m^6A mRNA Methylation: A New Circadian Pacesetter

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The purpose of m^6A methylation of RNA, first observed in the 1970s, has been a longstanding mystery. Fustin et al. now show that it regulates RNA processing and determines the period and oscillatory stability of the mammalian circadian clockwork.

Daily (circadian) rhythms, such as the sleep-wake cycle, adapt us to a 24 hr world. From fungi, to fruit flies, to humans, a canonical motif of the diverse clock mechanisms that drive such rhythms is a delayed feedback loop, in which circadian "clock genes" (*Per* and *Cry* in mammals) are suppressed by their protein products. This delay not only sets the period of the cycle and but also confers its intrinsic ability to oscillate. It is thought to arise from time-lags involved in transcription, translation, and protein shuttling into the nucleus (Koike et al., 2012; Meyer et al., 2006). Fustin et al. (2013) in this issue of *Cell* reveal a new point of control to circadian dynamics by showing that m^6A RNA methylation, a process of previously obscure function, sets the pace of circadian RNA processing and thereby determines clock speed and stability (Figure 1).

In contrast to its better known cousins, DNA and protein methylation (Sahar and Sassone-Corsi, 2013), the contribution of RNA-methylation to circadian or indeed any other physiological function, is uncharted territory. The recent description of transcriptome-wide m^6A RNA methylomes (Dominissini et al., 2012), and the association of mutations in m^6A RNA demethylase with particular metabolic diseases (Jia et al., 2011) has heightened the mystery as to the normal physiological functions of this extensive regulatory process.

The relative abundances of the methyl donor cosubstrate S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH), its inhibitory by-product, determine the methylation potential of a cell. The starting point for Fustin et al., therefore, was to test the circadian effect of tilting the SAM/SAH ratio using 3-deazaadenosine (DAA), an inhibitor of methylation. In cultured cell lines and in mice, inhibition of methylation lengthens the circadian period by 3 hr and 1 hr, respectively, monitored as bioluminescent circadian gene expression and rest-activity cycles. High-throughput sequencing shows that both circadian genes and RNA-processing genes are dysregulated in cells treated with DAA, the latter likely an autoregulatory response to disrupted RNA processing. m^6A-immunoprecipitation and high-throughput sequencing reveals a potential mechanism behind this: a number of circadian RNAs, including *Per1* and *Per2*, as well as clock output genes, carry m^6A sites and are downregulated by DAA. In contrast, a second methylation mark on circadian transcripts, m^7cap, which stabilizes the 5′ end of the RNA, is not affected by DAA.

What did these m^6A methylation events do to RNA processing and how might they be linked to the clock? Pulse-chase labeling of total RNA and targeted qPCR establishes that DAA prolongs nuclear retention of circadian RNAs, even in the absence of de novo transcription, despite an overall reduction in steady-state pre-mRNA and in cytoplasmic mRNA. This DAA-dependent stabilization of nuclear RNA is evident for *Per* and other circadian transcripts and is accompanied by a delayed decline of Per2 protein expression that is independent of any effect on translation rate.

Does DAA slow the clock because it extends the nuclear phase of RNA processing, specifically by altering RNA methylation marks? To test this, Fustin et al. use siRNA to reduce the activity of *Mettl3*, which encodes the SAM binding subunit of the relevant methyltransferase. They find a pronounced extension of circadian period length in cell lines that is associated with a reduction in the expression of m^6A methylation marks in circadian transcripts. Moreover, overexpression of wild-type *Mettl3* causes a period shortening when compared to catalytically dead Mettl3 (although the period of cells overexpressing either version of *Mettl3* is longer than is typically seen). The key result, however, is that suppression of Mettl3 delays the nuclear exit of mature *Per2* and *Bmal1* (*Arntl*) mRNA. Thus, silencing of m^6A methylation by Mettl3 suppression is sufficient to delay RNA processing and slow the clock.

Overall then, Fustin et al. have discovered a critical physiological function of m^6A methylation in setting the pace of the circadian cycle. This is a welcome addition to understanding how an oscillation of approximately 24 hr period is established biochemically but questions remain. Why is the contribution of m^6A methylation more significant on the falling rather than the rising phase of circadian transcription? Is this a simple consequence of the state of RNA or are other regulatory cofactors rhythmically expressed? Are some circadian factors, for example Per or Bmal1, more important than others for pacesetting by m^6A methylation? With circular processes such as the clock, cause and effect are often difficult to tease apart. And what is the relative contribution of m^7capping, inhibition of which also lengthens circadian period in a manner additive to that of m^6A suppression, generating an

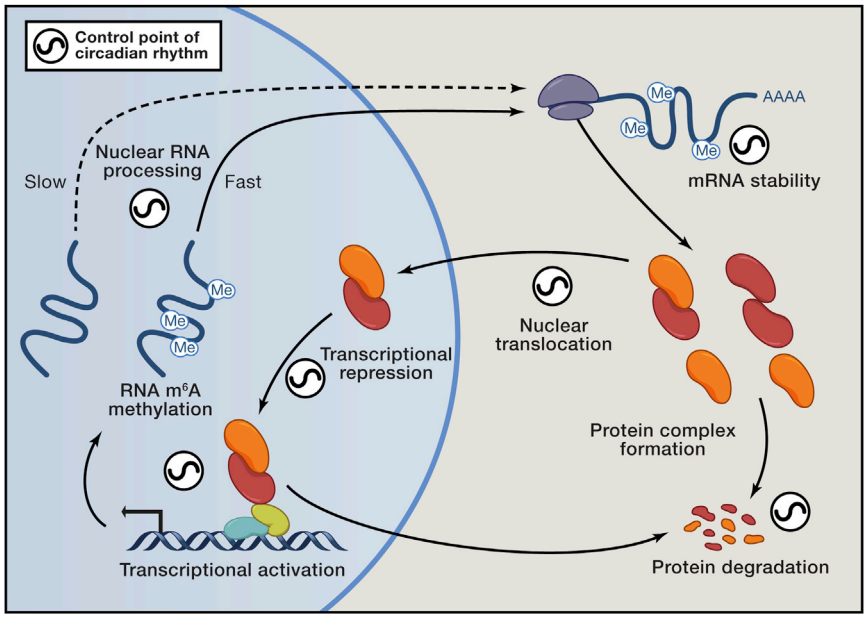

Figure 1. m^{6}A Methylation: An Early Pace-Setter in the Circadian Loop

Schematic view of the core feedback loop that constitutes the circadian molecular time-keeper. The sinusoidal symbol indicates control points for oscillation and period setting, including rates of clock gene transcription, mRNA poly-A tail length, protein complex formation, nuclear transfer, negative transcriptional feedback, and clock protein degradation. Pharmacological or genetic interference with all of these processes can speed or slow the clock, or suspend oscillations altogether. Fustin et al. now show that m^{6}A methylation puts mRNA production onto a fast-track for facilitating nuclear processing and so provides an early point of control in setting the dynamics of the feedback loop.

oscillation in cells with a remarkable ~31 hr aggregate period.

Lifting the lid on these events will provide a finely resolved understanding of circadian progression, but perhaps the more significant outcome of this study is to demonstrate a cellular function of m^{6}A methylation: namely to regulate the nuclear processing of mRNA. This means that RNA can be modified to carry more information beyond its familiar base sequence. Or to put it another way, the base sequence is not the sole intrinsic determinant of mRNA function. Depending on location (within long exons, around stop codons, within 3′ UTRs), m^{6}A methylation has been implicated in RNA splicing and translational control, and some m^{6}A-binding factors that may contribute to these processes have been identified (Dominissini et al., 2012). Fustin et al. say little about splicing, which is not a pronounced circadian phenotype (McGlincy et al., 2012), but show that m^{6}A methylation normally accelerates processing and nuclear export of RNA. Because the clock is so intimately involved in metabolic gene regulation (Green et al., 2008), the recent discovery that the fat mass and obesity-associated protein (FTO), mutations of which have the eponymous effect, is in fact the m^{6}A demethylase, provides a further possible bridge between RNA methylation and circadian gene expression. RNA methylation is, of course, not an exclusively circadian process, but perhaps the circadian pacemaker was the ideal place to reveal the (cryptic) contribution of m^{6}A methylation because of the long time course and "temporal discipline" of the component events of the clockwork. There may even be a general lesson here for cell biologists and biochemists with interests in particular processes that occur rapidly and with no temporal coherence within and between cells. Try working in synchronized tissue around circadian time when the events you wish to understand are teased out and made visible. As shown by Fustin et al., clocks can be a boon to more than clock biology.

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