Circadian rhythms synchronize mitosis in *Neurospora crassa*

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The cell cycle and the circadian clock communicate with each other, resulting in circadian-gated cell division cycles. Alterations in this network may lead to diseases such as cancer. Therefore, it is critical to identify molecular components that connect these two oscillators. However, molecular mechanisms between the clock and the cell cycle remain largely unknown. A model filamentous fungus, *Neurospora crassa*, is a multinucleate system used to elucidate molecular mechanisms of circadian rhythms, but not used to investigate the molecular coupling between these two oscillators. In this report, we show that a conserved coupling between the circadian clock and the cell cycle exists via serine/threonine protein kinase-29 (STK-29), the Neurospora homolog of mammalian WEE1 kinase. Based on this finding, we established a mathematical model that predicts circadian oscillations of cell cycle components and circadian clock-dependent synchronized nuclear divisions. We experimentally demonstrate that G1 and G2 cyclins, CLN-1 and CLB-1, respectively, oscillate in a circadian manner with bioluminescence reporters. The oscillations of clb-1 and stk-29 gene expression are abolished in a circadian arrhythmic frq<sup>ko</sup> mutant. Additionally, we show the light-induced phase shifts of a core circadian component, frq, as well as the gene expression of the cell cycle components clb-1 and stk-29, which may alter the timing of divisions. We then used a histone hH1-GFP reporter to observe nuclear divisions over time, and show that a large number of nuclear divisions occur in the evening. Our findings demonstrate the circadian clock-dependent molecular dynamics of cell cycle components that result in synchronized nuclear divisions in Neurospora.

Molecular mechanisms of circadian rhythms provide temporal information to other cellular processes, such as metabolism, to optimize their outcomes (1–3). For instance, circadian oscillations of rate-limiting genes in glucose metabolism suggest time-of-day specific regulatory mechanisms that maintain glucose homeostasis in mammals (3). Circadian clock-gated cell division cycles have been observed in various organisms, including mammals, indicating that cell divisions preferentially occur at specific times of the day (4–7). In the mouse liver, expression of the cell cycle kinase encoding gene, wee1, is directly activated by a heterodimeric circadian transcription factor, CLOCK-BMAL1, providing a molecular link between the cell cycle and circadian rhythms (5). This suggests that circadian clock-regulated WEE1 promotes periodic inhibition of mitotic cycles between G2 and M phase by phosphorylating and inactivating the mitotic cyclin-dependent kinase (CDK) (8). On the other hand, circadian-independent cell divisions have been reported in rat-l fibroblasts despite the fact that these cells maintain robust circadian rhythms (9). These data suggest that not all cells with circadian rhythms may display circadian-gated cell division cycles.

The multinucleate fungus *Neurospora crassa* has played a pivotal role in elucidating the molecular mechanism of circadian rhythms (10, 11). Briefly, circadian rhythms in *N. crassa* are regulated by positive and negative elements that create a time-delayed negative feedback loop (12). A heterodimeric transcription factor, White Collar Complex (WCC, which consists of WC-1 and WC-2), activates transcription of the frequency (*frq*) gene. Its product, FRQ protein, interacts with an RNA helicase, FRH (13), and inactivates the WCC by indirectly phosphorylating and removing WCC from the nucleus (14–16). FRQ is phosphorylated progressively over time, which makes it more susceptible to ubiquitination and degradation triggered by its conformational changes (17–19). The degradation of FRQ results in a new cycle of transcriptional activations by the WCC.

Previous studies in Neurospora showed asynchronous mitotic divisions, with no report of circadian-gated division cycles, despite the presence of robust circadian rhythms (20–22). On the other hand, although synchronous nuclear divisions are observed in other fungi, such as Aspergillus nidulans, it is unknown whether circadian rhythms play a role in the synchrony of their divisions (23). Recent use of GFP labeling has facilitated detailed observations of mitosis in germinating conidia, supporting models for asynchronous mitotic nuclear divisions (21, 24). These experiments, however, did not take into account the potential influence of circadian rhythms in mitotic division cycles. In Neurospora, robust circadian oscillations are observed in constant darkness (DD) or under entrainment regimens (e.g., light–dark cycles), but not in constant light (LL) conditions. There are no reports of experiments that address functional roles of circadian rhythms in mitotic divisions in the syncytium system.

Significance

Circadian rhythms provide temporal information to other cellular processes, such as metabolism. We investigate the coupling between the cell cycle and the circadian clock using mathematical modeling and experimentally validate model-driven predictions with a model filamentous fungus, *Neurospora crassa*. We demonstrate a conserved coupling mechanism between the cell cycle and the circadian clock in Neurospora as in mammals, which results in circadian clock-gated mitotic cycles. Furthermore, we observe circadian clock-dependent phase shifts of G1 and G2 cycles, which may alter the timing of divisions. Our work has large implications for the general understanding of the connection between the cell cycle and the circadian clock.

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The cell cycle regulation of Neurospora has yet to be investigated thoroughly because of some technical limitations, such as adequate methods to synchronize, image, and measure doubling times of nuclear divisions in growing mycelia. We explored the Neurospora genome (25) to find the homologs of key cell cycle regulators and found that Neurospora has a low number of predicted cyclins and CDKs. Neurospora has a single Cdk1 homolog (cdc-2, NCU09778), one G1 cyclin that resembles the sequence of the G1/S regulating budding yeast Cns (cln-1, NCU02114), and two B-type cyclins (clb-1, NCU02758, and clb-3, NCU01242) (26). There also exists a homolog of the CDK1 inhibitor WEE1 kinase (stk-29, NCU04326), which is regulated in a circadian manner in the mouse liver (5). Interactions between the above homologous proteins in budding and fission yeast have been well characterized, and their conservation among eukaryotes (27, 28) suggests they may be wired in a similar fashion in N. crassa.

Here, we investigate the molecular connection between the cell cycle and the circadian clock and functional consequences of this coupling in N. crassa. First, we show that there is a conserved connection between the cell cycle and the circadian clock in Neurospora as in mammals via STK-29, which is the Neurospora homolog of WEE1. Based on this finding and on the hypothesis of conserved cell cycle regulatory interactions, we use mathematical modeling to investigate molecular profiles of both cell cycle and circadian clock components. Our computational simulations predict circadian oscillations of cell cycle components, such as CLN-1 and CLB-1. We experimentally validate this prediction with luciferase bioluminescence reporters to track both cell cycle and circadian clock components in real time in vivo. Moreover, we demonstrate circadian clock-induced phase shifts of cell cycle components, which may alter the timing of divisions. The circadian oscillations of key cell cycle components suggest circadian clock-gated synchronized nuclear divisions. By observing nuclear morphology over time at 25 °C in DD, we indicate that most divisions occur in the evening. We propose that there is a significant coupling between the cell cycle and the circadian clock, which might result in immediate changes in the dynamics of cell cycle regulation upon alterations in circadian rhythms.

Results
There is a Conserved Coupling Between the Cell Cycle and the Circadian Clock in N. crassa as in Mus musculus. A heterodimeric circadian transcription factor, WCC, recognizes light-responsive elements (LREs) to activate target genes (13, 29, 30). We found four putative LREs (GAGATCC, CCGATCC, CGGATCC, and TCGATCT) within 1.75 kb of the stk-29 gene 5′ upstream region (Fig. 1A). To test WCC-dependent activation of stk-29, we performed a light induction experiment. WC-1 is also a photoreceptor that undergoes a light induction response, which is described by a sharp increase in its expression followed by a decrease to its basal level of expression when Neurospora is transferred from dark to light conditions (Fig. 1B). Light-induced WC-1 activates many downstream target genes by recognizing LREs (31). We observe light response from stk-29 mRNA in the wild type, which is abolished in the wc-1ko (Fig. 1C and D). In contrast, we do not observe a light response of cln-1 mRNA in wild-type strains (Fig. 1E). The WC-1–dependent light response of stk-29 indicates that stk-29 is activated by WCC and that it is a potential target for circadian regulation. To verify direct binding of WCC to the promoter of stk-29, we performed a WC-2 ChIP experiment and show that the WC-2 binds to the region close to LRE1 (Fig. 1F). Based on the finding that stk-29 is activated by WCC, we tested a mathematical model of the Neurospora circadian clock and cell cycle as a coupled oscillator and explored coupled dynamics (Figs. S1–S4 and Tables S1–S5).

cln-1 and clb-1 Gene Expression and Protein Abundance Show Circadian Clock-Dependent Oscillations. Our mathematical model predicts circadian oscillations of cell cycle components such as CLN-1 and CLB-1 proteins if intermediate to strong coupling exists between the circadian clock and the cell cycle (Figs. S1–S4). To validate circadian-dependent oscillations of cell cycle factors, we constructed bioluminescence reporters to track in vivo gene expression of cln-1 (NCU02114), clb-1 (NCU02758), stk-29 (NCU04326), and cdc-2 (NCU09778) in real time. Bioluminescence reporters were constructed by fusing the fully codon-optimized luciferase from firefly with a promoter of interest (32). Our data indicate that expression of cln-1, clb-1, and stk-29 from populations of Neurospora nuclei show circadian oscillations (Fig. 24). We also observe circadian oscillations of cln-1 and clb-1 mRNA expressions (Fig. S5). Expression of cdc-2, however, does not follow circadian regulation (Fig. 24). This is in accord with the cell cycle model that we adapted (33), which assumes constitutive expression of cdc-2. We then constructed translational bioluminescence reporters of CLN-1luc, CLB-1luc, and CDC-2luc by fusing luciferase to genes of interest as previously described for FRQluc (34), and followed protein abundances of CLN-1, CLB-1, and CDC-2. The abundance of both CLN-1 and CLB-1

![Figure 1](https://example.com/fig1.png)

**Fig. 1.** stk-29 mRNA shows WC-1–dependent light response, and WC-2 directly binds to the stk-29 promoter. (A) There are four LREs within 1.75 kb of the stk-29 gene 5′ upstream region. The first LRE, GAGATCC, is located 1.75 kb upstream (LRE1); the second LRE, CCGATCC, is located 1.2 kb upstream (LRE2); the third LRE, CCGATCG, is located 0.8 kb upstream (LRE3); and the fourth LRE, TCGATCT, is located −0.25 kb upstream (LRE4) of the stk-29 gene. (B) wc-1 mRNA undergoes light response when Neurospora is moved from dark to light conditions. (C and D) stk-29 mRNA shows light response in the wild type (C), which is abolished in wc-1ko (D). (E) cln-1 mRNA does not show light response in the wild type. The above data are relative units (R.U.) normalized with actin mRNA. The average ± SD is shown. The above data are representative of two or more independent experiments. (F) WC-2 directly binds to the promoter of stk-29. ChIP assay was performed on a wild-type strain (FGSC2489), with samples grown in the dark (0) or in response to a 15-min light pulse (15) using a polyclonal antibody that recognizes WC-2 protein and oligo specific for a region of the stk-29 promoter. A nonspecific IgG and a strain lacking the wc-2 gene (wc-2Δ) were used as controls. The results are an average of five experiments, and the error bars represent the SDs. The asterisks indicate a P value <0.001.
shows circadian oscillations with phase information similar to that of their gene expression profiles (Fig. 2B). The observed phase relationship between CLN-1 and CLB-1 is expected based on their cell cycle functions in G1 and G2/M phases, respectively. In contrast, the abundance of CDC-2 increases continuously over time, corresponding to the growth in mass of Neurospora, and does not exhibit circadian oscillations (Fig. 2B). The data suggest that CDC-2 is stable with a constant rate of expression, consistent with findings in budding yeast (35). Importantly, circadian oscillations of CLN-1 protein and clb-1 and stk-29 gene expression are lost in the frq ko strain, an arrhythmic mutant in which the circadian clock is nonfunctional (Fig. 2C). This indicates that the synchronized oscillations of cell cycle elements are under the influence of circadian rhythms.

Based on the above data, we hypothesized that the expression of cell cycle genes such as clb-1 might be altered in a circadian manner. We performed light-pulse experiments to phase-shift circadian rhythms and investigated the circadian-dependent phase shifts of cell cycle components. We tracked bioluminescence of frq, clb-1, and stk-29 gene expression after a 90-min light pulse at specific time points in DD. We observed ∼3–5-h phase advances and delays in the expression of frq, clb-1, and stk-29 when light pulses were given at DD32 (circadian time 23 (CT23)) and DD48 (CT16), respectively (Fig. 3). This demonstrates that the phases of clb-1 and stk-29 gene expression are influenced by phase

Fig. 2. cln-1, clb-1, and stk-29 demonstrate circadian oscillations. (A) cln-1, clb-1, stk-29, and cdc-2 promoters are fused to the codon-optimized firefly luciferase (32) for real-time analyses of their gene expressions in vivo. A strain carrying frq-luciferase reporter, an established core circadian component, is used as a positive control. (B) cln-1, clb-1, and cdc-2 genes are fused with the codon-optimized firefly luciferase for real-time observation of CLN-1, CLB-1, and CDC-2 protein abundances. (C) A strain housing clb-1–luciferase or stk-29–luciferase reporter is crossed with frq ko mutant resulting in clb-1–luciferase and stk-29–luciferase reporters in frq ko background that show loss of circadian oscillations of clb-1 and stk-29 gene expression. Similarly, the CLN-1 translational reporter is crossed with frq ko mutant, resulting in a CLN-1 translational reporter strain in frq ko background, which shows an arrhythmic phenotype. The above data are representative of three or more independent experiments. Arbitrary units (AU) are shown.

Fig. 3. clb-1 and stk-29 gene expressions indicate circadian clock-dependent phase shifts. (A–C) A 90-min light pulse is given at either DD32 (dashed black) or DD48 (solid black), and the phases of peak expressions of frq, clb-1, and stk-29 genes are compared with unperturbed data (frq, orange; clb-1, blue; stk-29, maroon) at the fourth peak of unperturbed data (dashed straight line). Corresponding peaks are labeled in each figure. The data shown represent three independent experiments. (D) A 90-min light stimulus at DD32 and DD48 creates ∼3–5-h phase advances and delays, respectively. The data are from three independent experiments (Fig. S6), and the average ± SD is shown. Arbitrary units (AU) are shown.

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changes of the circadian clock that are similar in degree and direction, which may alter the timing of nuclear divisions in *N. crassa*.

**Circadian Clock-Dependent Synchronized Nuclear Divisions Occur in the Mice of the Average Mammal.** The lack of circadian oscillations of clb-1 gene expression in *frq* ko does not necessarily indicate altered mitosis (Fig. 2C). Rather, it suggests asynchronous mitotic divisions uncoupled from circadian rhythms. To verify this, we investigated circadian clock-dependent synchronized nuclear divisions. In Neurospora, nuclei are visualized readily by using an *hH1-sgfp* strain in which histone H1 is fused to GFP (21, 24). By using this strain, the stages of the cell cycle can be visualized and categorized. We performed a time-course experiment under circadian conditions (i.e., DD at 25 °C) and classified the populations of nuclei into two categories: interphase and mitotic phase (Fig. 4A). At CT4, or during the subjective day, most nuclei are in interphase, as shown by round nuclear morphology (Fig. 4B). In contrast, many nuclei undergo mitosis at around CT17, which corresponds to late subjective evening (Fig. 4C). Although there is variability in mitotic stage, around 60% of nuclei are actively dividing in the evening (Fig. 4D). These data clearly demonstrate circadian oscillations in Neurospora mitotic divisions. The synchronized nuclear divisions are not observed in the *frq* ko strain (Fig. 4E), which indicates that circadian rhythms are necessary for this daily synchronization of cell cycles. These observations are in accord with the arrhythmic *clb-1* and *stk-29* gene expression in *frq* ko (Fig. 2C). We also used an established mitosis marker, phospho-histone H3 (pH3) antibody, as an independent measurement of mitosis (36, 37). We observed more pH3-positive nuclei at DD25 (CT15) than at DD35 (CT2) (Fig. S7 A and B).

The above experiments are performed by harvesting Neurospora from liquid culture media in DD and counting the number of nuclei present in fixed cells. It is important to note that we observe similar results via live cell imaging from Neurospora grown in defined solid agar media, in which we observe a second cycle of increased and decreased mitosis at DD47 and DD57, respectively (Fig. S8 and Movies S1–S4).

**Discussion**

In silico, we investigated various scenarios of coupled dynamics between the circadian clock and the cell cycle, which demonstrated circadian oscillations of cell cycle components if significant coupling exists between the two oscillators (Figs. S2–S4). We have demonstrated experimentally that elements of the cell cycle (e.g., *cln-1* and *clb-1*) undergo circadian oscillations, which manifest a circadian clock-dependent synchronized mitotic division in Neurospora. We also show that both *clb-1* and *stk-29* gene expression undergo light-dependent phase shifts in a length and direction similar to those of *frq* gene expression. This suggests circadian clock-dependent phase shifts of cell cycle components, which might be used to alter the timing of mitotic divisions.

The fundamental molecular regulatory architecture of circadian rhythms that highlight the time-delayed negative feedback mechanism is conserved from *N. crassa* to *M. musculus* (38). Coupling between circadian rhythms and the DNA damage response pathway is also conserved between Neurospora and mammals. Checkpoint kinase 2 (CHK2) is activated upon DNA damage and phosphorylates one of the core clock components (i.e., PER1 in mammals and FRQ in Neurospora), resulting in a subsequent degradation of PER1 or FRQ that leads to predominantly phase advances in circadian rhythms (39–43). We demonstrate that WC-2 binds to the promoter of *stk-29* (NCU04326) and that *stk-29* undergoes WC-1–dependent light-response and circadian oscillations, which shows conserved coupling between the cell cycle and circadian rhythms. The binding of WC-2 to the *stk-29* promoter was not reported in the recent WC-2 ChIP-sequencing data (44). This is probably a result of the low expression of *stk-29* and the less dramatic light response of *stk-29* compared with other targets. Further investigations are needed to understand the detailed dynamics of these connections as well as other possible coupling factors. We have shown circadian oscillations in a few cell cycle regulators. However, it is unclear whether these cycling components are genuine coupling components or mere reflections of the circadian-gated cell cycle determined by the currently known coupling factor STK-29. Recently, microarray data have suggested that several genes in cell cycle control show oscillatory behavior in Neurospora (45). Identification of other factors that couple the cell cycle and circadian rhythms will elucidate distinct points of interactions in which the circadian clock influences the cell cycle.

Identified conserved coupling components (i.e., CHK2 and WEE1) among the circadian clock, DNA damage response, and cell cycle mechanisms pose Neurospora as an ideal model organism to investigate the fundamental wiring of this network. However, one of the main disadvantages of Neurospora is that it is technically difficult to assess the doubling time of mitotic cycles in Neurospora mycelium grown on solid agar media. Previous measurements in liquid culture media showed a range in doubling time from 72 to 239 min, depending on growth conditions from young germinating conidia (46), which is in good agreement with our measurements in noncircadian conditions (e.g., LL) (Fig. S9). However, the doubling time in mature mycelium in circadian conditions (i.e., DD) might be different because of the presence of the circadian clock. Therefore, measuring the doubling time of nuclear divisions in DD for an extended period will be critical for future experiments. Real-time fluorescence and bioluminescence reporters, in addition to the use of
microfluidic devices for single-nucleus imaging, may facilitate measurement of doubling times for Neurospora growing in both liquid culture and solid agar media.

In this report, we demonstrate that many nuclear divisions occur during a specific window of circadian time. However, our experimental data also show that synchronized divisions are spread out over 6 h, with less frequent nuclear divisions also occurring at other times of the day (Fig. 4D). This does not imply that a single nucleus spends 6 h in the mitotic state; rather, our data suggest an increase in mitosis as a population within that 6-h window. It is also possible to hypothesize that a weak coupling might exist that enables the circadian clock to modulate the total abundance of CLB-1, which might allow more divisions during the evening in a threshold-dependent manner while keeping the cell cycle time short (Figs. S2B and S3B). Another hypothesis that might result in the observed phenotype is context-dependent (e.g., aging, nutrient conditions) weak to strong coupling. Our modeling work and other mathematical models predict quasi-periodic multimodal doubling times depending on the strength of the coupling and the frequency of the two oscillators (8, 47).

In our future work, we plan to assess the strength of the coupling and the doubling time by using both computational simulations and experiments observing both cell cycle and circadian components with bioluminescence assays. Our discovery of circadian clock-dependent synchronized mitotic cycles in Neurospora will serve as a stepping-stone for further investigations to uncover conserved principles of coupled mechanisms between the cell cycle and circadian rhythms.

Materials and Methods

Strains. Strains used for the experiments are a clock wild-type ras-1-ko (328-4) and three arrhythmic mutants from laboratories of Drs. J. Dunlap and G. O'Neill (Fig. S1). Strain 358-6 (ras-1-ko; clb-1-luc; A and 358-6 (ras-1-ko; frq-ko; a). The strain stk-29-luc;frq-ko is a progeny from a cross between stk-29-lucras-1-ko; A and 358-6 (ras-1-ko; frq-ko; a). The strain CLN-1-luc; frq-ko is a progeny from a cross between CLN-1-luc;frq-ko; and 358-6 (ras-1-ko; frq-ko; a). The strain hh1-lucsgfp;frq-ko is a cross progeny between hh1-lucsgfp (FGSC9518) and 358-6 (ras-1-ko; frq-ko; a).

Quantitative RT-PCR. Neurospora was grown in liquid culture media containing Vogel’s medium (pH 5.8) with 2% (w/v) glucose, 0.5% arginine, and 50 ng/mL biotin, and harvested as previously described (31). Total RNA was isolated using Tri Reagent (Molecular Research Center, Inc.), and quantitative RT-PCR (qRT-PCR) was performed as previously described (31). The actin mRNA is used to normalize real-time qRT-PCR data.

ChiP. ChiP was performed in a manner similar to methods previously described, with slight modifications (51, 52). One hundred-milliliter cultures of Neurospora mycelia were cross-linked with 1% formaldehyde for 15 min and then quenched with 0.1 M glycine for an additional 15 min. The Neurospora was lysed by filtration through a bed of Whatman paper, and the tissue was added to 10 mL FA lysis buffer (0.05 M Hepes, pH 7.4/0.5 M NaCl/0.001 M EDTA/1% Triton TX-100/0.1% SDS) containing protease inhibitors (0.002 mg/mL leupeptin, 0.002 mg/mL pepstatin A, 0.001 M PMSF). To improve cell disruptions, the tissue was subjected to a single sonication at 50% power and the cellular debris was removed by centrifugation at 2500 × g for 10 min. A chromatin-enriched fraction then was obtained by a high-speed centrifugation (20,000 × g for 30 min). The pellet was suspended in the lysis buffer and eluted two times with 50 mL 0.1 M sodium bicarbonate and 1.0% SDS. The cross-links were reversed by incubating for 4 h at 65 °C in the presence of 0.1 M NaCl. The DNA was recovered by treatment with proteinase K for 1 h followed by a phenol/ chloroform extraction, then suspended in 10 mM Tris, pH 7.5/1.0 mM EDTA. Two milliliters of the purified DNA was used in a quantitative PCR with primers specific to the stk-29 promoter.

Bioluminescence Assay. In all experiments, Neurospora was grown at 25 °C in constant white fluorescent light (LL) overnight before being transferred into constant darkness (DD) for time-course experiments. For bioluminescence assays, samples were grown with Vogel’s medium (pH 5.8) with 0.1% glucose, 0.17% arginine, 50 mg/mL biotin, 1.5% (w/v) agar, and 12.5 μM luciferin (Fig. 2). In vivo luciferase activity was collected for 10 min every hour with a PIXIS CCD camera from Princeton Instruments controlled with WinView/32 software from Roper Scientific. A 90-min pulse of white fluorescent light (80 μmol photons m⁻² s⁻¹) was given at indicated time points for phase-shift experiments, and in vivo luciferase activity was collected for 10 min every 2 h.

Microscopy. For microscopy experiments, Neurospora conidia suspensions were grown in 500-mL baffled flasks in liquid culture media containing Vogel’s medium (pH 5.8) with 2% (w/v) glucose, 0.5% arginine, and 50 ng/mL biotin. Neurospora was grown at 25 °C in constant white fluorescent light (LL) overnight before being transferred into constant dark (DD) for time-course experiments. Samples were grown on a shaker at 125 rpm. Random samples of mycelia were collected and fixated with 1% formaldehyde. 10% paraformaldehyde/PBS at indicated time points and observed under a confocal fluorescence microscope (Zeiss LSM710). Two to three slides were prepared from each time point, and four to six images of mycelia were captured from each slide. Nuclei from each image were analyzed to calculate the average number of nuclei undergoing mitosis.

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**Genes Dev**

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