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Achilles is a circadian clock-controlled gene that regulates immune function in *Drosophila*

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ABSTRACT

The circadian clock is a transcriptional/translational feedback loop that drives the rhythmic expression of downstream mRNAs. Termed "clock-controlled genes," these molecular outputs of the circadian clock orchestrate cellular, metabolic, and behavioral rhythms. As part of our on-going work to characterize key upstream regulators of circadian mRNA expression, we have identified a novel clock-controlled gene in *Drosophila melanogaster*, *Achilles* (*Achl*), which is rhythmic at the mRNA level in the brain and which represses expression of antimicrobial peptides in the immune system. *Achilles* knock-down in neurons dramatically elevates expression of crucial immune response genes, including *IM1* (*Immune induced molecule 1*), *Mtk* (*Metchnikowin*), and *Drs* (*Drosomysin*). As a result, flies with knocked-down *Achilles* expression are resistant to bacterial challenges. Meanwhile, no significant change in core clock gene expression and locomotor activity is observed, suggesting that *Achilles* influences rhythmic mRNA outputs rather than directly regulating the core timekeeping mechanism. Notably, *Achilles* knock-down in the absence of immune challenge significantly diminishes the fly's overall lifespan, indicating a behavioral or metabolic cost of constitutively activating this pathway. Together, our data demonstrate that (1) *Achilles* is a novel clock-controlled gene that (2) regulates the immune system, and (3) participates in signaling from neurons to immunological tissues.

1. Introduction

Circadian rhythms are internal timekeeping mechanisms that orchestrate daily oscillations of behavior, metabolism and physiology. In most living organisms, circadian rhythms play a profound role in the regulation of physiological behaviors, such as locomotor activity, sleep-wake cycle, body temperature, blood pressure, cardiovascular activity, muscle strength, feeding, glucose and lipid homeostasis, and alertness (Hastings et al., 2003). In addition, circadian rhythms regulate both adaptive and innate immunity and thereby influence resistance to infection (Scheiermann et al., 2013). Circadian rhythms are important for maintaining homeostasis by anticipating and adapting to predictable environmental changes. Consequently, disruption of circadian rhythms influences multiple pathologies, such as neurodegenerative diseases, cardiovascular diseases, obesity, diabetes, cancer, and depression (Halberg et al., 2006; Hastings et al., 2003; Klerman, 2005; Knutsson, 2003; Levi and Schibler, 2007; Wulff et al., 2010).

At the molecular level, circadian rhythms are regulated by core clock genes that underlie self-sustained 24-h feedback loops. In flies, two transcription factors, CLOCK (CLK) and CYCLE (CYC) compose the positive branch of the feedback loop while PERIOD (PER) and TIMELESS (TIM) compose the negative branch. CLK and CYC form heterodimers and bind to E-box elements located in the promoter regions of *per* and *tim*, promoting their expression. Once translated, PER and TIM dimerize and translocate into the nucleus, where they prevent CLK and CYC heterodimers from accessing

Abbreviations: Achl, *Achilles*; CCG, clock-controlled gene; RNA-seq, RNA-sequencing; AMP, antimicrobial peptide; CLK, CLOCK; CYC, CYCLE; PER, PERIOD; TIM, TIMELESS; TTFL, transcriptional-translational feedback loop; SCN, suprachiasmatic nuclei; qPCR, quantitative PCR; LD, 12 h light: 12 h dark; DD, constant darkness; ZT, zeitgeber time; CT, circadian time; DAM, *Drosophila* Activity Monitoring; RUM, RNA-seq Unified Mapper; RPKM, reads per kilobase per million mapped reads; CFU, colony forming unit; *IM1*, *Immune induced molecule 1*; *Mtk*, *Metchnikowin*; *Drs*, *Drosomysin*; *DptB*, *Diptericin B*; *Imd*, *immune deficiency*.

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E-box elements, thus decreasing the mRNA expression of *per* and *tim*. The degradation of PER and TIM resets the clock and thereby starts a new round of CLK and CYC activation. Well-studied protein modifications impose appropriate delay mechanisms, thus generating a transcriptional-translational feedback loop (TTFL) that occurs about every 24 h (Allada and Chung, 2010; Hardin, 2011; Ko and Takahashi, 2006).

In addition to promoting *per* and *tim* expression, CLK and CYC further drive the rhythmic expression of hundreds to thousands of downstream genes. Termed "clock-controlled genes (CCGs)," these rhythmic mRNAs are not involved in the core time-keeping mechanism but instead regulate physiological processes (Hastings et al., 2003). While the core clock genes are conserved in different tissues, CCGs are highly tissue-specific. Rhythmic transcriptome profiling in 12 different mouse organs shows little overlap of CCGs between different tissues, as expected, given how diverse these different tissues are in their physiological demands (Zhang et al., 2014). The observation that CCGs are largely tissue-specific is seen in flies as well as mammals, suggesting that it is a well-conserved aspect of circadian output pathways (Ceriani et al., 2002; Panda et al., 2002; Storch et al., 2002; Xu et al., 2011). Consequently, the disruption of core clock genes causes systematic rhythmic disorders, while the disruption of CCGs is more likely to be linked to local disorders (Hughes et al., 2012; Jeyaraj et al., 2012). Since the outputs of the circadian clock are ultimately responsible for the clock's influence on health and physiology, it is thus necessary to identify tissue-specific CCGs and to understand their regulatory mechanisms. For example, the study of cardiac specific CCGs revealed a role of rhythmic iron channels in arrhythmia development and susceptibility (Jeyaraj et al., 2012; Schroder et al., 2013). To this end, high-throughput microarray and RNA-sequencing (RNA-seq) have greatly accelerated our understanding of CCGs in diverse tissues as well as multiple cell types (Du et al., 2014; Filichkin and Mockler, 2012; Hughes et al., 2012; Keegan et al., 2007; McDonald and Rosbash, 2001; Menet et al., 2012). Furthermore, the use of these high-throughput approaches in genetically modified animals enables the understanding of their regulatory mechanisms as well as the contributions of principal oscillator and peripheral oscillators to the regulation of specific CCGs (Bugge et al., 2012; Koike et al., 2012; Meireles-Filho et al., 2014; Menet et al., 2014; Rey et al., 2011; Xu et al., 2011). Ongoing studies in our lab and others are aimed at identifying CCGs and understanding how they mediate clock output of physiological processes related to disease and therapeutics.

In animals, circadian rhythms are regulated in hierarchy. In both mammals and insects, there are neuron-based primary oscillators located in the brain. The primary oscillator in mammals resides in the suprachiasmatic nuclei (SCN), and in flies it is distributed among several diffuse clusters of neurons (Herzog, 2007; Nitabach and Taghert, 2008). In addition to the primary oscillator, there are multiple peripheral tissues that behave rhythmically. The principal oscillator integrates environmental signals and sends synchronizing cues to peripheral tissues through mechanisms that are the subject of active investigation (Hastings et al., 2003).

Circadian control of immunological defenses is one of the most dramatic examples of a pathway through which the circadian clock influences organismal health and fitness. In mammals, both principal arms of the immune system – innate and adaptive – are regulated by circadian rhythms. This is seen at both a molecular and cellular level (Curtis et al., 2015; Silver et al., 2012a,b). High-throughput analyses have revealed rhythmicity in many genes involved in the immune response (Keller et al., 2009). In addition, cytokines and chemokines, such as IL6 (interleukin 6), TNFα (tumor necrosis factors alpha) and CXCL 12 (Chemokine (C-X-C Motif) Ligand 12) are released into the circulation in a rhythmic manner. White blood cells, including T lymphocytes, natural killer cells, macrophages, monocytes and the precursor haematopoietic stem cells are released into the circulation in a rhythmic manner and respond to stimuli rhythmically (Ella et al., 2016; Gibbs et al., 2012; Labrecque and Cermakian, 2015; Lange et al., 2010; Mendez-Ferrer et al., 2008; Scheiermann et al., 2013). Together, these molecular and cellular rhythms influence organismal immunobiology in profound ways. Mice show differential resistance against infection at different times of the day. Inflammation, immune resistance, and the severity of autoimmune diseases are also found to vary throughout the day in a rhythmic manner (Carter et al., 2016; Curtis et al., 2014; Cutolo, 2012; Gibbs and Ray, 2013). The chronic disruption of circadian rhythms, including sleep deprivation, shift work, and jet lag can precipitate disease even in healthy individuals and exacerbate existing diseases, particularly inflammatory conditions (Ranjbaran et al., 2007).

Drosophila has been widely used as a model organism to study the mechanisms of immune response due to its relative simplicity and its genetic tractability. Some exotic defense mechanisms notwithstanding (Watson et al., 2005), the humoral immune system in *Drosophila* is highly conserved at a molecular level with its mammalian counterpart (Muller et al., 2008). The initial discovery of an immunological role for *Toll* in *Drosophila* revolutionized the study of mammalian pattern recognition receptors (Anderson, 2000; Hoffmann, 2003; Kimbrell and Beutler, 2001). The humoral immune system of *Drosophila* is divided into two major pathways, *Toll* pathway and *Imd* (*immune deficiency*) pathway. These two pathways combat different types of bacterial and fungal infections by distinguishing the pathogen-associated molecular patterns through pattern recognition proteins, activating the downstream AMPs (antimicrobial peptides) within the immune system, particularly within the fat body. AMPs are then secreted into the hemolymph to clear the infected pathogen (Hoffmann, 2003; Imler and Hoffmann, 2000). There are seven AMP families characterized in *Drosophila*: Drosomycin, Metchnikowin, Cecropins, Defensin, Attacins, Diptericin and Drosocin (Hetru et al., 2003). Similar to mammals, the immune response in *Drosophila* is also found to be rhythmic. Genes involved in immune response are rhythmically expressed, and flies infected with pathogenic bacteria at different times of the day show a rhythmic resistance peaking during the late night (Lee and Edery, 2008; McDonald and Rosbash, 2001; Stone et al., 2012). However, it is unclear how this is regulated at both a molecular and cellular level.

Here we show that *CG17386*, a previously uncharacterized clock-controlled gene is highly rhythmic in the fly head. Using whole-transcriptome profiling, we find that *CG17386* represses the expression of immune responsive genes. Neuron-specific *CG17386* knock-down results in dramatically elevated levels of crucial immune response genes, including AMPs. As a result, flies with knocked-down *CG17386* expression are more resistant to immune challenge with bacteria. Notably, *CG17386* knock-down in the absence of immune challenge significantly diminishes the fly's overall lifespan, indicating an energetic or metabolic cost of constitutively activating this pathway. Hereafter we refer to *CG17386* as *Achilles* (*Achl*), in recognition that its mutant phenotype protects flies against injury and infection, while simultaneously shortening their lifespan.

2. Materials and methods

2.1. Fly stocks and behavioral monitoring

Flies were maintained on standard food (Genesee Scientific, San Diego, California) at 25 °C in 12 h light: 12 h dark (LD) conditions. Humidity was maintained at roughly 50%. All fly stocks used were acquired from the Bloomington stock center: Elav-Gal4 strain: