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Light Regulates the Cell Cycle in Zebrafish

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Summary

The timing of cell proliferation is a key factor contributing to the regulation of normal growth. Daily rhythms of cell cycle progression have been documented in a wide range of organisms [1, 2]. However, little is known about how environmental, humoral, and cell-autonomous factors contribute to these rhythms. Here, we demonstrate that light plays a key role in cell cycle regulation in the zebrafish. Exposure of larvae to light-dark (LD) cycles causes a range of different cell types to enter S phase predominantly at the end of the day. When larvae are raised in constant darkness (DD), a low level of arrhythmic S phase is observed. In addition, light-entrained cell cycle rhythms persist for several days after transfer to DD, both observations pointing to the involvement of the circadian clock [3–6]. We show that the number of LD cycles experienced is essential for establishing this rhythm during larval development. Furthermore, we reveal that the same phenomenon exists in a zebrafish cell line. This represents the first example of a vertebrate cell culture system where circadian rhythms of the cell cycle are observed. Thus, we implicate the cell-autonomous circadian clock in the regulation of the vertebrate cell cycle by light.

Results and Discussion

In most organisms, light plays a major role in the synchronization of the circadian timing system with the environmental day-night cycle [7]. The circadian clock has been implicated in directing daily rhythms of cell division in several unicellular organisms and higher vertebrates [1, 2]. The vertebrate circadian clock is based on a complex hierarchy consisting of a small number of specialized central and multiple peripheral pacemakers [8]. The central pacemaker directs circadian rhythms of hormonal release implicated in growth control [9]. Furthermore, most cell types have been shown to possess their own circadian clocks [8]. Thus, cell proliferation could be regulated by the clock via humoral as well as cell-autonomous mechanisms. The zebrafish has properties that make it a valuable system for studying the links between cell proliferation, the circadian clock, and the environment. Zebrafish tissues and cells are directly light sensitive; thus, exposure of explanted tissues, and even cell lines, to LD cycles entrains rhythms of clock gene expression [10]. Zebrafish early larval stages show significant levels of cellular proliferation and the maturation of a functional circadian clock [3], but they have not yet established feeding behavior that is known to influence circadian clock function [11, 12]. We therefore decided to test the influence of light upon the timing of cell cycle progression in this vertebrate. We examined 6-day-old zebrafish larvae raised at 25°C. At this temperature, larvae hatch early during day 4 and start active daytime feeding around 9 days post fertilization. Groups of sibling larvae were raised either in forward or reversed light-dark cycles (LD or DL, respectively, with 12 hr light and 12 hr dark periods) or maintained in constant darkness (DD). The larvae were kept in thermostatically controlled water baths to avoid temperature variations that could influence cell proliferation. During day 6, starting at ZT3 (zeitgeber time 3, where ZT0 is defined as lights on), we treated larvae at six hourly intervals with the thymidine analog bromodeoxyuridine (BrdU). BrdU is incorporated into DNA only during its replication and so constitutes an unambiguous marker for S phase nuclei.

In a LD cycle (Figure 1A), abundant stained nuclei distributed uniformly over the larvae were observed at the ZT9 time point (3 hr before lights off), while only very low numbers were detected at ZT21 (3 hr before lights on). Larvae sacrificed at ZT3 and ZT15 showed intermediate numbers of S phase nuclei. Shifting the phase of the LD cycle by 12 hr in sibling larvae (DL conditions) led to a 12 hr shift of the rhythm (Figure 1A), thereby excluding the advancing developmental stage of the larvae from being responsible for driving this rhythmicity. In contrast, in DD at all four time points, similar low numbers of stained nuclei were observed (Figure 1A). These S phase nuclei were restricted to skin cells due to the method for BrdU staining used. In larvae of this age, the skin consists of a bilayered, ectodermally derived epidermis. S phase nuclei were counted over a section of the larval body between the posterior tip of the swim bladder and the anus (see Figure 1B). The data revealed a 15-fold difference between the peak and trough points in LD cycles and confirmed the low, nonoscillating levels in DD (Figure 1B). Therefore, the timing as well as the total number of cells in S phase each day seems to be a function of the ambient lighting

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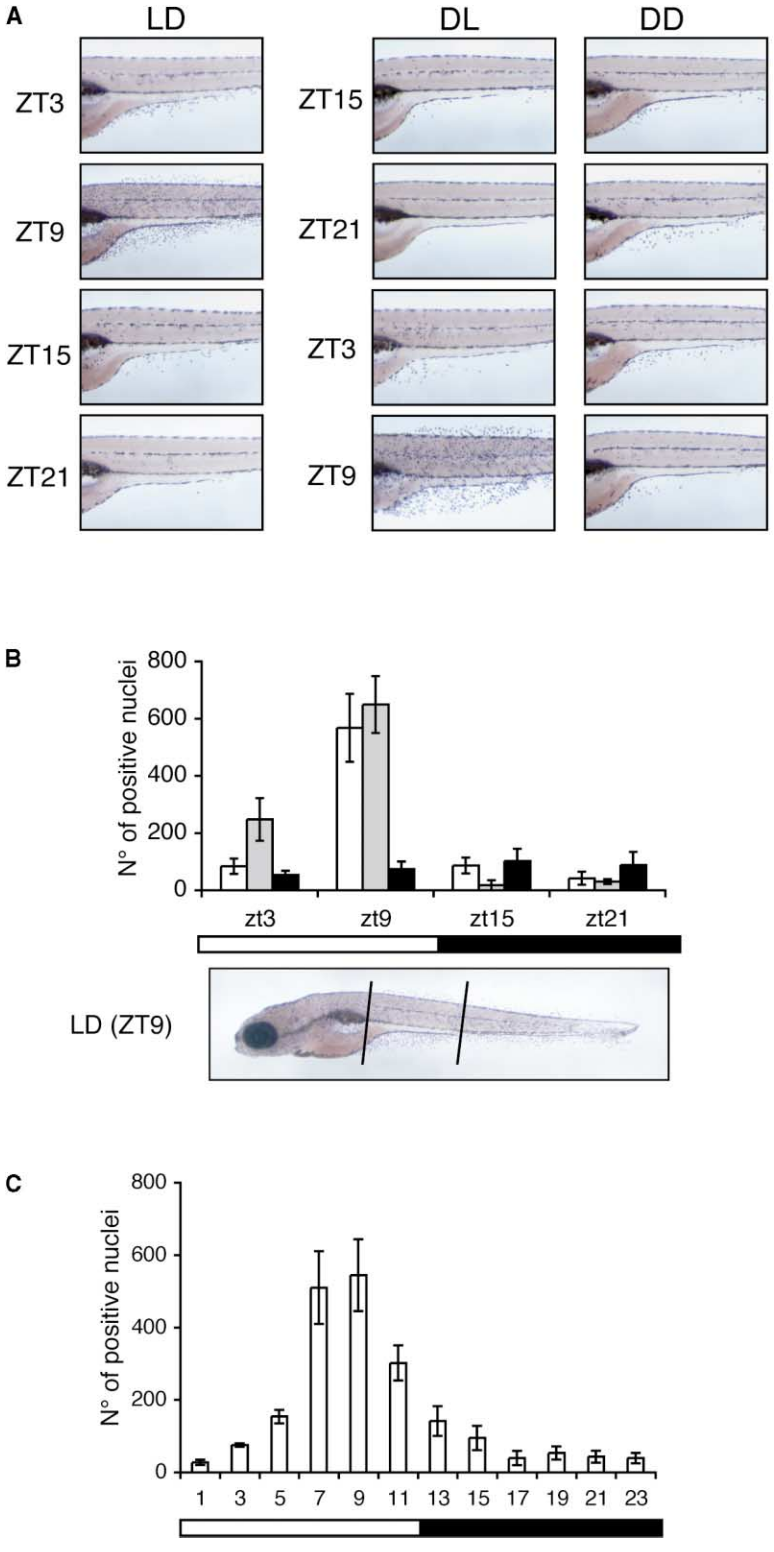


Figure 1. Light Cycles Regulate the Timing of S Phase in Zebrafish Larval Skin

(A) Whole-mount staining for BrdU incorporation in 6-day-old zebrafish larvae raised at 25°C under normal light-dark cycles (LD), reversed light-dark cycles (DL), or constant darkness (DD). Three hours after "lights on" for the LD larvae (zeitgeber time [ZT] = 3) larvae were incubated for 20 min with BrdU. This corresponded to ZT = 15 for the DL larvae. Larvae at the same time point in DD were also labeled. This procedure was repeated at three additional time points at 6 hr intervals.

(B) Quantification of BrdU-positive nuclei from the experiment shown in (A). At each time point, the mean number of positive skin nuclei lying between the posterior tip of the swim bladder and the anus calculated from 10 larvae per experiment (*y* axis) was plotted against ZT time (*x* axis) for LD (white bar), DL (gray bar), and DD (black bar). A horizontal white and black bar below the *x* axis denotes the light and dark periods. Below is shown a representative zebrafish larva stained for BrdU incorporation where two lines delimit the region in which nuclei were counted. Differences between peak and trough points for the LD and DL cycles are highly significant ($p << 0.0001$). Differences between DD values were not significant ($p \geq 0.39$).

(C) Mean numbers of BrdU-labeled nuclei from larvae raised in LD conditions but labeled each 2 hr during a 24 hr time course. *x* axis values represent ZT time.

conditions. These observations imply that exposure of zebrafish to LD cycles might constitute a mitogenic stimulus. A higher-resolution analysis was performed, labeling LD larvae each 2 hr (Figure 1C). The results of this analysis show that the period of elevated S phase lasts for approximately 8 hr with a peak 3 hr before the end of the day. Interestingly, in certain unicellular organisms, S phase is also timed to occur at the end of the day or during the night [13]. This has led to the hypothesis that the adaptive significance of daily timing of the cell cycle is to avoid DNA damage induced by UV radiation in sunlight during the critical step of DNA replication [13].

Time-of-day-dependent changes in the spectral composition of sunlight penetrating water may result in the observed late afternoon increase in S phase correlating with low UV exposure [14]. Alternatively, entry into S phase at the end of the day could ensure the appropriate timing of mitosis.

We wished to determine whether the light-directed rhythm of S phase was only a skin-cell-specific property. Therefore, we selected tissues derived from two other embryonic germ layers: the heart and the gut. The heart has been previously reported to contain a directly light-entrainable circadian clock [10] and, contrary to the skin, would be predicted to show low levels of cell proliferation. BrdU labeling of the hearts dissected from 6-day-old larvae raised in a LD cycle revealed a rhythm of S phase nuclei that was similar in timing to that documented in the skin but with a significantly lower amplitude (Figures 2A and 2B). In the gut, a high-amplitude rhythm of S phase was detected similar to that seen in the skin (data not shown). The presence of BrdU-labeled nuclei in these organs strongly suggests that this phenomenon exists in various cell types. Thus, the timing of cell proliferation in many tissues seems to be influenced by light. Interestingly, we have observed the highest-amplitude rhythms of S phase in tissues that manifest sustained high levels of cell proliferation and regeneration, suggesting the importance of this temporal regulation under conditions of rapid cell turnover in vivo. It is difficult to predict how changes in the rate of cell proliferation in such tissues would alter how rapidly they grow. However, it would be interesting to determine whether fish raised for long periods in constant darkness show reduced growth.

We next asked how this S phase rhythm is regulated. The prediction for a purely light-driven process would be that upon transfer to constant darkness, rhythmicity would rapidly be abolished. We therefore tested the persistence of light-entrained S phase rhythmicity in embryos that were raised for 5 days in LD and DL cycles and then transferred to DD. On the second and third days after transfer to DD, embryos were labeled and analyzed for BrdU incorporation (Figure 2C). Rhythmicity in the numbers of S phase nuclei continued during the time course of the analysis, although the amplitude of this rhythm was significantly reduced on the third cycle. Also, the period length of the S phase rhythm seems to be slightly longer than 24 hr, leading to a progressive shift of the peaks into the predicted subjective night (Figure 2C). These results are entirely diagnostic of the circadian timing system's involvement in directing the daily rhythms of cell cycle progression [4–6]. Furthermore, the arrhythmic S phase seen in larvae raised in DD conditions is consistent with previous reports of circadian clock outputs in zebrafish. Specifically, circadian rhythms of locomotor activity and melatonin release are not observed in zebrafish raised in constant darkness [3, 15, 16]. One can speculate that coupling cell cycle to the circadian clock might confer properties such as temperature compensation on the cell cycle [17]. Such a mechanism might dampen the influence of environmental fluctuations on the levels of cell proliferation.

We next investigated when during development the light-entrainable S phase rhythms first appeared. Prior to day 4, no 24 hr rhythm was detected, and the distribution of positive nuclei tended to be nonuniform (data not shown). Analysis of larvae raised in LD conditions at 25°C on day 4 revealed a low-amplitude (2- to 3-fold)

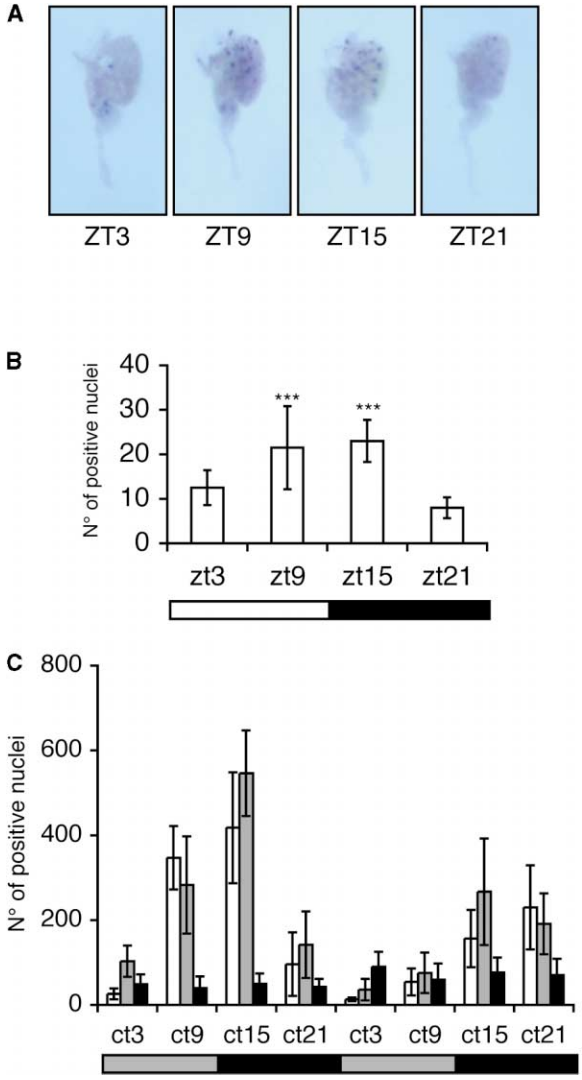
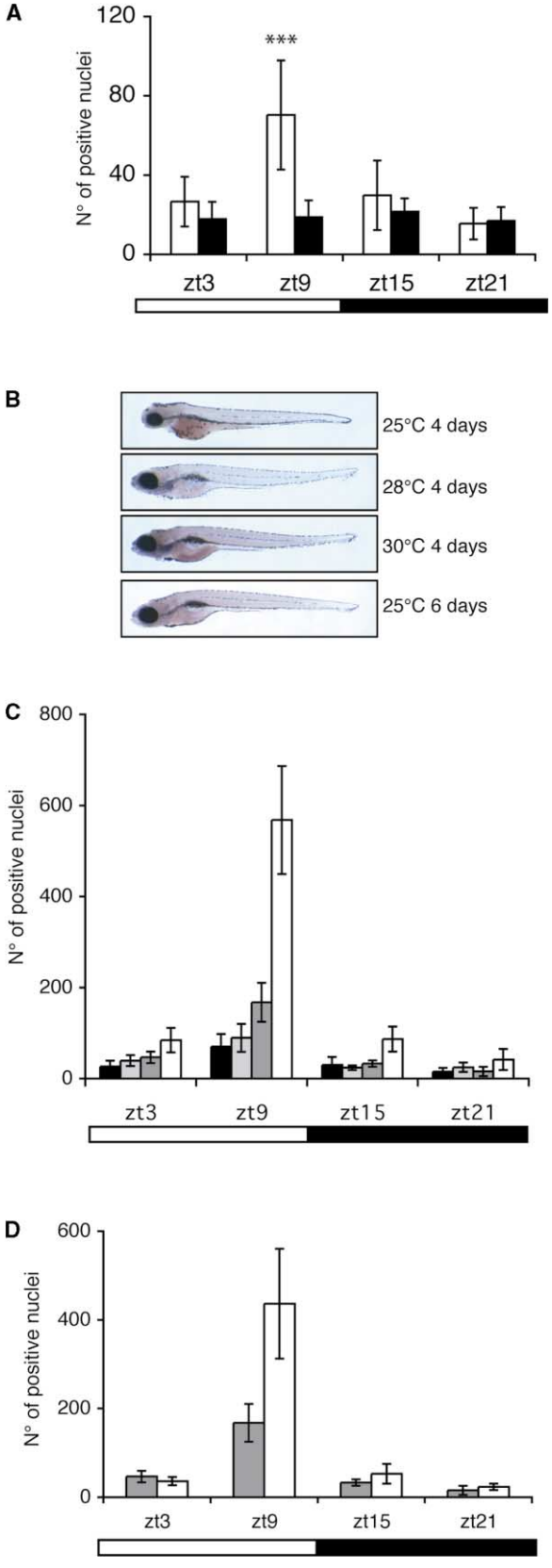


Figure 2. S Phase Rhythm in the Heart

(A) Representative hearts from 6-day-old, LD-entrained larvae sacrificed at the indicated ZT time points.

(B) Mean numbers of BrdU-positive nuclei calculated from ten hearts per point per experiment plotted against ZT time. Values at the peak points ZT9 and ZT15 are significantly different from the trough ZT21 point ($p \leq 0.0001$, indicated by ***). Differences between the ZT3 and ZT21 points are not significant.

(C) Light-entrained circadian clock regulates S phase in larvae raised under a light-dark cycle. Mean numbers of BrdU-positive nuclei in larvae raised for 5 days either in LD (white bars), DL (gray bars), or DD (black bars) and then transferred for 3 days into DD. Larvae were harvested at eight time points distributed over 48 hr at 6 hr intervals, starting from the beginning of the second day in DD. The gray and black bars below the graph denote the predicted duration of subjective day and night. Differences between the peak and trough points for both cycles following entrainment in LD and DL are highly significant ($p << 0.0001$). Differences between DD values were not significant.



rhythm with nuclei uniformly distributed over the entire body (Figure 3A). We then questioned whether the number of LD cycles experienced or the developmental stage contributed to the amplitude of light-entrainable S phase rhythms. Specifically, we tested whether accelerating development by raising the embryos at higher temperatures led to an earlier appearance of a high-amplitude rhythm. Initially, we raised sets of embryos for 4 days at 25°C, 28°C, or 30°C in LD conditions. The higher temperatures resulted in a significant acceleration of the rate of development and, consequently, hatching was advanced to 2.5 days post fertilization in 28°C fish and 1.5 days in 30°C fish. Examination revealed that the 4-day-old 28°C and 30°C larvae were comparable or slightly advanced in developmental stage relative to 6-day-old 25°C fish (Figure 3B). The 4-day-old 30°C larvae showed a low-amplitude S phase rhythm only slightly increased relative to that of 4-day-old 25°C and 28°C larvae (Figure 3C). These results contrast with the high-amplitude rhythm observed for 6-day-old larvae raised at 25°C (Figure 3C). Finally, examination of 5-day-old larvae raised at 30°C confirmed that the characteristic increase in rhythm amplitude did subsequently occur in the larvae raised at higher temperatures (Figure 3D). These results indicate that the number of LD cycles experienced and not the developmental stage is a major factor determining the time of appearance of a high-amplitude S phase rhythm. Circadian rhythms of clock gene (*zfperiod3*) expression have been documented in embryos raised in DD conditions and used as evidence for maternal inheritance of the circadian clock [18]. Previous reports have documented that clock outputs in the zebrafish such as rhythmic locomotor activity and melatonin release are only established following previous exposure to LD cycles [3, 15, 16]. Our data would tend to reinforce the notion that the development of circadian clock outputs in zebrafish is tightly linked with exposure to light cycles [3]. This could imply that the mechanism coupling the clock with its outputs may be directly affected by LD cycles.

We next wished to determine at which level light influences the timing of cell cycle progression. The activity of the neuroendocrine axis is under central pacemaker

Figure 3. Influence of the Number of LD Cycles on the Increase in the Amplitude of S Phase Rhythms during Development

(A) Mean numbers of BrdU-positive nuclei in 4-day old-larvae raised in LD and DD conditions at 25°C (white and black bars, respectively). Differences between the peak (ZT9) and trough points (ZT21) in LD were significant ($p < 0.0001$, indicated by ***). Differences between DD values were not significant.

(B) Images of 4-day-old BrdU-labeled larvae raised at 25°C, 28°C, and 30°C compared with a 6-day-old larva raised at 25°C.

(C) Mean numbers of BrdU-positive nuclei in 4-day-old larvae raised under LD conditions either at 25°C (black bars), 28°C (light gray bars), or 30°C (dark gray bars). These are compared with the results from 6-day-old larvae raised at 25°C (white bars). The peak values at 25°C and 28°C in 4-day-old fish were not significantly different ($p \geq 0.16$). However, peak values of 25°C and 28°C were both significantly different from 30°C ($p \leq 0.0001$).

(D) Mean numbers of BrdU-positive nuclei in larvae raised at 30°C at 4 days old (dark gray bars) and 5 days old (white bars). Peak values at ZT9 for day 4 and day 5 were significantly different ($p < 0.0001$).

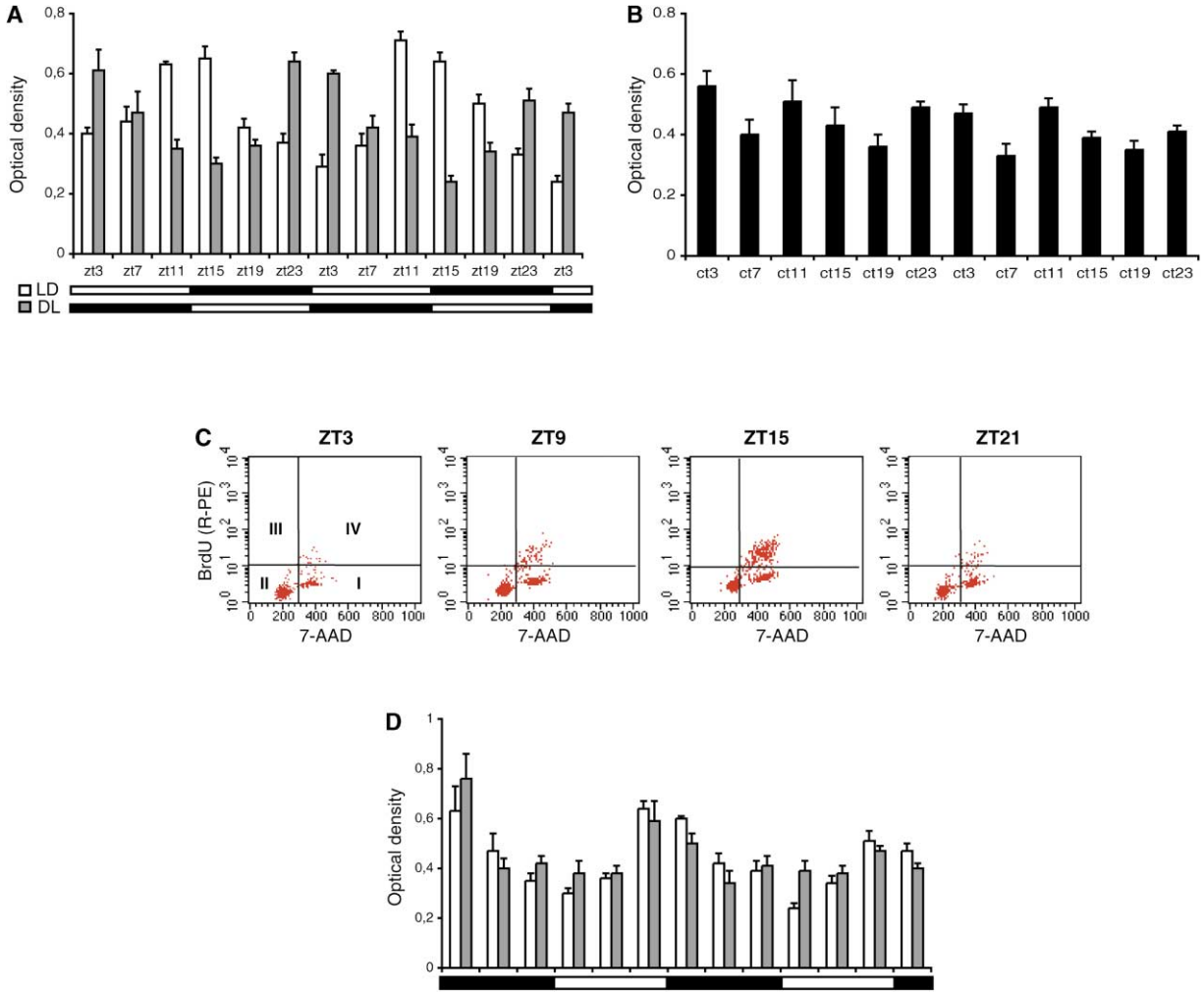


Figure 4. Light Entrains Circadian Rhythms of S Phase Cell Autonomously

(A) ELISA assay results of BrdU incorporation in PAC-2 cells maintained under normal or reversed light-dark cycles (LD, white bars; DL, gray bars). Both LD and DL optical density readings (OD_{450}) are plotted together on the same *x* axis, and each time point is labeled with the ZT time calculated relative to the LD cycle. White and black bars below show the timing of the light and dark periods for each cycle.

(B) ELISA data from cells maintained under DD conditions and harvested at the same time points as the LD and DL sets.

(C) FACS analysis of cells maintained under a LD cycle, labeled, and harvested at four time points. In each panel, dots represent single cells and are plotted against an *x* axis, quantifying, on a linear scale, 7-AAD fluorescence (cellular DNA content), and against a *y* axis, quantifying on a logarithmic scale, R-PE-conjugated BrdU antibody fluorescence (newly synthesized DNA). Quadrant II contains G0/G1 cells, S phase cells lie between quadrants III and IV, and G2/M cells predominantly occupy quadrants I and IV.

(D) ELISA results from cells incubated for 5 days in DL and then transferred to DD for 1 day before harvesting during a 48 hr period in DD (gray bars). Control cells were maintained under DL conditions up to and during the period of analysis (white bars).

For all ELISA and FACS results, differences between peak and trough values under light-dark cycles were highly significant ($p << 0.0001$).

control [19]. By this mechanism, circadian rhythms of hormone release direct many physiological processes including growth [9]. Thus, the possibility that such hormonal signals might direct timing of the cell cycle in zebrafish cannot be excluded. Alternatively, clock components might directly control cell cycle regulatory factors cell autonomously. Recently, the mPER2 clock protein in mouse has been implicated as a tumor suppressor [20], and the direct regulation of cell cycle regulatory genes by clock components has been documented during hepatic regeneration in mouse [21]. In order to determine the contribution of cell-autonomous regulation, we examined the effect of light on cell cycle progression in zebrafish embryo-derived cell cultures that have been previously reported to contain a directly light-entrainable circadian clock [10, 22]. This approach also allowed us to confirm the involvement of the zebrafish circadian clock in regulating cell cycle progression in a system more simple than the developing larva. PAC-2 cell cultures were seeded at subconfluence and exposed for 6 days to LD, DL, or DD conditions. The culture medium was then supplemented with BrdU at given time points and the number of labeled cells was measured using an ELISA assay. When cultures were exposed to LD cycles, they showed a robust 24 hr rhythm of S phase cells with a peak at the light-dark

transition, slightly later than that observed in the larvae. This rhythm was phase shifted by 12 hr in cells exposed to DL cycles (Figure 4A). A nonrhythmic profile was observed in cells maintained for an extended period in DD conditions (Figure 4B). This result was also confirmed by FACS analysis (Figure 4C). Cells were double labeled with BrdU and the DNA dye 7-Amino-Actinomycin D (7AAD), and subsequent sorting revealed the number of cells in G_0/G_1, S, and G2/M phases of the cell cycle. By this assay, the number of cells in S and G2/M phases showed the same rhythm observed in the ELISA analysis (Figure 4C and data not shown). As previously demonstrated in the zebrafish larvae, after transfer of LD- or DL-adapted cells to DD, rhythmic BrdU incorporation persisted on the second and third day with dampening (Figure 4D). The properties of these rhythms in cells cultured in the various LD and DD regimes are entirely consistent with previous reports of rhythmic clock gene expression in zebrafish cell lines maintained under equivalent conditions. Rhythms of *zfClock*, *zfperiod1*, *zfperiod3*, *zfBMAL1*, and *zfBMAL2* mRNA expression persist for 2 to 3 days after transfer from LD to DD, and on the fourth day, expression patterns are randomized [10, 22]. Our data strongly suggest a cell-autonomous contribution of the circadian clock to the light-regulated daily rhythms of S phase in zebrafish. Previously reported vertebrate cell culture models for the circadian clock are less attractive for studying this aspect of cell cycle regulation. Specifically, circadian clock rhythms in mammalian cell lines are only induced by transient exposure to either high serum concentrations or activators of various signaling pathways [23, 24]. All of these treatments have profound effects on cell proliferation independently of the circadian clock.

Conclusions

Here, we document a previously unknown facet of cell cycle regulation in the zebrafish, a widely used vertebrate model organism. Our results reveal that direct exposure to visible light determines the timing of S phase.

Our observations strongly implicate the circadian clock in mediating the effects of light on the cell cycle in zebrafish. (1) After exposure to LD cycles, S phase rhythms persist for several cycles upon transfer to constant darkness with a circadian period length [4–6]. (2) We reveal a crucial requirement for LD cycles in establishing these rhythms during development: the presence of arrhythmic S phase in larvae that have never been exposed to light and the increase in amplitude of S phase rhythms depending on the number of LD cycles experienced. This is consistent with previous reports documenting the maturation of other zebrafish clock outputs [3, 15, 16]. (3) Similar rhythms can be entrained by LD cycles in a zebrafish embryo-derived cell line and have properties that match well with previous reports of rhythmic clock gene expression in these cell cultures [10, 22].

The availability of cell lines with a light-entrainable clock gives the zebrafish a distinct advantage over other vertebrate models for studying the role of the circadian clock in the regulation of the cell cycle. For the first time, using these cells we have implicated a cell autonomous contribution of the circadian clock in generating rhythms of cell cycle progression.

Experimental Procedures

Raising Zebrafish Larvae

The zebrafish Tübingen strain was maintained and crossed according to standard methods. Fertilized eggs were collected within 2 hr of laying and rinsed well, and aliquots of 35 were transferred into 20 ml of E3 buffer [25] in 25cm^2 tissue culture flasks. Flasks were sealed and then submerged horizontally in large-volume, thermostatically controlled water baths to maintain a constant temperature. Larvae were illuminated with a tungsten light source (11μW/cm^2) connected to a programmable timer.

BrdU Labeling of Larvae

Larvae were incubated for 20 min in E3 buffer with a final concentration of 10 mM BrdU. Fixing and staining for BrdU incorporation was performed as described elsewhere [25]. To visualize BrdU incorporation of internal structures, 6-day-old larvae were bisected prior to the staining procedure followed by dissection of the organs. The mean and standard deviation of the number of positive nuclei were calculated, and single factor Anova analysis was used to assess statistical significance.

Cell Culture, ELISA, and FACS Assays

The establishment of the PAC-2 cell line has already been reported [26]. They were derived from 24-hr-old wild-type zebrafish embryos, and their growth properties indicate a fibroblast origin. Culture conditions were as previously described [10]. Cultures were illuminated while immersed in large-volume (60 liter), thermostatically controlled water baths with a tungsten light source (11 μW/cm^2) connected to a programmable timer.

A Biotrak cell proliferation ELISA assay kit (Amersham) was used to quantify BrdU incorporation. 1.5×10^4 cells per well were seeded in a 96-multiwell plate and incubated with 10 μM BrdU for 2 hr. They were then processed as recommended by the manufacturer. Plates were finally assayed on a multiwell plate reader (Sunrise reader, Tecan). Addition of BrdU to the culture medium during dark periods was performed under dim red light. For each experimental time point (minimum 16 wells), the mean OD_{450} measurement plus the standard deviation was calculated. Single-factor Anova analysis was used to assess the statistical significance of differences between peak and trough points.

For FACS analysis, after 2 hr labeling with 10 μM BrdU, 1×10^6 cells were harvested and DNA stained using 7-AAD (Via-PROBE, Becton Dickinson) and a R-PE-conjugated anti-BrdU antibody (Becton Dickinson) according to the manufacturer's instructions. Background fluorescence for the anti-BrdU antibody was assessed with an aliquot of cells stained by an R-PE-conjugated isotype control antibody (Becton Dickinson). FACS analysis was performed using a Becton Dickinson FACScalibur machine and data analyzed using Cellquest software.

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