). HSPB1 may protect from cardiac remodeling thus reducing the progression of human AF (Brundel et al. 2006; Yang et al. 2007). Drosophila, cardiomyocyte, and canine models of AF show that HSP-inducing compounds such as geranylgeranylacetone and their derivatives can prevent proteostasis and cardiac remodeling, thus attenuating AF progression (Brundel et al. 2006; Zhang et al. 2011; Hoogstra-Berends et al. 2012). Pharmacological induction of HSPs could potentially be considered for future studies in the treatment or prevention of HCM.

The cytochrome P450 (CYP) superfamily of enzymes are involved in the metabolism of fatty acids, xenobiotics, and therapeutic drugs. Several studies have shown that expression of CYP enzymes and therefore the production of endogenous metabolites are altered in patients with cardiovascular disease (Elbækai and El-Kadi 2006). The production of these endogenous CYP metabolites [epoxyeicosatrienoic acids (EETs), hydroxyeicosatetraenoic acids (HETEs), prostacyclin, and aldosterone] are involved in the regulation of heart contractility (Aspromonte et al. 2014; Rowland and Mangoni 2014). In a transgenic mouse model that overexpressed CYP2J2 in cardiomyocytes, cardioprotective EETs were generated that protected against arrhythmia susceptibility in cardiac hypertrophy (Westphal et al. 2013). On the other hand, elevated 20-HETE levels due to overexpression of CYP4A2 can lead to hypertension in rats (Wang et al. 2006). The results from our RNA-sequencing experiment when comparing the nonexercised WT-MYBPC3 flies to the exercised WT, A31P and R820W lines, revealed the dysregulation of CYP genes in both male and female flies, further implicating a role for these enzymes in maintaining cardiovascular health.

A previous study (Vu Manh et al. 2005) used the Gal4/UAS system in Drosophila as a model to study effects of MYBPC3 mutations associated with human familial HCM. Transgenic flies carrying human WT or two C-terminal truncated cMyBP-Cs were expressed in IFM to study transcriptional responses and flight phenotypes. Although the study only tested 3570 genes using expressed in IFM to study transcriptional responses and flight phenotypes, our RNA-sequencing experiment when comparing the nonexercised WT-MYBPC3 flies to the exercised WT, A31P and R820W lines, revealed the dysregulation of CYP genes in both male and female flies, further implicating a role for these enzymes in maintaining cardiovascular health. The processes associated with these overlapping genes are found among several of the genes that were dysregulated in our study, indicating that common biological processes are modified by different mutations in MYBPC3 known to cause HCM.

PPI networks derived using the differentially expressed genes revealed several hub proteins (degree > 10) (Supplementary Figure S5). Hub genes encoding these proteins include actins, which are involved in muscle contraction (Act57B, Act79B, Act87E, and Act88F); and the Hsps involved in protein folding (Hsp70Aa, Hsp70Bbb, and Hsp70Bc). The results of our RNAi–knockdown experiments revealed that the hub genes Act57B and Act79B displayed significantly reduced heart rates compared to the control line when knocked down, further implicating these genes in the disease etiology of HCM. Males harboring the mutant alleles showed more highly connected PPI networks than females and include a range of differentially expressed genes encoding hub proteins in a wide range of biological processes, including DNA replication, protein phosphorylation and histone acetylation/phosphorylation.

Several exercise studies in Drosophila have been published that assessed physical fitness using instruments such as the Power Tower (Piazza et al. 2009), TreadWheel (Mendez et al. 2016), Swing Boat (Berlandi et al. 2017), and the Rotating Exercise Quantification System (REQS; Watanabe and Riddle 2017). Exercise studies using the TreadWheel (Mendez et al. 2016) showed significant increases in fly protein content, which could be attributed to changes in protein metabolism (such as catabolism or synthesis). A recent GWAS study of the Drosophila melanogaster Genetic Reference Panel (DGRP) (Mackay et al. 2012) using the REQs method identified 81 variants in 47 genes associated with exercised-induced activity (Watanabe et al. 2020). These genes were significantly enriched for gene ontology categories relating to neuronal function (Prospa, Spec2, nuq, Naun, and Wnt) and protein processes (CG30463, Sh, CG33144, Ptp61F, sda, and CG6512). In our study, transcriptional responses to exercise induced up-regulation of genes involved in immune-response and proteolysis, an indication that exercise may be causing inflammation and muscle damage thereby eliciting a recovery response. The TreadWheel and REQs study together with this study reveal complex responses to exercise that are affected by sex and genotype that require further exploration.

In summary, we used RNAseq and bioinformatics tools to determine which processes are mis-regulated in the presence of mutant MYBPC3 alleles associated with feline HCM. Transcriptome analysis revealed significant downregulation of snoRNA genes in exercised female flies harboring the mutant alleles compared to flies that harbor the WT allele. Other processes that were affected included the unfolded protein response and immune/defense responses. These data suggest that mutant MYBPC3 alters transcriptional responses of genes that could be used as targets for therapeutic interventions. Transcriptionally differentially expressed genes are also candidate genes for future evaluation as genetic modifiers of HCM as well as candidate genes for genotype by exercise environment interaction effects on the manifestation of HCM, in cats as well as humans.

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