moter (*19*) or with antisense *Orca3* expression (Fig. 4B).

Thus, plants can regulate primary metabolic pathways coordinately with secondary metabolism using a single transcription factor. Because the biosynthesis of many secondary metabolites is induced by jasmonate, the identification of an AP2-domain protein as regulator of several genes involved in JA-responsive metabolism uncovers a control mechanism that may be operative in other stress-responsive plant metabolic pathways as well.

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9. L. van der Fits and J. Memelink, data not shown.
10. Ten micrograms of genomic DNA from line 46 were digested with Xba I, self-ligated, and electroporated into *Escherichia coli* strain NM554. Cells were selected on carbenicillin.
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14. One microgram of genomic *C. roseus* DNA was digested with Eco RI and self-ligated and inverse-PCR (I-PCR) was performed with primers OR5 and OR8 (Fig. 2B).
15. Northern blots were hybridized with *C. roseus* cDNAs as follows (see Fig. 1 legend for abbreviations; GenBank accession numbers are in parentheses): *Tdc* (Acc. No. X67662), *Str* (Acc. No. X61932), *Sgd* (Acc. No. AF112888), *G10h* (Acc. No. AJ251269), *Cpr* (Acc. No. X69791), *D4h* (Acc. No. U71604), *Dat* (Acc. No. AF053307), *Dxs* (Acc. No. AJ011840), *Asα* (Acc. No. AJ250008), *Geranylgeranyl pyrophosphate synthase* (*Ggpps*) (Acc. No. X92893), *Isochorismate synthase* (*Ics*) (Acc. No. AJ006065), and 40*S* ribosomal protein S9 (*Rps9*) (*12*).
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An Inherited Functional Circadian Clock in Zebrafish Embryos

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Circadian clocks are time-keeping systems found in most organisms. In zebrafish, expression of the clock gene *Period3* (*Per3*) oscillates throughout embryogenesis in the central nervous system and the retina. *Per3* rhythmic expression was free-running and was reset by light but not by the developmental delays caused by low temperature. The time of fertilization had no effect on *Per3* expression. *Per3* messenger RNA accumulates rhythmically in oocytes and persists in embryos. Our results establish that the circadian clock functions during early embryogenesis in zebrafish. Inheritance of maternal clock gene products suggests a mechanism of phase inheritance through ovogenesis.

Circadian rhythms in physiology and behavior allow living organisms to anticipate daily environmental changes (*1*). These rhythms are driven by endogenous circadian clocks synchronized to external time cues. All known circadian clocks, in organisms ranging from cyanobacteria to mammals, involve clock genes that interact to generate a molecular oscillator regulating output clock-controlled genes (*2, 3*). Studies in mammals have suggested that the circadian clock starts to function during late fetal and postnatal life (*4*). However, in lower vertebrate embryos, external development may require an earlier onset of the clock. In addition, although the mammalian fetal circadian rhythm is synchronized to that of the mother through maternal signals such as melatonin (*5*), such a mechanism cannot operate in embryos developing externally.

To analyze the development of the circadian clock in zebrafish, we isolated a homolog of the *Drosophila* clock gene *Period* (*dPer*), which encodes an essential component of the circadian clock (*6, 7*). Mammals have three homologs of *dPer* (*Per1*, *Per2*, and *Per3*), one of which, *Per2*, controls the mouse circadian clock (*6–11*). The isolated zebrafish cDNA contained an open reading frame predicted to encode a protein with a length of 1281 amino acids (Fig. 1A) (*12*). *Drosophila* and mammalian PER proteins share several conserved regions, including a PER-ARNT-SIM (PAS) dimerization domain, a cytoplasmic localization domain (CLD), a short region downstream of the $dPer^s$ mutation, and a COOH-terminal serine/threonine–glycine repeat (*7, 8*). These regions were also conserved in the zebrafish protein (Fig. 1B). Phylogenetic analysis indicated that the zebrafish protein was most closely related to mammalian PER3 and was thus designated zebrafish PER3 (Fig. 1C) (*10*).

Expression of *Per3* was determined in zebrafish embryos raised under a cycle of 14 hours of light and 10 hours of dark (LD 14:10). Adults were entrained to the same LD 14:10 cycle, and overnight crosses resulted in synchronous spawning and fertilization upon lights on [zeitgeber time 0 (ZT 0)], thus time 0 of embryogenesis corresponded to ZT 0. A robust circadian expression of *Per3* was detected in the central nervous system and the retina of embryos throughout development from 40 to 128 hours postfertilization (hpf), with maximum mRNA expression during the early light phase from ZT 0 to ZT 4 (Figs. 2 and 3A). This suggests that embryonic *Per3* expression is controlled by a transcriptional feedback loop similar to that described in *Drosophila* and mice (*13, 14*).

We also analyzed the expression of the clock-controlled gene *Rev-erbα*, a transcriptional repressor that is expressed in a strong circadian rhythm in the adult rat liver (*15–17*). In contrast to *Per3*, *Rev-erbα* showed a developmentally regulated circa-

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dian expression. From ZT 20 to ZT 0, *Rev-erbα* mRNA was detected in the epiphysis at 44 to 48 hpf, in the epiphysis and the retina at 68 to 76 hpf, and in the optic tectum of 96-hour-old embryos (Fig. 2). The *17β–hydroxysteroid dehydrogenase* (*17βHSD*) gene, which is also expressed in the epiphysis and the retina, did not show any rhythmicity. The *Per3* expression data show that a circadian clock functions during zebrafish embryogenesis. Yet, downstream results of this clock, such as the rhythmic expression of *Rev-erbα*, seem to require development. Epiphysis and retinotectal axonal connections may, for instance, be needed to supply appropriate regulatory signals for rhythmic *Rev-erbα* gene expression to the retina and optic tectum (*18, 19*).

To test whether the zebrafish embryonic circadian clock was responsive to photic entrainment, we transferred, after fertilization, embryos from adults kept in a LD 14:10 cycle to a LD 14:10 cycle that had been advanced by 8 hours. This resulted in a rapid synchronization of the clock to the light/dark cycle that was shifted 8 hours forward, with the maximum mRNA levels being observed exactly from ZT 0 to ZT 4 and from ZT 20 to ZT 0 for *Per3* (Fig. 3B) and *Rev-erbα* (*20*), respectively, like in control embryos (Figs. 2 and 3A). Next, embryos were subjected to a continuous resetting of their circadian clock by keeping them under a cycle of 8 hours of light and 8 hours of dark (LD 8:8) after fertilization. Surprisingly, this experiment resulted in an ultradian rhythmic expression of *Per3* with a period of ~16 hours, similar to that of the environmental light/dark cycle (Fig. 3C). These results indicate that a light resetting pathway of the zebrafish circadian clock functions during embryogenesis (*21*). The fast synchronization of the clock in LD 8:8 could additionally result from a direct light-driven mechanism. The flexibility of the zebrafish embryonic clock may reflect an adaptive mechanism related to the fast and external development of this species.

The ~24-hour free-running period of *Per3* expression in embryos kept under constant light (LL) or constant darkness (DD) indicated that the zebrafish embryonic circadian clock was driven by an endogenous oscillator (Fig. 3, D and E). The LL condition resulted in a phase delay of *Per3* expression as previously reported (*22*), whereas the phase in DD embryos was identical to that of LD 14:10 embryos, suggesting that this parameter could be controlled by a signal other than the light/dark cycle.

To analyze the role of early embryonic development in the onset of *Per3* circadian expression, we raised embryos at 23°C, a temperature at which development is ~25% slower than that of embryos kept at 28°C (*23*). To eliminate the strong entrainment by the light/dark cycle, we used LL conditions. Although the zebrafish is a poikilothermic species, the dramatic developmental retardation induced by low temperature did not alter the circadian clock (Fig. 3, D and F). These data suggest that the zebrafish embryonic clock does not depend on developmental timing.

To further investigate whether the onset of the circadian clock was dependent on the

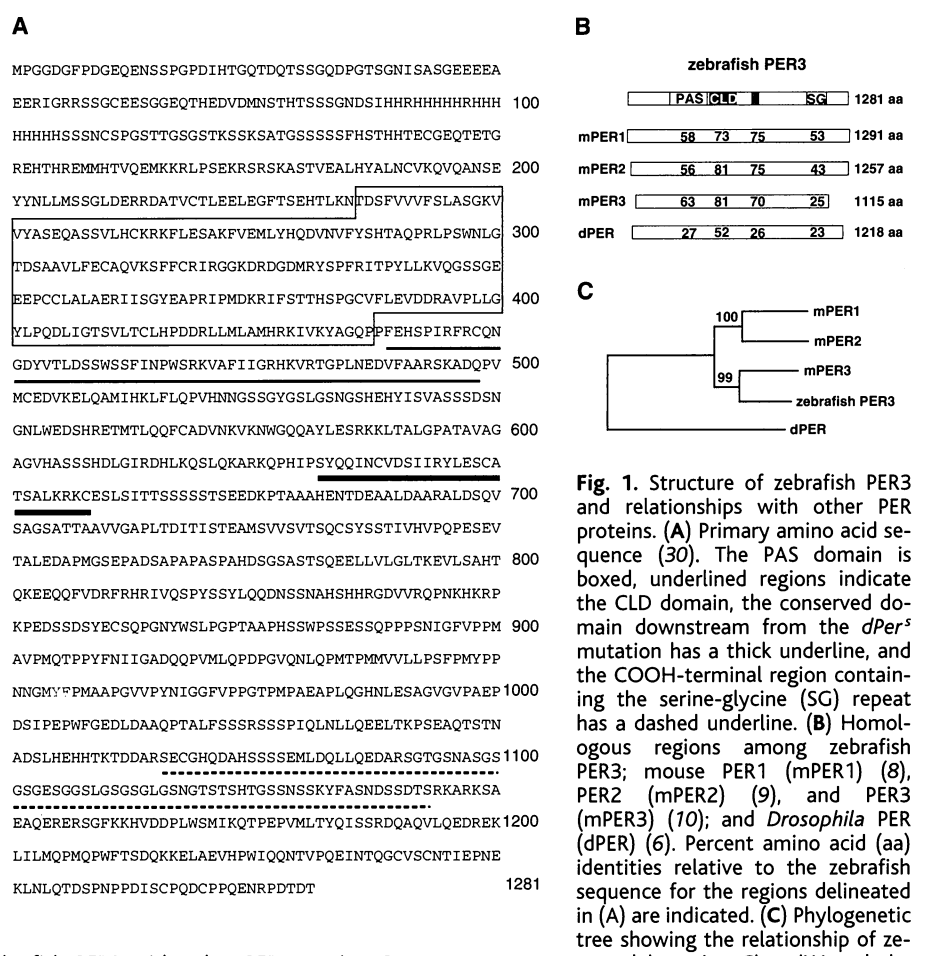

Fig. 1. Structure of zebrafish PER3 and relationships with other PER proteins. (**A**) Primary amino acid sequence (*30*). The PAS domain is boxed, underlined regions indicate the CLD domain, the conserved domain downstream from the *dPer*^{s} mutation has a thick underline, and the COOH-terminal region containing the serine-glycine (SG) repeat has a dashed underline. (**B**) Homologous regions among zebrafish PER3; mouse PER1 (mPER1) (*8*), PER2 (mPER2) (*9*), and PER3 (mPER3) (*10*); and *Drosophila* PER (dPER) (*6*). Percent amino acid (aa) identities relative to the zebrafish sequence for the regions delineated in (A) are indicated. (**C**) Phylogenetic tree showing the relationship of zebrafish PER3 with other PER proteins. Sequences were compared by using ClustalW and the neighbor-joining method; bootstrap values corresponding to 1000 replicate searches are indicated. European Molecular Biology Laboratory accession number for zebrafish *Per3* is AF254792.

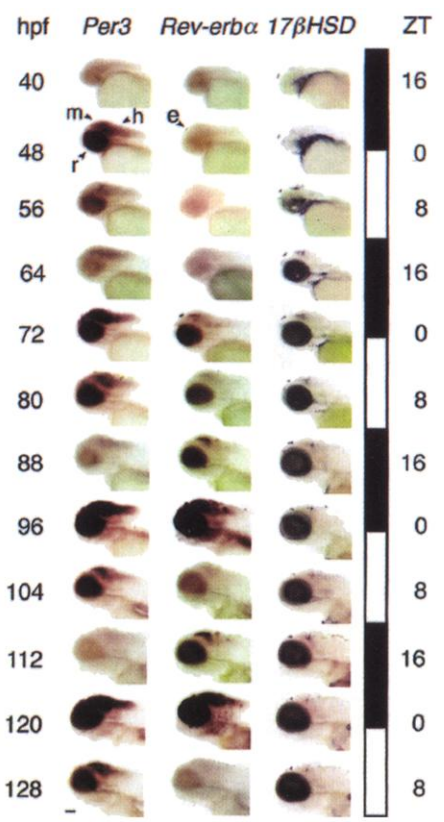

Fig. 2. Circadian expression of *Per3* and *Rev-erbα* during zebrafish embryonic development. Embryos raised in LD 14:10 were collected between 40 and 128 hpf and analyzed for *Per3* and *Rev-erbα* expression by whole-mount in situ hybridization (*28, 29, 31, 32*). The *17βHSD* gene was used as a control. White/black bars indicate the light/dark cycle. Scale bar, 100 μm. e, epiphysis; r, retina; m, midbrain, and h, hindbrain. One representative of three independent experiments is shown (see Web fig. 1, available at www.sciencemag.org/feature/data/1048812.shl).

timing of embryogenesis, we fertilized embryos from adults in the LD 14:10 cycle at either ZT 0 (control) or ZT 12 and analyzed them for *Per 3* expression. Both groups had the same phase of *Per3* circadian expression throughout embryogenesis (Fig. 3, D and G). This synchronous cycling demonstrates that, in zebrafish, the onset of circadian gene expression is independent of the time at which oocytes are fertilized. Because embryos were kept under constant conditions, it is difficult to understand how the circadian clock could be turned on at the same time or synchronized in both groups in the absence of any time cue. A possible explanation is that very early developing embryos inherit temporal information from the adults through ovogenesis. To test this hypothesis, we analyzed one-cell-stage to 24-hour-old embryos and unfertilized oocytes obtained from mature females kept under the LD 14:10 cycle. Results showed that the *Per3* mRNA level was high during the first 4 hours of development, corresponding to ZT 0 to ZT 4, then decreased during the gastrulation and segmentation stages (ZT 8 to ZT 16), increased specifically in the central nervous system and the hematopoietic territory at 20 to 24 hours (ZT 20 to ZT 0) (Fig. 4, A and B), and decreased again between 28 and 40 hours (not shown). The zebrafish midblastula transition (MBT), the stage at which transcription is activated, begins at cell cycle 10 (3 hours after fertilization at 28°C) (*24*). Thus, *Per3* mRNA detected from the 1-cell to the 256-cell stages (ZT 0 to ZT 2.5), is of maternal origin. A circadian rhythm of *Per3* mRNA accumulation was also observed in unfertilized oocytes, with a phase similar to that of the embryos (Fig. 4B). These data demonstrate that the circadian clock functions in very early zebrafish embryos. Notably, the rhythmic accumulation of clock gene mRNA such as *Per3* in the oocytes suggests that embryos synchronize their circadian clock by using maternal clock gene products. Thus, it is conceivable that, depending on the time of day, oocytes contain specific mRNA titers of the various clock genes, whose translated products are used for initiating the embryonic clock with a phase similar to that of the adults. This is supported by the observation that the *Per3* gene was not transcribed before ZT 20 in LD 14:10 embryos, indicating that this gene was under repression while zygotic transcription was activated. By such a mechanism, a readily synchronized clock is initiated by the embryo, irrespective of its developmental history, in the absence of communication with the mother. Conversely, activation of all clock genes upon activation of transcription at the MBT would require a delay for synchronizing the embryonic clock to the environment. The reason as to why embryos that are able to synchronize their clock to the light/dark cycle (*21*) would inherit the phase of their clock is unknown. Phase inheritance suggests that some rhythms may require synchronization during the first 24 hours of development before the

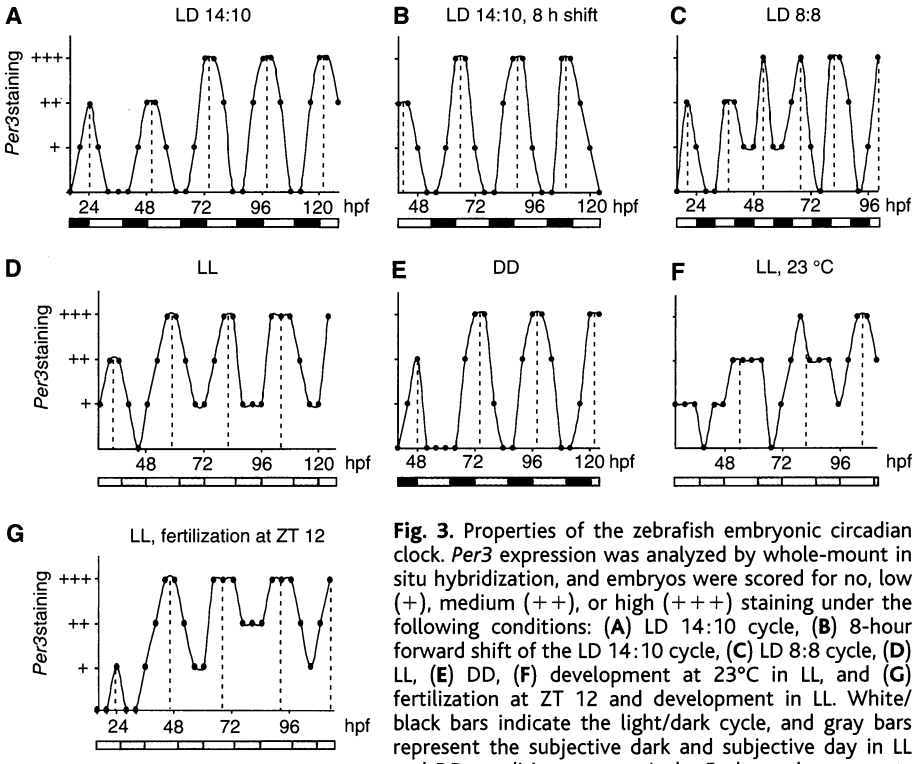

Fig. 3. Properties of the zebrafish embryonic circadian clock. *Per3* expression was analyzed by whole-mount in situ hybridization, and embryos were scored for no, low (+), medium (++), or high (+++) staining under the following conditions: (**A**) LD 14:10 cycle, (**B**) 8-hour forward shift of the LD 14:10 cycle, (**C**) LD 8:8 cycle, (**D**) LL, (**E**) DD, (**F**) development at 23°C in LL, and (**G**) fertilization at ZT 12 and development in LL. White/black bars indicate the light/dark cycle, and gray bars represent the subjective dark and subjective day in LL and DD conditions, respectively. Each graph represents one representative of at least two independent experiments (see Web fig. 2, available at www.sciencemag.org/feature/data/1048812.shl).

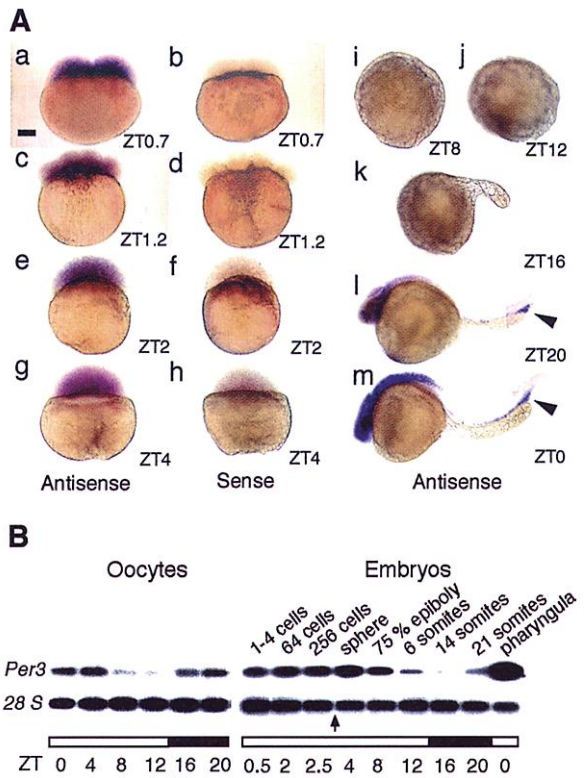

Fig. 4. *Per3* expression in early embryos and unfertilized oocytes. Embryos and mature females were maintained in LD 14:10 (*31*). (**A**) Whole-mount in situ hybridization of *Per3* mRNA in early embryos shows the following stages: cleavage (a through f), blastula (g and h), gastrulation (i), somitogenesis (j through l), and pharyngula (m). Arrows show the hematopoietic territory. The sense probe used at the cleavage and blastula stages showed no signal. Scale bar, 100 μm. (**B**) RT-PCR analysis of *Per3* mRNA in oocytes and early embryos (*32*). The arrow indicates the start of the MBT. 28*S* rRNA was used as a control. PCR product sizes were 357 and 151 base pairs for *Per3* and 28*S* rRNA, respectively. White/black bars indicate the light/dark cycle. One representative of two independent experiments is shown.

light resetting pathway becomes functional or, alternatively, that an oscillating clock cannot be entirely initiated de novo. In mammals, the circadian clock located in the suprachiasmatic nuclei (SCN) starts oscillating during late fetal life, and expression of the mouse clock genes *Per1* and *Per2* was observed in the SCN just before or at birth (*4, 9, 25*). However, the discovery of circadian oscillators in peripheral organs and in tissue culture cells indicates that circadian clock function does not necessarily require the completion of long and complex developmental processes such as vertebrate brain development (*10, 17, 26*). In *Drosophila*, *Per* is expressed throughout development, and the onset of circadian behavioral rhythm is light independent; however, its synchronization requires a light-entraining signal (*27*). Our data show that developing zebrafish embryos inherit maternal circadian clock gene products and perhaps also the phase of their clock.

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12. A zebrafish *Per3* polymerase chain reaction (PCR) probe was amplified with the primers 5′-CTGGG(C/T)TA(C/T)CTICC(C/T)CAGGA-3′ and 5′-CAG(T/C)T(C/G)ATCTGCTG(A/G)TAGGA-3′ and was used for screening a cDNA library of random-primed segmentation-stage zebrafish embryos and a cDNA library of oligo(dT)-primed maternal zebrafish embryos. A full-length and several overlapping independent cDNA clones were obtained and sequenced. The zebrafish *Rev-erbα* (NR1D1) probe was PCR amplified with the primers 5′-GT(T/G)GCITC(G/A)GG(C/A)TT(T/C)CA(C/T)TA-3′ and 5′-GC(A/G)AA(C/T)TC(C/T)ACCACCTC(A/C)CG-3′. The *17βHSD* probe was used as a control (*28*).
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30. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
31. The *AB zebrafish strain was kept at 28°C in a LD 14:10 cycle with lights on at 0900 hours (ZT 0). For most experiments, adults were crossed overnight, resulting in spawning and fertilization at about ZT 0, the next morning. Embryos were raised at 28°C in petri dishes containing 0.003% phenylthiourea to prevent pigmentation. Lighting conditions were either LD 14:10, LD 14:10 that was shifted 8 hours forward, LD 8:8, LL, or DD. Delayed development was obtained by keeping embryos at 23°C in LL. For desynchronizing development from the light/dark cycle, adults in LD 14:10 were crossed at ZT 3, and embryos fertilized at ZT 12 the same day were kept in LL. Embryos were fixed for 12 to 16 hours in 4% paraformaldehyde/phosphate–buffered saline at 4-hour intervals and stored in methanol. Unfertilized oocytes were obtained by squeezing mature females under anesthesia at 4-hour intervals.
32. Whole-mount in situ hybridization was performed according to Thisse *et al.* (*29*). Duration of staining was identical for all embryos. In Fig. 3, temporal expression profiles of *Per3* were determined by scoring 10 embryos per point for no, low, medium, or high staining with reference to the respective 40-, 56-, 80-, and 96-hpf time points from Fig. 2. No significant variation of the staining was observed. Reverse transcriptase–PCR (RT-PCR) detection of *Per3* mRNA and *28S* ribosomal RNA (rRNA) was carried out with reverse-transcribed total RNA and the following specific primers: 5′-CGGATCGGTACCTCAGTCCT-3′ and 5′-TCCATTATTATGAACAGGCT-3′ for *Per3* and 5′-CCTCACGATCCTTCTGGCTT-3′ and 5′-AATTCTGCTTCACAATGATA-3′ for *28S* rRNA. The cycle number was optimized for obtaining signals within the linear range. PCR products were analyzed by Southern blot and probed with a specific ^{32}P–end-labeled oligonucleotide.

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Requirement of the Spindle Checkpoint for Proper Chromosome Segregation in Budding Yeast Meiosis

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The spindle checkpoint was characterized in meiosis of budding yeast. In the absence of the checkpoint, the frequency of meiosis I missegregation increased with increasing chromosome length, reaching 19% for the longest chromosome. Meiosis I nondisjunction in spindle checkpoint mutants could be prevented by delaying the onset of anaphase. In a recombination-defective mutant (*spo11Δ*), the checkpoint delays the biochemical events of anaphase I, suggesting that chromosomes that are attached to microtubules but are not under tension can activate the spindle checkpoint. Spindle checkpoint mutants reduce the accuracy of chromosome segregation in meiosis I much more than that in meiosis II, suggesting that checkpoint defects may contribute to Down syndrome.

Meiosis I differs from mitosis and meiosis II. In meiosis I, the two sister centromeres remain attached to each other and move to one spindle pole, segregating away from the paired centromeres of the homologous chromosome (Fig. 1A) (*1*). We investigated the meiotic role of the spindle checkpoint, which keeps cells with misaligned chromosomes from starting anaphase by preventing the activation of the anaphase promoting complex (APC, also known as the cyclosome) (*2, 3*). The spindle checkpoint detects kinetochores that are not attached to microtubules (*4, 5*) and the absence of tension at kinetochores that are attached to microtubules (*6*). Mutations in the budding yeast *MAD1*, *MAD2*, *MAD3*, *BUB1*, *BUB3*, and *MPS1* genes eliminate the spindle checkpoint (*7, 8*).

To follow meiotic chromosome segregation, we targeted green fluorescent protein (GFP) to bind a specific chromosome. Tan-

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