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Extensive Oscillatory Gene Expression during *C. elegans* Larval Development

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SUMMARY

Oscillations are a key to achieving dynamic behavior and thus occur in biological systems as diverse as the beating heart, defecating worms, and nascent somites. Here we report pervasive, large-amplitude, and phase-locked oscillations of gene expression in developing *C. elegans* larvae, caused by periodic transcription. Nearly one fifth of detectably expressed transcripts oscillate with an 8 hr period, and hundreds change >10-fold. Oscillations are important for molting but occur in all phases, implying additional functions. Ribosome profiling reveals that periodic mRNA accumulation causes rhythmic translation, potentially facilitating transient protein accumulation as well as coordinated production of stable, complex structures such as the cuticle. Finally, large-amplitude oscillations in RNA sampled from whole worms indicate robust synchronization of gene expression programs across cells and tissues, suggesting that these oscillations will be a powerful new model to study coordinated gene expression in an animal.

INTRODUCTION

As a ubiquitous feature in biology, oscillations have been considered one of its fundamental dynamic principles that drive processes away from equilibrium (Hasty et al., 2010). Part of their utility stems from the broad range of timescales over which oscillations can drive dynamic behavior, from the millisecond periods of rhythmic neuronal spiking to the 24 hr periods of circadian clocks. Accordingly, the theoretic understanding and experimental design of oscillators has also been a major field of research in mathematical and synthetic biology (Hogenesch and Ueda, 2011; Tyson et al., 2008).

Biological oscillations drive not only rhythmic behaviors of cells, tissues, and organisms but also periodic gene expression programs. Circadian clocks in particular direct extensive rhythmic gene expression to help organisms anticipate environmental changes caused, for instance, by daily cycles of light and temperature (Hogenesch and Ueda, 2011). In mammals, separate, peripheral clocks act in distinct tissues. They are synchronized through a central pacemaker in the suprachiasmatic nucleus of the hypothalamus and drive rhythmic expression of large sets of genes (Mohawk et al., 2012). Interestingly, although individual peripheral clocks can direct oscillations in 3%–10% of active genes in a given tissue, there is little overlap in the sets of genes that undergo periodic expression in different tissues (Mohawk et al., 2012). As exemplified by the vertebrate segmentation clock, oscillators can also be utilized to drive periodic developmental events (Kageyama et al., 2012), but, generally, much less is known about rhythmic gene expression in developmental contexts than in circadian contexts. In *C. elegans*, only ~20 genes are known to be periodically expressed during development (Johnstone and Barry, 1996; Lassandro et al., 1994; Hashmi et al., 2004; Davis et al., 2004; Frand et al., 2005; Hao et al., 2006; Kostrouchova et al., 2001; Gissendanner et al., 2004; Jeon et al., 1999; Monsalve et al., 2011; McMahon et al., 2003). However, because systematic and quantitative studies have not been performed, the prevalence of periodic gene expression has remained unknown, and insights into general principles underlying the rhythmic expression patterns have been lacking.

Here, using genome-wide and temporally highly resolved gene expression studies, we reveal extensive periodic gene expression during *C. elegans* larval development, affecting a fifth of expressed genes. Our characterization reveals robust, transcriptionally driven oscillations across a continuum of phases that result in periodic translation, and, thus, promote periodic developmental processes such as molting. These results highlight an unanticipated dynamic and complexity of gene expression patterns during *C. elegans* development. Moreover, we propose that a unique combination of features makes these oscillations a powerful model to study coordinated gene expression in an animal.

RESULTS

The Expression of Thousands of Genes Oscillates Extensively during Development

To obtain insight into the dynamics of gene expression during *C. elegans* larval development, we performed a high-resolution and genome-wide time course analysis. Synchronized L1 stage larvae were placed on food at 25°C, and samples collected hourly over a 16 hr period that covered development from

L3 to the young adult stage. Unexpectedly, cross-correlation plots of gene expression profiles obtained by mRNA sequencing revealed a periodic increase in similarity, rather than a progressive decrease during development (Figure 1A). For instance, expression patterns at t = 21 hr were noticeably more similar to expression patterns at t = 27 hr and 28 hr than to those at 24 hr.

To identify trends in gene expression levels that could explain this observation, we performed principle component (PC) analysis. Three PCs explained ~92% of the variation (Figure 1B). The loadings of the first displayed a monotonic increase over time, thus revealing a set of genes whose expression is altered continuously along the time course. These are mostly related to germline development and function (see below).

The loadings of the second and third PCs displayed an oscillatory pattern with a period of 8 hr (Figure 1B). This suggested that a large number of genes oscillated at a common period but with different phases (Experimental Procedures). To test this possibility, we fitted a general cosine function with a fixed period of 8 hr and unknown phase and amplitude. Since the first PC explained a large part of the data, we included it as an additional component when performing the fit. Comparing PC1 to the oscillation amplitude for every gene revealed two distinct classes of dynamically changing genes with largely mutually exclusive membership: genes that increase their expression and those that oscillate (Figure 1C). This allowed us to categorize genes into "flat" (black), "rising" (green), and "oscillating" (red) based on empirically chosen cutoffs (Supplemental Experimental Procedures). The oscillating class contained the previously reported ~20 genes with periodic expression. However, oscillatory expression is much more pervasive in that this class comprised 2,718 genes, corresponding to 18.9% of 14,378 expressed genes (Table S1 available online).

Following cosine wave fitting, we could examine amplitudes and phases of oscillations more readily and observed large changes (Figure 1D; note that the amplitude of the cosine function equals half the magnitude of change between trough and peak expression). For instance, the levels of all 2,718 "oscillating" genes changed more than 2.1-fold, and those of >400 genes exceeded a 10-fold change. For comparison, comprehensive studies of circadian clocks revealed typical median expression changes of 2-fold (Hughes et al., 2009; Duffield, 2003). The extent of changes seen here is yet more striking when considering that we examined RNA from whole, undissected worms, not from specific tissues or cells.

A unifying theme among the previously identified, periodically expressed genes appears to be a connection to molting, as determined by the molecular nature of the encoded proteins and/or mutant phenotypes (Johnstone and Barry, 1996; McMahon et al., 2003; Lassandro et al., 1994; Hashmi et al., 2004; Davis et al., 2004; Frand et al., 2005; Hao et al., 2006; Kostrouchova et al., 2001; Gissendanner et al., 2004; Jeon et al., 1999; Monsalve et al., 2011). Molting occurs with an 8 hr periodicity at the end of each larval stage and involves the generation of a new cuticle and shedding of the old one (Monsalve and Frand, 2012). As we will discuss below, several classes of genes with roles in molting are also enriched among the oscillatory genes that we identify here. Nonetheless, rather than being restricted to the times of the molt, rhythmic gene expression occurred in all phases (Figure 1D; a full period corresponds to 8 hr or 360°, and a phase difference of 45° thus corresponds to a peak shift by 1 hr; the molt occurs roughly between 180° and 270°). This was also readily apparent when plotting the expression changes of all "oscillating" genes in a heatmap (Figure 1E). A somewhat larger number of genes were in the 90°, 135°, and 315° bins. RT-qPCR confirmed the oscillations and phases of expression for 18 genes (Figure 1F) in RNA sampled from an independent time course.

Oscillations Are Independent of Life History and Synchronization Procedure

To assess the robustness of the oscillations and potential effects of life history (Karp et al., 2011), we analyzed mRNA expression patterns in *C. elegans* that had exited from dauer diapause arrest. In contrast to continuous larval development, which proceeds from L1 through the L4 stage, dauer animals arrest development in an alternative L3 stage, for instance, in response to starvation (Hu, 2007). Synchronous exit from dauer can be induced by providing food to such dauer larvae, which will subsequently go through an L3-to-L4 molt and continue development through the L4 into the adult stage.

We placed dauer-arrested animals on food and collected hourly samples over 22 hr. Cross-correlation plots again revealed periodical similarity of the expression patterns (Figure 2A). A heatmap that displayed changes in the expression patterns of all genes that were periodically expressed following continuous development revealed strikingly similar oscillatory expression patterns for postdauer animals (Figure 2B). Thus, oscillations are highly robust and not substantially influenced by life history. This was in fact also true for the steadily upregulated genes, whereas more diversity was seen with the bulk of genes that did not follow either of these two patterns (Figure 2B).

Oscillations Are Locked in Phase over Time

To further explore the robustness of the oscillations, we followed them over time by preparing an extended time course where we sampled worms every 2 hr between 18 hr and 36 hr of development at 25°C, thus covering the entire L3 stage along with the later stages investigated in the first experiment. Beyond confirming that the genes with oscillatory expression during the L4 stage were also rhythmically expressed during the L3 stage (see Figure 5 below), this permitted us to examine whether phase relationships were maintained across these stages. Strikingly, and consistent with a robust oscillator, we found that phases were tightly locked between stages. Thus, peaks occurred in the same phase during the earlier and the later stage (Figure 2C).

Oscillations Are Not Temperature Compensated

Although 8 hr is a harmonic of the 24 hr circadian cycle, it is also the period of the molting cycle. Unlike circadian clocks, which are temperature compensated (Hogenesch and Ueda, 2011), the length of the molting cycle in *C. elegans* is strongly temperature dependent and approximately doubles when ambient

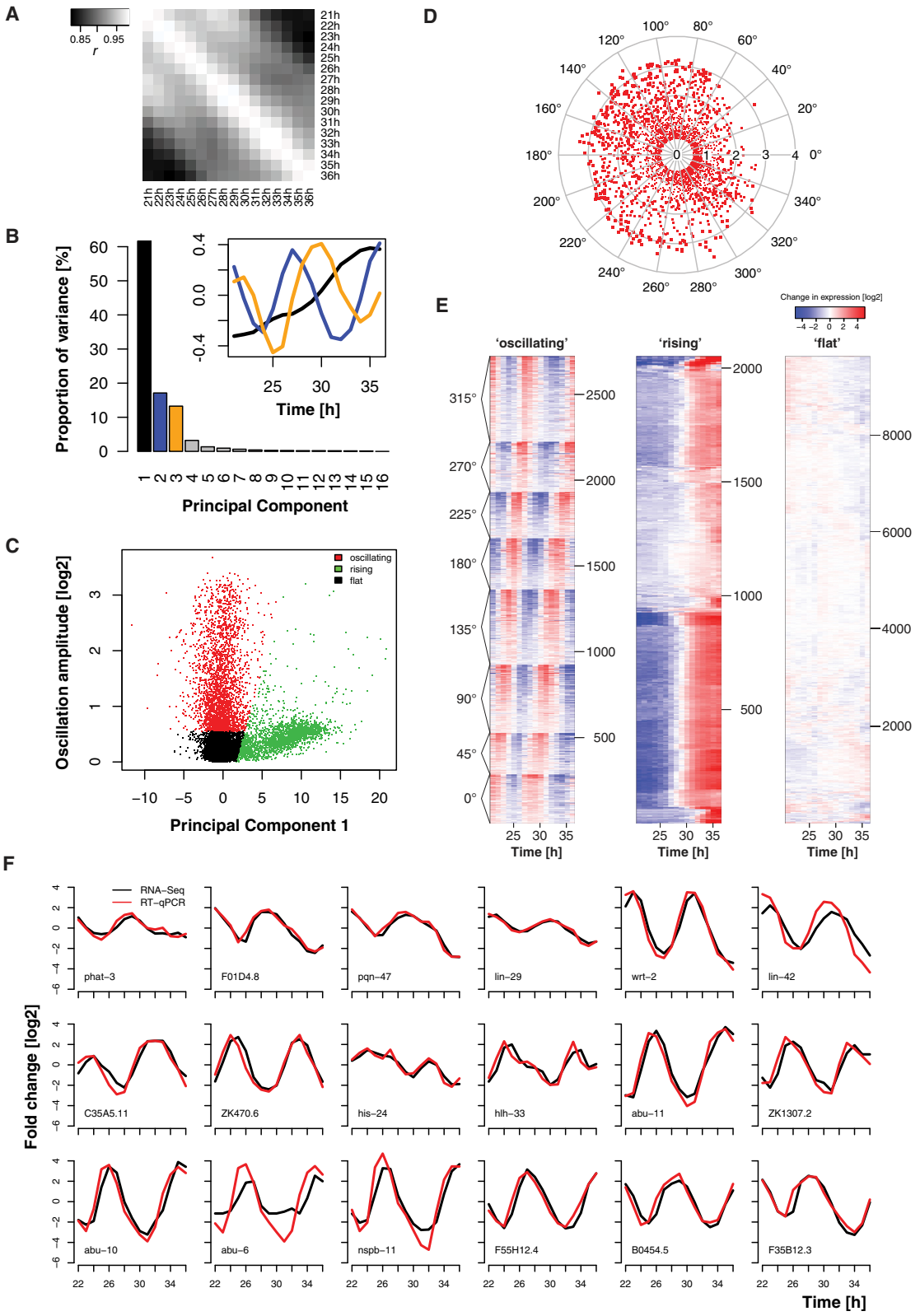


Figure 1. The Expression of Thousands of Genes Oscillates at an 8 hr Period

(A) Cross-correlation plot of gene expression patterns obtained by sequencing of mRNA sampled from *C. elegans* grown for the indicated time from L1 at 25°C. (B) Proportion of variance of gene expression profiles in (A) explained by 16 principle components (PCs). Inset shows PC1 through PC3 changes (loadings) over time.

(legend continued on next page)

temperature is decreased from 25°C to 15°C. To test whether the oscillator that drove the transcript oscillations that we had identified was compensated, we grew worms at 15°C and collected samples every 3 hr for 36 hr between late L3 and adult stage. RT-qPCR-based examination of the transcripts that oscillated with an ~8 hr period at 25°C (Figure 1F) revealed that the oscillations themselves were maintained but that the period now increased to ~18 hr (Figures 2D and 2E). This finding not only provides further evidence for the robustness of the oscillations but also reveals that, unlike circadian clocks, the underlying oscillator is not compensated. This is consistent with, yet does not prove, a mechanism important to keep developmental time.

Oscillations Occur in Diverse Somatic Tissues

To gain insight into the developmental function of the oscillations, we sought to determine where periodic gene expression occurred. To this end, we plotted the levels of all genes in the soma versus the levels in the gonad of young adult animals and color coded them according to the three categories: "oscillating," "rising," and "flat" (Figure 3A). Whereas genes whose expression neither oscillated nor increased steadily tended to be equally expressed in soma and gonad (Figures 3A and 3B), genes with steadily increasing expression exhibited typically higher levels in the gonad (Figures 3A and 3B). By contrast, "oscillating" genes were more highly expressed in the soma than the gonad (Figures 3A and 3B).

To confirm that somatic expression contributed significantly to the oscillations, we profiled gene expression of germline-less *glp-4* mutant animals across a developmental time course. Because *glp-4* animals experience a developmental delay, we harvested them at later time points than wild-type animals to obtain animals of comparable stages (Supplemental Experimental Procedures). Consistent with our hypothesis, we could readily observe periodic gene expression in *glp-4* animals (Figure 3C), despite the fact that these animals grow more asynchronously than wild-type animals, which will obscure oscillations. Collectively, these results suggest that periodic gene expression occurs preferentially in somatic tissues. Moreover, the extensive loss of upregulation among "rising" genes at the later time points of *glp-4* animal development demonstrates that these mostly correspond to germline-expressed genes.

The spatial expression patterns of some 2,000 *C. elegans* genes, mostly with human homologs, were recently inferred from an analysis of GFP expression driven by their promoters (Hunt-Newbury et al., 2007). Using these data, we found that epidermal cells (hypodermis and seam cells) were enriched for expression of oscillating genes whereas body wall muscle cells and neurons appeared to be depleted (Figure 3D). However, the extents of depletion and enrichment were generally small, not exceeding 1.2-fold, which reveals that oscillatory gene expression can occur in many somatic tissues.

Periodic Expression of Cuticular Collagen Genes May Facilitate Assembly of a Complex Structure

To identify molecular function and processes subject to periodic gene expression, we performed gene ontology (GO) term enrichment and depletion analysis (Ashburner et al., 2000). Consistent with oscillations as a preferentially somatic event, various terms related to function and development of the germline were strongly depleted (Table S2). Moreover, many of the most significantly depleted terms were linked to translation and the ribosome (e.g., among 123 expressed genes associated with "structural constituent of ribosome," mostly encoding ribosomal proteins, none was periodically expressed).

By contrast, GO:0003735 "structural constituent of cuticle," a term associated almost exclusively with cuticular collagen genes, was highly enriched (Figure 4A). Collagen genes had accounted for half of the ~20 genes previously known to undergo periodic expression (Johnstone and Barry, 1996; McMahon et al., 2003), but we were nonetheless surprised by the extent of oscillations: 91 of 126 expressed genes, or 72%, were periodically expressed. All but one of the remaining 35 genes increased expression across the time course, and these presumably represent adult-specific collagens.

Closer examination of expression phases further revealed that collagen genes preferentially peak between 180° and 270° (Figures 4B and 4C), the time when animals molt as determined by the occurrence of lethargus in a separate time course (data not shown). Nonetheless, different classes of collagens displayed noticeably dissimilar expression patterns: for members of the largest class, the *col* genes, expression peaked indeed mostly during the molt (Figure 4C). Cuticular collagens are assigned to this gene class solely by sequence homology. By contrast, collagens of the *rol* family were identified genetically through mutations that caused worms to display an abnormal movement (roller) phenotype due to helical distortions of the cuticle. *sqt* (squat) genes can be mutated to cause heterozygous roller and homozygous dumpy (short and fat) phenotypes. We find that expression of *sqt* and *rol* genes occurs in a highly coordinated manner, with peaks occurring all before the molt within a window of 10° (Figure 4C) and almost identical amplitudes ($\log_2$(A) ranging from 2.67 to 2.72). Taking further into account the specific genetic interactions (Kusch and Edgar, 1986) as well as sequence similarities among these cuticular proteins (Kramer et al., 1990), we propose that these proteins form a cuticular substructure.

Such a model is consistent with findings for the *dpy* (dumpy) collagen genes: individual DPY proteins contribute

(C) Plotting of oscillation amplitude, derived by cosine wave fitting, over PC1 reveals three separable classes of genes, the expression of which is "flat" (black), "rises over time" (green), or "oscillates" (red).
(D) A radar chart plotting oscillation amplitude over the phase of peak expression reveals that periodic gene expression occurs in all phases.
(E) Heatmap showing gene expression changes of genes assigned to classes as defined in (C). "Oscillating" genes were sorted by phase prior to plotting and assigned to indicated 45° bins (equaling 1 hr of development in this time course where one period corresponds to 8 hr).
(F) Genes from each bin were analyzed by RT-qPCR on RNA collected from a separate time course experiment. Expression patterns from the mRNA-seq and the RT-qPCR experiment are overlaid. Note that amplitudes in (C) and (D) are in $\log_2$ and, by definition, correspond to only half the change from peak to trough.

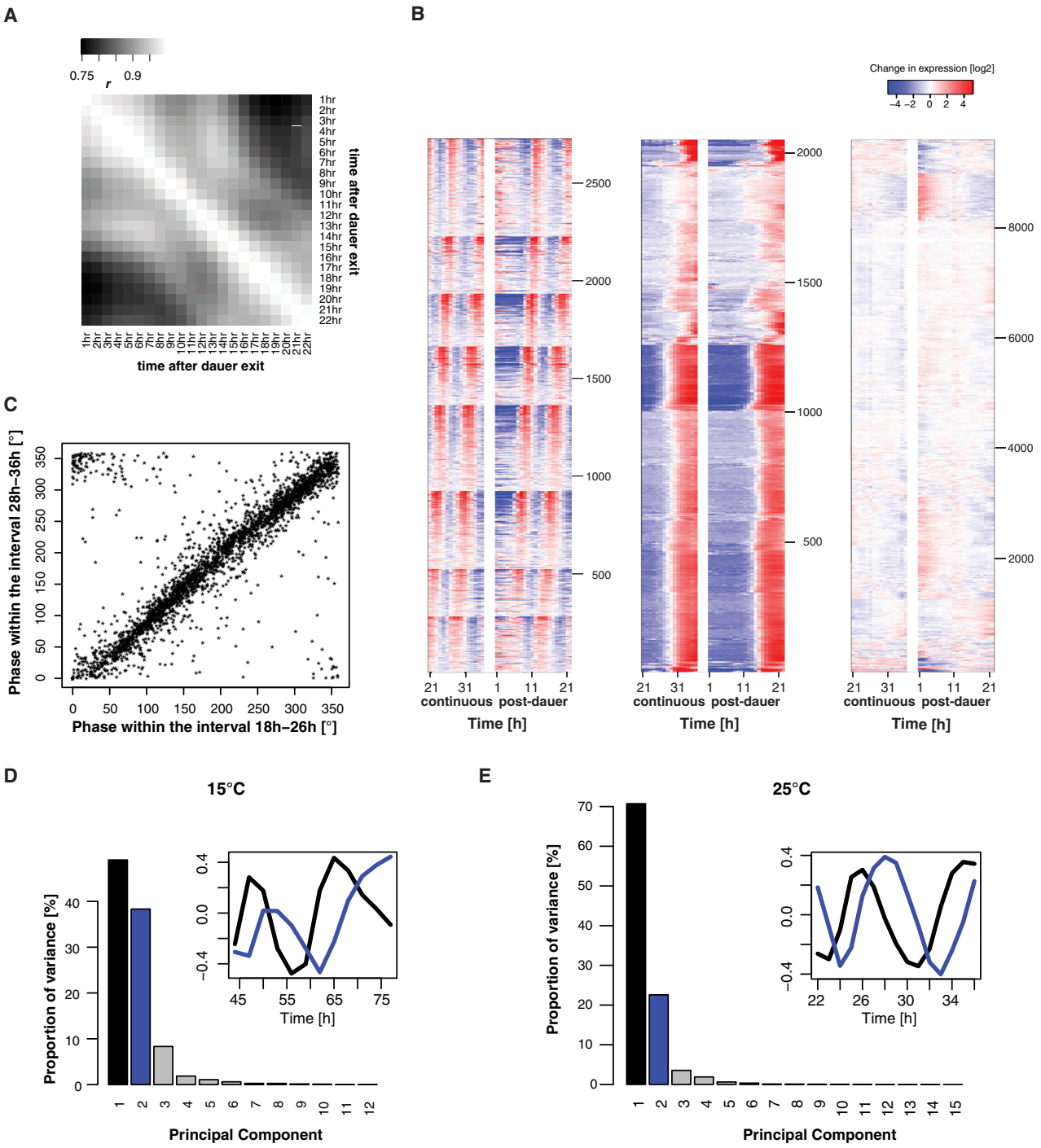


Figure 2. Periodic Gene Expression Is Robust and Phase Locked but Not Temperature Compensated

(A) Cross-correlation plot of gene expression patterns obtained by sequencing of mRNA sampled from *C. elegans* synchronously released from dauer and grown for the indicated time at 25°C.

(B) Heatmaps comparing side-by-side changes in expression for each gene in each of the three categories identified in Figure 1C, for animals grown continuously after L1 or transiently arrested in dauer prior to resuming development for the time indicated.

(C) Gene expression data were collected over an extended time course (Figure 5) and phases fitted separately for the first and the second half of the time course. A comparison of the two resulting phases in a scatterplot reveals close correlation of phases (circular correlation coefficient $r_C = 0.79$; Fisher and Lee, 1983). The points in the top left and bottom right corners are a consequence of the circular nature of the data, where 0° = 360°.

(D and E) Expression of the genes shown in Figure 1F was determined by RT-qPCR for animals grown for the indicated times at 15°C or 25°C, respectively. PC analysis demonstrates an increased period of oscillations at (D) 15°C relative to (E) 25°C. Data from Figure 1F were used for the 25°C time course PC analysis.

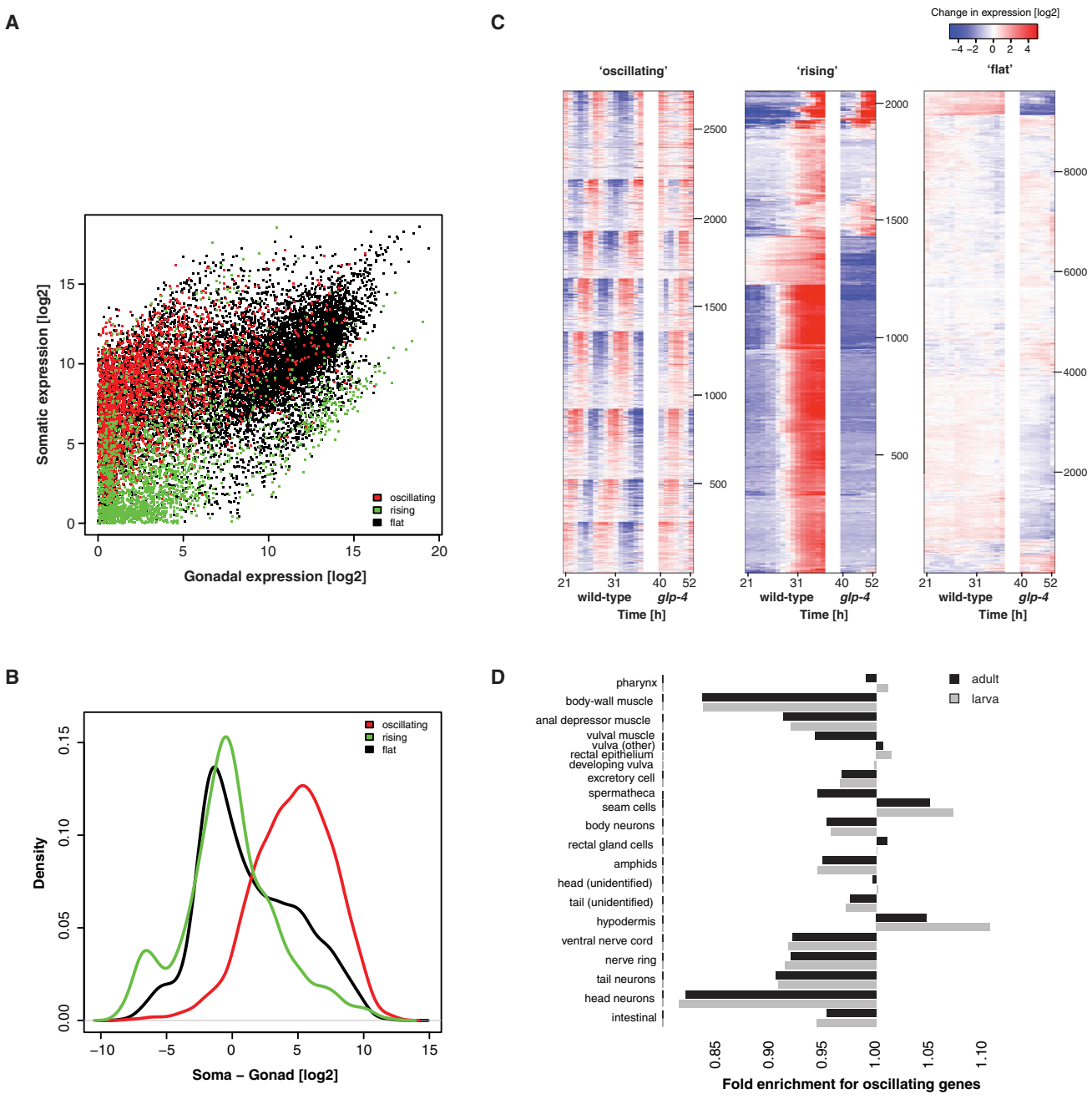


Figure 3. Oscillations Occur in Various Somatic Tissues

(A) Scatterplot comparing absolute gene expression in gonads and soma. Each dot corresponds to a gene and is color coded according to the categories in Figure 1C ("oscillating" is indicated by red, "rising" is indicated by green, and "flat" is indicated by black).

(B) Distributions of relative expression levels (comparing soma to gonad) for the categories shown in Figure 1C. Negative values denote enriched expression in the gonad and positive values denote enriched expression in the soma. "Oscillating" genes are shifted toward higher expression in the soma relative to the two other groups ($p << 2.2 \times 10^{-16}$; Kolmogorov-Smirnov test).

(C) Heatmaps comparing side-by-side changes in expression for each gene in each of the three categories identified in Figure 1C for wild-type and germline-less *glp-4* mutant animals. Because *glp-4* animals are developmentally delayed, they were sampled at later time points.

(D) Analysis of promoter activity (Hunt-Newbury et al., 2007) of "oscillating" genes reveals only modest enrichment for specific tissues within the soma.

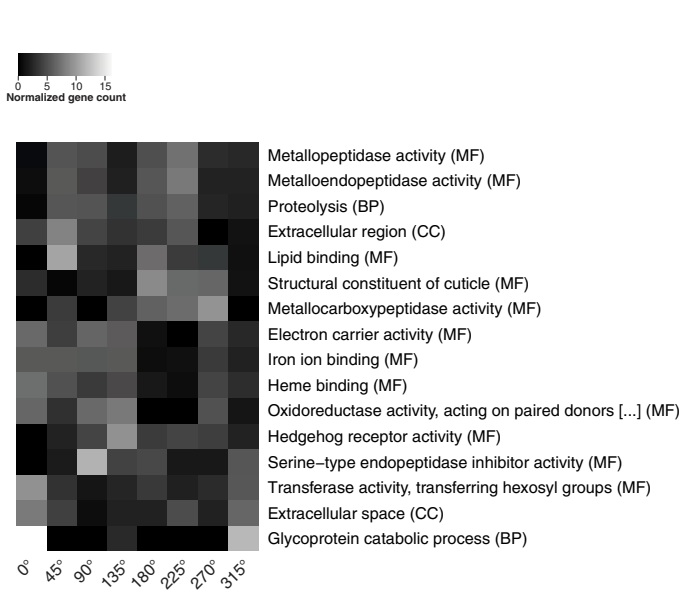
to one of two specific cuticular substructures (McMahon et al., 2003), and this functional specification coincides with expression in two separate clusters (Figure 4C; McMahon et al., 2003; Johnstone and Barry, 1996). The specific function of the "solitary" *dpy-17* is currently unknown and may be unique.

A

GO term	periodic expression[1]	p-value	fold enrichment
Structural constituent of cuticle (MF)	91/126	4.5e-58	3.82
Proteolysis (BP)	85/244	1.7e-22	1.84
Metallopeptidase activity (MF)	46/94	1.1e-19	2.59
Metalloendopeptidase activity (MF)	42/93	1.8e-16	2.39
Transferase activity, transferring hexosyl groups (MF)	38/82	1.7e-15	2.45
Serine-type endopeptidase inhibitor activity (MF)	26/43	1.2e-14	3.2
Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen (MF)	29/56	9.7e-14	2.74
Heme binding (MF)	41/109	7.5e-13	1.99
Hedgehog receptor activity (MF)	19/27	8.4e-13	3.72
Extracellular space (CC)	32/72	1.2e-12	2.35
Electron carrier activity (MF)	34/91	8.2e-11	1.98
Iron ion binding (MF)	38/111	1.3e-10	1.81
Extracellular region (CC)	33/88	1.4e-10	1.98
Glycoprotein catabolic process (BP)	10/11	3.2e-09	4.81
Metallocarboxypeptidase activity (MF)	10/13	6.8e-08	4.07
Lipid binding (MF)	18/40	8e-08	2.38

[1] periodically expressed/all expressed genes in category

B



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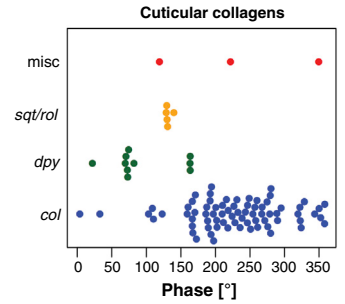

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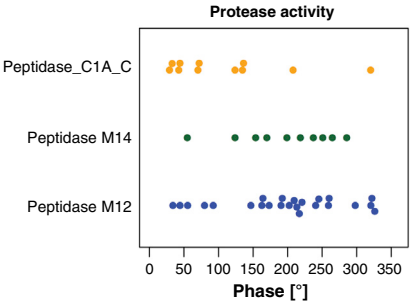

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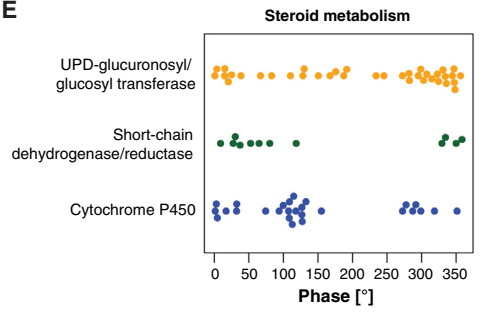

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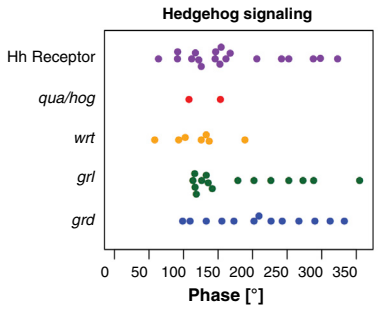

Figure 4. Specific Molecular Functions and Processes Are Enriched among Periodically Expressed Genes

(A and B) GO-term enrichment of "oscillating" genes.

(B) The heatmap shows the normalized phase distributions, in 45° bins, for all the oscillating genes that belong to enriched GO terms. Two normalization steps (Supplemental Experimental Procedures) correct for the fact that gene numbers vary across phase bins (Figure 1E) and GO terms (Figure 4A). MF indicates molecular function, CC indicates cellular compartmentalization, and BP indicates biological process.

(C–F) "Oscillating" genes coding for proteins of the indicated families were plotted by phase. The processes and/or gene families selected drive the following GO-term enrichments: (C) "structural constituent of the cuticle"; (D) "metallopeptidase acitivity," "metallocarboxypeptidase activity," "metalloendopeptidase activity," and "proteolysis"; (E) "electron carrier activity," "iron ion binding," "heme binding," and "oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen" (CYPs), and "transferase activity, transferring hexosyl groups" (UGTs); and (F) "Hedgehog receptor activity."

Table 1. Selected Gene Classes Enriched among Periodically Expressed Genes

Gene Class	Definition[a]	Periodic Expression[b]
Cuticular collagen	GO:0003735	72% (91/126)
Cytochrome P450 (CYP)	IPR001128	57% (27/47)
UDP-glucuronosyl/glucosyl transferase (UGT)	IPR002213	54% (37/69)
Short-chain dehydrogenase/reductase	WB: *dhs* class	40% (12/30)
Hedgehog receptor	GO:0008158	70% (19/27)
Hh-related genes: Warthog (*wrt*)	WB: *wrt* class	78% (7/9)
Hh-related genes: Ground-like (grl)	WB: *grl* class	83% (15/18)
Hh-related genes: Groundhog (*grd*)	WB: *grd* class	100% (13/13)
Hh-related genes: Quahog (*qua*)	WB: *qua* class	100% (1/1)
Hh-related genes: Hog only (*hog*)	WB: *hog* class	100% (1/1)
Metallopeptidase M14	GO:0004181	77% (10/13)
Astacin/peptidase M12	IPR001506	75% (24/32)
Peptidase C1A, papain C-terminal (Peptidase_C1A_C)	IPR000668	52% (11/21)

[a]GO indicates gene ontology; IPR indicates Interpro; WB indicates Wormbase.
[b]Percentage of detectably expressed genes. Numbers in brackets indicate the number of genes with oscillatory expression/number of genes detectably expressed.

We conclude that although collagen expression occurs preferentially during the molt, individual collagen genes reveal highly specific temporal expression patterns, which permits their incorporation into the cuticle at the right time and place.

Rhythmic Expression Frequently Occurs for Genes that Encode Proteases or Steroid Metabolism and Signaling Proteins

In addition to cuticular collagens, two other major themes became apparent following GO-term enrichment analysis of "oscillating" genes (Figure 4A). The first was proteolysis and resulted from frequent rhythmic expression among three peptidase families (Table 1; Figure 4D). These families contain peptidases with known functions in molting, for example, NAS-37 (Suzuki et al., 2004; Davis et al., 2004) and CPZ-1 (Hashmi et al., 2004), but the diversity of phases (Figure 4D) suggests roles beyond molting. Periodic protease activity may also be achieved or refined by rhythmic expression of protease inhibitors, as indicated by the enrichment of the GO-term "serine-type endopeptidase inhibitor activity" (Figures 4A and 4B).

The second theme comprised steroid hormone metabolism and signaling. Thus, cytochrome P450s (CYPs), UDP-glucuronosyl/glucosyl transferases (UGTs), and short chain dehydrogenases/reductases enzymes are enriched among oscillatory genes (Table 1; Figure 4E). These types of enzymes, and the steroid hormones they metabolize, play key roles in molting in insects (King et al., 2000; Iga and Kataoka, 2012) and, presumably, *C. elegans* (Entchev and Kurzchalia, 2005). Hence, their enrichment might reflect a function in molting hormone metabolism in *C. elegans*.

The Hedgehog (Hh) pathway genes of *C. elegans*, which frequently exhibit oscillatory expression (Figures 4A and 4F; Table 1), may also be linked to this theme. This is because they may function in sterol metabolism and signaling rather than canonical Hh signaling (Bürglin and Kuwabara, 2006) and because many of them are important for molting (Zugasti et al., 2005; Hao et al., 2006). Periodic expression was previously noted for four Hh-related genes (Hao et al., 2006), but we find it to be pervasive both among the genes in these families and the putative Hh receptors, which are characterized by the presence of a Patched-like domain (Table 1; Figure 4F). An attractive hypothesis is that oscillations can act as filters for signaling by helping to match specific receptor and ligand pairs through finely tuned coexpression, allowing them to execute distinct functions in molting and possibly other processes.

mRNA Level Oscillations Cause Rhythmic Protein Production

The examples provided above suggest that mRNA level oscillations could permit either transient protein accumulation or production of stable proteins at a given time when they are required for integration into a complex structure. However, both scenarios are based on the assumption that mRNA level oscillations cause rhythmic protein production. This assumption is not trivial, as evidenced by the discovery of extensive posttranscriptional regulation that refines and modifies circadian transcriptional oscillations (Hogenesch and Ueda, 2011). Hence, to rigorously test this notion, we examined whether transcript level oscillations in *C. elegans* larvae resulted in periodic transcript translation. We performed ribosome profiling to assess the translational status of cellular mRNAs (Ingolia et al., 2009) over a separate time course. We sequenced ribosome protected fragments (RPFs) as well as rRNA-depleted total RNA from worms collected every 2 hr during development from L3 to young adult stage. Confirming that RPFs reflect translation, they displayed robust phasing and were depleted from 5′ UTRs and, yet more extensively, 3′ UTRs (Figure S1), exactly as expected (Ingolia et al., 2009). Strikingly, the oscillations that occurred at a transcript level were also present at the translational level (Figure 5). Conversely, the oscillations seen at the translational level were well explained by transcript level oscillations. Hence, we conclude that periodic protein production is indeed the default result of periodic transcript accumulation in *C. elegans* larvae.

Oscillatory Gene Expression Is Driven by Periodic Transcription

Although the circadian clock was long considered to drive oscillations through periodic transcription, pre-mRNA level oscillations appear to be a poor predictor of mRNA level oscillations (Koike et al., 2012). On the other hand, the promoters of a few periodically expressed *C. elegans* genes have been shown to drive periodic accumulation of reporter proteins (Frand et al., 2005; Davis et al., 2004; Hao et al., 2006). We therefore wished to test quantitatively and comprehensively whether the present oscillator drives rhythmic accumulation of mRNAs through rhythmic transcription. Hence, we sequenced rRNA-depleted total RNA samples to determine pre-mRNA levels as a proxy of transcription. For this, we used the same RNA sample as those that we

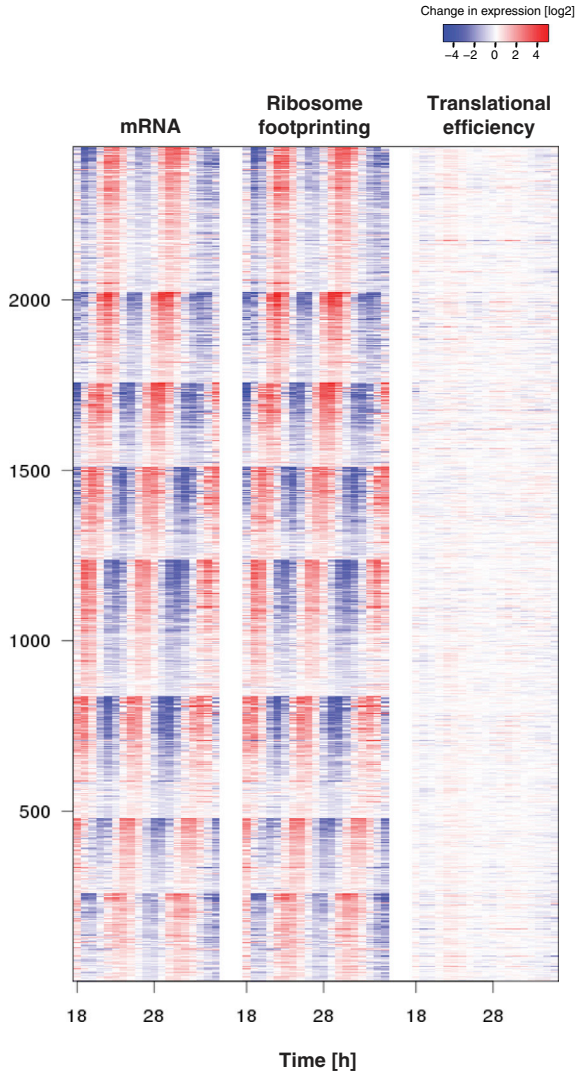

Figure 5. Rhythmic mRNA Accumulation Causes Periodic Translation

Ribosome profiling was performed to examine the translational status of expressed mRNAs and compared to mRNA expression analysis for "oscillating" genes. The timecouse was performed at 2 hr intervals, but the data were resampled at a 1 hr grid through spline interpolation for the heatmap to permit better comparison with the other data sets. "Translational efficiency" depicts the residual changes observed in the ribosome profiling data after subtracting mRNA expression data. See also Figure S1.

had subjected to mRNA sequencing (Figure 1). When analyzing transcripts for which sufficient intronic reads were available, we found that pre-mRNAs and mRNAs oscillated with highly comparable phases and amplitudes (Figures 6A and 6B). Consistent with transcriptional oscillations preceding mature transcript level oscillations, pre-mRNA peaks occurred on average some 15 min prior to mature mRNA peaks (Figure S2). We conclude that transcriptional regulation is a major contributor to the oscillations that we observe. At the same time, "oscillating" genes are found across all chromosomes and without any apparent clustering of genes according to phase of expression (Figure 6C), suggesting that there are no specific chromosomal domains that drive, or are particularly permissive to, periodic gene expression.

DISCUSSION

Extensive Oscillatory Gene Expression Serves a Developmental Function in *C. elegans* Larvae

We report here extensive oscillatory gene expression during *C. elegans* larval development. Superficially, the oscillations may appear reminiscent of transcriptional bursts (Raj and van Oudenaarden, 2008). However, we can rule out that the two are linked based on the fact that transcriptional bursts occur stochastically. Hence, they cannot be observed in ensembles of cells, let alone the populations of whole animals, comprised of numerous distinct cell types and tissues, which we studied here.

Formally, it is nonetheless possible that periodic gene expression does not serve a particular purpose but represents mere noise, perhaps as a side effect of another periodic process. The fact that oscillations involve high-amplitude expression level changes of a large number of genes, and that the process is highly robust, strongly argues against this possibility. Thousands of genes change >2-fold, and hundreds change >10-fold. If these changes reflected "noise" rather than function, it would be difficult to conceive mechanisms by which animals and their cells could prevent this noise from drowning the signal of "meaningful" changes in gene expression.

At the same time, oscillations were highly reproducible over three independent time courses examined by different detection technologies, independent of life history, and, although not temperature compensated, robust under different temperature regimens. Finally, we not only reproducibly observed oscillations for the same genes but also that these oscillations were also phase locked (i.e., the phase difference between pairs of different genes remained the same in different experiments). Kim et al. (2013) also recently observed extensive oscillatory gene expression but grouped oscillatory genes in a small number of distinct expression clusters instead of specifically examining phases and amplitudes. However, when we analyzed their data, we found a similar continuum of phases as in our data, and phase locking was maintained across stages (Figure S3). Summarily, the impressive robustness of the process thus provides additional strong evidence for a functional role.

A Role of Periodic Gene Expression in Molting

The above considerations support that widespread rhythmic gene expression in *C. elegans* serves a biological purpose. Because oscillations are not temperature compensated, they cannot function analogously to a circadian clock. Instead, they are likely to control a developmental process. The repetitious nature of molting makes it the strongest candidate, and gene expression oscillations and molting occur indeed both with the same periodicity.

Cuticular collagens are a particularly clear example of oscillatory expression of genes involved in molting. Consistent with cuticle generation during the molt, their expression peaks preferentially during, or in close temporal connection with, the molt. Surprisingly, however, this pattern is atypical (i.e., a continuum of gene expression phases is visible when examining the entirety

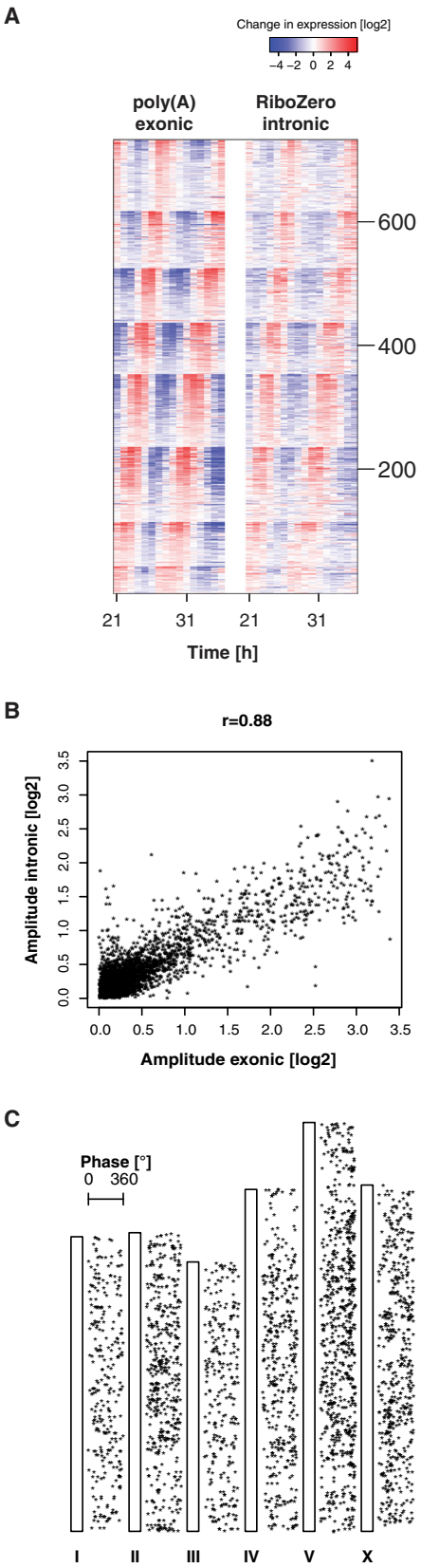

Figure 6. Oscillations Are Driven by Periodic Transcription

(A) Heatmaps comparing expression changes of "oscillating" genes at pre-mRNA levels ("RiboZero intronic"; obtained by sequencing of rRNA-depleted RNA and counting intronic reads) and mature mRNA levels ("Poly[A] exonic"; obtained by sequencing polyadenylated mRNA and counting exonic reads; Figure 1E). Genes for which sufficient pre-mRNA reads were detectable are shown. The same RNA samples were utilized for both sequencing reactions.

(B) Following cosine wave fitting of the data shown in Figure 6A, amplitudes derived for mature mRNA and pre-mRNA were plotted against one another.

(C) Genes were plotted by chromosomal location and phase. See also Figure S2.

of 2,718 genes). When combined with the fact that this number corresponds to nearly a fifth of expressed genes, it seems indeed likely that oscillations occur for many genes that are not required for the actual molting process. However, we speculate that extensive oscillatory gene expression permits coordination of molting with other cellular, developmental, or behavioral processes, which can be essential for viability (Ruaud and Bessereau, 2006). In fact, given the extensive and robust phase locking, it seems possible that the succession of periodically expressed genes may define a larval growth or development module in *C. elegans.*

The Utility of Gene Expression Oscillations Extends beyond Driving Transient Protein Accumulation

Atypical as the expression patterns of cuticular collagen genes relative to the overall patterns may be, they provide particularly interesting insights into the utility of periodic gene expression by highlighting a function that goes beyond achieving transient protein accumulation. Cuticular collagens are stable proteins that remain associated with the cuticle once incorporated. At the same time, the cuticle is a complex structure of several layers that need to be sequentially assembled (Page and Johnstone, 2007). Rhythmic and phase-locked collagen production then permits streamlining of this production through "just in time" delivery of individual components, facilitating faithful and efficient cuticle assembly. We propose that other oscillators may similarly be utilized to this end.

Phase Information Provides Insights into the Biology of Cuticle Synthesis

Johnstone and colleagues previously noticed the coexpression of *dpy* collagens destined for the same cuticular substructure (Johnstone and Barry, 1996; McMahon et al., 2003) and reported that collagens generally fell in three expression clusters termed "early," "intermediate," and "late" (Johnstone and Barry, 1996; McMahon et al., 2003). However, these conclusions were based on semiquantitative expression analysis of only ten genes. Our comprehensive analysis reveals a much more sophisticated choreography of collagen gene expression that involves a broad distribution of phases. This finding suggests the possibility that individual collagens may indeed be more functionally distinct than previously appreciated and that this, and not a need to achieve massive production of a generic collagen, has given rise to the large number of collagen genes present in *C. elegans*.

Another unexpected finding in our data is the observation of collagen expression after the molt. It had previously been assumed that cuticle synthesis was a molt-specific event (Page

and Johnstone, 2007) and that the larval growth that does occur between molts is facilitated by physical stretching of the flexible cuticle (Knight et al., 2002). Our data suggest the possibility that synthesis of specific collagens could additionally contribute to cuticle growth between molts. Consistent with this notion, cuticle regeneration can occur after physical injury (Pujol et al., 2008).

Rhythmic Transcription Drives mRNA Level Oscillations

Our finding that pre-mRNA expression patterns faithfully mirror mature mRNA oscillations reveals rhythmic transcription as a key driver of oscillations and implies a need for rhythmically modulated transcription factor activity. The diversity of expression phases potentially suggests the involvement of many different factors, and we observe periodic expression for 92 transcription factors, including several that are required for molting (data not shown).

Our efforts to identify motifs in the promoters of coexpressed genes have so far failed to yield convincing leads as to the identity of involved transcription factors (our unpublished data). However, *lin-42*, the rhythmically expressed *C. elegans* ortholog of the core circadian clock gene and transcriptional regulator *Period* (Jeon et al., 1999; Monsalve et al., 2011), is a particularly interesting candidate, since loss of *lin-42* activity causes arrhythmic molts (Monsalve et al., 2011). Whether LIN-42 indeed drives rhythmic transcription to time molts remains to be established, since no targets are known. Validating this hypothesis will presumably require the use of single-animal-based techniques, because arrhythmia, in population-based studies, will inevitably generate an appearance of decreased oscillations. At any rate, it will be of great interest to identify in future studies the transcription factors at the heart of these oscillations and understand how they crosstalk to one another. Possibly, this may occur in analogy with periodic transcription during the budding yeast cell cycle (Bähler, 2005), where transcription factors sequentially regulate one another (Simon et al., 2001) and, additionally, differ in their activities when present alone or in pairs (Kato et al., 2004). These two features may then suffice to achieve both phase locking and a continuum of expression phases. At the same time, we would like to emphasize that, although our data demonstrate that the oscillator manifests by driving extensive oscillatory transcription, it remains to be established whether the functionality of the core oscillator itself equally depends on transcription.

A Unique Combination of Features Defines a Model to Study Gene Expression Oscillations and Coordinated Gene Expression in an Animal

It has been emphasized that the study of diverse oscillators in various systems has been instrumental to identify unifying themes and idiosyncrasies (Hogenesch and Ueda, 2011; Tyson et al., 2008). We propose that the oscillations that we present here have a number of unusual characteristics that merit investigation. For instance, insights into the mechanisms that achieve robust phase locking and broad distribution of phases may be illuminating for our understanding of coordinated gene expression more generally. Moreover, a relatively short period and high amplitudes generate an interesting problem for gene expression kinetics: rapid induction needs to be balanced with rapid degradation. In other words, are the affected transcripts inherently unstable, which would necessitate yet higher transcription levels upon induction, or is their degradation rate increased once expression declines, and, if so, by which means? Finally, it is striking that oscillations are robustly detectable in RNA from whole animals, as this suggests the presence of effective mechanisms, yet to be uncovered, that coordinate oscillations spatially and temporally. The diversity of genetic and other tools available for *C. elegans* will permit extensive and productive investigation of this model oscillator to resolve these and other issues.

EXPERIMENTAL PROCEDURES

Worm Culture

Animals were grown and synchronized at the L1 or dauer stages according to standard procedures detailed in the Supplemental Experimental Procedures.

RNA Sequencing, Ribosome Profiling, and RT-qPCR

For RNA sequencing, RNA was extracted from extensively washed animals and extracted by freeze thawing or mortar and pestle in Tri Reagent. DNase-treated, quality-controlled RNA was used for preparation of mRNA sequencing or rRNA-depleted total RNA sequencing libraries using commercial kits and protocols. Ribosome profiling was performed following an adaptation of published protocols (Ingolia et al., 2012; Bazzini et al., 2012). RT-qPCR-based validation of mRNA-sequencing (seq) data was performed on a separately collected time course, with candidate genes chosen based on a wide distribution of their respective phases. All relevant steps are further detailed in the Supplemental Experimental Procedures.

Processing of the RNA-seq and Ribosome Profiling Data

All the RNA-seq data (50 bp read length) were mapped to the *C. elegans* genome (ce6) using the spliced alignment algorithm SpliceMap included with the R package QuasR (Au et al., 2010), as detailed in the Supplemental Experimental Procedures.

For ribosome profiling, bowtie (Langmead et al., 2009) was used to map the ~30 bp ribosome-protected fragments obtained after 3′ adaptor removal to the *C. elegans* genome (ce6), allowing only uniquely mapping reads. Gene expression quantification was performed by counting reads and analyzing them, as detailed in the Supplemental Experimental Procedures.

Principal Component Analysis and Cosine Curve Fitting

After mean normalization of the $\log_2$ gene expression levels, we performed PC analysis using the function princomp in R with default parameters. A combination of PC2 and PC3 permitted representation of sinusoidal waves with an 8 hr period and any phase angle (Supplemental Experimental Procedures). Hence, for each gene we fitted a separate cosine curve with a known period of 8 hr and thus a frequency $\omega = 2 \cdot \pi/8$ hr and unknown variables C and φ. Since $(C \cdot \cos(\omega t + \varphi) = A \cdot \cos(\omega t) - B \cdot \sin(\omega t)$ with $A = C \cdot \cos(\varphi)$ and $B = C \cdot \sin(\varphi))$, we performed the fit using a linear regression including the two components $\cos(\omega t)$ and $-\sin(\omega t)$ as regressors. Because a large proportion of the variance in the data was explained by the nonperiodic first principal component PC1, we included it as a separate regressor during the fit. Based on a scatterplot comparing PC1 to the oscillation amplitude, we classified the genes into three categories, oscillating, rising, and flat. A detailed description is provided in the Supplemental Experimental Procedures.

GO Enrichment Analysis

GO annotations for *C. elegans* were downloaded from http://www.geneontology.org/gene-associations/gene_association.wb.gz, and enrichments and depletions were calculated, as detailed in in the Supplemental Experimental Procedures. To display the normalized phase distributions for all the oscillating genes that belong to enriched GO terms in a heatmap, we binned the phases into eight equally sized intervals and corrected for gene number variations across phase bins and GO terms (Supplemental Experimental Procedures).

Tissue Enrichment Analysis

To determine whether periodic gene expression occurred preferentially in the soma or the germline, we examined mRNA-seq data obtained for gonads dissected out of wild-type young adult animals and for germline-less *glp4ts* mutant young adult animals, kindly provided by Dr. Rafal Ciosk (C. Scheckel, D.G., and R. Ciosk, unpublished data). *C. elegans* promoter::GFP fusions expression data (Hunt-Newbury et al., 2007) were obtained from http://gfpweb.aecom.yu.edu and analyzed for those cell types that had at least 100 expressed genes, as detailed in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

All sequencing and ribosome profiling data generated for this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE52910 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE52910).

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures, three tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.molcel.2013.12.013.

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