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Circadian rhythm disruption linked to skeletal muscle dysfunction in the Mexican Cavefish

Luke Olsen[1,2], Jaya Krishnan[1], Charles Banks[1], Huzaifa Hassan[1], and Nicolas Rohner[1,2,*]

It has become clear that circadian clocks in peripheral tissues play important functions. Disruption of the circadian clock in skeletal muscle, for example, results in insulin resistance, sarcomere disorganization, and muscle weakness[1]. Interestingly, cavefish, which exhibit a disrupted central clock, exhibit similar muscle phenotypes[2–4], raising the question of whether they are caused by alterations to central or peripheral clocks. Here, we demonstrate a loss in clock function in the skeletal muscle of the Mexican Cavefish *Astyanax mexicanus* that is associated with reduced rhythmicity of a large number of genes and disrupted nocturnal protein catabolism. Some of the identified genes are associated with metabolic dysfunction in humans.

We dissected skeletal muscle from adult surface fish and cavefish every 4 hours spanning 24 hours and performed bulk RNA-sequencing (Figure 1A). Using maSigPro and JTK_Cycle programs we identified 440 and 370 circadian-regulated genes within the skeletal muscle of surface fish and cavefish, respectively. Confirming the accuracy of our pipeline, gene-ontology (GO) enrichment analysis revealed 'circadian rhythm' and 'rhythmic process' as the most enriched pathways within both surface fish and cavefish datasets (Figure S1A,B in Supplemental information, published with this article online). We identified multiple canonical clock genes within both populations, such as *ciarta* and *per1a*. However, of these shared clock genes, almost all had changes in either amplitude or time of peak expression within cavefish relative to surface fish (Figure 1B). Notably, the core clock gene *bmal1*, a crucial regulator of muscle function[5], lacked rhythmicity within cavefish skeletal muscle, findings confirmed via qPCR (Figures 1B and S1C).

We next sought to determine the downstream affected gene families, termed 'clock-controlled genes'. We performed k-means clustering of the 440 circadian-regulated genes within surface fish muscle, here considered canonical clock-controlled genes. We identified 4 clusters peaking during early (Circadian Time (CT) 0–4; clusters 1 and 4), mid (CT 8; cluster 3), or late (CT 16–20; cluster 2) hours (Figure S1D). Of the 4 clusters, ~60% of the genes fell into cluster 2, with GO-enrichment analysis revealing pathways enriched for protein turnover, most notably the 'proteasome complex' (Figure S1E). Strikingly, ~93% of the genes within cluster 2 lacked rhythmicity within cavefish muscle, findings confirmed in a separate RNA-sequencing experiment (Figures 1C and Figure S2). Importantly, contrasting the cluster 2 genes against a recent whole-body circadian transcriptome of *A. mexicanus*[4] confirmed that the cluster 2 gene-set oscillate in a muscle-specific manner (only 4 shared genes between datasets) and, subsequently, that their loss of rhythmicity within cavefish is a muscle-specific phenomenon.

The most striking example of cavefish-specific disruption in clock-controlled genes were within the ubiquitin-proteasomal system. For example, multiple such genes peaked at night exclusively within surface fish muscle, suggesting enhanced nocturnal protein catabolism (Figure 1D). In fact, proteomic analysis of muscle collected at CT0 and CT16 revealed that surface fish had 3-fold more downregulated proteins at CT16 relative to CT0 as compared to cavefish (Supplemental information). To determine heritability of the proteasomal phenotype, we performed bulk RNA-sequencing of skeletal muscle from cavefish–surface hybrids collected at CT0, CT8, and CT16. We observed an intermediate nocturnal increase in proteasomal gene expression in the F1 fish (Figure 1E), suggesting that the proteasomal gene rhythmicity is an incomplete dominant, potentially multigenic trait.

Seeking to determine transcriptional regulators of the cluster 2 gene set, we conducted Ingenuity Pathway Analysis and found that *nfe2l2* (*Nrf2*) and *nfe2l1* (*Nrf1*) are predicted upstream regulators. Intriguingly, both *nfe2l2* and *nfe2l1* were under circadian control exclusively within surface fish muscle (albeit only within our JTK_Cycle dataset). Our analysis further revealed Bmal, a transcriptional regulator of nfe2l2 and nfe2l1[6], as the most significantly upregulated 'causal network' of the cluster 2 genes, suggesting rhythmicity of Bmal transcriptionally regulates *nfe2l2* and *nfe2l1* expression and, subsequently, proteolytic gene expression — all of which are absent within cavefish muscle (Figure S1F,G). Notably, querying the *bmal* sequence from wild-caught fish revealed a cave-specific, non-synonymous mutation within its basic helix-loop-helix domain (human homolog: D93G), a mutation residing two residues away from a Bmal1 disrupting mutation[7], thus a potential candidate underlying disrupted cavefish *bmal1* activity and cluster 2 gene-set rhythmicity.

Circadian control of protein turnover is crucial for muscle quality. Metabolomic analysis from our group underscores this relationship, finding reduced cavefish muscle free amino acid levels coupled with decreased muscle mass and contractility[2,8]. For example, the anabolic amino acid leucine is decreased ~2-fold within cavefish skeletal muscle, with its transporter (*slc7a8*) lacking rhythmicity exclusively within cavefish (Figure S1H). Interestingly, *slc7a8* similarly loses rhythmicity within muscle of aged mice and following exposure of human muscle cells to palmitate, conditions resulting in muscle dysfunction.

In agreement, contrasting genome-wide association studies (GWAS) against the disrupted cluster 2 gene-set revealed that ~23% of the 261 genes are associated with type 2 diabetes, obesity, and triglyceride levels, traits contributing to muscle atrophy and weakness. In fact, the most arrhythmic proteasomal gene within cavefish muscle, *psma3*, is associated with five GWAS single nucleotide polymorphisms for increased hip-to-waist ratio and type 2 diabetes (Figure 1F). These findings highlight the evolutionary link between nocturnal protein turnover and muscle homeostasis and reveal many known and novel candidate genes associated with circadian disruption and muscle function.

SUPPLEMENTAL INFORMATION

Supplemental information contains two figures and experimental procedures, and can be

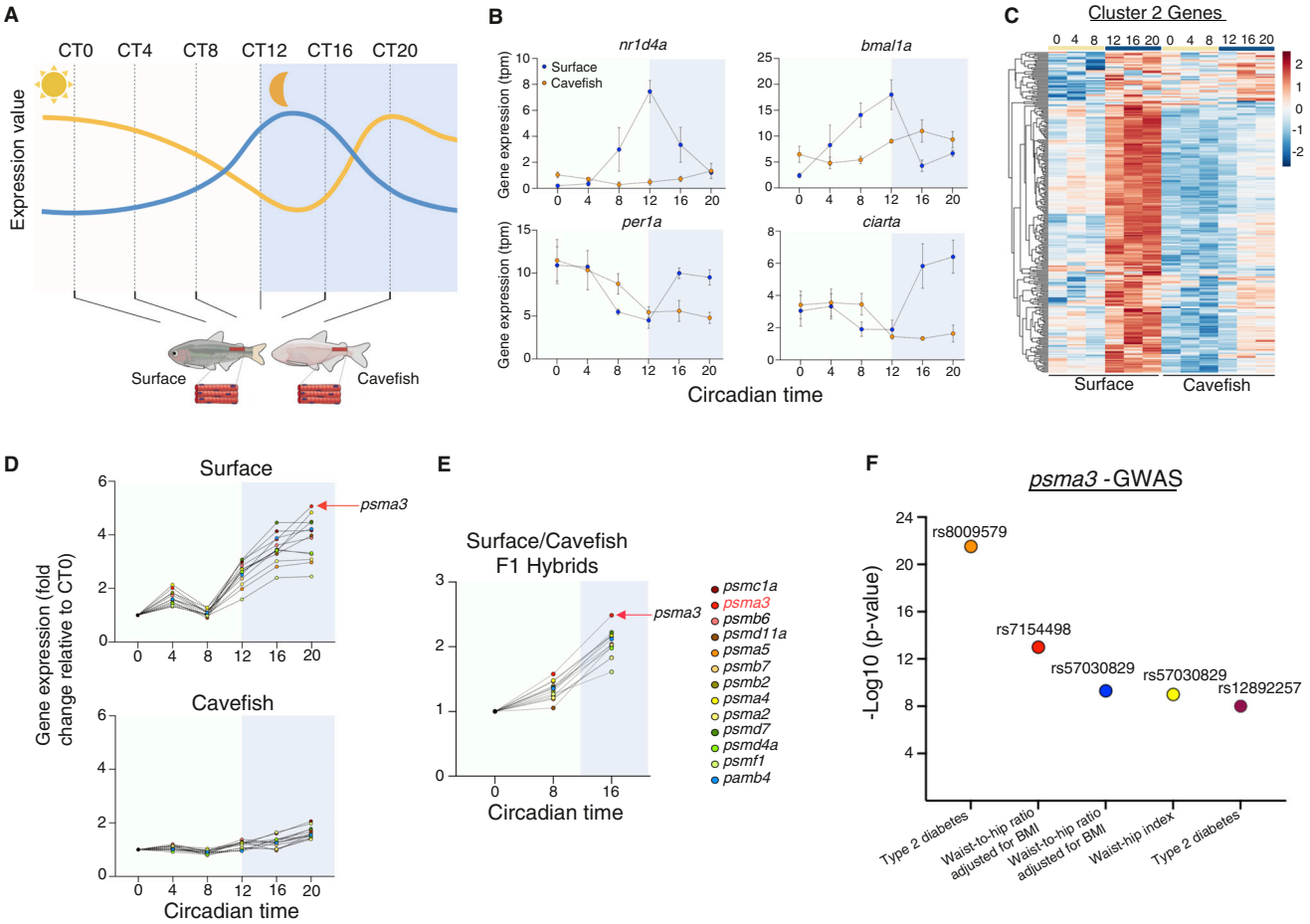

Figure 1. *Astyanax mexicanus* muscle circadian rhythm.
(A) Schematic of tissue collection from cavefish and surface fish. Timepoints are presented as circadian time (CT) with CT0 being 0600 and CT20 being 0200. N = 3 for all analyses. Orange and blue lines represent the rhythmic (blue) and disrupted (orange) rhythmicity of a given gene. (B) Gene expression of canonical clock genes between the surface fish (blue circles) and cavefish (orange circles). Data are presented as ± SEM. (C) Heatmap of all genes identified within surface fish cluster 2 gene-set and the respective cavefish genes. (D) Circadian expression of proteasomal genes identified within surface fish cluster 2 gene-set for (D) surface fish and cavefish and (E) cavefish/surface F1 hybrids. (F) Single nucleotide polymorphisms of the *psma3* gene (including the psma3 antisense RNA 1) associated with increased prevalence of obesity-related traits and Type 2 diabetes in humans (additional sourcing from https://www.ebi.ac.uk/gwas/genes/PSMA3). The variant/risk alleles are placed above each data point. All statistical analysis can be found in Supplemental information.

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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[1]Stowers Institute for Medical Research, Kansas City, MO 64110, USA. [2]Department of Cell Biology & Physiology, University of Kansas Medical Center, Kansas City, KS 66103, USA.
*E-mail: nro@stowers.org