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Misregulation of *Drosophila* Myc Disrupts Circadian Behavior and Metabolism

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SUMMARY

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

The authors declare no competing interests.

SUPPLEMENTAL INFORMATION

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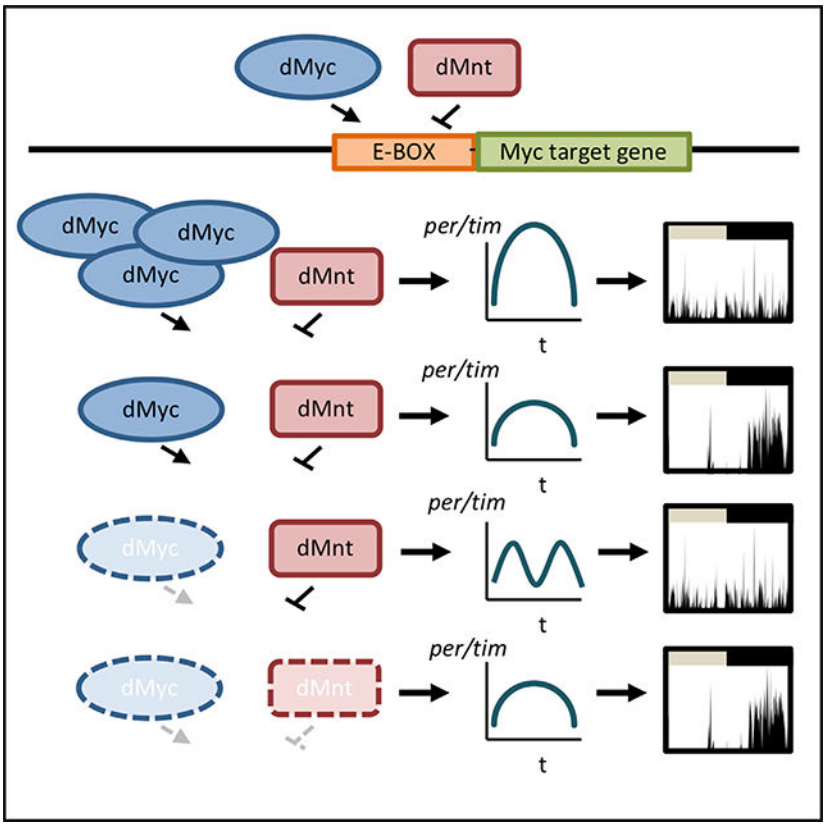
Drosophila Myc (dMyc) is highly conserved and functions as a transcription factor similar to mammalian Myc. We previously found that oncogenic Myc disrupts the molecular clock in cancer cells. Here, we demonstrate that misregulation of dMyc expression affects *Drosophila* circadian behavior. *dMyc* overexpression results in a high percentage of arrhythmic flies, concomitant with increases in the expression of clock genes *cyc*, *tim*, *cry*, and *cwo*. Conversely, flies with hypomorphic mutations in *dMyc* exhibit considerable arrhythmia, which can be rescued by loss of *dMnt*, a suppressor of dMyc activity. Metabolic profiling of fly heads revealed that loss of *dMyc* and its overexpression alter steady-state metabolite levels and have opposing effects on histidine, the histamine precursor, which is rescued in *dMyc* mutants by ablation of *dMnt* and could contribute to effects of dMyc on locomotor behavior. Our results demonstrate a role of dMyc in modulating *Drosophila* circadian clock, behavior, and metabolism.

In Brief

The human MYC oncogene is involved in many cancers and disrupts the clock in cancer cells. Hsieh et al. show that dMyc, the fruit fly homolog of human MYC, plays a role in fly daily sleep-wake cycles, such that increased or decreased dMyc activity disrupts circadian behavior and metabolism.

Graphical Abstract



INTRODUCTION

Circadian rhythms are 24-h cycles of sleep-wake, fasting-feeding, body temperature, and metabolism (Asher and Schibler, 2011; Bass and Takahashi, 2010; Sahar and Sassone-Corsi, 2012). Genetic regulation of circadian rhythms by the clock, which is found broadly from cyanobacteria to humans, was first discovered in *Drosophila* and can be entrained by external cues such as light and food (Dubowy and Sehgal, 2017). The molecular clock consists of transcription-translation feedback loops, in which clock genes autoregulate their own expression. In the major loop, the *Drosophila period* (*per*) and *timeless* (*tim*) genes encode proteins that rhythmically inhibit activity of their transcriptional activators, CLOCK (CLK) and CYCLE (CYC), to maintain cycles of gene expression (Dubowy and Sehgal, 2017). Light entrains this loop via degradation of TIM by Cryptochrome (CRY), a light-sensitive photoreceptor (Koh et al., 2006; Peschel et al., 2006). In a second feedback loop, CLK drives expression of its own activator and repressor, PDP1 and VRILLE (VRI), respectively. Transcriptional control also includes the Clockwork orange (CWO) protein, which contributes to repression by PER and TIM (Zhou et al., 2016).

The molecular clock in *Drosophila* is expressed in the large and small ventrolateral neurons (lLNvs and sLNvs, respectively), which serve as the central clock regulators similar to the suprachiasmatic nucleus (SCN) in mammals (Rieger et al., 2006). Of these, the sLNvs are crucial in maintaining circadian rhythms in constant darkness (Grima et al., 2004; Stoleru et al., 2005). Both lLNvs and sLNvs express neuropeptide pigment-dispersing factor (PDF), which synchronizes clock neurons in the fly brain. Loss of PDF desynchronizes clock neurons and alters circadian locomotor behavior (Hyun et al., 2005; Renn et al., 1999; Yoshii et al., 2009; Zhang et al., 2010).

CLK and CYC are basic helix-loop-helix (HLH) proteins that activate transcription of target genes by binding to specific DNA sequences, E boxes. Other HLH proteins include the MYC onco-protein, which transcriptionally orchestrates cell growth, cell cycle, and metabolism (Hsieh et al., 2015; Stine et al., 2015), and has a conserved role in *Drosophila* (Demontis and Perrimon, 2009; Gallant, 2013; Grewal et al., 2005). dMyc overexpression in *Drosophila* S2 cells stimulates glycolysis and suppresses oxidative phosphorylation (de la Cova et al., 2014). Ectopic expression of *Drosophila* Myc (dMyc) in flies results in larger body size (de la Cova et al., 2004) associated with increased cell size (Johnston et al., 1999) or apoptosis (de la Cova et al., 2004; Montero et al., 2008; Moreno and Basler, 2004). Conversely, loss of dMyc function underlies the *diminutive* phenotype with smaller cell and organismal body size (Pierce et al., 2004).

MYC overexpression in mammalian cancer cells can suppress oscillation of *BMAL1* (homolog of *Drosophila* CYC) by inducing the BMAL1 repressor, *REV-ERBα* (Altman et al., 2015), and inhibiting MIZ-1 (Altman et al., 2017; Shostak et al., 2016). However, the role of dMyc in *Drosophila* circadian behavior, molecular clock, and metabolism is unknown. Here, we demonstrate that misregulation of dMyc expression perturbs circadian locomotor behavior, expression of clock genes, and metabolism in flies. Additional *dMnt* loss is able to reverse alterations of behavior, gene expression, and specific metabolite levels in hypomorphic *dMyc* flies. Our results demonstrate a role of dMyc in modulating

Drosophila circadian locomotor behavior potentially through directly regulating components of the core molecular clock, metabolism, as well as an effect on the clock output PDF.

RESULTS

Overexpression of dMyc Disrupts Circadian Locomotor Activity

To study the effect of dMyc overexpression, we ectopically expressed dMyc with three different drivers (*tim*-Gal4, *Pdf*-Gal4, cry^{24}*Pdf*-Gal4) using the GAL4/UAS (upstream activating sequence) system. To counter possible apoptotic effects of dMyc, *p35*, the *Drosophila* homolog of *BCL2*, was co-expressed (Greer et al., 2013). We examined transcript levels of clock genes in whole-head extracts of *tim*-Gal4 > UAS-*Myc*+*p35* flies, compared to Gal4 control flies (*tim*-Gal4 > +) and flies overexpressing p35 (*tim*-Gal4 > UAS-*p35*). We found that *dMyc* mRNA is expressed rhythmically and peaked at zeitgeber time 14 (ZT14) in Gal4 control flies (*tim*-Gal4 > +) (Figure S1A, dMyc inset), suggesting that *dMyc*, similar to mammalian *Myc*, may be clock-regulated (Fu et al., 2002). Similar to mammalian Myc (Altman et al., 2015), overexpressed dMyc increased expression of several clock genes, including *cyc*, *cwo*, *per*, *tim*, and *vri* (Figure S1A). Immunoblots of whole-head lysates also showed a significant increase in PER protein levels (Figure 1A). But unlike mammalian Myc, dMyc did not suppress the *BMAL1* (*ARNTL*) homolog *Clk* or *cyc* mRNA (Figure S1A). To evaluate whether dMyc directly binds clock genes, we analyzed dMyc chromatin immunoprecipitation-sequencing (ChIP-seq) data from *Drosophila melanogaster* Kc cells (Yang et al., 2013) and found endogenous dMyc binding at the promoters of *cyc*, *cwo*, *pdp*, and *vri*, but not of *per* and *tim* (Figure S1C).

Given that dMyc increased expression of several clock genes, we then sought to determine the effects of dMyc overexpression on fly behavior. We assayed locomotor rhythms under constant darkness for flies with *dMyc* driven by different promoters: *tim*-Gal4 driving dMyc and p35 in all clock cells, *Pdf*-Gal4 in ventral lateral neurons, or cry^{24}*Pdf*-Gal4 in ventral lateral neurons and cells expressing cryptochrome (Baik et al., 2017). dMyc overexpression in all clock cells (*tim*-Gal4 > *Myc*+*p35*) resulted in a lower percentage of rhythmic flies (30%) compared to the Gal4 control (56%) (Table 1; Figures S2C and S2D). Additional control with p35 overexpression was performed in *tim*-Gal4 flies with isogenic background. As expected, dMyc overexpression in *tim*-Gal4 (*w*/Y;*tim*-G/+;+/UAS-*p35* versus *w*/Y;*tim*-G/+;+/UAS-*Myc*, UAS-*p35*) also showed a moderate effect on rhythmicity (91% versus 77% rhythmic, respectively; Table 1). Restricted dMyc expression in PDF-positive ventral lateral neurons (*Pdf*-Gal4 > *Myc*+*p35*) was similarly associated with weaker rhythms, with 87% of the flies rhythmic compared to 100% rhythmic in control flies. Rhythm strength was also significantly decreased in rhythmic flies (Table 1; Figures S2A and S2B). Notably, use of both cry^{24}-Gal4 and *Pdf*-Gal4 to drive dMyc expression (cry^{24}*Pdf*-G > *Myc*+*p35*) resulted in arrhythmia in about 70% of the flies and weaker rhythm in the rhythmic flies (Table 1; Figure 1B). These observations indicate that dMyc overexpression perturbs *Drosophila* circadian behavior. Perturbation of rhythmic behavior, however, is not always associated with the same changes in clock gene expression. Although head extracts of cry^{24}*Pdf*-G > *Myc+p35* flies showed overall higher levels of *cry*, *cyc*, *cwo*, and *tim* transcripts (Figure S1B), the oscillation and expression of other clock genes were largely unchanged.

Our results from using three different drivers suggest that where, rather than how much dMyc is overexpressed, affects circadian behavior. With up to 500-fold increase in *dMyc* transcript and significant elevation of dMyc protein in *tim*-G > *Myc*+*p35* flies, the percentage of rhythmic flies only dropped from 56% to 30% (Figure S1A; Table 1). However, *cry*^{24}*Pdf*-G > *Myc*+*p35* flies showed a severely arrhythmic phenotype, although *dMyc* transcripts were only 3-fold higher than controls (Figure S1B; Table 1). These results suggest that dMyc-mediated disruption of circadian behavior in flies may be dependent on where dMyc is expressed.

Previously, we documented that MYC suppresses *BMAL1* through upregulation of *REV-ERBα* (Altman et al., 2015). Although the *Drosophila REV-ERBα* homolog *Ecdysone-induced protein 75B* (*E75*) suppresses *Clk*, the *BMAL1* homolog (Kumar et al., 2014), we found that dMyc did not change the levels of four mRNA isoforms of *E75* in *cry*^{24}*Pdf*-G > *Myc*+*p35* flies (Figure S2E).

dMyc-Overexpressing Flies Show Reduced PDF Expression but Preserved Axonal Morphology at the Dorsal Termini of Central Pacemaker Neurons (sLNvs)

Integrity of sLNvs and their secretion of the PDF neuropeptide are essential for rhythmic behavior in *Drosophila*, such that loss of *Pdf* neurons or expression result in arrhythmic flies (Renn et al., 1999). PDF expression in the dorsal axon of sLNvs is particularly relevant, with rhythmic axonal fasciculation observed at the terminus of this axon (Fernández et al., 2008). Intriguingly, overexpression of another E-box transcriptional factor Mef2 alters circadian sLNv dorsal axonal fasciculation and causes arrhythmic *Drosophila* behavior (Sivachenko et al., 2013). We thus asked whether ectopic dMyc alters sLNv morphology or PDF expression, rendering *cry*^{24}*Pdf*-G > *Myc*+*p35* flies arrhythmic.

We visualized PDF in the cell bodies and axons by immunohistochemistry (IHC) at two different time points—ZT1 (an hour after lights on) and ZT13 (an hour after lights off). While there was no significant change in PDF staining in the cell bodies of both lLNvs and sLNvs, PDF expression was reduced in the dorsal projection of sLNvs in *cry*^{24}*Pdf*-G > *Myc*+*p35* flies compared to controls (Figure 1D, arrowheads). To investigate whether reduced PDF expression is associated with dysmorphic dorsal projection, we expressed membrane-labeled GFP (mCD8-GFP) under the control of a *Pdf*- and *cry*-specific promoter in dMyc-overexpressing or control flies. The mouse lymphocyte transmembrane protein (mCD8) fused to GFP labels the cell surface, particularly axons and dendrites due to their high surface area (Lee and Luo, 1999). Although *cry*^{24}*Pdf*-G > *Myc*+*p35* flies exhibit less intense PDF staining at dorsal termini, the mCD8-labeled axons and their fasciculation are preserved (Figure 1C, rectangle), suggesting that dMyc overexpression does not affect axonal morphology but, instead, reduces PDF expression in the dorsal projection of sLNv cells. Because loss of PDF expression in the dorsal projection has been observed in *Clk* and *cyc* mutant flies (Park et al., 2000), we surmise that ectopic dMyc alters CLK-mediated PDF expression and contributes to dysrhythmia in these flies.

Hypomorphic dMyc Flies Exhibit Arrhythmic Behavior

Per2 is reported to regulate endogenous mouse *Myc* expression (Fu et al., 2002). However, whether endogenous *Myc* affects the circadian clock is not known. To investigate whether loss of *dMyc* affects *Drosophila* circadian locomotor behavior, we used hypomorphic *dMyc* alleles, dm^{P0} (P-element insertion 100 bp upstream of the transcription start site of dMyc) and dm^{4} (two exon deletion dMyc-null allele) (Figure 2A) (Pierce et al., 2004). Male dm^{P0}/Y flies are viable but exhibit smaller body size (Figure 2B), and dm^{4} mutations are lethal at the early larval stage (Johnston et al., 1999). Assay of circadian locomotor behavior of dm^{P0}/Y flies in constant darkness revealed robust rhythms in 86% of the sibling controls (*w*/Y), but only in 41% of dMyc mutants, with other mutant flies showing weak rhythms or arrhythmia (Table 1; Figures 2C–2E). These data demonstrate that endogenous *dMyc* function is necessary for normal circadian behavior.

dMnt Mutation Rescues Arrhythmicity in dMyc Mutant Flies

To determine whether dMyc transcriptional activity is involved in rhythmic behavior, we used flies mutant for MNT (dMnt; a homolog of mammalian MNT), a transcription repressor that antagonizes MYC function by dimerizing with the MYC-binding partner MAX (Hooker and Hurlin, 2006). Loss of *dMnt* in *dMyc*-null flies rescues growth arrest from the early larval to pupal stage (Loo et al., 2005; Pierce et al., 2008). Further knockdown of *Mnt* compensates for loss of *Myc* and accelerates cell proliferation in *Myc*-null cells, presumably by relieving transcriptional repression (Nilsson et al., 2004).

Therefore, we asked whether loss of *dMnt* is able to rescue the arrhythmic phenotype in *dMyc* hypomorphs. Because dm^{4}/Y and $dm^{4}dmnt^{1}$/Y are inviable as adult males, we crossed male *dMyc* hypomorphic flies (dm^{P0}/Y) with either wild-type females (w^{ISO31}), females heterozygous for a *dMyc*-null allele (dm^{4}/FM7), or females carrying a *dMyc*-null allele along with a *dMnt* mutation ($dm^{4}dmnt^{1}$/FM7) to generate female heterozygotes dm^{P0}/+, dm^{P0}/dm^{4}, and $dm^{P0}/dm^{4}dmnt^{1}$, respectively. Consistent with the smaller body size observed in dm^{P0}/Y flies, dm^{P0}/dm^{4} flies also have smaller body size compared to dm^{P0}/+ flies (Figure 2F). Strikingly, the smaller body size was reversed in $dm^{P0}/dm^{4}dmnt^{1}$ flies that also carry the *dMnt* mutation (Figure 2F), suggesting loss of the *dMyc* suppressor *dMnt* is sufficient to de-repress *dMyc*-driven growth pathways.

Consistent with the arrhythmic behavior we observed in *dMyc* hypomorphic male flies (dm^{P0}/Y), dm^{P0}/dm^{4} flies exhibit substantial arrhythmicity compared to dm^{P0}/+ flies (30% rhythmic versus 94%) (Table 1; Figures 2G–2I). Notably, among the 30% rhythmic dm^{P0}/dm^{4} flies, the rhythm strength was also significantly reduced (Table 1; Figure 2I). Strikingly, loss of *dMnt* in the dm^{4} background rescued the rhythmicity from 30% to 63% (Table 1). Furthermore, reduced rhythm strength in rhythmic dm^{P0}/dm^{4} flies was rescued in $dm^{P0}/dm^{4}dmnt^{1}$ flies (Table 1; Figure 2I). Of note, there is no statistically significant difference in period length between *w*/Y and dm^{P0}/Y flies or dm^{P0}/+ and dm^{P0}/dm^{4} flies (Table 1; Figures 2D and 2H), suggesting altered endogenous *dMyc* activity has minimal effect on period length.

Endogenous dMyc Activity Affects Molecular Oscillations of *per* and *tim*

We explored the mechanism of arrhythmic behavior in dm^{P0}/dm^{4} flies by determining whether oscillation of *per* and *tim* is altered in brains of these flies (Zeng et al., 1996). As expected under constant darkness, *dMyc* mutants showed significantly lower *dMyc* transcript levels and dampened oscillations (Figure S3A). Loss of *dMnt* in hypomorphic *dMyc* background ($dm^{P0}/dm^{4}dmnt^{1}$) did not affect the *dMyc* transcript level nor rescue *dMyc* oscillations (Figure S3A). Next, we examined whether decreased dMyc activity affects the cycling of *per* and *tim* mRNA levels. In $dm^{P0}/+$ flies, *per* and *tim* mRNA have an oscillation pattern similar to that of wild-type controls with a peak at circadian time 10 (CT10) (CT reflects time in constant darkness conditions and roughly corresponds to the equivalent ZT times in 24-h light/dark cycles) (Jang et al., 2015). In dm^{P0}/dm^{4} flies, instead of peaking at CT10, both *per* and *tim* mRNA levels display a trough at CT10 (Figures S3B and S3C). Notably, the peak of *per* and *tim* transcripts at CT10 re-emerged in $dm^{P0}/dm^{4}dmnt^{1}$ flies.

We assayed the protein levels of dMyc and PER in the whole heads of dMyc hypomorphic flies $dm^{P0}/+$, dm^{P0}/dm^{4} at ZT2, ZT8, ZT14, and ZT20 during entrainment and maintained in a light/dark cycle (Figure S3D). dMyc was not detected, and PER protein expression was not significantly changed between dm^{P0}/dm^{4} flies versus $dm^{P0}/+$ flies (Figure S3D). We then assayed the expression of PER proteins in the whole brains of $dm^{P0}/+$, dm^{P0}/dm^{4}, and $dm^{P0}/dm^{4}dmnt^{1}$ flies, based on their rhythmicity, at CT2, CT6, CT10, CT14, CT18, and CT22 in constant darkness. Consistent with the mRNA expression profile, PER was lower in dm^{P0}/dm^{4} flies at times of peak expression in wild-type controls ($dm^{P0}/+$) (Figures S3E and S3F). PER levels were rescued in $dm^{P0}/dm^{4}dmnt^{1}$ flies, corroborating the observation with per transcripts (Figure S3B). Interestingly, among the dm^{P0}/dm^{4} mutants, arrhythmic flies appear to have even lower PER expression than rhythmic flies (Figures S3E–S3G), suggesting that altered PER expression in hypomorphic *dMyc* flies may be linked to arrhythmicity.

Oscillation of PER Expression Is Altered in sLNvs of dMyc Hypomorphic Flies

As mentioned, ventral lateral neurons are central clock cells in *Drosophila* that are crucial for maintaining rhythmic behavior. To investigate whether clock protein oscillation is preserved in ventral lateral neurons, particularly in arrhythmic flies, we compared PER protein levels in dissected fly brains from rhythmic $dm^{P0}/+$, dm^{P0}/dm^{4}, arrhythmic dm^{P0}/dm^{4} and $dm^{P0}/dm^{4}dmnt^{1}$ flies by immunohistochemistry. We focused on sLNvs, because they have robust PER oscillation, with nuclear PER expression reaching its peak at CT2 and trough at CT14. Although not statistically significant, nuclear PER accumulation in sLNvs was reduced in dm^{P0}/dm^{4} flies relative to $dm^{P0}/+$ flies at CT2 (Figures S4A and S4B). The reduced PER nuclear accumulation was rescued in $dm^{P0}/dm^{4}dmnt^{1}$ flies (Figures S4A and S4B). In contrast, nuclear PER expression in sLNvs was increased at CT8 in dm^{P0}/dm^{4} flies relative to $dm^{P0}/+$ flies (Figures S4A and S4B). The elevated PER nuclear expression was similarly reversed in $dm^{P0}/dm^{4}dmnt^{1}$ flies (Figures S4A and S4B). This observation echoes the oscillation pattern of *per* transcript in whole brain where at CT10, instead of reaching the peak as in $dm^{P0}/+$ flies, the level is reduced in dm^{P0}/dm^{4} flies (Figure S3B). Altogether, these data demonstrate that loss of endogenous dMyc in

Drosophila perturbs *per* oscillation and disrupts circadian behavior, which can be rescued by further deletion of dMnt.

Overexpressed dMyc Alters Metabolism in Fly Heads

Mammalian Myc is known to orchestrate a number of metabolic pathways in proliferating cells (Hsieh et al., 2015; Stine et al., 2015). As alteration of cellular energetic status, such as the ratio of NAD^+/NADH or ATP/AMP, provides feedback to the circadian clock (Lamia et al., 2009; Rutter et al., 2002), we hypothesized that overexpression of dMyc alters metabolism in flies, which in turn contributes to increased arrhythmic behavior. To test this idea, metabolites in cry^{24}*Pdf*-G > *Myc*+*p35* and control fly heads were extracted and analyzed by mass spectrometry. While the majority of amino acids did not change with dMyc overexpression, we noted that the relative enrichment of valine, L-cystathionine, and sarcosine was significantly reduced (Figure 3A). In addition, significant increase of histidine was observed in cry^{24}*Pdf*-G > *Myc*+*p35* flies compared to controls (Figure 3A). Furthermore, glutamine was slightly lowered with dMyc overexpression. These observations indicate that dMyc affects metabolism broadly in the fly head even when its expression is limited just to *cry*- and *Pdf*-expressing cells.

Loss of *dMyc* Alters Fly Head Metabolites in a Time-Dependent Manner that Is Partially Rescued by Further Loss of *dMnt*

Given the metabolic dysregulation observed in *dMyc*-overexpressing flies, we investigated whether there are comparable metabolic alterations in *dMyc* hypomorphic flies, particularly those metabolites or metabolic pathways that can be rescued by loss of *dMnt*. Flies (dm^{P0}/+, dm^{P0}/dm^4, and $dm^{P0}/dm^4 dmnt^1$) were harvested at two times, ZT3 and ZT15, after light/dark entrainment. We calculated the ratio of the difference of metabolites between dm^{P0}/dm^4 flies versus dm^{P0}/+ flies and dm^{P0}/dm^4 flies versus $dm^{P0}/dm^4 dmnt^1$ flies [(dm^{P0}/+ – dm^{P0}/dm^4)/($dm^{P0}/dm^4 dmnt^1$ – dm^{P0}/dm^4)] at ZT3. We searched for metabolites that were significantly reduced in the *dMyc* hypomorph flies but rescued by loss of *dMnt* by focusing on metabolites that exhibited a ratio between 0.5 and 1.5. We subsequently ranked the metabolites from the ratio closer to 0.5, representing the largest magnitude of rescue from loss of *dMnt* (Figures 3B and 3C). A heatmap was graphed based on the log value of each metabolite that was further normalized to yield a value between 0 and 1. At ZT3, selected amino acids (histidine, leucine/isoleucine, glutamate, tyrosine), neurotransmitters (glutamate, gamma-aminobutyric acid [GABA]), tricarboxylic acid cycle (TCA) cycle intermediates (citrate/isocitrate, fumarate, alpha-ketoglutarate), and oxidized energy transfer molecules (NADP+, cyclic AMP [cAMP]) were greatly reduced in dm^{P0}/dm^4 flies compared to dm^{P0}/+ flies and were rescued in $dm^{P0}/dm^4 dmnt^1$ flies (Figure 3B). At ZT15, the majority of amino acids (aspartate, serine, tyrosine, methionine, glutamine, threonine, lysine) were instead increased in dm^{P0}/dm^4 flies and were rescued in $dm^{P0}/dm^4 dmnt^1$ flies, demonstrating the abundance of certain type of metabolites at specific time points are different in dm^{P0}/dm^4 flies compared to either dm^{P0}/+ flies or $dm^{P0}/dm^4 dmnt^1$ flies.

Putative Link between dMyc Regulation of Histidine Metabolism and Circadian Activity

Altered metabolites that could affect circadian activity include histamine, which is derived from histidine through histidine decarboxylase, Hdc, and glutamate that is produced from

glutamine by glutaminase, GLS. Both histamine and glutamate have been shown to affect wakefulness (Abe et al., 2004; Zimmerman et al., 2017). Our data demonstrated that the ion count of glutamine is higher and glutamate is lower in the dm^{P0}/dm^{4} flies, consistent with decreased conversion from glutamine to glutamate (Figures 3B, S5B, and S5C). These changes suggest a possible link to *Drosophila GLS*, which if diminished in low Myc states would decrease the conversion of glutamine to glutamate. Hence, we sought to determine whether *Drosophila GLS* is directly regulated by dMyc. We took advantage of the publicly available data of dMyc ChIP-seq and found that the endogenous dMyc binds to the promoter of *GLS* in *Drosophila melanogaster* Kc cells (Figure S5A) (Yang et al., 2013). However, a time-course study of *GLS* mRNA levels in *tim*-G > *Myc*+*p35* with increased dMyc expression versus *tim*-G flies (Figures S5D and S5E) and of decreased dMyc activity in $dm^{P0}/+$ versus dm^{P0}/dm^{4} flies (Figures S5F and S5G) does not corroborate GLS as a dMyc target in *Drosophila* heads.

dMyc regulation of histidine metabolism, however, could affect circadian behavior. Histidine levels were significantly increased in *dMyc*-overexpressing flies (Figure 3A) and remarkably reduced in *dMyc* hypomorph flies (Figure 3B). Hence, we sought to determine whether dMyc regulates Hdc, which converts histidine to histamine, or the histidine transporters CG13743 and CG30394. dMyc target genes are expected to be bound by dMyc and have increased mRNA levels with dMyc gain of function and decreased levels with hypomorphic dMyc. Endogenous dMyc does not bind to the promoter site of Hdc from the dMyc ChIP-seq in *Drosophila melanogaster* Kc cells (Figure S5H) (Yang et al., 2013). By contrast, endogenous dMyc binds to the promoter regions of both CG13743 and CG30394 (Figure S5H) (Yang et al., 2013). Intriguingly, the mRNA levels of CG13743 and CG30394 differ in response to dMyc levels with CG30394 behaving as a dMyc target (Figures S5I–S5P). While both CG13743 and CG30394 mRNA levels seem to oscillate and peaked at ZT10 in *tim*-G and $dm^{P0}/+$ flies (Figures S5I, S5K, S5M, and S5O), only CG30394 was increased in *tim*-G > *Myc*+*p35* flies versus *tim*-G flies (Figures S5M and S5N) and decreased in dm^{P0}/dm^{4} flies versus $dm^{P0}/+$ flies (Figures S5O and S5P). Furthermore, it is notable that misregulation of dMyc expression resulted in disruption of CG30394 oscillation (Figures S5M and S5O). These observations demonstrate a significant difference of histidine levels and expression of the histidine transporter CG30394 at different times of the day with misregulation of dMyc expression, suggesting that dMyc regulation of histidine metabolism could be a potential link between dMyc-driven metabolism and circadian behavior.

DISCUSSION

In this report, we establish that misregulation of *dMyc* expression disrupts *Drosophila* circadian activity, adding to its pleiotropic roles in regulating cell growth, cell cycle, apoptosis, and metabolism (de la Cova et al., 2004, 2014; Gallant, 2013; Johnston et al., 1999; Montero et al., 2008; Moreno and Basler, 2004).

We report here that gain or loss of function of *dMyc* reduced rhythmicity and demonstrate a role of *dMyc* in *Drosophila* circadian behavior. We observed that overexpressing *dMyc* specifically in *Pdf* and *cry*-expressing cells results in loss of rhythm in more than 70% of flies. High levels of *dMyc* are associated with decreased expression of the neuropeptide PDF

in dorsal projections of the sLNvs, which likely contributes to arrhythmicity. Moreover, we found that loss of endogenous *dMyc* significantly impairs rhythmicity that can be rescued by additional loss of the *dMyc* transcriptional antagonist *dMnt*. Through gene expression analysis, we found that low dMyc activity alters transcript oscillations of circadian clock genes, *per* and *tim*, and protein oscillation of PER in fly brain extracts as well as sLNvs. We also observe metabolic derangement resulting from loss of dMyc activity that could be rescued by concomitant loss of dMnt activity.

Unlike mammalian Myc (Altman et al., 2015), which suppresses *BMAL1* expression, overexpression of *dMyc* in *tim*-positive cells increased expression of the *Drosophila* homolog of *BMAL1*, *cyc*. Furthermore, while mammalian REV-ERBα, repressor of BMAL1, was increased by Myc, its fly counterpart, *E75*, was unaffected by elevated dMyc in *Pdf* and *cry*-expressing cells. In addition to *cyc*, overexpressed dMyc induced mRNA levels of several E-box-containing, negative regulators of the clock—*per*, *tim*, *vri*, and *cwo*—and also increased PER protein expression in the fly heads. By examining publicly available data of dMyc ChIP-seq, we found that the endogenous dMyc directly binds to the promoter regions of *cyc*, *pdp*, *vri*, and *cwo* of *Drosophila melanogaster* Kc cells, suggesting that dMyc dysregulation of some clock genes contributes to dysrhythmia.

Although we found that *dMyc* overexpression affects circadian behavior, the arrhythmic behavior depended significante on background strain and the chosen promoter for ectopic *dMyc* expression. *dMyc* transcript and protein levels increased significante in *tim*-G > Myc +p35 flies; however, the percentage of rhythmic flies only marginally dropped. By contrast, cry^{24}*Pdf*-G > Myc+p35 flies showed a severely arrhythmic phenotype, although *dMyc* transcripts were only moderately higher than controls. It is notable that ectopic *dMyc* expression also affects the expression of the neuropeptide PDF, central to fly circadian behavior. Because distinct subset of MYC target genes can differ as a function of MYC levels (Lorenzin et al., 2016), it is possible that the effect of ectopic *dMyc* expression on circadian behavior and clock genes is contextual. These observations suggest that dMyc may affect behavior through complex mechanisms in regulation of clock and metabolic gene expression.

Endogenous *Drosophila* Myc in Molecular Clock

Although dMyc has no prior known connection to the clock in flies, the increased arrhythmicity in *dMyc* mutant flies suggests that endogenous dMyc is involved in circadian homeostasis. In *tim*-Gal4 and cry^{24}*Pdf*-Gal4 control flies, *dMyc* mRNA oscillates in a circadian fashion, suggesting dMyc is regulated by the *Drosophila* clock. Prior studies using microarray analysis to compare the mRNA expression profile of PDF neurons with that of ELAV (marker for the majority of neurons)-expressing neurons revealed that *dMyc* was highly enriched in PDF neurons, including both sLNvs (2.96 at ZT12 and 3.1 at ZT0 fold enrichment relative to ELAV neurons) and lLNvs (2.44 at ZT0 but not enriched at ZT12), and cycles within lLNvs (2.7-fold down at ZT12 compared to ZT0), suggesting a potential role of dMyc in circadian regulation (Kula-Eversole et al., 2010). Furthermore, given the observations of relatively stronger effects of dMyc on *per/tim* mRNA cycling in fly brains

than on the PER protein oscillation in sLNvs, the action of dMyc in non-Pdf cells may also be crucial to maintain rhythmicity.

Drosophila Myc and Mnt

Our observation that loss of *dMnt* is able to rescue the arrhythmicity of *dMyc* mutant flies supports the notion that dMyc target genes are involved in circadian behavior. Mnt belongs to the extended Myc network, which includes Max, Mxd, Mlx, MondoA, and ChREBP (Conacci-Sorrell et al., 2014). Mnt represses Myc activities by interacting with Myc-binding partner Max and displacing Myc from binding Max (Conacci-Sorrell et al., 2014; Link et al., 2012). Although Myc was not linked to the molecular clock until recently (Altman et al., 2015), *dMnt* (isoform B) was found to be bound and regulated by CLK, and its mRNAs oscillate with peak expression at ZT14 (Abruzzi et al., 2011). Notably, *dMnt* expression was increased in sLNvs (2.2 at ZT12) and cycles within lLNvs (2.09-fold up at ZT12 compared to ZT0) (Kula-Eversole et al., 2010). The circadian expression of *dMnt* perhaps plays a role in maintaining the persistent oscillation of *per* and *tim* mRNAs in *dMyc* hypomorphic fly brains when *dMyc* level is low. The inverse abundance of dMyc and dMnt in PDF neurons at different times (ZT0 for *dMyc* and ZT12 for *dMnt*) may provide a potential explanation on the phase shift of *per* and *tim* transcripts, further supporting the hypothesis that both dMyc and dMnt are involved in the fly molecular clock.

Other members of the extended Myc network participate in metabolism and may affect circadian rhythm. MondoA was reported to be required for Myc-induced tumorigenesis both *in vitro* and *in vivo* (Carroll et al., 2015). MondoA, a glucose- 6-phosphate sensor, heterodimerizes with Mlx to regulate glucose-sensing, glutamine uptake, and glutamine-derived lipid biosynthesis (Carroll et al., 2015). *Mondo* and *Mlx,* the *Drosophila* orthologs of *ChREBP/MondoA* and *Mlx,* respectively, have also been shown to be crucial in sugar-sensing and metabolism (Havula et al., 2013; Mattila et al., 2015; McFerrin and Atchley, 2011). Interestingly, the direct target of *Drosophila* Mondo-Mlx, *cabut* (*cbt)*, was found to repress gluconeogenesis enzyme *pepck,* and genes involved in lipolysis and glycerol metabolism with glucose stimulation (Bartok et al., 2015). Intriguingly, 85% of *cbt*-overexpressing flies exhibit arrhythmic or weakly rhythmic circadian behavior (Bartok et al., 2015).

Misregulation of *Drosophila* Myc Affects Metabolism

The metabolic alteration we observed in cry^{24}*Pdf*-G > *Myc*+*p35* flies and *dMyc* hypomorphic flies supports the role of dMyc in metabolism, as documented for mammalian MYC. Specifically, we surmise that alterations of glutamine, glutamate, and histidine levels by misregulated dMyc expression could affect fly circadian behavior. Previous studies demonstrated that Myc directly regulates mammalian glutamine metabolism to promote tumor growth (Gao et al., 2009; Xiang et al., 2015), but our data do not support a role for dMyc in regulating glutaminase, which converts glutamine to glutamate, a neurotransmitter, in fly head. However, among the metabolites that were reduced through loss of *dMyc* and rescued by *dMnt,* histidine also stood out, and its derivative histamine was previously shown to influence sleep-wake cycles (Oh et al., 2013; Parmentier et al., 2002). Histidine can be converted to histamine, which induces wakefulness in both mice and fruit flies (Oh et al.,

2013; Parmentier et al., 2002; Thakkar, 2011). Interestingly, a recent study uncovered that the growth of dMyc-dependent neural dedifferentiation-derived clones in fruit flies is sensitive to histidine deprivation (Froldi et al., 2019). In that study, the mutant *nerfin-1* clone (nerfin-1 is required for *Drosophila* neurons to maintain a differentiated state) that was generated in a Myc P0 (hypomorphic) heterozygous background failed to respond to dietary histidine supplementation, which can be rescued by dMyc overexpression. This result suggests that dMyc is critical for histidine import or utilization. Endogenous dMyc does not bind the Hdc promoter in *Drosophila melanogaster* Kc cells, suggesting that Hdc is not a direct dMyc target. We found, however, that dMyc bound histidine transporter genes, CG13743 and CG30394 (Yang et al., 2013), but only the expression of CG30394 behaved as expected for a dMyc target. The elevated histidine level in dMyc-overexpression flies and diminished histidine level in dMyc-hypomorphic flies support the hypothesis that dMyc upregulates histidine transporter to increase histidine import. As such, further studies are required to understand the putative connection between alteration of histidine metabolism with circadian behavior.

Collectively, our observations support the hypothesis that ectopic dMyc can affect the clock and that endogenous dMyc activity is essential for normal *Drosophila* circadian behavior. Furthermore, we surmise that similar to mammalian MYC, dMyc is able to induce negative regulators of the circadian clock and alter metabolism. These foundational observations linking dMyc to the *Drosophila* clock provide a path for additional studies, which will hopefully further link MYC with the circadian clock and metabolism in various organisms and settings.

STAR★METHODS

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Chi Van Dang (cdang@wistar.org). *Drosophila* lines generated in this study can be requested by contacting Dr. Amita Sehgal. This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly stocks—*Pdf*-Gal4, *tim*-Gal4, cry^{24}-Gal4, dm^{P0}, UAS-*p35* and UAS-*mCD8::GFP* lines were provided by the Bloomington *Drosophila* Stock Center. UAS-*Myc* UAS-*p35* and dm^{4} were kindly shared by Dr. Julie Secombe (Albert Einstein College of Medicine, NY). dm^{4} $dmnt^{1}$ lines were provided by Dr. Robert N. Eisenman's lab. dm^{4} and dm^{4} $dmnt^{1}$ lines were balanced with FM7i, *Act*-GFP. cry^{24}-Gal4; *Pdf*-Gal4 lines were generated from crossing cry^{24}-Gal4 to *Pdf*-Gal4 flies. dm^{P0}/Y and its sibling control *w*/Y were generated by backcrossing dm^{P0} line from into isogenic $w^{[1118]}$ line (w^{ISO31}). cry^{24}-Gal4; *Pdf*-Gal4 > GFP^{CD8} lines were generated from crossing cry^{24}-Gal4; *Pdf*-Gal4 to UAS-*mCD8::GFP* flies. Flies that used for the experiments were grown at 25°C.

METHOD DETAILS

Locomotor activity assay—Circadian locomotor activity assays were measured using the *Drosophila* Activity Monitoring System (Trikinetics). Approximately 5- to 7-day-old adult flies were entrained to light-dark (LD) 12 hour: 12 hour (12:12) cycles for three days, then transferred to constant darkness (DD) for 7–8 days. Locomotor activity data were analyzed using Clocklab software (ActiMetrics). Individual periods were calculated from 7–8 days activity data during DD using chi-square periodogram. Rhythm strength was determined by Fast Fourier Transform (FFT) analysis, then categorized as arrhythmic (< 0.01), weakly rhythmic (0.01–0.03) and rhythmic (> 0.05). Periods and rhythm strength were only calculated from rhythmic and weakly rhythmic flies.

Immunohistochemistry—Five-day-old adult flies were entrained to LD 12:12 cycle for 3 days and then transferred to DD. Brain dissection was done on day 4 or day 5 in DD. Adult fly brains were dissected and fixed for 30–60 min in 4% paraformaldehyde made in PBS with 0.3% Triton-X(PBST). Fixed brains were quickly rinsed twice in PBST, washed 3 × 10 min with PBST, followed by blocking with 5% normal donkey serum in PBST (NDST) for 1 hour. Brains were then incubated with primary antibody diluted in NDST at 4°C overnight. Brain samples were then washed 3 × 10 min with PBST, incubated with secondary antibody diluted in NDST at room temperature for 2 hours, washed with 3 × 10 min PBST again and mounted with Vectashield. Five to ten brains of each genotype at each time point were processed. A Leica TCS SP5 confocal microscope was used to acquire images for immunostained brain. Primary antibodies used include: rabbit anti-PDF (C7; Developmental Studies Hybridoma Bank) and Guinea Pig anti-Per (GP1140). Secondary antibodies used include: Alexa Fluor 488 goat anti-rabbit IgG (Life Technologies), Alexa Fluor 488 goat anti-Guinea Pig IgG (Life Technologies), Cy3 donkey anti-rabbit (Jackson ImmunoResearch Laboratories, West Grove, PA), Cy3 donkey anti-mouse (Jackson ImmunoResearch Laboratories). Quantitative analysis was performed by measuring integrated density on section of the largest area of sLNv with Fiji software. The data for each genotype/rhythmicity is an average value of approximately 40 sLNvs from 10 brain hemispheres.

Quantitative real-time PCR—Five- to seven- day old adult flies were entrained to LD 12:12 cycle for 3–5 days. Approximately 20 flies were collected on dry ice at each time points on the last day of LD for heads. Ten brains of each genotype and at each time point were dissected on the fourth day of DD after 3 days of LD. Brains were dissected in cold RNase-free PBS and spun down at 4C to remove PBS within 30 minutes. Adult fly heads or brains were homogenized in TRIzol® Reagent using VMR Pellet Mixers for 10 s. Total RNA was then extracted according to manufacturer's instruction (TRIzol; Life Technologies) and cDNA was made by using TaqMan Reverse Transcription Reagents (Life Technologies). cDNA was used as template for quantitative real time PCR (RT-PCR) with specific *Drosophila* primers. RT-PCRs were performed using the ViiA 7 or QuantStudio™ 6 Flex Real-time PCR system (Applied Biosystems) with a SYBR Green kit (Life Technologies). Relative mRNA expression levels were normalized to actin and analyzed using absolute quantification method. Standard curve used for quantification was determined by serial dilution of mixed samples from all time points. Delta-Delta Ct method was also used in some of the experiments. Primers used in the experiments were *dMyc* forward (5′-

GAGCAACAACAGGCCATCGATATAG-3′), *dMyc* reverse (5′-CCTTCAGACTGGATCGTTTGCG-3′), *per* forward (5′-CGTCAATCCATGGTCCCG-3′), *per* reverse (5′-CCTGAAAGACGCGATGGTG-3′), *tim* forward (5′-GCTGCGCCTTGTTTTCCTT-3′), *tim* reverse (5′-ATGATGTTCAGATCCTGCTGGA-3′), *Pdp1* forward (5′-TTTGAACAGCTTGAAAGCGC-3′), *Pdp1* reverse (5'-GAGATTTCCTGCCTGAGCTGG-3'), *vri* forward (5′-CGACTCTCTCGATGAACGGC-3′), *vri* reverse (5′-ACGGATGCAAGTTAGAAGCCTC-3′), *Actin* forward (5′-GCGCGGTTACTCTTTCACCA-3′), *Actin* reverse (5′-ATGTCACGGACGATTTCACG-3′), *cry* forward (5′-GCAGTACGTCCCGGAGTTGA-3′), *cry* reverse (5′-AGGGCTCGTGAACAAATTCCT-3′), *Clk* forward (5′-TTCTCGATGGTGTTCTCGGTG-3′), *Clk* reverse (5′-AGTTCGCAAAGCCAACGG-3′), *cyc* forward (5′-GTACGTTTCCGATTCGGTGT-3′), *cyc* reverse (5′-CTCTGTGGAACGTCGGTCTT-3′), *cwo* forward (5′-CAGGACTTTTGCCACC GACTATTG-3′), *cwo* reverse (5′-CTGCCGCCGCCTGCTGAC-3′), *E75*-RA forward (5′-CGGTAATCTGCACATTGTCGC-3′), *E75*-RA reverse (5′-TGCTTCAGCTGTTGGCTCTTG-3'), *E75*-RB forward (5′-TGCAACATCATCCGGAGGAT-3′), *E75*-RB reverse (5′-TCCTCCAGATGCAGCATCTCA-3′), *E75*-RC forward (5′-GACTTCTGTGATCTGCAGCACG-3′), *E75*-RC reverse (5′-CGACCTGTTACCCCCAAAATG-3′), *E75*-RD forward (5′-GCGAAGAACTCCCGATATTGAA-3′), *E75*-RD reverse (5′-TGAACTCACAGGTCTCGAGGTG-3′), *GLS* forward (5′-CGAGTGGCAGGAACTTCAA-3′), *GLS* reverse (5′-TGACAAGGGCGTTCATCAG-3′), *CG13743* forward (5′-ATACCCTACGCCCTTCACA-3′), *CG13743* reverse (5′-TGACCATCAGGATGAGTGAGTA-3′), *CG30394* forward (5′-CTC CAATGCCTACCCATCTTC-3′), *CG30394* reverse (5′-TGTCGAGACTCTGGTTGTTAATG-3′)

Western blot analysis—Five- to seven- day old adult flies were entrained to LD 12:12 cycle for 3–5 days. Approximately 8–10 flies were collected on dry ice at each time point on the last day of LD. Ten brains of each genotype and at each time point were dissected on the fourth day of DD after 3 days of LD. Brains were dissected in cold PBS and spun down at 4C to remove PBS within 30 minutes. Adult fly heads or brains were lysed and homogenized in 1X Passive Lysis Buffer (Promega) diluted in PBS supplemented with protease inhibitors and phosphatase inhibitor okadaic acid. Sample buffer (Thermo Cat: 39000) was added to each sample. All samples were then boiled and spinned. Equal volume of supernatant from each sample was loaded into the Criterion pre-cast gradient gels (Bio-Rad, Hercules, CA, USA). Proteins were transferred onto nitrocellulose membranes using iBlot® Gel transfer system (Thermo). Primary antibodies used include: mouse anti-dMyc (from Eisenman lab), rabbit anti-α-Tubulin (Cell Signaling, Danvers, MA, USA) and Guinea Pig anti-PER (GP1140). Secondary antibodies used include: Peroxidase AffiniPure Goat Anti-Guinea Pig IgG (Jackson ImmunoResearch Laboratories), Goat anti-Mouse IgG1, HRP conjugate (Thermo). Immunoblots were then developed using ECL substrate (Thermo).

UCSC Genome Browser and Publicly Available Genomic Data—For *Drosophila melanogaster* Kc cell ChIP-Seq for endogenous dMyc peaks, publicly available data from Yang et al. was used [GEO accession GSE39521 (Yang et al., 2013) access date 2–13-2019]. dMyc peak tracks from GEO accession numbers GSM970847 were downloaded and analyzed on UCSC genome browser *D. melanogaster* Apr. 2006 (BDGP R5/dm3) assembly. The resulting images were exported using the Genome browser's website and cropped for display.

Fly metabolites analysis by Gas Chromatography mass spectrometry (GC-MS)—Fly heads and bodies were separated before metabolite extraction. 100μL of cold methanol was added to the fly samples and homogenized using VMR homogenizer for 10 s. 100μL of cold methanol and chloroform was then added, followed by sonication on a power setting of 2 and a duty cycle of 20% for 1 minute. Nor-valine was also added to methanol-chloroform mix alone for comparison. The samples were centrifuged at 8,000 rpm at 4°C for 10 min. The metabolite-containing supernatant was evaporated under nitrogen at 40°C. 100 μL of N-tertbutyldimethylsilyl- N-methyltrifluoroacetamide (MTBSTFA, Regis, Morton Grove, IL, USA) and 100 μL of acetonitrile were added to the dried residue. The samples were heated in 4 mL sealed glass vials at 70°C for 90 minutes. The resulting silylated metabolites were transferred to 1.5ml Eppendorf tubes and centrifuged at 13,000 rpm to remove insoluble materials. One micro-liter of the supernatants was analyzed with an Agilent 7890A/5975A GC-MS system with a DB-5 column (Agilent, Santa Clara, CA, USA). Mass spectra were quantified with the MSD ChemStation software (Agilent).

Fly metabolites analysis by Liquid chromatography mass spectrometry (LC-MS)—Fly heads and bodies were separated before metabolite extraction. 600μL of cold 2:1 methanol:chloroform was added to the fly samples and homogenized in a bead-based tissue homogenizer at 25Hz for 4 minutes (TissuLyser II, QIAGEN, Hilden, Germany). 200μLof both water and chloroform was then added, followed by centrifugation at 18787×g for 7 minutes at 4°C. 350μL of the upper layer, comprising the aqueous layer, was separated and dried down overnight under vacuum. Samples were resuspended in 50μL of 50:50 water:acetonitrile for injection onto the mass spectrometer. Liquid chromatography conditions and mass spectrometer parameters for hydrophilic interaction chromatography (HILIC) analysis for small polar metabolites were executed as previously reported (Rhoades and Weljie, 2016). Then, 5 μL injections were performed for each sample in duplicate on a Waters Acquity H-Class UPLC coupled to a Waters TQ-S micro mass spectrometer (Milford, MA), using an XBridge BEH Amide column for chromatographic separation (2.1 × 150 mm, 2.5 μm). The LC solvents consisted of 95:5 water:acetonitrile with 20 mM ammonium acetate at pH 9 (mobile phase A) and acetonitrile for mobile phase B. The gradient was changed from 15% to 70% mobile phase A over 5 min at 0.15 mL/min, followed by an isocratic hold for 10 min. The column was washed in 98% mobile phase A and then re-equilibrated in starting conditions for 5 min before the next injection. The MS operated in ion-switching mode with a capillary voltage of 3 kV for electrospray positive mode (ESI+) and 2 kV for negative mode (ESI−). The desolvation gas flow was set to 900 L/h and desolvation temperature at 450°C, with the source temperature set at 150°C. Metabolites were detected using multiple reaction monitoring (MRM), with mass transitions

and voltages optimized as previously described. LC-MS chromatograms were processed using TargetLynx under Mas-sLynx version 4.1. Ion counts were exported and processed in R (version 3.3), and normalized by the number of fly heads collected per genotype before calculation of ratios and generation of heatmaps.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical methods of experiments and the number of biological replicates are described in figure legends. Error bars represent standard error of the mean (SEM). All statistical analyses were performed using Microsoft Excel or GraphPad Prism and the statistical significance (p value) was determined using a two-tailed Student's t test. Locomotor activity data were analyzed and quantified using Clocklab software (ActiMetrics). Circadian periods were calculated using chi-square periodogram. Rhythm strength was determined by Fast Fourier Transform analysis. Quantification of the western blot band intensities were measured using Image Studio Lite Version 4.0.21, according to the manufacturer's instruction. Quantitative analysis of the confocal images was performed by measuring integrated density on a section of the largest area of the neuron of interest with Fiji software. Quantification of the mass spectrometry was described in details in method details.

DATA AND CODE AVAILABILITY

This study did not generate/analyze datasets/code.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *Drosophila* Myc expression affects circadian locomotor behavior
- Loss of dMyc inhibitor dMnt rescues dysrhythmia caused by hypomorphic dMyc
- Perturbed metabolism by misregulated dMyc may underlie altered circadian rhythm

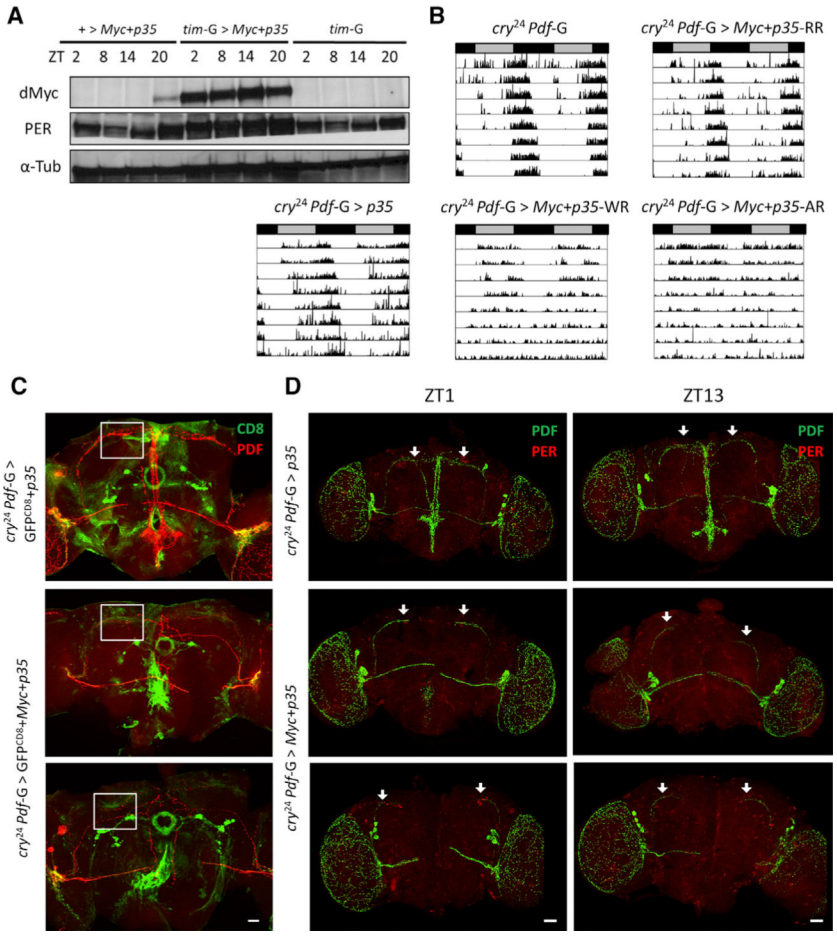

Figure 1. PER Immunoblot, Circadian Locomotor Rhythms, and PDF Expression of sLNvs in dMyc-Overexpressing Flies

(A) dMyc and PER protein levels determined by immunoblot in heads of *tim*-G > *Myc*+*p35* flies compared to *tim*-G and + > *Myc*+*p35* flies. Flies were entrained in light/dark 12:12-h cycle for 3–5 days. On the last day of entrainment, flies were snap frozen every 4–6 h and the heads were used to extract protein. α-Tubulin (α-Tub) is the loading control. Data are representative of three or more biological replicates. (B) Representative actogram of cry^{24}*Pdf*-G, cry^{24}*Pdf*-G > *p35*, cry^{24}*Pdf*-G > *Myc*+*p35* rhythmic (RR), cry^{24}*Pdf*-G > *Myc* +*p35* weakly rhythmic (WR), and cry^{24}*Pdf*-G > *Myc*+*p35* arrhythmic (AR). Flies were entrained to a light/dark cycle for 3 days before being monitored in constant darkness over 8 days.

(C and D) Decreased PDF expression in dorsal projections from sLNvs in dMyc-overexpressing flies with intact neuronal processes. Representative confocal image of brains from control flies (cry^{24}*Pdf*-G > *p35*) and dMyc-overexpressing flies (cry^{24}*Pdf*-G > *Myc* +*p35*) with membrane-labeled GFP (mCD8-GFP) (C) or without mCD8-GFP (D) subjected to immunofluorescence staining using antibodies directed against PDF (red in C; green in D) and PER (red in D) proteins at ZT1 and ZT13. A total of 12 brains from control flies and 34 brains from dMyc-overexpressing flies with mCD8-GFP were dissected, and the observations were consistent. Rectangles in (C) and arrows in (D): dorsal projections from sLNvs. Scale bars, 40 μm.

See also Figures S1, S2, and S5, and Table 1.

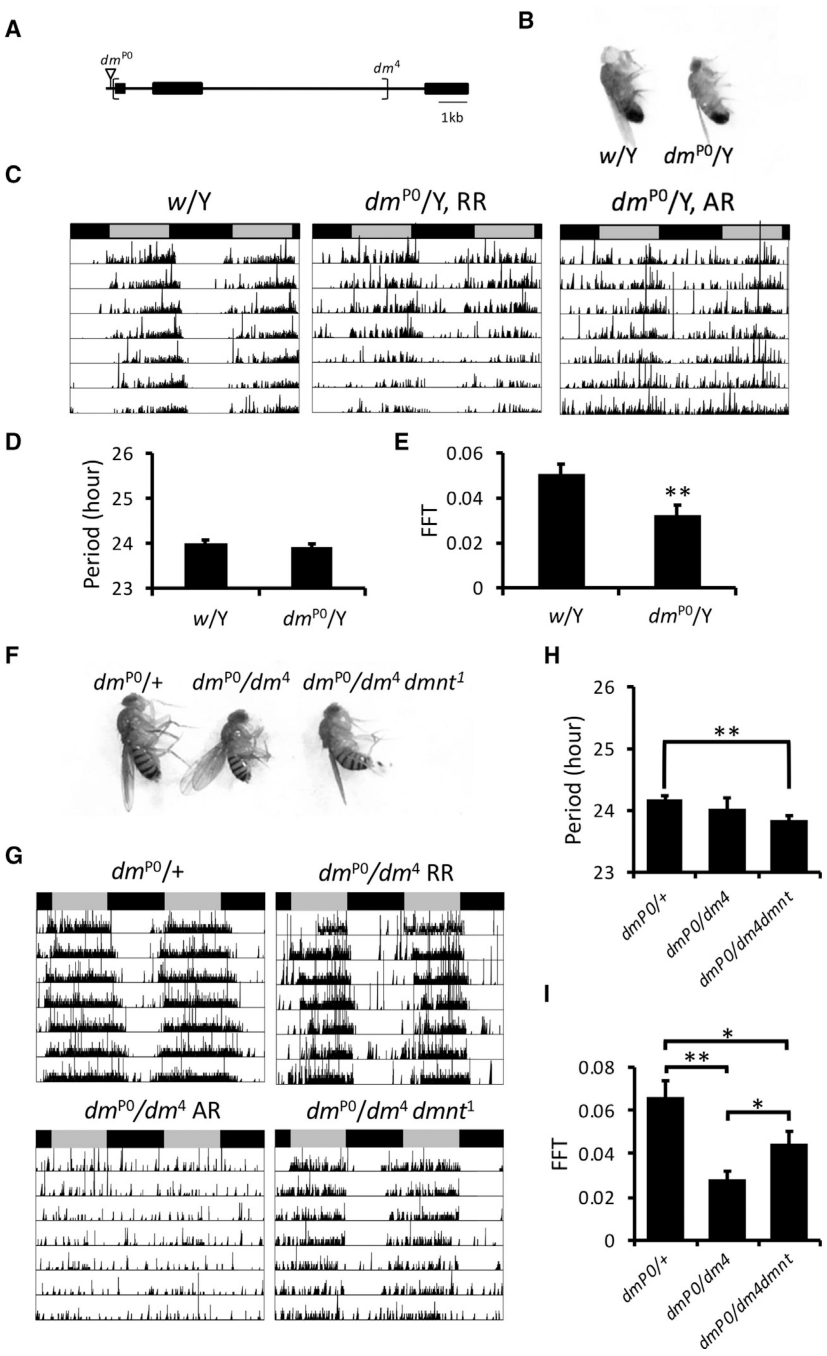

Figure 2. Hypomorphic *dMyc* Flies Exhibit Arrhythmic Behavior

(A) Schematic illustration (modified from Pierce et al., 2004) of the dm^{P0} and dm^{4} alleles of the *dMyc* gene.

(B) Picture of a dm^{P0}/Y fly versus its sibling control *w*/Y.

C) Representative actogram of *w*/Y, rhythmic (RR) dm^{P0}/Y, and arrhythmic (AR) dm^{P0}/Y flies. Flies were entrained to a light/dark cycle for 3 days before being monitored in constant darkness over 7 days.

(D and E) Period length (D) and rhythm strength (E) of rhythmic dm^{P0}/Y flies compared with control flies *w*/Y.

(F) Picture of dm^{P0}/+, dm^{P0}/dm^{4}, and $dm^{P0}/dm^{4}dMnt^{1}$ flies.

(G) Representative actogram of dm^{P0}/+, rhythmic (RR) dm^{P0}/dm^{4}, arrhythmic (AR) dm^{P0}/dm^{4}, and $dm^{P0}/dm^{4}dMnt^{1}$.

(H and I) Period length (H) and rhythm strength (I) of dm^{P0}/+, rhythmic dm^{P0}/dm^{4} (dm^{P0}/dm^{4}), and $dm^{P0}/dm^{4}dMnt^{1}$ (*dmP0/dm4dmnt*) flies. Data are represented as mean ± SEM. *p < 0.05 and **p < 0.001 by Student's t test.

See also Figures S3 and S4, and Table 1.

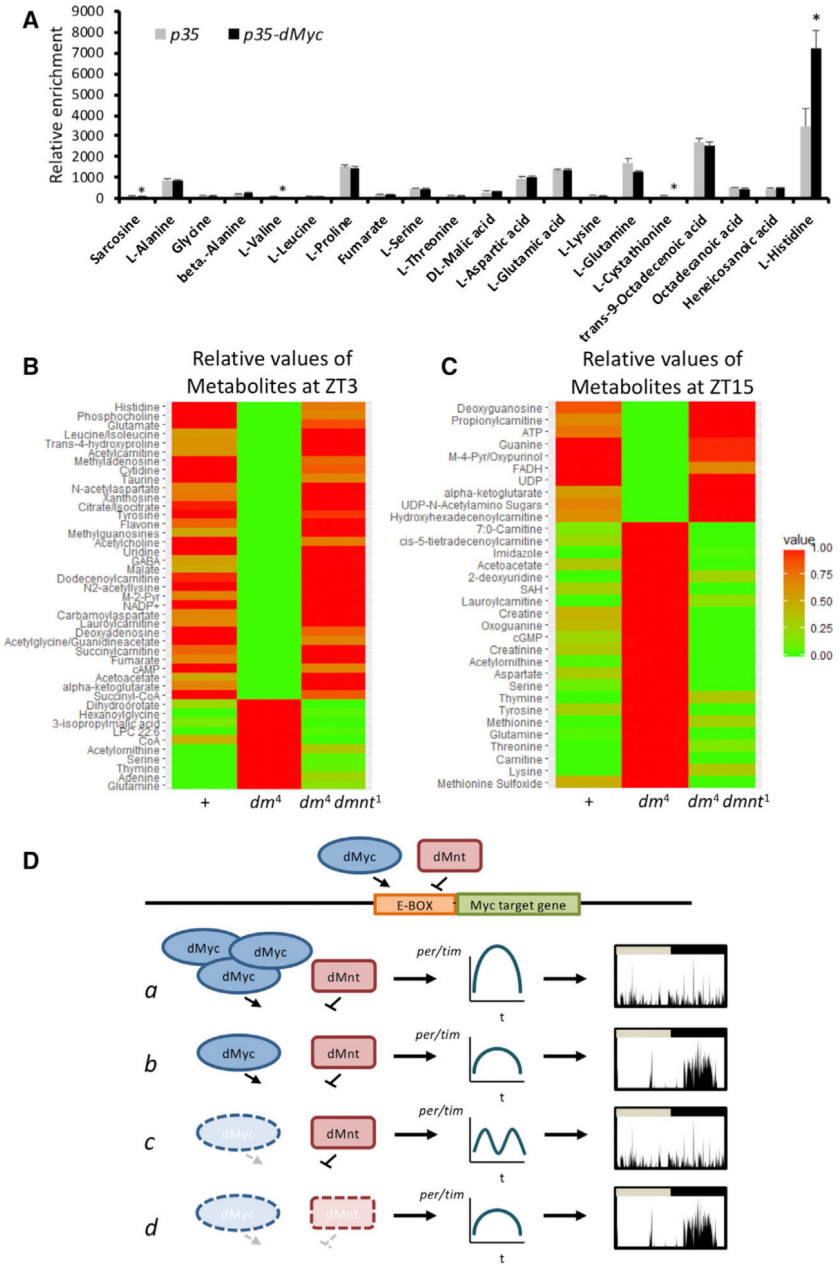

Figure 3. Metabolic Alteration in *dMyc*-Overexpressing and *dMyc* Hypomorphic Flies

(A) Relative enrichment of metabolites detected by gas chromatography-mass spectrometry (GC-MS) compared with Nor-leucine extracted from heads of control flies (cry^{24}*Pdf*-G > *p35*) or dMyc-overexpressing flies (cry^{24}*Pdf*-G > *Myc*+*p35*). Each data point is averaged from four pooled head extracts. Data are represented as mean ± SEM. *p < 0.05 and **p < 0.001 by Student's t test.

(B and C) Heatmap of metabolites (log value with normalized value between 0 and 1), detected by liquid chromatography-mass spectrometry (LC-MS) extracted from heads of dm^{P0}/+ (+), dm^{P0}/dm^{4} (dm^{4}), and $dm^{P0}/dm^{4}dMnt^{1}$ ($dm^{4}dMnt^{1}$) flies at ZT3 (B) and ZT15 (C).

(D) Model for the effect of dMyc and dMnt on the *Drosophila* molecular clock and locomotor activity. Schematic diagram of how dMyc and dMnt act on E-box-driven dMyc target genes (top). The bottom shows *per*/*tim* transcript oscillation (second column) and fly actogram (third column) in flies with dMyc overexpression (*a*), normal level of dMyc (*b*), low level of dMyc (*c*), and low level of dMyc with loss of dMnt (*d*).
See also Figure S5.

Table 1.

Circadian Locomotor Rhythmicity of Flies with Misregulated *dMyc* Expression

Genotype[a]	n	No. Rhythmic	% Rhythmic	Period (h) ± SEM	FFT ± SEM[b]
Circadian Locomotor Rhythmicity of *dMyc*-Overexpressing Flies in Constant Darkness					
w/Y;+;UAS-*Myc*, UAS-*p35*/+	32	32	100	23.80 ± 0.04	0.04 ± 0.004
w/Y;*Pdf*-G/+;*Pdf*-G/+	32	32	100	24.01 ± 0.04	0.06 ± 0.005
w/Y;*Pdf*-G/+;*Pdf*-G/UAS-*Myc*, UAS-*p35*	30	26	87	24.13 ± 0.10	0.02 ± 0.002[c]
cry^{24}-G/Y;*Pdf*-G/+;+	31	31	100	24.49 ± 0.05	0.10 ± 0.008
cry^{24}-G/Y;*Pdf*-G/+;+/UAS-*p35*	28	25	89	24.60 ± 0.05	0.04 ± 0.004
cry^{24}-G/Y;*Pdf*-G/+;+/UAS-*Myc*, UAS-*p35*	26	8	31	24.46 ± 0.07	0.03 ± 0.004[d]
w/Y;TUG/+;+	32	32	100	24.49 ± 0.04	0.05 ± 0.005
w/Y;TUG/+;+/UAS-*Myc*, UAS-*p35*	32	32	100	23.97 ± 0.06[e]	0.07 ± 0.009[f]
yw/Y;*tim*-G/+;+	54	30	56	25.32 ± 0.11	0.01 ± 0.001
yw/Y;*tim*-G/+;+/UAS-*Myc*, UAS-*p35*	69	21	30	24.77 ± 0.07[g]	0.01 ± 0.001
w/Y;*tim*-G/+;+/UAS-*p35*	32	29	91	24.52 ± 0.08	0.04 ± 0.004
w/Y;*tim*-G/+;+/UAS-*Myc*, UAS-*p35*	64	49	77	24.06 ± 0.05[h]	0.04 ± 0.003
Circadian Locomotor Rhythmicity of *dMyc* Mutant Flies in Constant Darkness					
w/Y; + ; +	28	24	86	24.01 ± 0.06	0.05 ± 0.004
dm^{P0}/Y; + ; +	32	13	41	23.91 ± 0.03	0.03 ± 0.005[i]
dm^{P0}/+; *bw*^{1}/+ ; *st*^{1}/+	31	29	94	24.17 ± 0.07	0.066 ± 0.008
dm^{P0}/*dm*^{4}; *bw*^{1}/+ ; *st*^{1}/+	30	9	30	24.03 ± 0.17	0.028 ± 0.004[j]
dm^{P0}/*dm*^{4} *dmnt*^{1}; *bw*^{1}/+ ; *st*^{1}/+	30	19	63	23.84 ± 0.07[k]	0.045 ± 0.006[l]

[a] *w*: *w*^{ISO31} isogenic white color eye mutant; *yw*: *y*^{1},*w*^{1118} yellow color body and white color eye mutant; *tim*-G: *tim*-Gal4; TUG: *tim*-(UAS)-Gal4; *cry*^{24}-G: *cry*-Gal4 (line 24); *Pdf*-G: *Pdf*-Gal4; *bw*^{1}: brown color eye mutant; *st*^{1}: light orange eye mutant.

[b] FFT, fast Fourier transform. Flies with FFT value > 0.01 are counted as rhythmic.

[c] $p < 0.005$ compared to *w*/Y;*Pdf*-G/+;*Pdf*-G/+ control, by Student's t test.

[d] $p < 0.05$ compared to *cry*^{24}-G/Y;*Pdf*-G/+;+ control, by Student's t test.

[e] $p < 0.005$ compared with *w*/Y;TUG/+;+ control, by Student's t test.

[f] $p < 0.05$ compared with *w*/Y;TUG/+;+ control, by Student's t test.

[g] $p < 0.005$ compared with *yw*/Y;*tim*-G/+;+, by Student's t test.

[h] $p < 0.005$ compared with *w*/Y;*tim*-G/+;+/UAS-*p35*, by Student's t test.

[i] $p < 0.01$ compared with *w*/Y; + ; + control, by Student's t test.

[j] $p < 0.005$ compared with *dm*^{P0}/+; *bw*^{1}/+ ; *st*^{1}/+ control, by Student's t test. $p < 0.05$ compared with *dm*^{P0}/*dm*^{4} *dmnt*^{1}; *bw*^{1}/+ ; *st*^{1}/+, by Student's t test.

[k] $p < 0.005$ compared with *dm*^{P0}/+; *bw*^{1}/+ ; *st*^{1}/+ control, by Student's t test.

[l] $p < 0.05$ compared with *dm*^{P0}/+; *bw*^{1}/+ ; *st*^{1}/+ control, by Student's t test.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse anti-dMyc	Eisenman Laboratory	Greer et al., 2013
Rabbit anti-α-Tubulin	Cell Signaling Technology	Cat#2144
Guinea Pig anti-PER (GP1140)	Cocalico Biologicals	Garbe et al., 2013
Rabbit anti-PDF (C7)	Developmental Studies Hybridoma Bank	AB_760350
Peroxidase AffiniPure Goat Anti-Guinea Pig IgG	Jackson ImmunoResearch Laboratories	AB_2337402
Goat anti-Mouse IgG1, HRP conjugate	Invitrogen	Cat#A10551
Chemicals, Peptides, and Recombinant Proteins		
Pierce ECL Western Blotting Substrate	Thermo Scientific	Cat#32106
TRIzol reagent	Invitrogen	15596018
Reverse transcription kit	Invitrogen	N8080234
Passive Lysis 5X Buffer	Promega	Cat#E1941
Pierce Lane Marker Reducing Sample Buffer	Thermo Scientific	Cat#39000
N-tertbutyldimethylsilyl-N-methyltrifluoroacetamide	Regis Technologies	Cat#77377–52–7
Critical Commercial Assays		
Power SYBR Green PCR Master Mix	Thermo Fisher	4368708
Deposited Data		
Drosophila melanogaster Kc cell ChIP-Seq	Yang et al., 2013	GEO: GSE39521 (GSM970847)
Experimental Models: Organisms/Strains		
D. melanogaster: Iso31	Sehgal Laboratory Stocks	N/A
D. melanogaster: w; *Pdf*-GAL4;*Pdf*-GAL4	Bloomington Drosophila Stock Center	BDSC_80939
D. melanogaster: yw; *tim*-GAL4	Sehgal Laboratory Stocks	N/A
D. melanogaster: w; *tim*-GAL4	Bloomington Drosophila Stock Center	BDSC_80941
D. melanogaster: cry^{24}-GAL4	Bloomington Drosophila Stock Center	BDSC_24774
D. melanogaster: w; UAS-*Myc* UAS-*p35*	Gift of Julie Secombe	Greer et al., 2013
D. melanogaster: w; UAS-*mCD8::GFP*	Bloomington Drosophila Stock Center	BDSC_ 5137
D. melanogaster: w; *tim* (UAS)-GAL4	Sehgal Laboratory Stocks	N/A
D. melanogaster: w; UAS-*p35*	Bloomington Drosophila Stock Center	BDSC_5073
D. melanogaster: cry^{24}-GAL4; *Pdf*-GAL4	This study	N/A
D. melanogaster: dm^{P0};*bw*^{1};*st*^{1}	Bloomington Drosophila Stock Center	BDSC_11298
D. melanogaster: dm^{P0}	This study	N/A
D. melanogaster: dm^{4}/FM7i, *Act*-GFP	Gift of Julie Secombe	Pierce et al., 2008
D. melanogaster: dm^{4}*dmnt*^{1}/FM7i, *Act*-GFP	Eisenman Laboratory Stocks	Pierce et al., 2008

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Oligonucleotides		
For rtPCR primers, see method details section ***Quantitative real-time PCR***	N/A	N/A
Software and Algorithms		
Image Studio software, version 2.0	LI-COR	https://www.licor.com/bio/products/software/image_studio_lite/
Fiji	Fiji	https://fiji.sc/
Prism	GraphPad	N/A
Excel	Microsoft Office	N/A
Clocklab software	ActiMetrics	https://www.actimetrics.com/products/clocklab/
DAMSystem3 Data Collection Software	TRIKINETICS	https://trikinetics.com/
MSD ChemStation software	Agilent	https://www.agilent.com/en/products/software-informatics/massspec-workstations/gc-msd-chemstation-software
MassLynx version 4.1	Waters	https://www.waters.com/waters/en_US/MassLynx-MS-Software/nav.htm?locale=en_US&cid=513662