

A Role for the *Drosophila* Fragile X-Related Gene in Circadian Output

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Summary

Mutations that abolish expression of an X-linked gene, *FMR1*, result in the pathogenesis of fragile X syndrome, the most common form of inherited mental retardation [1, 2]. To understand the normal function of the FMR1 protein, we have produced fly strains bearing deletions in a *Drosophila* homolog of *FMR1* (*dfmr1*). Since fragile X patients show a number of abnormal behaviors including sleep problems [3, 4], we investigated whether a loss-of-function mutation of *dfmr1* affect circadian behavior [5–8]. Here we show that under constant darkness (DD), a lack of *dfmr1*

expression causes arrhythmic locomotor activity, but in light:dark cycles, their behavioral rhythms appear normal. In addition, the clock-controlled eclosion rhythm is normal in DFMR1-deficient flies. These results suggest that DFMR1 plays a critical role in the circadian output pathway regulating locomotor activity in *Drosophila*.

Results and Discussion

The fruit fly *Drosophila* has proven to be a powerful tool for the genetic dissection of biochemical pathways for human neurological diseases [9, 10]. Recently, a *Drosophila* homolog of *FMR1* (the abbreviation “*dfmr1*” will be used hereafter) has been identified [11]. *Drosophila* and vertebrate FMR1 proteins share a number of topographical landmarks, including two types of RNA binding motifs [11]. To obtain fly strains bearing mutations in *dfmr1*, we mobilized a P{EP} element [12] inserted near the *dfmr1* transcriptional start site on the EP(3)3422 chromosome to produce several partial deletions of *dfmr1* (Figure 1A). One such mutation was selected for further characterization. We refer to this mutation as *dfmr1*^{B55}. In this mutant, imprecise excision of the EP element generated a 2.5 kb deletion of genomic DNA, which included exons 2, 3, and 4 of the *dfmr1* gene. The deletion removed the translational start codon and the first 59 codons (Figure 1A). The homozygous *dfmr1*^{B55} mutant proceeds into adulthood without expressing a discernible morphological defect. Western blot analysis with anti-DFMR1 antibodies revealed that the DFMR1 protein was expressed throughout development in wild-type controls (Figure 1B). In contrast, there is no DFMR1 protein of the expected size, 85 kDa, in homozygous *dfmr1*^{B55} mutant flies (Figure 1B). These results show that *dfmr1*^{B55} is a null mutation of the *dfmr1* gene.

To test for behavioral effects of the mutation, *dfmr1*^{B55} flies were assayed for circadian locomotor activity [13]. Over the course of 24 hr in light:dark (LD) cycles, wild-type flies are entrained to (or synchronized with) LD cycling and exhibit a substantial locomotor activity rise during the second half of the day (e.g., [14]). Homozygous *dfmr1*^{B55} flies appeared to behave in this manner (i.e., before lights-off) and exhibited 24 hr periodicity under such LD (data not shown). Clear anticipations of lights-off suggest that the LD behavior of *dfmr1*^{B55} flies is truly clock dependent and not simply masking behavior. In contrast, locomotor behavior of *dfmr1*^{B55} in constant darkness was arrhythmic. Actograms of *dfmr1*^{B55} individuals showed that most mutants lost rhythmicity within a couple of days after transfer to DD (Figure 2B). By periodogram analysis, 86% of *dfmr1*^{B55} flies exhibited arrhythmicity in DD for 15 days (Table 1). Of the 50 *dfmr1* mutant flies tested, the 7 “escapers” that were rhythmic appear to have a wild-type 24 hr rhythm (Table 1). Although period length of escapers in clock mutants are usually affected (e.g., see [15, 16]), the *dfmr1*^{B55} mutation does not appear to affect period length of the escapers. To confirm that disruption of the *dfmr1* gene is directly

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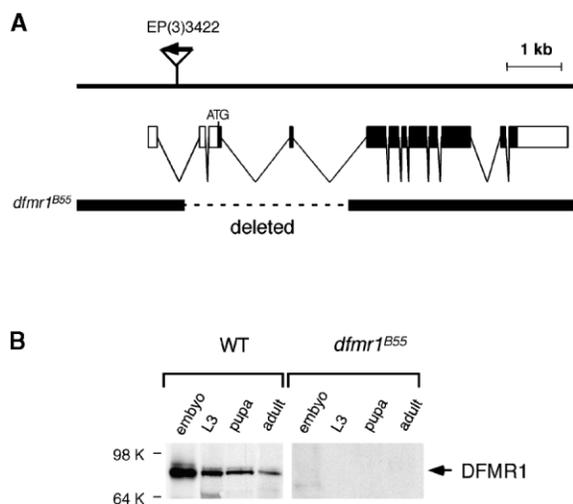


Figure 1. Characterization of the *dfmr1* Deletion Mutant *dfmr1^{B55}*
(A) Structure of the *dfmr1* locus is shown. Exons are indicated with open boxes, and the closed portion is the protein coding region. The translation initiation start site is located in exon 3. The position of EP(3)3422 is represented as triangle with an arrow pointing in the direction of GAL4-induced transcription. The deleted genomic region of the *dfmr1^{B55}* chromosome is shown. Sequence information from the Berkeley *Drosophila* Genome Project (BDGP; [37]) reveals that the *dfmr1* locus is positioned in a small region on the cytological location 85F11-12, on the right arm of the third chromosome. There is no other gene that overlaps in the *dfmr1* locus.
(B) Developmental Western blot analysis with anti-DFMR1 antibodies reveals that there is no DFMR1 protein of the expected size of 85 kDa in homozygous *dfmr1^{B55}* mutant flies.

responsible for the circadian phenotype, we transformed mutant flies with a P element containing wild-type *dfmr1* genomic sequences. The arrhythmic locomotor activity phenotype in DD was ameliorated by an introduction of a *dfmr1* minigene (Figure 2C and Table 1). This demonstrates that the arrhythmic phenotype is caused by the *dfmr1^{B55}* mutation rather than a second site mutation elsewhere on the chromosome. The “rescued” flies have altered periods, with 1 hr longer than wild-type (Figure 2C and Table 1). This might be due to dosage effects on period of the *dfmr1* locus as is often the case for many clock mutants (e.g., [17, 18]). In fact, expression levels of *dfmr1* mRNA in heads of the rescued flies appear to be significantly higher than those in wild-type flies (data not shown).

To investigate whether other rhythms are also affected in flies bearing DFMR1 deficiencies, homozygous *dfmr1^{B55}* flies were tested for an independent manifestation of circadian output, eclosion (emergence of the adult fly from the pupal case) [5]. Although eclosion occurs only once in the lifetime of an individual fly, it occurs repeatedly and rhythmically in a population of flies of diverse ages [19, 20]. In stark contrast to what was found for locomotor activity (Figure 2), homozygous *dfmr1^{B55}* flies emerged from their pupal cases in a circadian-gated manner with a phase and amplitude very similar to those observed in normal flies (Figure 3). Under the experimental conditions used in this study we clearly observed normal eclosion rhythm even at day 5 of DD. Therefore, rhythmic eclosion in the *dfmr1^{B55}* mutant persists with a high amplitude after prolonged incubations

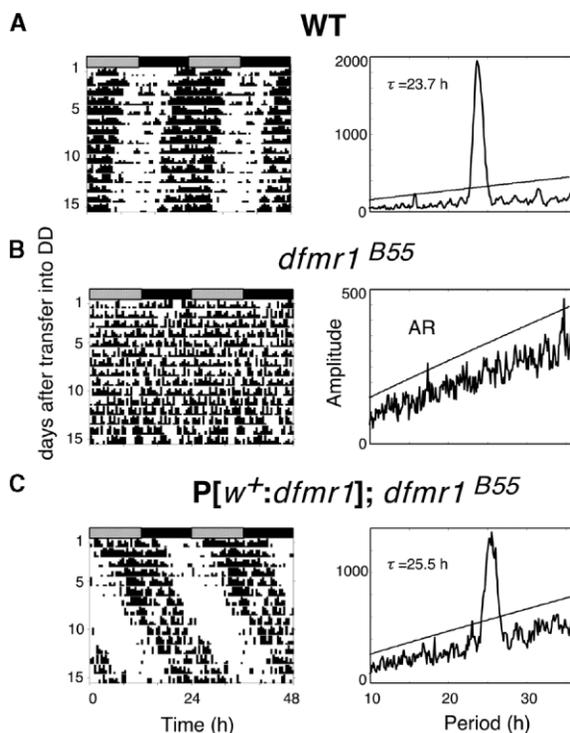


Figure 2. *dfmr1^{B55}* Flies Are Arrhythmic

Representative locomotor activity records of three individual flies, (A) one yellow-white wild-type (wt), (B) one *dfmr1^{B55}*, and (C) one *dfmr1^{B55}* with a *dfmr1* minigene ($P[w^+ : dfmr1]; dfmr1^{B55}$), in constant darkness (DD). Adult flies were entrained to cycles of 12 hr light:12 hr dark cycle (12L:12D) for at least 2 days, and their locomotor activity was measured subsequently in DD. The data are double plotted: activity from days 1 and 2 is on the first line, days 2 and 3 on the second, and so on. The dark bars indicate the subjective night and the gray bars indicate the subjective day. The results of chi-square periodogram analysis are shown in each right panel. The line indicates 99% confidence level. During DD, most *dfmr1^{B55}* flies lost locomotor activity rhythm and the rhythmicity was rescued by an introduction of a *dfmr1* minigene. The genomic rescue construct contained a 12 kb *Bam*HI-*Kpn*I fragment of the *dfmr1* locus.

in DD, showing that the period of eclosion rhythm is not affected by the mutation. Thus, these results indicate that DFMR1 is not universally required for the manifestation of overt circadian rhythms.

To investigate whether the *dfmr1^{B55}* mutation changes the molecular oscillation of known clock components

Table 1. *dfmr1^{B55}* Causes Arrhythmicity

Genotype	$\tau \pm$ SD	% AR	n
Wild-type	23.5 \pm 0.3	7.9	63
<i>w; +; dfmr1^{B55}</i>	23.8 \pm 0.5	86.0	50
<i>w; P[w⁺: dfmr1]; dfmr1^{B55}</i>	24.8 \pm 1.6	28.8	52

The genotypes of normal, mutant, and transgenic flies are listed at left. The *y w* flies were used as a wild-type (wt). Flies were entrained in a 12 hr light:12 hr dark cycle (12L:12D) for at least 2 days and then transferred to constant darkness (DD) for 15 days. Period length was calculated from all data collected using chi-square periodogram analysis (ClockLab, Actimetrics, Inc.) The genomic rescue construct contained a 12 kb fragment of the *dfmr1* locus (see Figure 1A). τ indicates free-running period, means \pm SD in DD. Percent AR indicates percentage of flies that are arrhythmic.

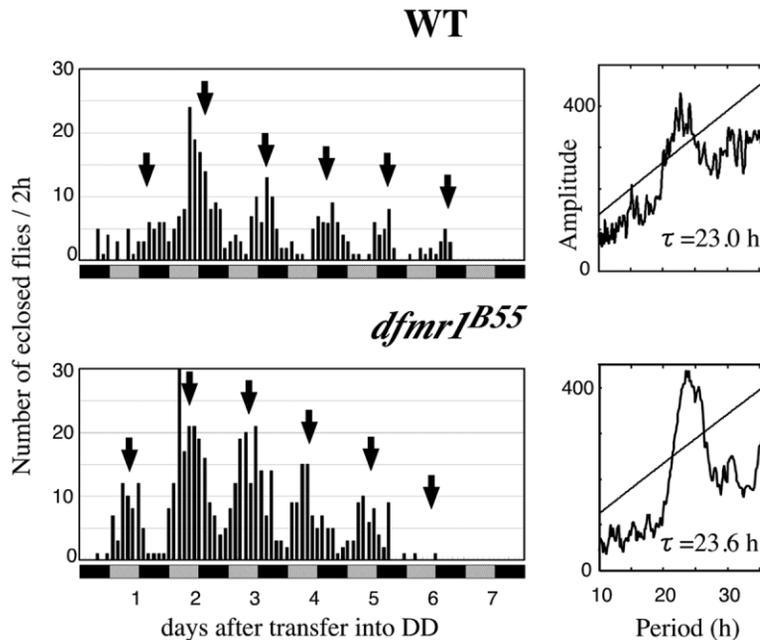


Figure 3. Normal Eclosion Rhythm in *dfmr1^{B55}* Mutants

Eclosion rhythm is not affected by the *dfmr1^{B55}* mutation. Eclosion profiles of yellow-white wild-type (WT) and *dfmr1^{B55}* flies in DD are shown. The number of flies eclosing from their pupal cases is plotted as a function of circadian time. Black and hatched horizontal bars at the bottom of the panels represent subjective night and day, respectively. For eclosion assays, larvae were reared in 12L:12D, then pupae were transferred to DD at 25°C. Pupae were glued to disks that subsequently were placed onto the eclosion monitor as described previously [19]. Emerging flies were counted using an eclosion monitor (TriKinetics, Inc.) in DD. The activity period was determined by chi-square periodogram analysis (ClockLab, Actimetrics, Inc.).

[5–8], we looked at PER and TIM protein time courses on Western blots using protein extracts that were prepared from heads of flies [21, 22]. Flies were entrained for three LD cycles and then transferred to DD conditions. We assayed the last day of LD and subsequent 3 days of DD for wild-type and *dfmr1^{B55}* genotypes (Figure 4). In wild-type flies, PER and TIM undergo daily fluctuations in abundance and electrophoretic mobility (Figure 4A). These results are similar to those previously observed for wild-type flies [7, 8]. Differences in the apparent molecular weight of PER are due to the circadian regulation of its phosphorylation [7, 8]. In LD, both proteins oscillated in *dfmr1^{B55}* flies as they did in wild-type controls (Figure 4A). These results, together with the observation that the LD behavior of the *dfmr1^{B55}* flies appears normal, suggest that DFMR1-deficient flies have an active circadian pacemaker that can be entrained by light-dark cycles. However, in DD, phase differences of PER and TIM protein accumulation between the genotypes could be detected (Figure 4B). For *dfmr1^{B55}* in DD, transitions from high-molecular type to low-molecular type PER are not as sharp as they are for wild-type controls. For TIM, the levels of expression of the protein are not grossly altered for the two genotypes, and the oscillation in *dfmr1^{B55}* flies remains quite robust even in the third day of DD conditions when most of the flies display arrhythmic locomotor activity (Figure 2). However, TIM oscillates with a slight phase delay in *dfmr1^{B55}* in DD, indicating that *dfmr1^{B55}* alters expression of TIM as for PER. Altered cycling of PER and TIM in *dfmr1^{B55}* flies in DD could be due to a measure of the developing desynchrony among many fly heads because most flies are behaviorally arrhythmic (Figure 2). However, we could not exclude the possibility that there might be feedback effects of the DFMR1-mediated output pathway on the pacemaker.

To determine the pattern of expression of DFMR1, the levels of DFMR1 protein accumulating during a circadian cycle were examined by Western blot analysis (Figure 4C). No significant difference of DFMR1 protein levels

was detected in head extracts from each of the time points examined in both LD and DD conditions, showing that levels of DFMR1 protein are not under circadian control.

The signaling mechanism that mediates output from central clock proteins to behavior is poorly understood [5]. Several output genes have been identified so far in *Drosophila* [23–25]. The circadian phenotype displayed by *dfmr1^{B55}* flies is reminiscent of what happen in flies deficient in either protein kinase A (PKA) or NF1, the protein product of the *neurofibromatosis-1* gene. However, whether mutations in *dfmr1* and PKA or NF1 lead to arrhythmic activity by similar or different pathways is currently not clear. An intriguing finding is that DFMR1-deficient flies manifest normal eclosion rhythms (Figure 3), suggesting that the daily timing of developmental rhythm might not require DFMR1. These results suggest that eclosion and locomotor rhythms are mediated by different neurons that use the same pacemaker molecules [26].

How, then, might DFMR1 participate in an output pathway associated with the manifestation of overt locomotor activity rhythms? DFMR1 is a cytoplasmic RNA binding protein associated with ribosomes, as is the case for mammalian FMR1 [2, 27]. Therefore, DFMR1 could regulate posttranscriptionally the expression of specific target mRNAs that control output functions. Given the recent findings showing that a secreted neuropeptide, pigment-dispersing factor (PDF) is a critical circadian mediator that couples a molecular clock to circadian rhythms in locomotor activity and can in turn influence function of the clock [28–31], it will be important to follow the fate of this peptide in *dfmr1^{B55}* flies. Alternatively, since in both the human and *Drosophila*, the fragile X protein (FMR1 and DFMR1) has been found to have a role in synaptic growth [32, 33], DFMR1 might regulate expression of mRNAs required for synaptic function and structure such as mRNA for microtubule-associated protein MAP1B [33]. Proteins that affect neu-

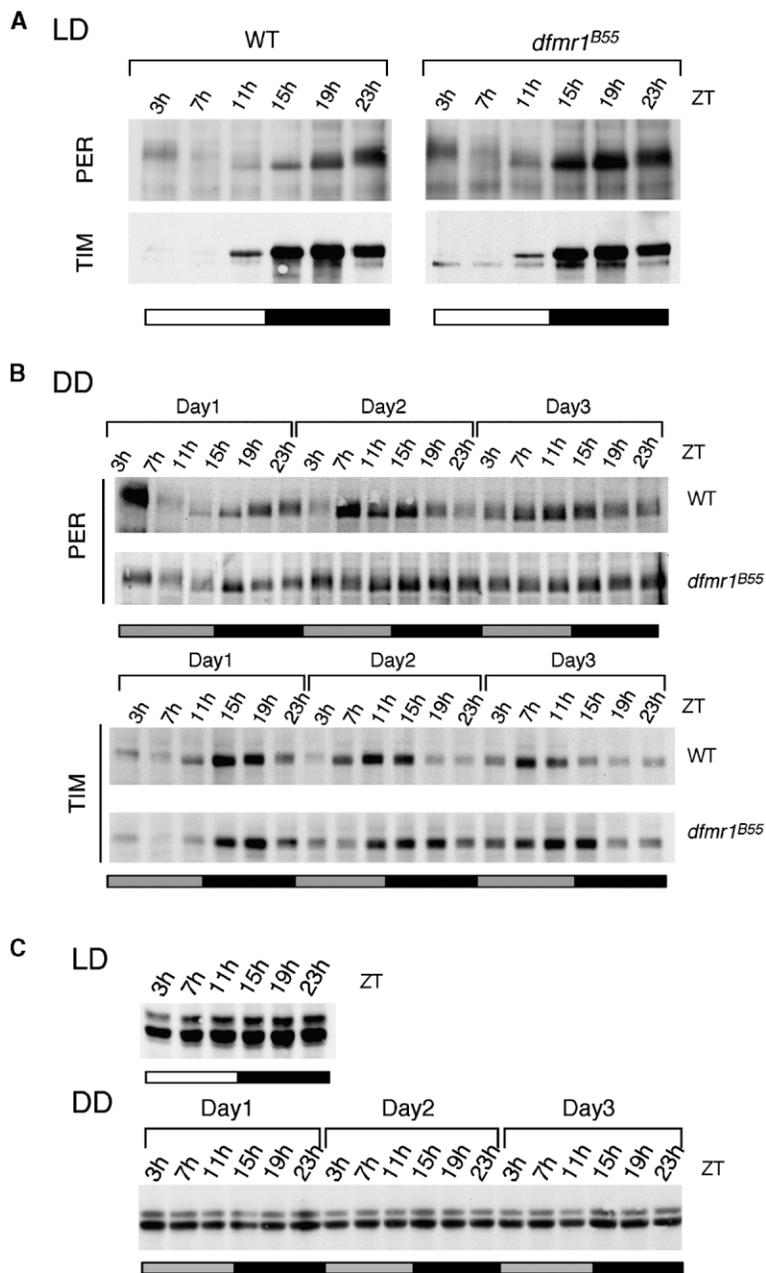


Figure 4. The Timing of PER and TIM Protein Oscillations Is Altered in *dfmr1^{B55}* Adult Heads in DD

(A and B) Western blot analysis of TIM and PER proteins from wild-type (yellow-white:wt) and *dfmr1^{B55}* head extracts, collected at various times (indicated at top) of day during an LD cycle (A), or collected at various times over 3 days during an DD cycle (B). The light cycle is indicated by the open (light) and filled (dark) bars in (A), and bars below in (B) represent prior LD cycles. Note the successive phase changes in DD for wild-type (wt) and *dfmr1^{B55}*, respectively. A crossreading non-specific band (not shown) indicated equal loading. Experiments for wild-type (wt) and *dfmr1^{B55}* were performed three times.

(C) DFMR1 protein levels are not under circadian control. DFMR1 protein levels are constant during the course of a daily cycle. Wild-type (wt) fly heads were collected during one day in LD and after the subsequent 3 days in DD, as reflected by the bar underneath, with time shown at top in 24 hr cycles. Blotted protein extracts were probed with an anti-DFMR1 antibody, and with anti-ribosomal protein P0 [38] to ensure equal loading (data not shown). DFMR1 levels were constant during days assayed. Identical results were obtained when we used two different anti-DFMR1 antibodies (data not shown).

ronal development and/or function are expected to affect circadian rhythms that are driven by neuronal pacemakers. For example, similar results to those found in *dfmr1^{B55}* flies were obtained when synaptic transmission was blocked using the tetanus-toxin light chain in *per/tim*-expressing cells, i.e., less effect on locomotor activity during LD but largely arrhythmic during constant dark conditions [34].

It is tempting to speculate that sleep problems observed in fragile X patients [4] are attributable to alterations of circadian rhythmicity because sleep propensity is modulated by a circadian clock [35]. Since the molecular mechanisms involved in the generation of circadian rhythms are remarkably similar between *Drosophila* and mammals [5, 36], our *Drosophila* model of fragile X syndrome provides insight into the sleep-wake cycles of

animals. The discovery of modifiers involved in DFMR1-mediated regulation of circadian rhythms reveals additional molecular mechanisms in the fragile X syndrome.

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