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Elizabeth Anne Bowman, Emory University
Christopher Ray Bowman, Emory University
Jeong H. Ahn, Emory University
William Kelly, Emory University

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Phosphorylation of RNA polymerase II is independent of P-TEFb in the C. elegans germline

Elizabeth Anne Bowman¹, Christopher Ray Bowman², Jeong H. Ahn¹ and William G. Kelly¹,*

SUMMARY
RNA polymerase II (Pol II) elongation in metazoans is thought to require phosphorylation of serine 2 (Ser2-P) of the Pol II C-terminal domain (CTD) by the P-TEFb complex, CDK-9/cyclin T. Another Ser2 kinase complex, CDK-12/cyclin K, which requires upstream CDK-9 activity has been identified in Drosophila and human cells. We show that regulation of Ser2-P in C. elegans soma is similar to other metazoan systems, but Ser2-P in the germline is independent of CDK-9, and largely requires only CDK-12. The observed differences are not due to differential tissue expression as both kinases and their cyclin partners are ubiquitously expressed. Surprisingly, loss of CDK-9 from germ cells has little effect on Ser2-P, yet CDK-9 is essential for germline development. By contrast, loss of CDK-12 and Ser2-P specifically from germ cells has little impact on germline development or function, although significant loss of co-transcriptional H3K36 trimethylation is observed. These results show a reduced requirement for Pol II Ser2-P in germline development and suggest that generating Ser2-P is not the essential role of CDK-9 in these cells. Transcriptional elongation in the C. elegans germline thus appears to be uniquely regulated, which may be a novel facet of germline identity.

KEY WORDS: C. elegans, CDK-9, CDK-12

INTRODUCTION
The large subunit of RNA polymerase II (Pol II) contains a C-terminal domain (CTD) that is composed of repeats of a heptapeptide sequence with consensus Y_{1}S_{2}P_{3}T_{4}S_{5}P_{6}S_{7} (42 in C. elegans). The phosphorylation states of specific residues in the repeats are thought to be important for proper mRNA synthesis. Pol II initially recruited to genes is hypophosphorylated on the CTD. Serine 5 of the repeat (Ser5) becomes phosphorylated during initiation, and serine 2 (Ser2) is phosphorylated on elongating Pol II (Egloff and Murphy, 2008; Zhou et al., 2012). Dephosphorylation of both Ser2 and Ser5 is important for recycling of Pol II complexes for reinitiation (Cho et al., 1999). The different phosphorylation marks are thought to help recruit various Pol II transcription factors, RNA processing machinery and histone/nucleosome modifiers at the proper time during the transcription cycle (Meinhart et al., 2005; Egloff and Murphy, 2008).

The transition between initiation and elongation is a regulated process in many metazoans, controlled by positive and negative regulatory factors that interact with Pol II (Adelman and Lis, 2012). Pol II elongation is inhibited by the combined activity of two complexes, DSIF (DRB-sensitivity inducing factors Spt4 and Spt5) and NELF (negative elongation factor). Phosphorylation of these factors releases inhibition of Pol II elongation and this appears tightly linked to Pol II CTD Ser2 phosphorylation (Yamaguchi et al., 2013). Whereas DSIF is highly conserved among eukaryotes, NELF is absent in many eukaryotes, including C. elegans (Narita et al., 2003). It is unclear whether Pol II elongation is a regulated step of transcription in this system, as Pol II pausing is not commonly detected during normal development (Whittle et al., 2008; Baugh et al., 2009; Krueis et al., 2013).

In budding yeast, Ser2 and DSIF phosphorylation requires two kinases, Bur1 and Ctk1. Bur1 phosphorylates both DSIF and Ser2, but Ctk1 is responsible for the majority of Ser2 phosphorylation (Ser2-P) (Qiu et al., 2009). In metazoans, the P-TEFb complex, which is composed of Cdk9 and cyclin T, has been considered the sole DSIF/NELF and Pol II Ser2 kinase because loss of Cdk9 activity leads to loss of Ser2-P (Wood and Shilatifard, 2006). However, the closest metazoan homolog of yeast Ctk1, Cdk12, was shown to be required for a large fraction (~60%) of Ser2-P in Drosophila and humans (Bartkowiak et al., 2010). It was thus suggested that Ser2-P in metazoans may match the elongation process in budding yeast: Cdk9 phosphorylates the Pol II CTD and DSIF (and NELF when present), and these events precede and are required for Cdk12-mediated phosphorylation of Ser2 on elongating Pol II (Bartkowiak and Greenleaf, 2011). This model could explain the requirement for Cdk9 for all Ser2-P observed in metazoans.

Here, we show that Ser2-P is differentially regulated in the somatic and germ cell lineages in C. elegans. Ser2-P regulation in the soma requires CDK-9 acting upstream of CDK-12 for Ser2-P; however, Ser2-P in the germline is independent of CDK-9 and instead requires only CDK-12. Surprisingly, CDK-12-dependent Ser2-P is not required for germline development. These results show that Ser2-P is not an obligate component of Pol II-mediated gene expression in all tissues, and further suggest that fundamental differences in transcriptional regulation may be important features of soma and germline distinction.

MATERIALS AND METHODS
Strains used
Strains used: wild-type N2 (Bristol); cdk-9(tm2884) I/hT2 qIS48; cdk-12(tm3846) I/V/qC1 qIS26; cdk-12(ok3664) IV/qC1 qIS26; cdt-1(gk241) III; mes-4(bn83) V/Dnt1-GFP; dpy-18(e364) pie-1(zu127) III/qC1; rrf-1(pk1417) I; rrf-3(1426) II; and met-1(h4337) I. Transgenic lines were created for this study (see supplementary material Tables S2, S3). Some strains were provided by the CGC (Minneapolis, MN, USA), funded by NIH Office of Research Infrastructure Programs (P40 OD010440), and the National Bioresource Project for the Experimental Animal ‘Nematode C. elegans’ (Kawada-cho, Shinjuku-ku, Tokyo). Worms were grown at 20°C except where indicated.

¹Department of Biology, Emory University, Atlanta, GA 30322, USA. ²School of Medicine, Emory University, Atlanta, GA 30322, USA.
*Author for correspondence (bkelly@emory.edu)

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RNA interference

RNAi knock down for all experiments, except where indicated (supplementary material Fig. S7), was performed by soaking and feeding as previously described (Furushashi et al., 2010) with the following modifications: 1 µg/ml dsRNA was used and animals were grown on RNAi plates for 24 hours before analysis. RNAi was considered efficient if 100% embryonic lethality (for cdk-9, cyclin T or fcp-1) or larval arrest (for cdk-12 and cck-1) was observed.

Immunofluorescence

Immunofluorescence analyses on methanol/formaldehyde-fixed specimens was performed as previously described (Seydoux and Dunn, 1997) using the following antibodies: rat anti-Ser2-P (3E10, 1/10 dilution), rat anti-Ser5-P (3E8, 1/10), rat anti-Ser7-P (4E12, 1/10) (Chapman et al., 2007), goat anti-PIE-1 (cN-19, 1/100, Santa Cruz Biotech, Dallas, TX, USA, sc9245), rabbit anti-PGL-1 (1/10,000, Kawasaki et al., 1998) and mouse anti-H3K36me3 [CMA333, 0.25 µg/ml (Furushashi et al., 2010)]. Methanol/aceton fixation (Schaner et al., 2003) was used for anti-GFP (Millipore MAB3580, 1/200), anti-AMA-1 (AMA-1 N-terminal, 1/10,000; Novus Biological, Littleton, CO, USA, #38520002), anti-H3K4me2 [CMA303, 1/20 (Kimura et al., 2008)] and anti-P-granules [OIC1D4, 1/4 (Strome and Wood, 1983)]. Incubation with primary antibodies was for 12-16 hours at 4°C. Secondary antibodies included Alexa Fluor 594- and Alexa Fluor 488-conjugated donkey antibodies (1/500) (Molecular Probes, Eugene, OR, USA).

Specimens were examined on a Leica DMRXA microscope equipped with a Q-Imaging Retiga-SRV CCD camera (Hamamatsu Photonics, Hamamatsu, Japan) using Simple PCI software (Hamamatsu Photonics). Fiji (Schindelin et al., 2012) was used for quantification of raw immunofluorescence data. Average nuclear signal intensity was measured for Z2/Z2 or three representative nuclei. Average background signal, identified as non-nuclear staining from three surrounding representative regions, was subtracted. Standard error was determined according to Ashcroft and Pereira (Ashcroft et al., 2012) was used for quantification of raw immunofluorescence data. Average nuclear signal intensity was measured for Z2/Z2 or three representative nuclei. Average background signal, identified as non-nuclear staining from three surrounding representative regions, was subtracted. Standard error was determined according to Ashcroft and Pereira (Ashcroft et al., 2012). Non-overlapping z-stacks were taken of each embryo. The DAPI channel of each layer was transformed into a binary image, the Watershed protocol was applied to separate individual nuclei, and the number of particles (nuclei, 200-2000 pixel units) was counted.

Quantification of anti-Ser2-P was performed using the 3E10 antibody rather than H5 antibody because the H5 antibody recognizes an AMA-1-independent signal in mitotic nuclei that interferes with quantitative approaches (Walker et al., 2004); however, reported results were consistent between both antibodies.

Protein isolation and western blot

Seventy-five young adult worms following RNAi were transferred to 5 µl 1× PBS in a 1.5 ml microfuge tube. Lysis buffer (75 µl) [100 mM Tris HCl (pH 8.0), 3 mM MgCl2, 300 µM KCl, 0.1% NP40, 20% glycercol, 40 mM DTT] was added before snap freezing in liquid nitrogen. Samples were thawed and refrozen four times, and 45 µl chloroform added before spinning for 15 minutes at 4°C. Nucleic acids were precipitated with isopropanol and resuspended in 100 µl nuclease-free water. The RNasey kit (Qiagen, Valencia, CA, USA) and DNase treatment was used. RNA (1 µg) was used for cDNA synthesis using iScript cDNA synthesis kit (Bio-Rad) following manufacturer’s instructions. cDNA corresponding to 50 ng RNA was used for qPCR analysis using SsoFast (Bio-Rad) following manufacturer’s instructions on a CFX96 (Bio-Rad) machine. Transcript levels were normalized to either 18S or U6 levels for each sample. The average of two technical replicates from three biological samples was plotted with s.e.m.

Brod size analysis

Twenty worms of each genotype were transferred to individual plates at the L4 stage, moved every 7 hours and the number of embryos on each plate were counted until no offspring were produced. After 21 hours, unhatched embryos on plates were counted as dead. Adults that died during analysis were excluded from results. cdk-12(ok3664)/cdk-12(ok3664); cdk-12:pal-1 3’UTR (cdk-12 mutant) animals analyzed were the offspring of balanced cdk-12 mutants maintained to prevent suppressor selection.

RESULTS

Tissue-specific requirements for CDK-9 and CDK-12 in Ser2 phosphorylation

Previous studies have indicated that CDK-9 activity is required for all detectable Ser2-P in early C. elegans embryos (Shim et al., 2002), but Ser2-P is minimally affected by CDK-9 depletion in the two primordial germ cells (PGCs), Z2/Z3 (Furushashi et al., 2010). Although these initial studies implicated TLK-1 as a Ser2 kinase in the germ cells, the cell-cycle defects in ttk-1(RNAi) embryos made it difficult to conclude this was a direct effect (Furushashi et al., 2010). To further investigate embryonic Ser2 kinase requirements, we depleted other components of the CDK-9/cyclin T complex as well as homologs of the newly described CDK-12/cyclin K Ser2 kinase complex (supplementary material Fig. S1).

As previously observed, cdk-9(RNAi) resulted in loss of detectable Ser2-P in all embryonic somatic cells, but significant levels of Ser2-P remained unaffected by this treatment in Z2/Z3 (Fig. 1A,B; supplementary material Fig. S2A). Loss of the two
cyclin T paralogs yielded identical results to cdk-9(RNAi) (Fig. 1A; supplementary material Fig. S2E). Knock down of either CDK-9 or cyclin T caused defective gastrulation and early embryonic arrest at ~120 cells (supplementary material Fig. S3). These phenotypes correlate with defective somatic zygotic transcription and also occur with depletion of the large subunit of Pol II, AMA-1 (Powell-Coffman et al., 1996). Thus, although CDK-9 and cyclin T are important for both zygotic transcription and Ser2-P in somatic cells, neither is required for Ser2-P in Z2/Z3.

CDK-12 is a CTD kinase responsible for significant Ser2-P in both Drosophila and human cells (Bartkowiak et al., 2010; Blazek et al., 2011; Kohoutek and Blazek, 2012). A previously uncharacterized gene, B0285.1, is the closest homolog to Drosophila CDK12 in C. elegans (supplementary material Fig. S1). In contrast to cdk-9(RNAi), cdk-12(RNAi) embryos had undetectable Ser2-P levels in the PGCs, and a ~60% reduction of Ser2-P in embryonic somatic nuclei (Fig. 1A,B; supplementary material Fig. S2A). The RNAi conditions used were efficient and specific for each gene (supplementary material Fig. S4A).

Cyclin K is the cyclin partner of CDK-12 in other systems (Bartkowiak et al., 2010; Blazek et al., 2011; Kohoutek and Blazek, 2012). RNAi of the closest sequence homolog to Drosophila cyclin K, F43D2.1 (hereafter named cnck-1), phenocopies cdk-12(RNAi), and is likely the cyclin partner of CDK-12 (Fig. 1A,B; supplementary material Fig. S2F). Knock down of either CDK-12 or cyclin K results in early larval arrest, but no specific developmental defects were observed other than what appeared to be arrested growth (supplementary material Fig. S3).

These results demonstrate that CDK-12, rather than CDK-9, is the predominant Ser2 kinase in embryonic germ cells. The Ser2-P antibody used in this study [3E10 antibody (Chapman et al., 2007)] is specific for Pol II in C. elegans, as ama-1(RNAi) depletes all antibody binding (Furuhashi et al., 2010). Effects observed with cdk-12(RNAi) are specific for Ser2 in the embryo, as another phosphoepitope, Ser5-P, was unaffected by cdk-12(RNAi). We observed decreases in Ser5-P with cdk-9(RNAi) (supplementary material Fig. S2B,D), which could be an indirect effect, but CDK-9 has been reported to phosphorylate Ser5 in human cells (Glover-Cutter et al., 2009).

Decreased Ser2-P after cdk-12 knockdown could be an indirect result of increased Pol II CTD phosphatase activity. Knockdown of the best characterized Pol II CTD Ser2 phosphatase, FCP-1, resulted in increases in Ser2-P levels in embryonic somatic and germline nuclei (Fig. 1C,D; supplementary material Fig. S2G). However, RNAi against fcp-1 in combination with cdk-12(RNAi) caused a Ser2-P reduction in Z2/Z3 similar to cdk-12(RNAi) alone, so reduction of Ser2-P in these cells is not due to an ectopic increase in FCP-1 activity (Fig. 1C,D; supplementary material Fig. S2G).

**Embryonic germline-soma conversion yields switch in kinase dependency**

The maternal protein PIE-1 inhibits Ser2 phosphorylation and Pol II transcription in the C. elegans embryonic germline precursor cells, called P-cells (Ghosh and Seydoux, 2008). PIE-1 is proposed to inhibit Ser2 phosphorylation by sequestering P-TEFb until PIE-1 is degraded at the birth of Z2/Z3, and degradation of PIE-1 in Z2/Z3 correlates with Ser2-P appearance in these cells [Fig. 2A (Seydoux and Dunn, 1997)]. However, our results suggest that the germline Ser2-P kinase inhibited by PIE-1 is likely to be CDK-12/cyclin K. Indeed, delaying PIE-1 degradation in Z2/Z3 by knocking down ZIF-1, a protein involved in PIE-1 degradation, caused a delay in appearance of CDK-12-dependent Ser2-P in Z2/Z3 (Fig. 2A-C).

We next analyzed the germline correlation with CDK-12-dependent Ser2-P. Loss of PIE-1 in the P-cells results in a transformation of germline into soma and premature Ser2-P appearance. We tested whether the ectopic Ser2-P that appears in P-cells of pie-1(zu127) mutants is CDK-12 dependent. Transformation from germline into soma and premature Ser2-P (Fig. 2D). This further illustrates a tight correlation between germline identity and CDK-9-independent Ser2-P regulation.
CDK-12-dependent Ser2-P is not essential for early germ cell proliferation

We next examined the requirement for CDK-12 in post-embryonic germ cells. Z2/Z3 are born in the embryo after about 2 hours of development (~100 cells), remain mitotically quiescent through the remaining 10 hours of embryogenesis and re-enter the cell cycle after hatching (Sulston et al., 1983). cdk-12(RNAi) embryos arrest after hatching, so we examined germ-line Ser2-P in L1 larval offspring of RNAi-treated animals. cdk-12(RNAi) larvae had decreased Ser2-P in somatic nuclei and no detectable Ser2-P in the dividing germ cells (Fig. 3A,B). Thus, CDK-12 is required for Ser2-P in post-embryonic germ cells. The Ser2-P observed in cdk-12(RNAi) soma is presumably due to CDK-9 activity, although this could not be verified because of early embryonic lethality from cdk-9(RNAi).

Importantly, the cdk-12(RNAi) larvae examined arise from embryos with no history of detectable Ser2-P in their germ cells. Despite absence of Ser2-P, the larval germ cells showed some proliferation before arresting with the rest of the somatic lineages in the larvae (supplementary material Fig. S3). The larval arrest appeared to be related to a lack of growth, as the animals moved and fed normally but did not progress into further larval stages and eventually died.

CDK-12 is the predominant germ line Ser2 kinase

We next examined the requirements for CDK-9 and CDK-12 in adult germ cells. The adult gonad is a syncytium of germ cell nuclei
produced from a pool of proliferating stem cells at the distal end of the gonad, and these cells progress sequentially through meiosis and gametogenesis as they migrate toward the proximal end. Thus, the adult gonad contains germ cells linearly arranged in discrete, sequential stages of development that are highly transcriptionally active. We targeted each kinase by RNAi and used CDK-9:mCherry and CDK-12:GFP transgenes (described below) to assess RNAi knockdown. By initiating RNAi treatment at the last larval (L4) stage, we obtained adults with loss of detectable kinase protein in younger cells in the distal end of the gonad, and near complete loss in older nuclei at the proximal end (supplementary material Fig. S4B). Kinase RNAi in presence or absence of transgenes yielded similar results.

Surprisingly, although cdk-9(RNAi) caused loss of CDK-9:mCherry below detection, this had little effect on Ser2-P (Fig. 3B,C; supplementary material Fig. S5G). By contrast, cdk-12(RNAi) caused near complete loss of Ser2-P in all germ cells (Fig. 3B,C). Some Ser2-P remained detectable in older proximal nuclei in cdk-12(RNAi) gonads, presumably owing to CDK-12 protein produced before RNAi treatment was maximal. Similar RNAi results were observed in male gonads (data not shown). A substantial decrease of Ser2-P in cdk-12(RNAi) germ cells, which represent roughly half of the total nuclei in an adult worm, was also apparent by western blot analyses (supplementary material Fig. S5A). Thus, Ser2-P in germ cells at all stages of the germ cell cycle is largely independent of CDK-9/P-TEFb and dependent on CDK-12.

Importantly, neither cdk-9(RNAi) nor cdk-12(RNAi) significantly affected Ser5-P levels in adult germ cells (supplementary material Fig. S5C). By contrast, cdk-12(RNAi) caused a significant reduction of Ser7-P levels (supplementary material Fig. S5D), but it is unclear whether this is a direct effect.

CDK-9, CDK-12 and cyclin partners are ubiquitously expressed

Tissue-specific differences in Ser2 kinase requirements could be due to differences in expression of the kinases and/or their cyclin partners. In the absence of available antibodies for these components in C. elegans, we analyzed their expression using low copy, integrated, fluorescently tagged transgenes under control of their endogenous promoters and 3’UTRs (Frokjær-Jensen et al., 2008).

All components tested were ubiquitously expressed (Fig. 4A). The expression of the CDK-9, CDK-12 and cyclin T (CIT-1.2) transgenes recapitulate endogenous protein function because the transgenes rescue their respective mutant strains (a cyclin K mutation is unavailable; supplementary material Table S1). The kinase activity of both CDK-9 and CDK-12 is essential, as
transgenes expressing kinase-inactivated point mutants did not rescue (data not shown). Thus, tissue-specific expression is unlikely to underlie the different kinase requirements observed.

We further analyzed the subnuclear distribution of the fusion proteins in adult germ cells. The X chromosome is transcriptionally suppressed in meiotic germ cells in *C. elegans* (Kelly et al., 2002). Both CDK-9 and CDK-12 and their component cyclins are associated with autosomal chromatin but largely excluded from the X chromosome until late oogenesis, when X-linked transcription activates (Fig. 4B,C; cyclin T/K data not shown). Thus, although only CDK-12 activity is required for Ser2-P in the germline, both the CDK-9/cyclin T and CDK-12/cyclin K complexes are distributed in low-resolution patterns that correlate with active transcription.

Both cyclin T paralogs are ubiquitously expressed; however, the transgenic expression of each appeared reduced in the adult gonad and embryonic germ cells relative to somatic expression (Fig. 4A; supplementary material Fig. S6A,B). We increased cyclin T expression in germ cells to test whether reduced expression correlates with the decreased requirement on CDK-9/cyclin T for Ser2-P. We observed no increase in CDK-9-dependent Ser2-P in the germline as a result of increased cyclin T expression (supplementary material Fig. S6C,D).

Tissue-specific differences in Ser2 kinase requirements could also be due to the presence or absence of other factors involved in transcription elongation. We examined the expression of SPT-5, the major functional component of DSIF in *C. elegans* and other systems (Swanson and Winston, 1992; Yamaguchi et al., 1999; Shim et al., 2002). As observed for the CTD kinases and cyclins, SPT-5:GFP is ubiquitously expressed and also excluded from the X chromosome in adult meiotic germ cells (Fig. 4D and data not shown). Thus, tissue-specific differences in Pol II Ser2 phosphorylation are not likely due to differential expression of the kinases, cyclins or the DSIF complex.

**CDK-9, but not CDK-12, is required for proper germ line development or transcription**

We next examined whether either kinase is essential for germline development. The RNAi analyses described above were performed on late larval (L4) worms, after substantial germline development had occurred, so we performed RNAi starting at the L1 stage. *cdk-9(RNAi)* in L1 worms results in larval arrest so the effect on germline development could not be assessed. *cdk-12(RNAi)* in wild-type animals resulted in sterility of nearly all animals, but the sterility was not observed when *cdk-12(RNAi)* was performed in *rrf-1(pk1417)* mutant animals (supplementary material Fig. S7). RNAi is ineffective in *rrf-1* mutant soma, resulting in largely germline-specific RNAi effects (Sijen et al., 2001; Kumsta and Hansen, 2012). The sterility observed in *cdk-12(RNAi)* worms thus appears to be an indirect effect due to CDK-12 knockdown in soma rather than a direct effect of knockdown in the germline. The converse experiment is not readily feasible [mutants defective in germline-specific RNAi have fertility defects on their own (Kumsta and Hansen, 2012)], but we also observed increased sterility after *cdk12(RNAi)* in *rrf-3(pk1427)* worms, a strain with enhanced somatic RNAi but not enhanced germline RNAi [supplementary material Fig. S7 (Sijen et al., 2001)].

To definitively determine whether CDK-9 and CDK-12 are essential for germline development, we developed a method to specifically remove CDK-9 and CDK-12 activity from only the germline. Homozygous *cdk-9* and *cdk-12* deletions mutants arrest early in larval development because of somatic requirements, so we provided kinase expression solely in somatic tissues to allow larval growth and adult development. To do this, the endogenous 3′UTRs of the rescuing kinase transgenes were replaced with the 3′UTR of the pal-1 gene, which prevents translation in the germline until late oogenesis in the adult (Merritt et al., 2008). The CDK-9 and CDK-12 transgenes with the *pal-1* 3′UTR show ubiquitous somatic expression, but no expression in post-embryonic germ cells until late in adult oogenesis (Fig. 5A). Expressing the *kinase:pal-1* 3′UTR transgenes in the respective mutants therefore allows rescue of kinase function in all somatic lineages, but no rescue in post-embryonic germline development.

Expression of the *kinase:pal-1* 3′UTR transgenes fully rescued somatic development in the respective mutants: the transgene-rescued mutants grew to adults in parallel with their heterozygous siblings and showed no obvious somatic phenotypes. The absence of CDK-9 or CDK-12 activities in germ cells, however, had very different consequences.

The absence of CDK-9 in germ cells caused dramatic sterility, with animals producing only ~50-100 germ cells per gonad (~1/10th normal size, Fig. 5B). The germ cells produced, however, exhibited robust Ser2-P levels, further supporting the conclusion that Ser2-P in germ cells does not require CDK-9 (Fig. 5C). No CDK-9::mCherry is detectable in germ cells at any larval stage, suggesting that some proliferation can still occur in the absence of CDK-9 activity. Although persistence of trace amounts of maternally derived CDK-9 mRNA and/or protein cannot be ruled out, its activity would have been at very low levels and distributed among a significant number of nuclei.

Surprisingly, *cdk-12(ok3664)* animals expressing the *cdk-12:pal-1* 3′UTR transgene showed few germline defects. Neither CDK-12::GFP nor Ser2-P was detected at any post-embryonic germ cell stage until late oogenesis (Fig. 5D,E); yet these animals produced large numbers of functional germ cells and were fertile (Fig. 5D). Therefore, neither CDK-12 nor Ser2-P phosphorylation is essential for germ cell proliferation and development at normal temperatures. Interestingly, 100% of homozygotes expressing the *cdk-12:pal-1* 3′UTR transgene were sterile when raised at 25°C instead of 20°C (data not shown). The interpretation of this is unclear because these animals also showed misregulation of the *pal-1* 3′UTR transgene in the germline, which complicates the analysis.

Fertility of animals lacking CDK-12 at 20°C, however, was not completely normal. Homozygous *cdk-12(ok3664)* animals expressing the *cdk-12:pal-1* 3′UTR transgene produced fewer offspring than their heterozygous siblings and fully rescued *cdk-12(ok3664)* animals (Fig. 6A). However, no dramatic rise in embryonic lethality was observed in any strain (Fig. 6A). Therefore, neither CDK-12 nor Ser2-P is crucial for germline development.

We further examined transcript levels of a panel of germline-specific genes in *cdk-12:pal-1* 3′UTR transgene rescued mutants. Surprisingly, no substantial decrease in transcript was observed for any of the 14 genes assayed, most of which actually tended towards higher levels of expression (Fig. 6B). Therefore, neither CDK-12 nor Ser2-P is crucial for germline transcription.

**CDK-12 is required for normal H3K36me3 levels in germ cells**

We did not observe dramatic defects in RNA production in germlines lacking detectible Ser2-P, so we next examined whether loss of Ser2-P results in defects in another process linked to this mark. Ser2-P has been shown to regulate histone H3 lysine 36 trimethylation (H3K36me3) levels by recruiting the K36 methyltransferase Set2 during Pol II transcription elongation (Kizer et al., 2005). *C. elegans* has two H3K36 methyltransferases (HMT):
MET-1, a transcription-dependent K36 HMT and MES-4, a transcription-independent H3K36 HMT (Bender et al., 2006; Furuhashi et al., 2010; Rechtsteiner et al., 2010). We tested whether transcription-dependent (i.e. MET-1 dependent) H3K36me3 in the germline is dependent on CDK-12.

Previous studies have shown that H3K36 dimethylation (H3K36me2) in the adult germline requires MES-4 (Bender et al., 2006). We found that mes-4(RNAi) or mes-4(bn85) mutants still exhibit substantial levels of H3K36 trimethylation (H3K36me3) in adult germ cells (Fig. 7A,C; supplementary material Fig. S8A,C). All the offspring of the mes-4(RNAi) animals were sterile, indicating efficient knockdown of MES-4, which is essential for fertility (Garvin et al., 1998). No H3K36me3 was detected in met-1(n4337); mes-4(RNAi) animals (data not shown). The H3K36me3 in mes-4(RNAi) germlines is thus due to transcription-dependent activities of MET-1, allowing us to assess the requirement for CDK-12 in transcription-dependent H3K36me3.

The decreased Ser2-P in cdk-12:pal-1 3' UTR germ cells correlated with a significant decrease in MET-1-dependent H3K36me3 (Fig. 7A,B). Both total H3K36me3 levels and MES-4-independent H3K36me3 levels showed significant decreases (Fig. 7A,C). Similar results were obtained with cdk-12(RNAi) in mes-4(bn85) mutants (supplementary material Fig. S8A,C). Thus, normal levels of H3K36me3 in the C. elegans germline require Ser2-P produced by CDK-12.

We also observed decreases in H3K36me3 levels after cdk-9(RNAi) in mes-4(bn85) mutant germ cells (supplementary material Fig. S8A,C). This was surprising, as loss of CDK-9 has little effect on Ser2-P levels in germ cells. Given the severe germ cell phenotypes caused by decreased CDK-9, this may be an indirect effect on H3K36me3 levels, although a similar decrease in H3K36me3 levels has been observed in yeast Bur1 mutants (Chu et al., 2006).

**DISCUSSION**

**Tissue-specific Ser2-P regulation**

Although phosphorylation of Ser2 of the Pol II CTD has been studied for over 20 years, the role of CDK-12 in this process has only recently been appreciated (Bartkowiak et al., 2010). This is understandable considering that the loss of P-TEFb (CDK-9/cyclin T) activity in most analyses caused loss of Ser2-P, thereby obviating an apparent requirement for additional kinases. The severe effect on Ser2-P observed with loss of CDK-9 indicates a role for P-TEFb upstream of CDK-12 (Bartkowiak and Greenleaf, 2011). Somatic tissues in C. elegans embryos match this model: knock down of P-TEFb results in complete loss of Ser2-P. By contrast, CDK-12/cyclin K knock down results in a 60% decrease in Ser2-P, a decrease remarkably consistent with that observed in other systems (Bartkowiak et al., 2010; Blazek et al., 2011).

Ser2-P phosphorylation in soma also agrees with current models of Ser2-P regulation in the transcription cycle (Fig. 8A). CDK-9, cyclin T and P-TEFb knock down cause defective gastrulation, a phenotype associated with defective zygotic transcription in C. elegans (Powell-Coffman et al., 1996). Although this phenotype could be due to other functions of these proteins, it seems likely that these effects are due to defective Pol II transcription, i.e. CDK-9 knock

**Fig. 5. CDK-9, but not CDK-12 or Ser2-P, is essential for germline development.** (A) Expression of both pal-1 3'UTR-tagged kinases (only cdk-9/pal-1 3'UTR is shown) is observed in all somatic cells, but not in germ cells until late oogenesis. White outline surrounds adult germline, blue outline surrounds start of transgene expression. (B) Expression of cdk-9/pal-1 3'UTR in homozygous cdk-9(tm2884) young adult hermaphrodite worms. Boxed region is expanded in inset. White oval surrounds germ cells. Arrows indicate somatic gonad nuclei. (C) Anti-Ser2-P immunofluorescence analyses of cdk-9(tm2884), cdk-9/pal-1 3'UTR hermaphrodite ovaries (outlined). (D) Expression of cdk-12/pal-1 3'UTR in homozygous cdk-12(ok3664) young adult hermaphrodite worms. White outline surrounds gonad. (E) Anti-Ser2-P immunofluorescence analysis of cdk-12(ok3664), cdk-12/pal-1 3'UTR hermaphrodite gonads. Distal and proximal ends of the gonad are marked by ‘d’ and ‘p’, respectively.
down prevents proper Pol II elongation, whereas FCP-1 prevents proper Pol II recycling, in agreement with the current models (Cho et al., 1999; Fuda et al., 2012).

Our results show that transcription regulation in the C. elegans germline dramatically differs from current models of metazoan transcription elongation: CDK-9 does not play a major role in phosphorylation of Ser2, and Ser2-P added by CDK-12 does not require upstream CDK-9 activity (Fig. 8B). This is the first demonstration of Pol II Ser2-P not requiring, directly or indirectly, CDK-9/P-TEFb activity in a multicellular organism. Furthermore, the pie-1 mutant demonstrates that this is a fundamental difference between somatic and germline Pol II regulation, as transformation of germline to soma is accompanied by a switch from CDK-12- to CDK-9-dependent regulation of Ser2-P.

Unique regulation of Pol II kinases is a common feature of germ cell specification in many organisms (Nakamura and Seydoux, 2008), yet we were surprised to identify P-TEFb-independent Ser2-P in the C. elegans PGCs, Z2/Z3. Previous studies have suggested that inhibition of P-TEFb by PIE-1 is important for transcriptional repression in the germline precursors that give rise to the PGCs, Z2/Z3 (Zhang et al., 2003; Ghosh and Seydoux, 2008). PIE-1 degradation at the birth of Z2/Z3 was modeled to release P-TEFb inhibition. We find instead that PIE-1 activity correlates with CDK-12 repression in the P-cells, suggesting that PIE-1 may regulate Pol II by inhibiting multiple CDK kinases. Indeed previous studies have shown that PIE-1 also inhibits Ser5 phosphorylation by CDK-7, which may be more crucial for suppression of Pol II in the P-cells (Ghosh and Seydoux, 2008).

**Kinase requirements in development**

Although animals lacking CDK-9 and CDK-12 function arrest at different points, both kinases are essential for normal somatic development and growth. Their kinase activities are essential for somatic development, as kinase-dead point mutants do not rescue the lethality of deletion mutants. Although both of these kinases play roles in somatic Ser2-P levels, interpretation of this should be made with caution as it is unclear whether Pol II is the sole, or even the essential, target for either CDK-9 or CDK-12 in somatic tissues.

In the germline, CDK-9 is required for normal germline development, yet paradoxically is not significantly required for its most-studied activity: phosphorylation of Ser2 in the Pol II CTD. CDK-9 has other well-characterized roles in transcriptional regulation (Pirngruber et al., 2009), and the association of CDK-9 with transcriptionally active chromatin in the adult gonad indicates it has a role in germline transcription. However, whether the essential function of CDK-9 in the germline is restricted to transcription regulation is unclear.

Because Ser2-P in germ cells does not require CDK-9, we had the unique opportunity to assess the requirement for Ser2 phosphorylation in transcription apart from any other essential roles of CDK-9. Surprisingly, the high levels of Ser2-P in germ cells, which are dependent on CDK-12, were not required for germline development or fertility. Germlines lacking CDK-12 develop germline stem cells, progress successively through meiosis, and produce functional gametes and substantial numbers of viable offspring. Although brood size is reduced, transcription does not appear to be. Thus, neither CDK-12 nor Ser2-P is required for substantial transcriptional activity at any stage of germ cell development in C. elegans. Therefore, our results indicate that CTD phosphoepitopes may not always be accurate indicators of different stages of transcriptional regulation; e.g. absence of Ser2-P may not always reflect an absence of elongating Pol II.

CTD phosphorylation provides docking sites on elongating Pol II for complexes involved in transcript processing and nucleosome dynamics. Given the essential and predominant role of co-/post-transcriptional processing in C. elegans germ cells, it is unlikely these mechanisms are grossly affected in the cdk-12 mutant at optimal growth conditions. We observed a dramatic decrease in co-transcriptional H3K36me3 caused by loss of CDK-12. The role of H3K36 methylation, which is largely limited to gene bodies, decreases chromatin remodeling activity in the wake of Pol II elongation and helps restrict initiation to the correct promoter (Buratowski and Kim, 2010; Venkatesh et al., 2012). Co-transcriptional H3K36me3 by MET-1 is neither essential for viability nor for fertility under normal growth conditions in C. elegans (Andersen and Horvitz, 2007), but its absence could affect chromatin integrity and transcription fidelity, and more dramatically impact the transcriptome during post-embryonic growth or during stress. Pol II holoenzyme associations with RNA processing factors or other chromatin-modifying complexes could also be more dependent on Ser2-P during growth or stress. Germ cell development appears more reliant on CDK-12 activity at higher temperature, as the cdk-12(ok3664) mutant rescued with the cdk-12:pal-1 3’UTR construct is sterile at 25°C. This requirement under
stress is similar to that observed in budding yeast, where deletion of Ctk1, the major Ser2 kinase, causes cold-sensitive growth (Lee and Greenleaf, 1991).

We observed very small but quantifiable amounts (<5% in germ line cdk-12 mutants) of Ser2-P in cdk-12(RNAi) and mutant germ cells. This small signal could represent CDK-9-dependent Ser2-P that occurs in a subset of loci that are essential for germ cell development and viability. Indeed, this could explain the requirement of CDK-9 for fertility: the germ line/soma difference in kinase requirements may reflect a difference in gene-specific requirements for Ser2-P regulation. It is even possible that the residual H3K36me3 observed in germ cells lacking CDK-12 could be due to low levels of CDK-9 dependent Ser2-P. Although this is possible, the loss of 95% of detectable Ser2-P does not dramatically affect germline gene expression, so the requirement for CDK-9 for fertility does not generally equate with a requirement for Ser2-P. Importantly, decreased correlation between transcription and Ser2-P may be a general property of pluripotent cells, as Ser2-P is undetectable in melanocyte stem cells and in other stem cells (Freter et al., 2010). In any case, regulation of Ser2-P in C. elegans germ cells is clearly unique from other described systems: the majority of Ser2-P is not downstream of CDK-9 activity and is not required for germ cell development.

Mechanism behind tissue-specific kinase requirements on Ser2-P

The regulation of Ser2-P in embryonic soma matches the current model of metazoan Ser2-P regulation: CDK-9 is required ‘upstream’ of CDK-12. Previous studies suggest that the upstream role of CDK-9 in metazoans is to alleviate negative regulation causing promoter-proximal ‘paused’ polymerase, i.e. Pol II that has initiated transcription but cannot progress to elongation. Pausing is largely mediated by the negative regulator, NELF (Yamaguchi et al., 2013). C. elegans does not have homologs to NELF components. Furthermore, paused polymerase signatures are also largely absent from C. elegans Pol II profiles, excluding a few hundred genes in starved C. elegans larvae (Baugh et al., 2009; Kruesi et al., 2013). Thus, although the upstream requirement of CDK-9 for somatic Ser2-P in embryos cannot be directly related to NELF-mediated pausing in other systems, it is intriguing to speculate that there may be another mechanism of Pol II elongation regulation in embryonic soma employing DSIF in combination with soma-specific factors.

By contrast, germline regulation of Ser2-P more closely matches that in yeast where the CDK-9 homolog, Bur1, is not
required for Ser2 phosphorylation by the CDK-12 homolog Ctk1 (Qiu et al., 2009). The similarity between this tissue and yeast is particularly interesting as both organisms lack NELF-mediated pausing, and thus do not appear to require CDK-9/Bur1 for the progression of initiated Pol II into elongation and CDK-12/Ctk1-mediated Ser2-P. As in C. elegans germline, Bur1/CDK-9 is essential in yeast, yet the essential target is still unclear in both organisms.

The striking differences between these two modes of Ser2 regulation likely reflect inherent differences in regulating the transition between Pol II initiation and elongation in soma versus germline. Control of this transition may be more important in somatic development, in which the spatial and temporal regulation of tissue-specific gene expression is tightly regulated. The transition to elongation may not be as tightly regulated in C. elegans germ cells, which predominantly rely on post-transcriptional regulation for spatial and temporal protein patterns (Merritt et al., 2008). There is also substantial epigenetic contribution to the guidance of germ cell transcription in C. elegans that might decrease reliance on dynamic promoter regulation by kinases. For example, histone methylation contributes to an ‘epigenetic memory’ of transcription and chromatin organization produced in germ cells of the previous generation, and this memory is essential for normal germ line development (Furuhashi et al., 2010; Rechtsteiner et al., 2010; Gaydos et al., 2012). A structural memory of open chromatin, reinforced by active transcription in each generation that is stabilized in the germ line through successive generations, may obviate the need for the complications of multi-step transcriptional control seen in somatic development.

In summary, we have identified novel and tissue-specific regulation of Ser2-P in C. elegans. We believe that this demonstration of P-TEFb-independent Ser2-P in the C. elegans germline, the first to our knowledge in a multicellular organism, will provide a unique model for understanding the role of Ser2-P in transcription regulation during development. It is also intriguing to speculate that the different kinase requirements for Ser2-P and germline-specific modes of Pol II elongation regulation may be related to germline immortality.

**Note added in proof**

A recent publication (Cecere et al., 2013) has proposed that the ZFP1(AF10)/DOT-1 complex may play a role in Pol II transcription elongation regulation and might be involved in Pol II pausing in C. elegans.

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**Competing interests statement**

The authors declare no competing financial interests.

**Author contributions**

E.A.B. conceived the project, designed and performed most experiments, and prepared the manuscript. C.R.B. performed fcp-1 RNAi experiments and helped with quantification of RNAi experiments. J.H.A. contributed to the larval RNAi experiments. W.G.K. supervised the study and preparation of the manuscript.

**Supplementary material**

Supplementary material available online at http://dev.biologists.org/lookup/suppl?tid=10.1242/dev.095778/-DC1

**References**


Pol II phosphorylation in C. elegans


