subunit (5 units) or cAMP (10 μM; Sigma) with or without a PKA inhibitor (PKI_{5-24}, 500 nM, Calbiochem). Phosphorylation at room temperature was initiated with 33 μM Mg–adenosine triphosphate (ATP) containing 10% [γ-^{32}P]ATP (NEN Life Sciences, Boston) and terminated after 5 min at room temperature with 5 μl of stop solution (4% SDS and 0.25 M dithiothreitol). Samples were size-fractionated by SDS-PAGE (10% gel).

26. Adult murine cardiomyocytes were isolated (*18*). Cells were plated in petri dishes placed on the stage of an inverted microscope (IMT-2, Olympus), and currents (myocytes and CHO cells) were recorded using the whole-cell patch-clamp technique (*19*) with local perfusion (*20*) and solutions previously described for CHO cells (*15*) and murine myocytes (*21*). Voltage pulse protocols are described in figure legends. Statistical significance was assessed with Student's *t* test for simple comparisons and analysis of variance (ANOVA) for multiple comparisons; differences at $P < 0.05$ were considered to be significant.

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Calcium, Calmodulin, and CaMKII Requirement for Initiation of Centrosome Duplication in *Xenopus* Egg Extracts

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Aberrant centrosome duplication is observed in many tumor cells and may contribute to genomic instability through the formation of multipolar mitotic spindles. Cyclin-dependent kinase 2 (Cdk2) is required for multiple rounds of centrosome duplication in *Xenopus* egg extracts but not for the initial round of replication. Egg extracts undergo periodic oscillations in the level of free calcium. We show here that chelation of calcium in egg extracts or specific inactivation of calcium/calmodulin–dependent protein kinase II (CaMKII) blocks even initial centrosome duplication, whereas inactivation of Cdk2 does not. Duplication can be restored to inhibited extracts by addition of CaMKII and calmodulin. These results indicate that calcium, calmodulin, and CaMKII are required for an essential step in initiation of centrosome duplication. Our data suggest that calcium oscillations in the cell cycle may be linked to centrosome duplication.

Centrosome duplication occurs once and only once during the cell cycle, ensuring the formation of bipolar spindles that distribute replicated chromosomes equally to daughter cells. Overduplication of centrosomes causes multipolar spindles that are thought to lead to genomic instability. More than two centrosomes have been observed in many different types of tumor cells (*1*), and the absence of the tumor suppressor protein p53 correlates with the presence of multiple centrosomes in the cell (*2*). Thus, the process of centrosome duplication is likely to be important in the etiology of cancer.

There are links between the cell cycle and the centrosome duplication cycle. Centrosome duplication generally occurs at the G_1-S transition (*3*), and if S phase is prolonged in mammalian cells and in embryonic systems including cycling *Xenopus* egg extracts, centrosomes can reproduce multiple times. Cdk2 is identified as a direct link between the cell cycle and centrosome duplication (*4*–*7*). The cyclin E–Cdk2 complex regulates DNA replication, and its activity is greatest at the G_1 to S phase transition. Cdk2 is also required for multiple rounds of centrosome duplication in *Xenopus* egg extracts and embryos (*4, 5*) and in somatic cells (*6, 7*). Nucleophosmin (also called B23) and the mouse Mps1p-like kinase (mMps1p) were recently identified as possible centrosomal substrates for cyclin E–Cdk2 (*8, 9*).

Calcium and calmodulin (CaM) are required for cells to traverse the G_1-S, G_2-M, and metaphase-anaphase boundaries of the cell cycle (*10*). Calcium/calmodulin–dependent protein kinase II (CaMKII), which has a wide tissue distribution (*11*), has been suggested to be a target of calcium and calmodulin at the G_2-M transition in various cell types (*10*). In addition, periodic calcium oscillations occur in each cell cycle in dividing *Xenopus* embryos (*12*) and in cycling egg extracts (*13*). In particular, calcium oscillations occur at the G_1-S boundary and near the G_2-M transition. These times correlate with centrosome duplication at the G_1-S boundary and centrosome separation at the G_2-M transition. Moreover, calcium-modulated proteins, including CaMKII and centrin [a homolog of Cdc31p, a protein essential for duplication of yeast centrosomes (*14*)], are localized in the centrosome (*15, 16*). Thus, we examined the role of calcium in centrosome duplication using *Xenopus* egg extracts arrested in S phase by aphidicolin in which centrosome duplication starts after addition of sperm nuclei (*4*).

Addition of the calcium chelator BAPTA [1,2-bis(*O*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid] (*17*) caused depolymerization of microtubules in the extracts (*18*) and prevented detection of centrosomes by polarization microscopy. Therefore, centrosome duplication in the presence of BAPTA was visualized by immunofluorescence with an antibody to γ-tubulin, a well-characterized component of the centrosome (*19*). Extracts incubated with sperm nuclei were subjected to microtubule depolymerization with nocodazole, and the centrosomes were centrifuged onto a cover slip for staining (*20, 21*). The number of γ-tubulin–staining foci increased during a 6-hour incubation (Fig. 1A), and staining with antibody to either α-tubulin or centrin, a component of the centriole (*14*), confirmed that each γ-tubulin–staining focus (>90%) corresponded to one centriole (Fig. 1B). Because each centrosome contains two centrioles, the number of centrosomes is half the number of γ-tubulin–staining foci. A fourfold increase in centrosomes was detected during the 6-hour incubation (Fig. 1C, control), indicating that centrosomes duplicate twice in these extracts in approximately 6 hours.

In the presence of 2 mM BAPTA, no increase in centrosomes was evident during the 6-hour incubation (Fig. 1C), even though 20 mM EGTA had no effect on centrosome duplication (*18*). Considering that BAPTA chelates calcium more rapidly than does EGTA (*17*), this suggests that rapid oscillations rather than prolonged changes in the concentration of free calcium are necessary for centrosome duplication. Inositol 1,4,5-trisphosphate (IP_3) is a second messenger that releases calcium from intracellular stores (*22*). To determine whether calcium oscillations are mediated by the IP_3 receptor, we used heparin, an IP_3 receptor antagonist (*23*) that blocks calcium oscillations in *Xenopus* egg extracts (*13*). Heparin effectively inhibited centrosome duplication in the extracts (Fig. 1C). Moreover, after centrosomes had

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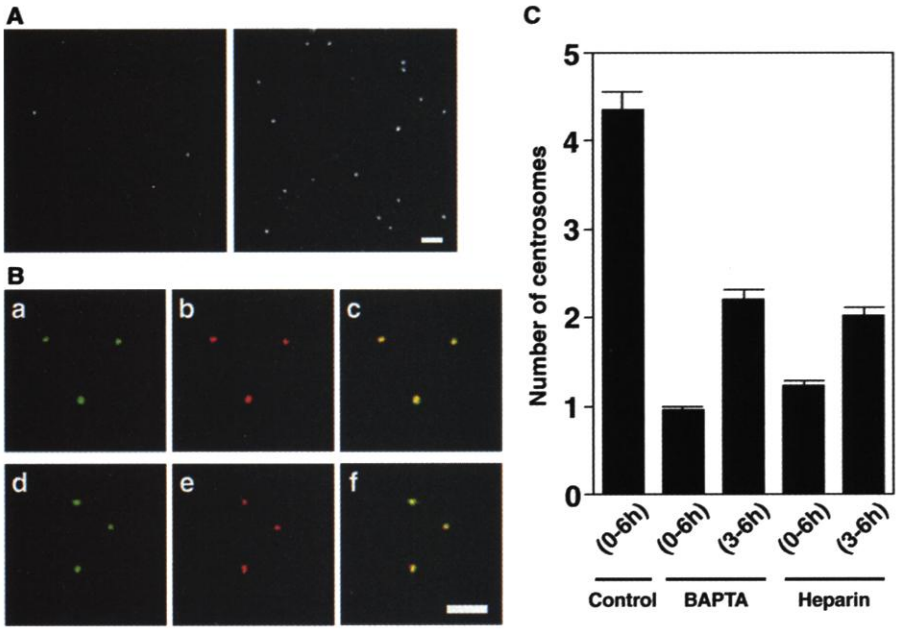

Fig. 1. (**A**) γ-Tubulin–staining foci in S phase–arrested *Xenopus* egg extracts, 1 hour (left) and 6 hours (right) after addition of demembranated sperm nuclei. Scale bar, 10 μm. (**B**) Immunostaining with antibodies to the indicated centriolar components. Shown are γ-tubulin–staining foci 6 hours after addition of sperm nuclei: (a) γ-tubulin localization; (b) α-tubulin localization; (c) merged image of (a) and (b); (d) γ-tubulin localization; (e) centrin localization; (f) merged image of (d) and (e). Scale bar, 5 μm. (**C**) Chelation of free calcium in extracts blocks initiation of centrosome duplication. Quantification of centrosome duplication 6 hours after addition of sperm nuclei. BAPTA (2 mM) or heparin (150 μg/ml) were added immediately after addition of sperm nuclei (0 to 6 hours) or 3 hours after addition of sperm nuclei (3 to 6 hours). The number of centrosomes is presented as half of the number of γ-tubulin–staining foci averaged over more than five different sites on each cover slip divided by the number of sperm nuclei added in each experiment. The data are shown as means ± SEM of three independent experiments.

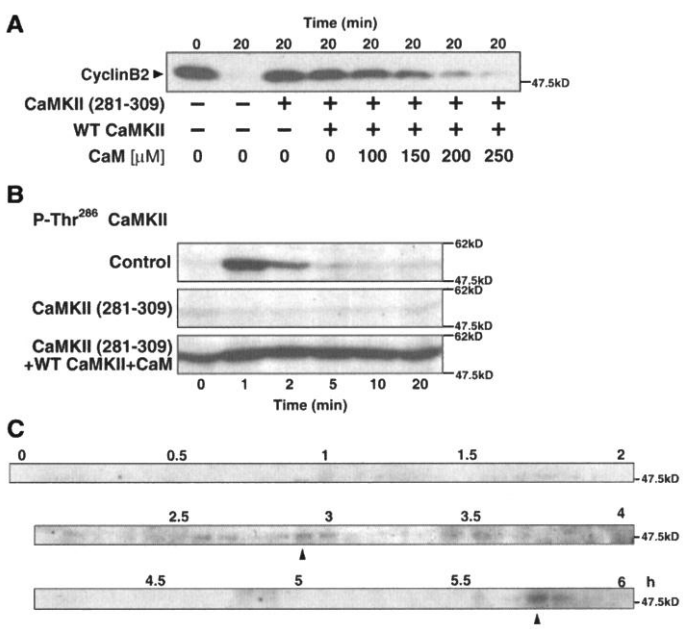

Fig. 2. Specificity of CaMKII inhibition. (**A**) CaMKII-dependent cyclin B degradation. Cyclin B2 was immunoblotted at the indicated times after calcium addition to metaphase-arrested CSF extracts. Each lane represents 1 μl of extract. Inhibition of cyclin B2 degradation by 0.4 mM CaMKII(281–309) (third lane from left) is reversed by addition of WT CaMKII (2 μg/ml) and the indicated concentrations of CaM (rightmost five lanes). (**B**) Immunoblotting of CSF extracts with anti-phospho-Thr^{286} of CaMKIIα after calcium addition. Each lane represents 1 μl taken from the same extract at the indicated times after calcium addition. Inhibition of CaMKII-autophosphorylation (control) by 0.4 mM CaMKII(281–309) was reversed by addition of WT CaMKII (2 μg/ml) and CaM (250 μM), as indicated. (**C**) Immunoblotting of aphidicolin-treated cycling extracts with anti-phospho-Thr^{286} of CaMKIIα. Each lane shows 1 μl of the extracts taken every 5 min after addition of demembranated sperm nuclei. Arrowheads indicate peaks of CaMKII activity.

duplicated once, addition of BAPTA or heparin stopped the second round of duplication (Fig. 1C). These results suggest that a transient increase in the concentration of intracellular free calcium is required for initiation of centrosome duplication.

One potential target of calcium is CaMKII. This kinase functions in calcium-dependent activation of the anaphase-promoting complex and cyclin B degradation during exit from mitosis in *Xenopus* egg extracts (*24*). To suppress CaMKII activity, we used a specific pseudosubstrate inhibitor peptide corresponding to the autoinhibitory domain of CaMKII (amino acids 281 to 309), which specifically inhibits CaMKII (*25*). This peptide is slightly longer than the one used by Lorca *et al.* (*24*) to inhibit CaMKII in egg extracts. After calcium addition to *Xenopus* egg extracts arrested in metaphase by cytostatic factor (CSF), addition of 0.4 mM CaMKII(281–309) inhibited cyclin B degradation (*24, 26*) (Fig. 2A). Moreover, immunoblots with an antibody that specifically recognizes CaMKIIα phosphorylated on Thr^{286} showed that the inhibitor peptide eliminated the phosphorylation of Thr^{286}, which reflects activation of CaMKII after binding calcium-CaM (*27*) (Fig. 2B). Thus, the CaMKII(281–309) inhibitor peptide completely inhibits CaMKII activity in *Xenopus* egg extracts.

When CaMKII(281–309) was added to the in vitro centrosome duplication system at the beginning of the assay, all duplication, including the first round, was blocked. Like BAPTA or heparin, addition of CaMKII(281–309) in the midst of the assay stopped the second round of centrosome duplication (Fig. 3A), which suggests that CaMKII activity is required for the initiation of each round of centrosome duplication. Consistent with the two rounds of centrosome duplication, immunoblots of phosphorylated Thr^{286} of CaMKIIα showed two peaks of CaMKII activity at the times when centrosomes duplicated (Fig. 2C, arrowheads), which were not detected in the presence of CaMKII(281–309) (*18*). Under polarization microscopy, centrosome duplication was temporally correlated with peaks of CaMKII activity (*18*).

The specificity of regulation by CaMKII was evaluated by determining whether the effects of CaMKII(281–309) could be suppressed by addition of the CaMKII holoenzyme (WT CaMKII) (*28, 29*), calcium, and CaM. WT CaMKII plus CaM restored cyclin B degradation upon calcium addition to inhibited extracts in a dose-dependent manner (Fig. 2A) and also reversed inhibition of CaMKIIα autophosphorylation by the inhibitor peptide (Fig. 2B). Most important, whereas CaMKII(281–309) blocked centrosome duplication, the addition of WT CaMKII and CaM restored duplication (Fig. 3B). These results reveal a specific require-

ment for CaMKII in the initiation of centrosome duplication.

We compared the effects on centrosome duplication of inhibiting CaMKII versus the only Cdk2 complex at this stage of development, cyclin E–Cdk2 (*4*). When cyclin E–Cdk2 was inactivated by Δ34Xic1, a form of the Cdk inhibitor $p27^{Xic1}$ specific for cyclin E–Cdk2 (*30*), centrosome formation was limited to a single doubling (Fig. 3C) (*31*). As an alternative method for inhibition of cyclin E–Cdk2 activity, we used catalytically inactive Cdc25A(C432A) to prevent dephosphorylation of Tyr^{15} in Cdk2 (*32*). Periodic changes in Tyr^{15} phosphorylation of Cdk2 were detected in the S phase–arrested extracts (*33*) as seen in the embryonic cell cycle (*32*). Addition of Cdc25A(C432A) led to accumulation of an inactive Tyr^{15}-phosphorylated form of Cdk2 (*33*) and again limited centrosome duplication to a single doubling (Fig. 3C). Thus, in contrast to CaMKII, inactivation of cyclin E–Cdk2 does not inhibit the first round of centrosome duplication. Taken together, these results suggest that CaMKII is required for initiating an essential step in the centrosome duplication process.

To exclude the possibility that the requirement of CaMKII for centrosome duplication is due to an effect on tubulin polymerization, we demonstrated that in the presence of CaMKII(281–309) microtubules grew from centrosomes to an extent equivalent to that seen in control extracts or in the presence of Δ34Xic1 (Fig. 4A) (*34*). We also confirmed by polarization microscopy (*4*) that the microtubule arrays around the centrosomes were normal (Fig. 4B).

The arrest of the centrosome duplication process at two different points by CaMKII(281–309) and Δ34Xic1 suggests that CaMKII and cyclin E–Cdk2 control discrete steps in the duplication process. In somatic cells, inactivation of Cdk2 completely inhibits the initiation of centrosome duplication (*6, 7*), and overexpression of Cdk2 (*6*) or cyclin A (*7*) induces duplication in G_1 phase–arrested cells. However, in *Xenopus* egg extracts and embryos, inactivation of cyclin E–Cdk2 did not inhibit the first round of centrosome duplication but blocked additional rounds of duplication (*4, 5*) (Fig. 3C). It has been suggested that Cdk2 may be a licensing factor (*4*) that restores reproductive components to daughter centrosomes but is not the initiator of the duplication event itself. Cdk2 has been proposed to regulate centrosome duplication through binding of nucleophosmin (*8*) or stabilization of mMps1p (*9*). However, in egg extracts in which cyclin E–Cdk2 was inhibited, nucleophosmin was not detected in the centrosome with antibodies to *Xenopus* nucleophosmin (*35*), and licensing is therefore unlikely to reflect any effects of nucleophosmin binding. Lack of an inhibitor of centrosome duplication may contribute to the rapidity of centrosome duplication in early embryonic cell cycles, which lack G_1 and G_2 phases.

Our results indicate that a functional con-

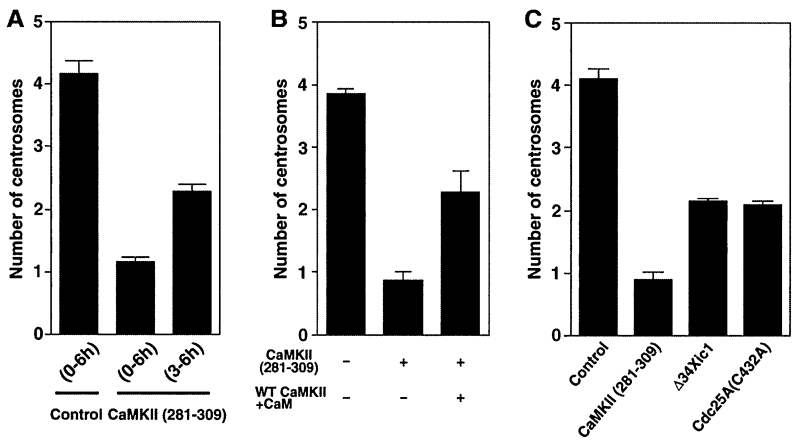

Fig. 3. Quantification of centrosome duplication 6 hours after addition of demembranated sperm nuclei. (**A**) Inactivation of CaMKII blocks initiation of each round of centrosome duplication. CaMKII(281–309) inhibitory peptide (0.4 mM) was added immediately before addition of sperm nuclei (0 to 6 hours) or 3 hours after addition of sperm nuclei (3 to 6 hours). (**B**) WT CaMKII and CaM suppress inhibition of centrosome duplication. CaMKII(281–309) (0.4 mM), without or with WT CaMKII (2 μg/ml) and CaM (250 μM), was added immediately before addition of sperm nuclei. (**C**) Comparison of CaMKII and cyclin E–Cdk2 requirement for centrosome duplication. CaMKII(281–309) inhibitory peptide (0.4 mM), Δ34Xic1 (175 nM), or Cdc25A(C432A) (260 nM) was added immediately before addition of sperm nuclei.

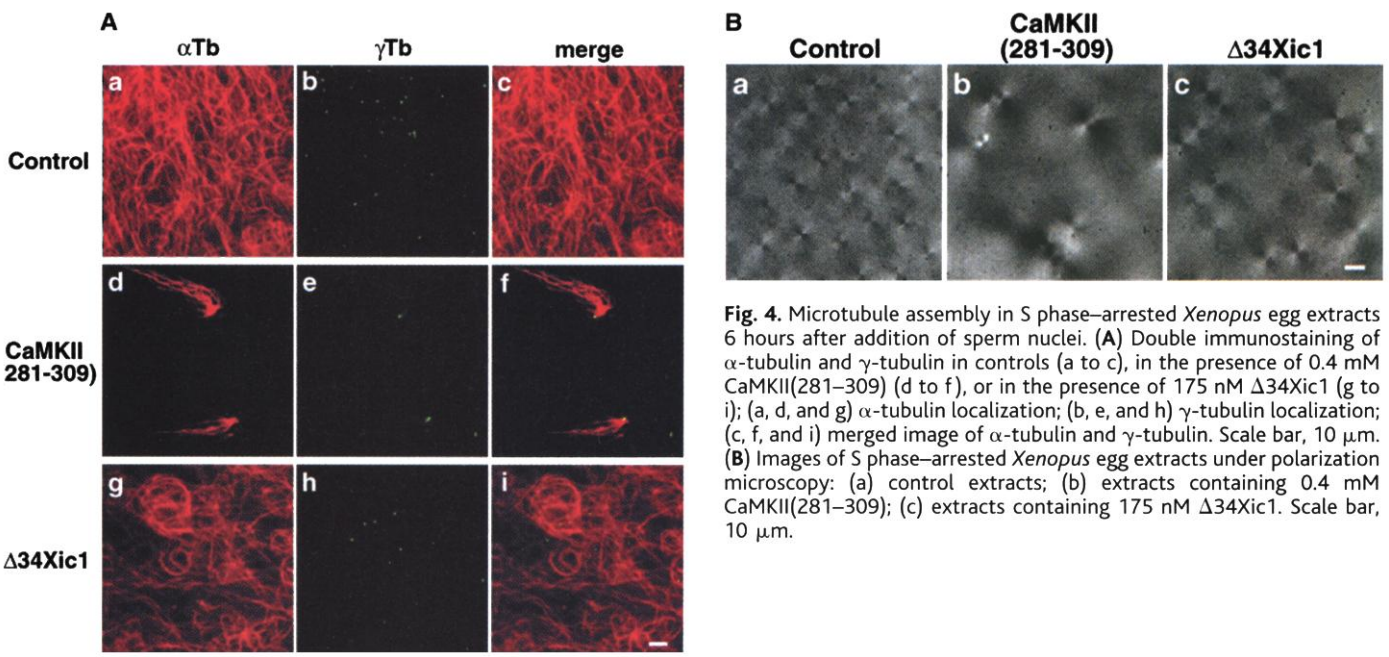

Fig. 4. Microtubule assembly in S phase–arrested *Xenopus* egg extracts 6 hours after addition of sperm nuclei. (**A**) Double immunostaining of α-tubulin and γ-tubulin in controls (a to c), in the presence of 0.4 mM CaMKII(281–309) (d to f), or in the presence of 175 nM Δ34Xic1 (g to i); (a, d, and g) α-tubulin localization; (b, e, and h) γ-tubulin localization; (c, f, and i) merged image of α-tubulin and γ-tubulin. Scale bar, 10 μm. (**B**) Images of S phase–arrested *Xenopus* egg extracts under polarization microscopy: (a) control extracts; (b) extracts containing 0.4 mM CaMKII(281–309); (c) extracts containing 175 nM Δ34Xic1. Scale bar, 10 μm.

sequence of calcium oscillations in the cell cycle is to trigger centrosome duplication, and in particular we suggest that IP_3-dependent calcium oscillations regulate the initiation of centrosome duplication through CaMKII activation. Consistent with the requirement for CaMKII to trigger centrosome duplication, chelation of calcium in cycling *Xenopus* egg extracts blocks the cell cycle before S phase entry, when centrosome duplication starts (*13*). CaMKII is localized on centrosomes (*15, 16*) and phosphorylates several centrosomal proteins in vitro (*16*). These proteins may be candidate substrate(s) for initiation of centrosome duplication by CaMKII.

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20. The S phase–arrested extracts were prepared as described (*4*). Briefly, freshly made cycling *Xenopus* egg extracts were aliquoted into 25 μl per tube. Aphidicolin (10 μg/ml, CalBiochem) and 400 demembranated sperm nuclei per microliter of extract were added before S phase started and the extracts were incubated for 1 or 6 hours at 2°C. For analysis, the S phase–arrested extracts were treated with nocodazole (30 μg/ml, CalBiochem) for 15 min at 20°C, and kept on ice for 15 min. Centrosomes were then centrifuged onto a cover slip as described (*21*) and fixed with 1:1 methanol/acetone for 10 min at –20°C. For centrosome duplication assays, γ-tubulin monoclonal antibody (mAb) (Sigma) was used. For double immunostaining of centrioles, we used a rabbit polyclonal antibody to γ-tubulin (Sigma), α-tubulin mAb (Sigma), and centrin mAb 20H5 (a gift of M. Winey) (*14*).
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26. CaMKII(281–309) (CalBiochem) was added to CSF extracts on ice. After addition of calcium to 0.6 mM, the extracts were incubated at room temperature for the indicated time. For cyclin B immunoblots, a sheep affinity-purified polyclonal antibody to cyclin B2 was used. For immunoblots of phospho-Thr^{286} in CaMKIIα, the membrane was incubated in 2% (v/v) goat serum (Sigma) containing rabbit anti-ACTIVE CaMKII polyclonal antibody (Promega). After washing in 0.5 M NaCl, 20 mM tris-Cl (pH 7.5), and 0.05% (v/v) Tween-20, the immunoblot was developed with anti-ACTIVE qualified, horseradish peroxidase–conjugated donkey antibody to rabbit immunoglobulin G (Promega).
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34. After incubation of the S phase–arrested extracts (*4*) for 6 hours, centrosomes and microtubules were centrifuged onto cover slips at room temperature without depolymerization of microtubules. The cover slip was fixed with 99% methanol at –20°C for 5 min and then stained with a rabbit polyclonal antibody to γ-tubulin (Sigma) and α-tubulin mAb (Sigma).
35. Y. Matsumoto, J. L. Maller, unpublished data. Immunofluorescence staining was performed using mAbs (No-185 and No-63) to NO38, a *Xenopus* homolog of nucleophosmin (B23) (*36*). The antibodies were a kind gift of M. S. Schmidt-Zachmann. The extracts were fixed with methanol, methanol/acetone (1:1), or 10% methanol/10% formaldehyde, as described (*8*).
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37. We thank G. Sluder for providing adaptors for spinning down centrosomes from extracts and for help in setting up polarization microscopy in this laboratory; M. Winey for the antibody to centrin; M. D. Browning for the CaM kinase II purified from rat brain; M. S. Schmidt-Zachmann for the antibodies to NO38; members of this laboratory for helpful discussions and support, particularly F. Taieb for advice on the CaMKII experiments; and P. Eyers and E. Erikson for critical reading of the manuscript. Recombinant baculovirus-infected Sf9 cells were produced in the Tissue Culture/Monoclonal Antibody Core Facility at the University of Colorado Cancer Center (CA46934). Y.M. is an Associate and J.L.M. is an Investigator of the Howard Hughes Medical Institute.

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Regulation of Life-Span by Germ-Line Stem Cells in *Caenorhabditis elegans*

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The germ line of the nematode *Caenorhabditis elegans* influences life-span; when the germ-line precursor cells are removed, life-span is increased dramatically. We find that neither sperm, nor oocytes, nor meiotic precursor cells are responsible for this effect. Rather life-span is influenced by the proliferating germ-line stem cells. These cells, as well as a downstream transcriptional regulator, act in the adult to influence aging, indicating that the aging process remains plastic during adulthood. We propose that the germ-line stem cells affect life-span by influencing the production of, or the response to, a steroid hormone that promotes longevity.

Killing the germ-line precursor cells, Z2 and Z3, extends the life-span of *C. elegans* by ~60% (*1*). This longevity is not a result of sterility, because removing the entire reproductive system (germ line and somatic gonad) has no effect on life-span. In order for germ line–ablated animals to live longer than normal, they require DAF-12, a nuclear hormone receptor, and DAF-16, a forkhead-family transcription factor. We found that this effect could be reproduced genetically: *mes-1(bn7)* mutants, which lack germ cells, were long lived (Fig. 1A), as were *glp-1(q158)* mutants (Fig. 1B) (*2, 3*). *glp-1* encodes the receptor for a germ-line proliferation signal that is produced by the distal tip cells of the somatic gonad (*4–7*). In *glp-1(q158)* mutants, Z2 and Z3 generate only a few germ cells, which then enter meiosis and differentiate as sperm (*7*). In both mutants, life-span extension was suppressed by a *daf-16* null mutation and by ablation of the somatic gonad precursor cells (Fig. 1) (*8, 9*). Many other mutants with defective germ-line proliferation were also long lived [Web table 1, experiments A and B (*10*); (*9*)].

The germ-line precursors are stem cells that divide continuously during development.

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