entraînable oscillateur. Note that the different rhythmique frq mutants show strain-specific responses to short light pulses. In laboratory experiments, the effect of light on the Neurospora clock, even at moonlight levels, is so strong that it apparently stops the oscillator. In nature, such a clock would be no more than an hourglass timer, functioning only during the darkest nights. The light-cycle experiments shown here were done in constant temperature, but when light and temperature are given in anti-phase in a 24 h cycle (cold with light and warm with dark), temperature dominates as the zeitgeber. When frq is exposed to temperature cycles (22–27°C) in constant light (which results in arrhythmicity in constant temperature), conidiation is robustly rhythmic (data not shown). Thus, temperature cycles ‘gate’ light transduction, enabling the Neurospora clock to continue throughout the day. Our results show that FRQ is unnecessary for entraining an oscillator with generic circadian properties in temperature cycles. However, without FRQ, the circadian clock cannot synchronize to light-dark cycles and self-sustained rhythmicity is almost never seen, except under special conditions. As part of a circadianly regulated light input pathway, FRQ apparently supplies the clock with sufficient amplitude for self-sustained rhythmicity. (frq) remains arrhythmic after release from temperature entrainment and sets the period in the circadian range. This role of FRQ is especially relevant in view of the recent reports of clock-regulated light transduction (via cryptochrome) to the clock in flies and mammals.

Methods

Strains and media. bd, bd frq and bd frq are standard lab strains (provided by the Dunlap Lab, Dartmouth Medical School). bd frq was obtained from the Fungal Genetics Stock Center. Solid media for glass tubes contained 1× Vogels salts, 0.3% glucose, 0.5% l-arginine, 2% agar and 10 ng biotin per ml. Liquid media was the same except for 0.5% glucose and the lack of agar.

Zeitgeber cycles. Depending on the zeitgeber, each cycle of light was spent in low temperature or darkness, the other half in high temperature or light (Lumilux Interna, Osram). Temperature cycles were created in constant temperature (26°C) and set the period in the circadian range. This role of FRQ is especially relevant in view of the recent reports of clock-regulated light transduction (via cryptochrome) to the clock in flies and mammals.

mRNA analysis. Glass tubes and liquid cultures were inoculated and incubated for 1 day in constant light and ambient laboratory temperature (23 ± 1°C) before transfer to the zeitgeber cycle (low temperature or darkness).

Data analysis. Images of glass tubes were digitized with an Apple Color One scanner, stored as PICT files and analysed with CHRONO. Conidiation was quantified by the number of white pixels in each vertical line of the image and expressed as deviation around the non-rhythmic trend. 3–6 tubes were analysed for each time series and variable. Onsets of conidiation were defined as expressed as deviation around the non-rhythmic trend. 3–6 tubes were inoculated and incubated for 1 day in constant light and ambient laboratory temperature (23 ± 1°C) before transfer to the zeitgeber cycle (low temperature or darkness).
Figure 1  The *gon-1* gene is required for morphogenesis of the gonad. L1–L4, first to fourth larval stages. Arrows, direction of arm extension. a, Wild-type hermaphrodite. The gonad possesses two U-shaped ovarioles extending symmetrically from central gonadal structures that include uterus (ut), spermatheca (st) and sheath (sh). Hermaphrodite leader cells, called distal tip cells (DTCs) lead arm extension (for a review, see ref. 23). b, The *gon-1* gene is required for gonadal shape. In *gon-1* mutants, arm extension does not occur, and the gonad develops as a disorganized mass of somatic and germline tissues.

![Diagram of gonad development](image)

Figure 2  The *gon-1* gene and protein. a, Molecular cloning. Top, genetic map: *gon-1* maps to LG IV between Tc1 insertions pKP5151 and pKP614. Middle, *gon-1* transcript F25H8.3 spans two cosmids (F25H8 and T13H10); T13H10 rescues *gon-1* (q518), as does a deleted version that removes the smaller transcript T13H10.1. Below, the *gon-1* transcript, deduced by comparing genomic and cDNA sequences. Black boxes, exons; translation start and stop sites are indicated. b, Domains of *GON-1* and other family members. Prodomain, thin line; MP, metalloprotease (red); conserved cysteine-rich regions (CR1, dark grey) and CR2 (light grey); TSP1, thrombospondin type 1 motif (bright yellow); TSP1-like motifs, amino acids 889–1,923 (light yellow). Arrows, *gon-1* mutations: q518 (TG→GA at amino acid 591), e2551 (TG→GA at amino acid 1,069), e2547 (TG→GA at amino acid 1,199), q18 (TG→GA at amino acid 1,234) W→stop; e1254 (CA→GA at amino acid 1,345) R→stop. c, *GON-1* sequence alignment with two MPT family members. Colour coding as in b; black boxes, identical amino acids; grey boxes, similar amino acids. Alignment created by GCG Pileup program, with modification. In the metalloprotease domain (red), amino acids that are critical for enzymatic activity are marked by asterisks. In the TSP1 domain, amino acids that are conserved in vertebrate TSP1 repeats are marked by plus symbols. Filled triangle, site of *gon-1* (q518). Only the first two TSP1-like motifs are shown; the consensus sequence for these repeats is W-X_{4–5}-W-X_{2}-C-X_{4–5}-X-G-X_{3}-R-X_{3}-C-X_{4–27}C-X_{8–12}-C-X_{3–4}-C.
multiple thrombospondin type-1-like repeats. This motif architecture is typical of a small family of genes that include bovine procollagen I N-protease (PINP), which cleaves collagen, and murine ADAMTS-1, the expression of which correlates with tumour cell progression. We find that gon-1 is expressed in two sites, leader cells and muscle, and that expression in each site has a unique role in forming the gonad. We speculate that GON-1 controls morphogenesis by remodelling basement membranes and that regulation of its activity is crucial for achieving organ shape.

The C. elegans hermaphrodite gonad has two U-shaped arms that extend from the somatic gonadal structures (Fig. 1a). The shape of the male gonad is similar except that it has one arm. During gonadogenesis, elongation of the growing gonadal arms is controlled by leader cells, which are called distal tip cells (DTCs) in hermaphrodites and the linker cell (LC) in males. The gon-1 gene is required for both elongation of the gonadal arms and morphogenesis of somatic gonadal structures (Fig. 1b). To investigate gon-1 function, we cloned the gene by a combination of genetic mapping, mutant rescue and RNA-mediated interference (Fig. 2a, legend). We confirmed the predicted gene F25H8.3 as gon-1 by identifying molecular lesions in five gon-1 mutant alleles (Fig. 2b, c; see below). The GON-1 protein bears a signal sequence at its amino terminus, but no predicted transmembrane domain, suggesting that it is secreted (Fig. 2c).

The gon-1 protein possesses a predicted metalloprotease (MP) domain (Fig. 2c, red bar) which is most similar to members of the reprolysin subfamily. A potential furin cleavage site is located at the N-terminal border of the metalloprotease domain (Fig. 2c, green bar). GON-1 and the reprolysins share a common zinc-binding active site with the larger metzincin superfamily. Amino-acid conservation within the active site, together with the known crystal structure of several superfamily members, reveals those amino acids that are essential for enzymatic activity (marked by asterisks in Fig. 2c).

To determine where and when GON-1 is expressed during development, we generated a transgene, called gon-1 5′::GFP, carrying a predicted gon-1 promoter fused to the coding region for green fluorescent protein (GFP). gon-1 5′::GFP drives GFP expression in the leader cells of both hermaphrodites and males during the period of most active gonadal-arm elongation (Fig. 3). In hermaphrodites, GFP is not observed in gonads from first-stage (L1) larvae (Fig. 3a, b). Its first gonadal expression occurs in L2 and is limited to the DTCs (Fig. 3c, d). GFP continues to be expressed in DTCs through L4 (Fig. 3e, f), but is faint or not detectable in the adult (Fig. 3g, h). GFP is similarly expressed in the male linker cell (Fig. 3i, j; data not shown). In addition to its expression in leader cells, the gon-1 promoter also drives GFP in muscle cells throughout development (Fig. 3k; data not shown).

To explore the significance of gon-1 expression in leader cells and in muscle, we placed gon-1 coding sequences under the control of either of two heterologous promoters. The lag-2 promoter drives expression in the hermaphrodite DTC, whereas the unc-54 promoter drives expression in body-wall muscle. For both transgenes, effects were examined in a gon-1 null (0) background. We found that lag-2 5′::GON-1 rescues arm extension and fertility (5/6 lines) (Fig. 4c), but unc-54 5′::GON-1 does not (0/11 lines). However, unc-54 5′::GON-1 does affect gonadal shape. Whereas the gonadal tissues of gon-1 mutants are not consolidated in a single mass (Fig. 4b), those of gon-1(0); unc-54 5′::GON-1 form a discrete mass that

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**Figure 3** The gon-1 promoter drives GFP expression in leader cells and in muscle. All animals are wild type and carry gon-1 5′::GFP. a, c, f, h, Nomarski; b, d, f, g, i, GFP fluorescence. Same animal in each left-right pair. Arrows, leader cells. a, b, No GFP in early gonad (arrow, progenitor of leader cell). c–f, GFP is expressed in leader cells during active elongation. g, h, GFP is not expressed in adult leader cell. i, j, Male leader cell expresses GFP. k, GFP is expressed in all muscles as well as in the leader cells. Body wall muscle extends length of body in four stripes, which are twisted because of the roller phenotype (see Methods). px, Pharynx; arrows, DTCs.
is expanded in all axes (Fig. 4d). Therefore, muscle expression of gon-1 permits the contained growth and expansion of gonadal tissues within a basement membrane. Furthermore, because GON-1 expressed in muscle can act non-autonomously on the gonad, we conclude that GON-1 is secreted and can act over a distance. We propose that, in wild-type animals, gon-1 must be expressed in leader cells to achieve a localized activity that directs elongation of gonadal arms along the proximal–distal axis. In addition, gon-1 expression in muscle may provide a diffuse activity that is important for expansion of the gonad along dorsal–ventral and left–right axes. By this model, the combination of localized and dispersed activities is important in defining the final shape of the organ.

Figure 4  gon-1 expression is critical for organ shape. a–d, Differential interference contrast (DIC) microscopy of adult hermaphrodites. Solid triangle, vulva; e, embryo; oo, oocyte. a, Wild type. Gonadal arm has normal U-shape. b, gon-1(0). No arm extension and gonadal tissues are irregularly shaped. gp, gonad proper; gb, gonadal tissue bulging from gp. c, gon-1(0) with gon-1(+) expressed in DTC. Gonad can develop with U-shaped arm. Some animals are fertile, but more commonly, somatic gonadal structures (for example, uterus) are poorly formed and animals are sterile. Furthermore, the distal gonadal arms are thinner than normal. d, gon-1(0) with gon-1(+) expressed in body wall muscle. Gonadal tissue is more coherent than in gon-1 mutants and has expanded in all three axes.

We suggest that the GON-1 metalloprotease directs expansion of the gonad by remodelling the basement membrane. GON-1 belongs to a small gene family of secreted metalloproteases with one canonical TSPt1 domain, multiple TSPt1-like motifs and two conserved cysteine-rich regions (Fig. 2b). Based on this conserved architecture, we suggest the name MPT (for MetalloProtease with TSPt1 repeats) for the family. Bovine P1NP can proteolise the N-terminal propeptide from collagen I (refs 3, 15). We show that metalloprotease activity is required for GON-1 function and propose that, like P1NP, it may cleave components of the extracellular matrix. Murine expression of adamants-1 RNA correlates with tumour cell progression1. Given the role of GON-1 in promoting cell migration of the C. elegans leader cell, we suggest that MPT proteins may be involved more generally in cell migrations that must pass through extracellular matrix and that, in cancerous tissues, a change in MPT regulation may promote metastasis.

The GON-1 metalloprotease drives arm extension during organogenesis in C. elegans, and localization of its activity has a profound effect on organ shape. We suggest that similar activities may control organ morphogenesis throughout the animal kingdom, and previous notions that are supported in vitro experiments. For example, antibodies recognizing matrix metalloprotease 9 (MMP9) can block branching of the ureter bud during kidney development16, and inhibitors of MMPs block the invasion of endothelial cells into a fibrin matrix in assays for angiogenesis17,18. Based on these observations and our analysis of GON-1, we suggest that the matrix metalloproteases are critical modulators of organogenesis. With the identification of GON-1 in C. elegans, the functions of MPT metalloproteases can be analysed in vivo during organogenesis using the full force of molecular genetics available in this model system.

Methods

Mapping and rescue of gon-1. Four-factor mapping placed gon-1 between Tc1 insertions pKP614 and pKP5151. Specifically, two out of ten fertile Unc recombinants from unc-24 gon-1 dpy-20/pKP5151 mothers did not carry the Tc1, placing gon-1 right of pKP5151. One of 52 fertile Dpy recombinants from unc-24 gon-1 dpy-20/pKP614 was negative for the Tc1, placing gon-1 left of pKP614. Tc1 insertions were scored by polymerase chain reaction (PCR) as previously described14. For rescue experiments, 10 μg ml−1 cosmids plus 100 μg ml−1 pRF4 were co-injected into unc24 gon-1/gon-4 dpy-20 as described previously18. From 13 F1 roller lines injected with the T13H10 cosmids, we identified 3 rescued fertile Unc progeny. pJK651 (a deleted version of T13H10), was constructed by digesting with KpnI and religating the backbone. pJK651 rescued at a similar efficiency as T13H10. RNA-mediated interference (RNAi) was performed with both single-stranded and double-stranded RNA. Approximately 2 μg ml−1 RNA corresponding to a 1 kilobase (kb) portion of each predicted transcript on T13H10 were injected into N2 gonads as described18. Phenocopy was scored as absence of DTC migration. Molecular lesions were identified by direct sequencing of PCR products spanning the gon-1 gene from single-mutant worms11. Sequencing was done on an ABI sequencer. Complementary DNAs were isolated from the following libraries: ARB1 and ARB2 (gifts from R. Barstead) and LAE1 (gift from A. Puoti). cDNAs spanning the remaining gaps were isolated by performing PCR after reverse transcription of RNA1. The 5' end was identified using an SL1-specific primer and nested primers within the predicted transcript19. For gon-1; smg-1 double mutants, non-Unc progeny from gon-1/nt1[unc-7(n754 dm) let-7]]; smg-1[+861] mothers were isolated and scored for fertility and arm extension. nT1[unc-7(n754 dm) let-7]] is a dominant Unc balancer for gon-1.

Transgene experiments. To construct gon-1 5'::GFP, we performed Expanded High-Fidelity PCR (Boehringer Mannheim) with restriction-site-tagged primers that spanned –7 to –11,547 relative to the initiator ATG of the gon-1 genomic DNA sequence. This region contains 4 kb of gon-1 5' flanking region and 8 kb of the gon-1 transcribed region preceding the first AUG (two large introns, one small intron and the 500 nucleotides of 5’UTR (Fig. 2a)). All PCR reactions used pJK651 as template. The gon-1 5' PCR product was cloned into the pPD95.77 vector (from A. Fire). The resulting gon-1 5'::GFP construct was co-injected at 5 μg ml−1 with pRF4 (100 μg ml−1), Stably transmitting roller
lines were scored for fluorescence. To construct the gon-1(+)-minigene, we performed high-fidelity PCR with restriction-site-tagged primers spanning −7 to +5,276 of gon-1 gDNA. To construct the gon-1 (E → A) mutant minigene, we made primers that introduced a T→G mutation at +2,546 and an A→C mutation at +2,551. The first mutation is translationally silent and introduces a SphI restriction site whereas the second mutation causes an E→A transition in the gon-1 metalloprotease active site. We used these primers to amplify either half of the wild-type minigene and spliced the resulting products using the new SphI site. Sequencing of gon-1(+) and gon-1(E → A) showed no second-site mutations. The two minigenes were cloned into pPD49.26 (from A. Fire) together with the lag-2 5′ promoter from pHK35 (ref. 13). The lag-2 5′-gon-1(+) and lag-2 5′-gon-1(E → A) constructs were injected at 1 μg ml−1 (together with pRF4 (50 μg ml−1) into unc-24(e138) gon-1(q516/87) gon-4(c2575) dpy-20(e1282) hermaphrodites. Roller Unc animals from stably transmitting roller lines were scored for arm extension and fertility in both F2 and F3 generations. The unc-54::gon-1(+) transgene was constructed by cutting the gon-1(+) minigene from lag-2 5′::gon-1(+) and splicing it downstream of the unc-54 promoter in pdP30.35 (from A. Fire). The resulting transgene was co-injected at both 1 μg ml−1 (50 μg ml−1 pRF4) and 5 μg ml−1 (100 μg ml−1 pRF4) into gon-1 heterozygotes and scored as above. Effects on gon-1 gonadal structure were mild at 1 μg ml−1 and stronger at 5 μg ml−2.

Received 24 March; accepted 27 April 1999.


25. Acknowledgements. We thank A. Cudron for providing cosmid bacteria, R. Barstad and A. Piuot for cDNA libraries, T. Bruce for SAGE and expression vectors, D. Goo for lag-2 promoter, J. Nance and J. Provancher for the Caenorhabditis Genetics Center for worm strains, and the many lab members who provided thoughts and criticisms during the course of this work. R.B. is a MD/PhD student and was an NIH Molecular Biosciences trainee. J.K. is an investigator with the Howard Hughes Medical Institute and has been supported by grants from NIH and NSF.

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