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Transcription reactivation steps stimulated by oocyte maturation in *C. elegans*

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Abstract

Developing oocytes produce materials that will support early embryonic development then cease transcription before fertilization. Later, a distinct transcription program is established in the embryo. Little is understood about how these global gene regulation transitions are effected. We have investigated in *C. elegans* how oocyte transcription is influenced by maturation, a process that releases meiotic arrest and prepares for fertilization. By monitoring transcription-associated phosphorylation of the RNA polymerase II (Pol II) C-terminal domain (CTD), we find that oocyte transcription shuts down independently of maturation. Surprisingly, maturation signals then induce CTD phosphorylation that is associated specifically with transcription initiation steps and accumulates to high levels when expression of the CTD phosphatase FCP-1 is inhibited. This CTD phosphorylation is also uncovered when a ubiquitylation pathway is blocked, or when maturation is stimulated precociously. CTD phosphorylation is similarly detected during embryonic mitosis, when transcription is also largely silenced. We conclude that oocyte maturation signals induce abortive transcription events in which FCP-1 may recycle phosphorylated Pol II and that analogous processes may occur during mitosis. Our findings suggest that maturation signals may initiate preparations for embryonic transcription, possibly as part of a broader program that begins the transition from maternal to zygotic gene expression.

Keywords: Oocyte; Germ cell; Oocyte maturation; Meiosis; *C. elegans*; Transcription; CTD; CTD phosphatase; FCP-1; Ubiquitylation

Introduction

Oocytes retain the developmental potential of totipotent stem cells, but are also specialized cells that undergo extensive preparations for fertilization and the early stages of embryogenesis. During most of their development oocytes are highly active transcriptionally, producing mRNAs for their own needs or to be stored for the embryo, but eventually they terminate mRNA production prior to fertilization (Fig. 1A) (Davidson, 1986). Embryonic development initially relies entirely upon these maternal gene products, until a different program of zygotic transcription is initiated at a species-specific stage (Baugh et al., 2003; Seydoux and Fire, 1994; Zeng and Schultz, 2005). It is not known how these events are regulated, or what processes are involved in preparing for a rapid and coordinated induction of zygotic transcription.

Phosphorylation of the RNA polymerase II (Pol II) large subunit C-terminal domain (CTD) provides an indirect indicator of mRNA transcription activity. During transcription, the CTD undergoes a cycle of phosphorylation and dephosphorylation and acts as a scaffold that mechanistically couples transcription with downstream mRNA production steps (Fig. 1B) (Bentley, 2005; Buratowski, 2003; Meinhart et al., 2005; Orphanides and Reinberg, 2002). During the initiation phase of transcription the CTD repeat (YSPTSPS) is phosphorylated on Ser5 by the TFIIH kinase CDK-7. Subsequently, during elongation and other post-initiation steps, the balance of CTD phosphorylation shifts to Ser2. These phosphorylation events can be detected by staining with antibodies that recognize these respective forms of the CTD (Bregman et al., 1995; Leatherman et al., 2002; Martinho et al., 2004; Seydoux and Dunn, 1997; Walker et al., 2004). For example, in developing *C. elegans* oocytes,

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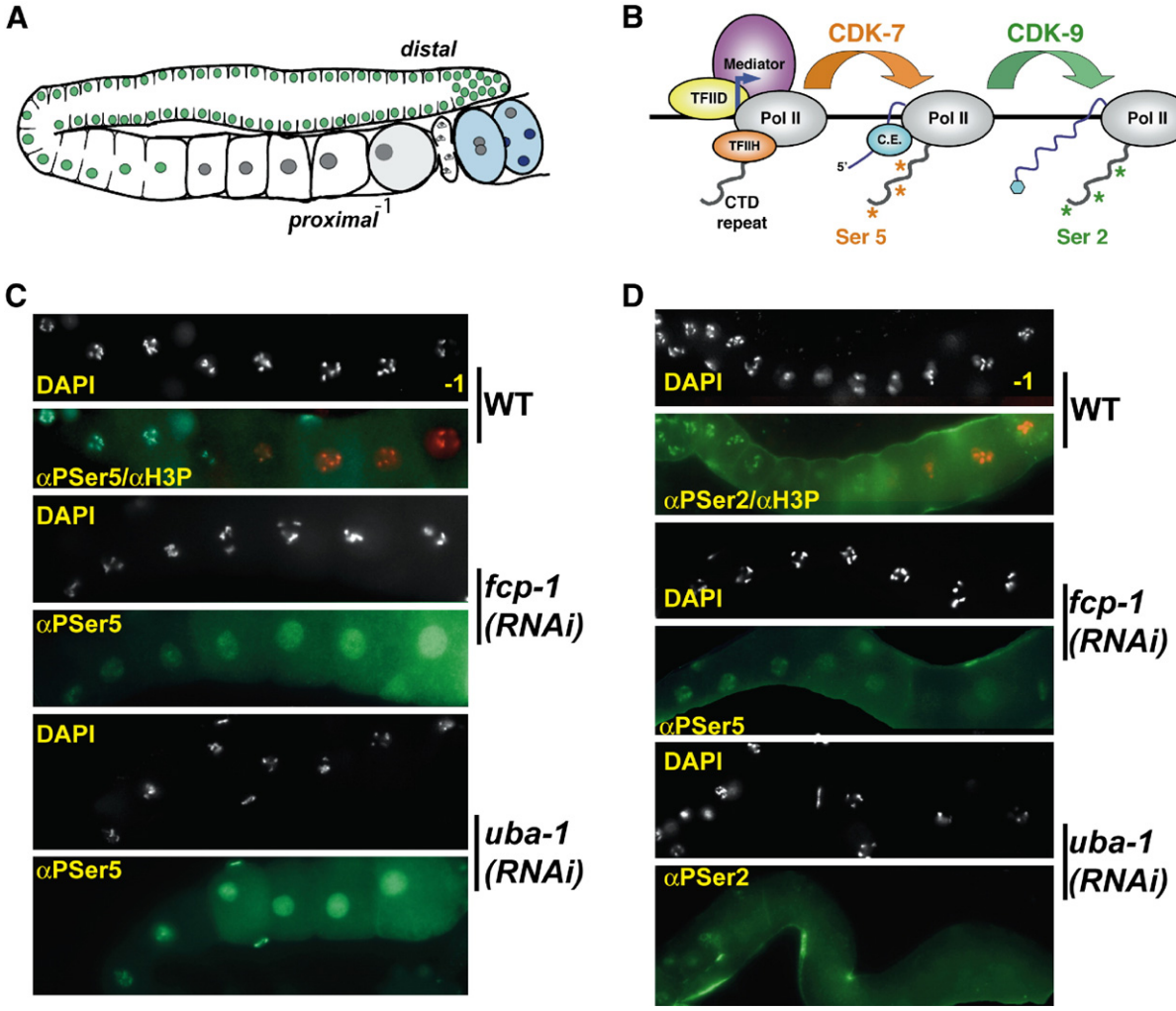

Fig. 1. Pol II CTD phosphorylation in proximal oocytes. (A) An adult *C. elegans* hermaphrodite gonad arm, in which oogenesis proceeds distally to proximally (relative to the uterus) in an assembly-line fashion (Greenstein, 2005; Schedl, 1997). A stem cell population at the distal end gives rise to approximately 160 sperm during larval development, then during adulthood produces only oocytes. Transcriptionally active nuclei, which are apparent from the distal mitotic region through the diplotene stage (Kelly et al., 2002; Schisa et al., 2001) (see panels C and D), are marked in green. Proximal oocyte nuclei that have entered diakinesis and are transcriptionally silent are shown in gray. The most proximal oocyte, which is designated as -1, assumes a rounded shape shortly before ovulation into the spermatheca, where fertilization occurs. Embryos (blue) initiate transcription in somatic nuclei (dark blue) at the 4-cell stage. (B) RNA Pol II transcription cycle (Bentley, 2005; Meinhart et al., 2005; Orphanides and Reinberg, 2002). A partial PIC is shown at a promoter. The PIC components that are shown are brought together specifically at promoters. The multi-copy CTD repeat (52 in human, 38 in *C. elegans*) must be unphosphorylated for Pol II to be recruited into the PIC, which also includes a set of general transcription factors and the Mediator complex. During initiation, the CTD repeat is phosphorylated on Ser5 by CDK-7, a subunit of the general transcription factor TFIIH. This modification is required for promoter clearance and recruitment of the 5′ capping enzyme (C. E.), after which Ser2 is phosphorylated by CDK-9. Ser2 phosphorylation promotes transcription elongation and is important for recruiting factors involved in mRNA processing. (C) Proximal oocytes of wild type and RNAi gonads (top panels) stained with DAPI (white), and antibodies to PSer5 (αPSer5) (green) and phospho-histone H3 (Ser10) (αH3P) (red). αH3P staining of RNAi gonads is not shown either here or in panel D, but was indistinguishable from wild type. The -1 oocyte is pictured farthest to the right here and in subsequent figures, unless otherwise noted. Identical exposure times are shown within the panel sets here and in all other figures. (D) Proximal oocytes of wild type and RNAi gonads stained with DAPI (white), and antibodies to PSer2 (αPSer2) (green) and αH3P (red, shown for wild type only).

phospho-CTD Ser5 (PSer5) staining parallels transcription activity, as detected by UTP incorporation (Figs. 1A, C) (Kelly et al., 2002; Schisa et al., 2001). This staining is readily apparent through the diplotene stage of Meiosis I then becomes undetectable as transcription shuts down.

In most species, oocytes arrest development late during the prophase of Meiosis I until an extrinsic signal stimulates maturation, a process that releases this arrest and prepares for fertilization (Greenstein, 2005; Masui and Clarke, 1979). This strategy allows organisms to maintain only a small pool of oocytes in a fertilization-competent state. In *C. elegans* oocyte maturation is triggered by a diffusable ligand from sperm and occurs continuously as long as sperm are present (Fig. 1A) (McCarter et al., 1999; Miller et al., 2003). Maturation involves conserved changes in cellular architecture that include nuclear envelope breakdown and rearrangement of the cortical cytoskeleton (Greenstein, 2005). In *C. elegans* maturation signals also lead to marking of some maternal proteins for eventual degradation, suggesting that maturation may involve additional processes that prepare for embryogenesis (Stitzel et al., 2006).

In *Xenopus* oocytes, fertilization stimulates a global dephosphorylation of the CTD that appears to require the conserved CTD phosphatase FCP-1 (Palancade et al., 2001), raising the question of whether transcriptional silencing in *C. elegans* oocytes might similarly involve FCP-1 or signals from sperm. FCP-1 is generally important for mRNA transcription, at least in part because it recycles phosphorylated Pol II for reinitiation, but its functions are not fully understood (Cho et al., 1999; Kobor et al., 1999; Meinhart et al., 2005). In yeast FCP-1 localizes to active transcription complexes and appears to dephosphorylate Ser2 during elongation (Cho et al., 2001; Meinhart et al., 2005). Yeast FCP-1 also robustly dephosphorylates Ser5 in vitro (Kong et al., 2005), but its role in Ser5 phosphorylation in vivo is still debated (Cho et al., 2001; Meinhart et al., 2005; Schroeder et al., 2000).

Here we have investigated the role of maturation and FCP-1 in the shutdown of transcription that occurs during *C. elegans* oogenesis. We show that oocyte transcription ceases independently of maturation signals, which specifically *stimulate* early steps in the transcription process. These steps are detectable as Ser5 phosphorylation that accumulates in developing oocytes after RNA interference (RNAi) knockdown of FCP-1, and is dependent upon multiple transcription initiation factors. Transcription- and maturation-associated CTD phosphorylation is also apparent after RNAi knockdown of a ubiquitylation pathway or when maturation is induced precociously. FCP-1 and this ubiquitylation pathway similarly prevent accumulation of Ser5 phosphorylation in transcriptionally silent embryonic mitotic and germline cells. Our results identify a previously undescribed function for oocyte maturation, induction of incomplete transcription steps that may prepare for zygotic gene activation (ZGA). They also suggest parallels between how transcription is shut down in oocytes and during mitosis and show that *fcp-1* is required for Ser5 dephosphorylation in these contexts.

Materials and methods

Strains

The following strains were used: N2 (wild type); JH1288 (*cdk-7(ax224)*) (Wallenfang and Seydoux, 2002); CB4108 (*fog-2(q71)*) (McCarter et al., 1999); DG1650 (*vab-1(dx31)/mIn1[dpy-10(e128) mIs14]; fog-2(q71); ceh-18(mg57)*) (Miller et al., 2003); TX183 (*oma-1(zu405te33)/nT1[unc-?(n754) let-?]IV; oma-2(te51)/nT1*) (Detwiler et al., 2001); SD939 (*mpk-1(ga111)*) (Lackner and Kim, 1998).

RNAi

Double stranded RNA was produced from expressed sequence tags (gift of Yuji Kohara) or PCR products generated from a *C. elegans* library (gift of Marc Vidal), except for *wee-1.3*, which was provided by Andy Golden. To perform *wee-1.3* RNAi, L4 hermaphrodites were placed on dsRNA-producing bacteria as described (Walker et al., 2004), then gonads were dissected and examined 18 and 30 h later. For all other immunofluorescence experiments, RNAi was performed by dsRNA injection (1 μg/ml). Those RNAi gonads were dissected at 18–20 (*uba-1*, *ubc-2*, *rpt-4*) or 22–24 (*fcp-1*, *rpb-2*) h after injection. At these times RNAi hermaphrodites reproducibly continued to produce embryos that uniformly failed to hatch, indicating that the RNAi penetrance was consistent. To perform RNAi in *cdk-7(ax224)* animals, injected worms were placed at 24 °C (non-permissive) or 15 °C (permissive) for 18 h before antibody staining. In DG1650 (*vab-1(dx31)/mIn1[dpy-10(e128) mIs14]; fog-2(q71); ceh-18(mg57)),* GFP (+) unmated females were injected, then oocyte maturation rates were determined 18 h later as described (McCarter et al., 1999). To determine whether regulation of Ser5 phosphorylation was epistatic to *oma-1* and *oma-2*, we injected *uba-1* dsRNA into *oma-1(zu405te33); oma-2(te51)* homozygotes (non-Unc, sterile). In western blot experiments RNAi was performed by feeding.

In each combinatorial RNAi experiment, a control sample was analyzed in which total input dsRNA amounts were maintained as constant (1 mg/ml) by addition of an unrelated dsRNA. This control dsRNA (*glp-1* or *skn-1*) did not obviously affect the late stages of oogenesis, but induced an embryonic phenotype that could be scored readily (Bowerman et al., 1992; Priess et al., 1987), making it possible to see that these dsRNAs served as valid controls that were processed by the RNAi machinery. For example, to compare effects of *fcp-1* and *fcp-1; ama-1* RNAi, injection samples contained either *fcp-1* and control dsRNA at 0.5 mg/ml each, or *fcp-1* and *ama-1* dsRNA at 0.5 mg/ml each, respectively. No diminution of *fcp-1* or *uba-1* oocyte or embryonic RNAi phenotypes was observed in any of these individual control experiments. In striking contrast, in multiple experiments the essential transcription factor dsRNAs that we assayed consistently affected these phenotypes as described in the text.

Quantitative (q)RT-PCR

For each sample, total RNA was extracted (Tri-reagent, Sigma) from 12 to 14 dissected gonads, half of which was used to synthesize cDNA (SuperScript II, Invitrogen). PCR was performed using the SYBR Green PCR Master mix (Applied Biosystems) according to the manufacturer's instructions, and reactions were run on a DNA Engine Opticon® 2 system (MJ Research). *fcp-1* forward (5′ ATGGACACAATTGGGAAAGC 3′) and reverse (5′ CCCATCATCATCCTCATCCT 3′) primers produced an amplicon of 108 bp. The normalization control actin (*act-1*): forward (5′ TACCCAATTGAGCACGGTAT 3′) and reverse (5′ TTAGCCTTTGGATTGAGTGG 3′) primers produced a 140 bp amplicon. Relative RNA levels were determined using the 2(−Delta Delta *C*(*T*)) method (Livak and Schmittgen, 2001).

Immunohistochemistry and image analysis

Antibodies used were αPol II (ARNA3, Research Diagnostics), αphosphohistone H3 (Ser10) (Upstate Biologicals), FK2 (Biomol), MAPK-YT (Sigma), αPSer2 (H5, Covance), αPSer5 (H14, Covance) and αUnP (8WG16, Covance), which recognizes the unphosphorylated CTD (Bregman et al., 1995). H5 and H14 specifically recognize the Ser2- and Ser5-phosphorylated forms of the Pol II CTD in vitro and in vivo (Bregman et al., 1995; Buratowski, 2003; Walker et al., 2004). Immunostaining was performed essentially as described (Walker et al., 2004). Images were captured on a Zeiss Axioplan, with an AxioCam color camera set to linear gain. Some gonad images were cut out of their background and pasted onto a black panel. Adjustments in brightness and contrast (to aid in visualization of images) were performed uniformly to all images in a panel. 4″-6-Diamidino-2-phenylindole (DAPI) staining was converted to black and white in all images.

Immunoblots

Extracts from RNAi embryos were obtained as in Walker et al. (2004). Lysates were separated on 6% Tris–acetate SDS gels (Invitrogen), blotted according to manufacturer's instructions and visualized with Enhanced Chemiluminesence (Amersham).

Results

Oocyte transcription ceases independently of maturation

We have investigated how transcription is regulated during the late stages of oogenesis by examining CTD phosphoryla-

tion. In proximal oocytes PSer5 staining disappears in parallel to appearance of staining for phospho-Ser10 of histone 3 (H3P) (Hsu et al., 2000) (Fig. 1C). H3P staining appears on chromosomes as they condense during mitosis, and in meiotic prophase this epitope similarly marks entry into the diakinesis stage, during which chromosomes become more condensed. Evidence from *C. elegans* embryos and other systems indicates that CTD Ser2 phosphorylation is essential for and indicative of occurrence of transcription steps downstream of initiation (Fig. 1B) (Meinhart et al., 2005; Seydoux and Dunn, 1997; Shim et al., 2002). Accordingly, in proximal oocytes phospho-CTD Ser2 (PSer2) staining is detected in a similar pattern to PSer5 (Fig. 1D; data not shown), supporting the idea that transcription is silenced as *C. elegans* oocytes progress into diakinesis.

Because *C. elegans* oocytes are continuously exposed to maturation signals in the presence of sperm (McCarter et al., 1999), the above experiments leave open the question of whether maturation signals might be required for this transcription shutdown. We investigated this possibility by examining CTD phosphorylation in *fog-2(q71)* animals, which are either male or female. In unmated *fog-2(q71)* females, which lack the maturation signal from sperm, oocytes arrest meiotic progression in diakinesis. These diakinetic oocytes complete late stages of oocyte development (nucleolar disappearance, distal nuclear positioning), but undergo maturation-dependent processes (nuclear envelope breakdown, cortical rearrangements, ovulation) only at a very low frequency. In unmated *fog-2(q71)* females all proximal oocytes that have entered diakinesis lack PSer5 and PSer2 staining similarly to wild type (Fig. 2; data not shown), indicating that in *C. elegans* transcription ceases in diakinesis independently of the maturation signal from sperm.

Accumulation of transcription-associated CTD phosphorylation in diakinetic oocytes that lack FCP-1

To investigate whether the FCP-1 phosphatase is important for shutting down oocyte transcription, we knocked down its expression by RNAi. Hermaphrodites that were injected with *fcp-1* dsRNA produced oocytes that entered diakinesis appropriately, as indicated by DAPI and H3P staining, and gave rise to near-normal numbers of embryos at the times analyzed (Fig. 1C; Materials and methods). This indicates that most aspects of the late stages of oogenesis occurred normally in *fcp-1(RNAi)* animals. In striking contrast to wild type, however, in *fcp-1 (RNAi)* animals Ser5 phosphorylation was consistently present at high levels in all proximal oocyte nuclei (Fig. 1C). Importantly, in diakinetic *fcp-1(RNAi)* oocytes, the levels of PSer5 staining also consistently increased distally-to-proximally as these cells developed (Fig. 1C; Supplementary Table 1). This progressive increase in PSer5 staining indicates that the bulk of this CTD phosphorylation did not perdure from earlier stages and suggests instead that the CTD repeat was continuously being phosphorylated on Ser5 in these cells.

If the Ser5 phosphorylation detected in *fcp-1(RNAi)* diakinetic oocytes occurred in the context of mRNA transcription events, it should depend upon factors that are required for transcription initiation. For example, this phosphorylation should require essential subunits of Pol II along with components of the transcription pre-initiation complex (PIC) (Fig. 1B) (Orphanides and Reinberg, 2002). The PIC is formed by recruitment of multiple separate protein complexes and must be assembled at promoters before transcription can occur. This CTD phosphorylation should also be mediated by the CDK-7 kinase, which is recruited to promoters separately from Pol II and phosphorylates Ser5 specifically in the context of the assembled PIC (Orphanides and Reinberg, 2002).

To test these possibilities, we used RNAi to inhibit expression of *fcp-1* simultaneously with subunits of these essential transcription machinery components. In these experiments we maintained the total amounts of injected dsRNA as constant by addition of a control dsRNA where appropriate (*glp-1* or *skn-1*; see Materials and methods). Quantitative RT-PCR indicated that the *fcp-1* mRNA was depleted from the gonad comparably by introduction of *fcp-1* dsRNA either alone, or when mixed with a control or essential transcription factor dsRNA (Supplementary Fig. 1). The Ser5 phosphorylation seen in *fcp-1(RNAi)* proximal oocytes was not diminished by co-injection of either control dsRNA that we tested (not shown). In

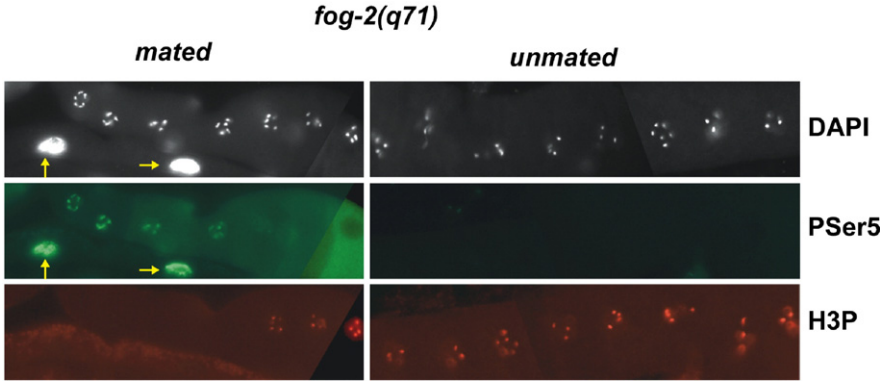

Fig. 2. Oocyte transcription shuts down independently of sperm-dependent maturation signals. Proximal *fog-2(q71)* oocytes are shown as in Fig. 1. Unmated *fog-2 (q71)* females accumulated many oocytes in diakinesis, all of which were H3P-positive and none of which showed nuclear PSer5 staining. The cytoplasmic αPSer5 staining apparent in the -1 oocyte in the mated sample was seen in some experiments. This staining was not detected by antibodies to two other AMA-1 (Pol II large subunit) epitopes (Fig. 4D; data not shown) and did not disappear when AMA-1 was depleted by RNAi, indicating that it was background. Intestinal nuclei are indicated by arrows.

contrast, this PSer5 staining was consistently abolished not only by knockdown of the Pol II large subunit AMA-1, which includes the CTD epitope, but also by simultaneous RNAi of either a different Pol II subunit (RPB-2) or the CDK-7 kinase (Fig. 3A). Significantly, this phosphorylation similarly required the Mediator subunit RGR-1 and the TFIID component TAF-4, each of which is recruited to the PIC separately from Pol II (Kornberg, 2005; Woychik and Hampsey, 2002) and is generally essential for transcription in *C. elegans* (Shim et al., 2002; Walker et al., 2001). This dependence upon CDK-7 and these various distinct PIC subunits argues strongly that the Ser5 phosphorylation accumulating in *fcp-1(RNAi)* diakinetic oocytes was generated in the context of *bona fide* transcription initiation complexes.

We next investigated whether *fcp-1(RNAi)* diakinetic oocytes also accumulated high levels of Ser2-phosphorylated Pol II, a marker of transcription elongation (Fig. 1B). After *fcp-1* RNAi Ser2-phosphorylated Pol II was detectable in diakinetic oocytes, but was present at very low levels that did not increase in parallel to PSer5 staining (Figs. 1C, D). A similar discordance between PSer5 and PSer2 staining was previously seen in *C. elegans* embryos that lack the mRNA capping enzyme, which is thought to be required for Pol II to progress to Ser2 phosphorylation and transcription elongation (Takagi et al., 2003). This suggests that most of the Ser5-phosphorylated Pol II produced in *fcp-1(RNAi)* diakinetic oocytes did not progress to the elongation phase. Interestingly, in diakinetic *fcp-1(RNAi)* oocytes, PSer5 staining seemed to fill the entire nucleoplasm, in contrast to its association with chromosomes at earlier stages in these and wild type hermaphrodites (Fig. 1C). Analysis with confocal microscopy indicated that this PSer5 staining was not excluded from chromatin (data not shown), but was present throughout the nucleus. PSer2 staining was present in a similar distribution in diakinetic *fcp-1(RNAi)* oocytes (Fig. 1D). The diffuseness of these staining patterns suggests that they may reflect phosphorylated Pol II that was generated during abortive transcription steps and is no longer associated with transcription complexes.

Sperm-dependent signals are required for CTD Ser5 phosphorylation to accumulate in diakinetic fcp-1(RNAi) oocytes

The striking finding that Ser5-phosphorylated Pol II continued to accumulate as diakinetic *fcp-1(RNAi)* oocytes developed raised the question of whether this phosphorylation might be stimulated by maturation signals. While the morphologic changes that are characteristic of maturation are seen in the -1 oocyte (Fig. 1A) (McCarter et al., 1999), induction of a mitogen-activated protein kinase (MAPK) signal that is

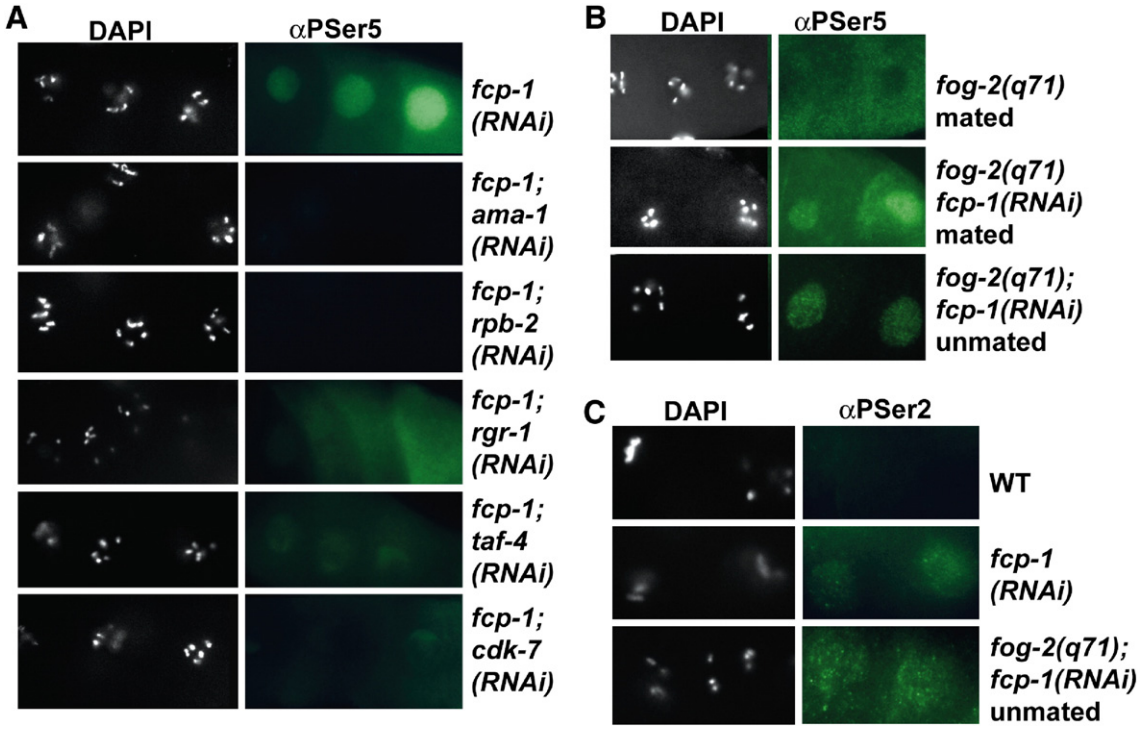

Fig. 3. Ser5 phosphorylation in diakinetic *fcp-1(RNAi)* oocytes requires transcription initiation factors and maturation signals. (A) Proximal oocytes in the indicated RNAi backgrounds were stained with DAPI and αPSer5. In each combinatorial RNAi experiment, input RNA amounts were carefully maintained by addition of a control dsRNA where appropriate (Materials and methods). (B) Maturation signals stimulate de novo CTD phosphorylation in diakinetic *fcp-1(RNAi)* oocytes. Mated *fog-2(q71)* and mated or unmated *fog-2(q71); fcp-1(RNAi)* gonads were stained with DAPI and αPSer5. PSer5 staining was present at low levels and did not increase progressively in unmated *fcp-1(RNAi)*; *fog-2* gonads, which contain approximately 8–10 oocytes in diakinesis. (C) PSer2 is present at low levels in diakinetic *fcp-1 (RNAi)* oocytes. Wild type, *fcp-1(RNAi),* or *fog-2(q71); fcp-1(RNAi)* gonads were stained with DAPI and αPSer2. Similarly low and variable levels of PSer2 staining were observed in diakinetic oocytes in *fcp-1(RNAi)* and mated or unmated *fog-2(q71); fcp-1(RNAi)* animals (Supplementary Table 2; data not shown). This PSer2 staining was abolished by *ama-1* (Pol II large subunit) RNAi (data not shown).

associated with maturation is detected earlier during diakinesis (Miller et al., 2003), indicating that these more distal oocytes sense and respond to sperm-dependent maturation signals.

We investigated the effects of sperm-dependent signals by comparing PSer5 staining in mated and unmated *fog-2(q71); fcp-1(RNAi)* females. In *fog-2(q71); fcp-1(RNAi)* females that had been mated, PSer5 staining was present at high levels and consistently accumulated distally-to-proximally in diakinetic oocytes (Fig. 3B), as was seen when *fcp-1* RNAi was performed in the wild type (Figs. 1C, 3A). In striking contrast, in unmated *fog-2(q71); fcp-1(RNAi)* females diakinetic oocytes showed low levels of Ser5-phosphorylated Pol II (Fig. 3B) that, importantly, did not increase in intensity distally-to-proximally. Similarly low levels of PSer2 staining were seen in diakinetic oocytes in unmated *fog-2(q71); fcp-1(RNAi)* females and *fcp-1(RNAi)* hermaphrodites (Fig. 3C), suggesting that this Ser2 phosphorylation is not globally influenced by the presence of sperm. It is possible that the PSer5 staining seen in unmated *fog-2(q71); fcp-1(RNAi)* diakinetic oocytes (Fig. 3B) might have perdured from the transcriptionally active diplotene stage. Significantly, however, our findings show that sperm-dependent signals are required for the bulk of PSer5 staining that is present in diakinetic *fcp-1(RNAi)* oocytes, as well as for the characteristic distal-to-proximal increase in that staining (Fig. 3B). We conclude that the Ser5-phosphorylated Pol II that accumulates in diakinetic oocytes when the FCP-1 phosphatase is lacking is generated de novo in response to maturation signals.

Accumulation of transcription-associated CTD phosphorylation in diakinetic oocytes is inhibited by a ubiquitylation/proteasome pathway

In a small-scale RNAi screen of other protein modification factors (data not shown), we observed that robust PSer5 staining also appeared in diakinetic oocytes after interference with a ubiquitylation pathway. Ubiquitylation requires an activating enzyme (E1), conjugating factor (E2) and target-specific ligase (E3) (Harper et al., 2002). Hermaphrodites in which the single *C. elegans* E1 (UBA-1) had been knocked down by RNAi laid eggs that failed to hatch, but consistently produced oocytes that appeared normal, entered diakinesis and were fertilized appropriately (Fig. 1C; Materials and methods). High levels of nucleoplasmic PSer5 staining were uniformly present in these diakinetic *uba-1(RNAi)* oocytes, although this staining did not increase progressively in the striking fashion seen in *fcp-1 (RNAi)* oocytes (Figs. 1C, 4A). Ser5 phosphorylation similarly appeared in diakinetic oocytes after RNAi knockdown of the E2 UBC-2 (Ubc4/5) (Fig. 4B), suggesting that this effect derives from interference with a specific ubiquitylation pathway.

UBA-1 and UBC-2 regulate meiotic divisions by functioning upstream of anaphase-promoting complex (APC) components (Frazier et al., 2004; Harper et al., 2002), but RNAi knockdown of APC^{Cdc20} or APC^{Cdh1} E3 subunits did not affect Ser5 phosphorylation in oocytes (Supplementary Fig. 2). Ubiquitylation can target proteins to the proteasome for either degradation or chaperone activities (Ferdous et al., 2001), and Ser5 phosphorylation appeared in proximal oocytes after RNAi knockdown of the proteasome component RPT-4 (Sug2) (Weeda et al., 1997) (Fig. 4B). Pol II staining did not detectably decrease in wild type proximal oocytes and was not increased by *uba-1* or *rpt-4* RNAi, however (Fig. 4D). The PSer5 staining seen in diakinetic oocytes after interference with this ubiquitin/proteasome pathway thus did not appear to derive from pleiotropic effects on either the cell cycle, or Pol II levels.

Significantly, as observed in *fcp-1(RNAi)* hermaphrodites, the PSer5 staining seen in *uba-1(RNAi)* diakinetic oocytes depended upon *ama-1*, *rpb-2*, *cdk-7*, *rgr-1* and *taf-4* (Figs. 4B, C), arguing that it was generated in the context of a transcription initiation complex. Ser2 phosphorylation remained undetectable in proximal oocytes after *uba-1*, *ubc-2* or *rpt-4* RNAi however (Fig. 1D; data not shown), indicating that these transcription events were not completed and that these proximal oocytes have shut down transcription at the appropriate stage. Consistent with this model, an early embryonic reporter transgene was not expressed earlier than normal in *uba-1 (RNAi)* embryos (Supplementary Fig. 3).

As seen in *fcp-1(RNAi)* oocytes, after *uba-1* RNAi robust PSer5 staining was present during diakinesis in mated but not unmated *fog-2(q71)* females, suggesting that this PSer5 staining depended upon oocyte maturation signals (Fig. 5A). This PSer5 staining similarly did not appear after *uba-1* RNAi was performed in *oma-1(zu405te33); oma-2(te51)* hermaphrodites (Fig. 5B), in which diakinetic oocytes initiate but fail to complete maturation (Detwiler et al., 2001). PSer5 staining was also not detected in diakinetic oocytes in *uba-1(RNAi); mpk-1 (ga111)* hermaphrodites (Supplementary Fig. 4) (Lackner and Kim, 1998), which fail to undergo maturation because the essential MAPK signal is blocked (M.-H. Lee, M. Ohmachi, E. Lambie, R. Francis, T. Schedl, personal communication). Oocyte maturation signals are therefore required for this PSer5 staining to appear in diakinetic oocytes after interference with *ubc-2*-dependent ubiquitylation.

Induction of CTD Ser5 phosphorylation by precocious oocyte maturation

We next examined whether constitutive or precocious stimulation of oocyte maturation is sufficient to allow PSer5 to accumulate in diakinetic oocytes. Parallel mechanisms involving *vab-1* and *ceh-18* are needed to prevent maturation from occurring constitutively in the absence of the sperm signal, so that maturation proceeds continuously in unmated *vab-1 (dx31); ceh-18(mg57); fog-2(q71)* females (Miller et al., 2003). Remarkably, robust nucleoplasmic PSer5 staining was present in diakinetic *vab-1(dx31); ceh-18(mg57); fog-2(q71)* oocytes (Fig. 5C). Even higher PSer5 staining levels were apparent during diakinesis after RNAi knockdown of *wee-1.3* (Fig. 5C), which prevents maturation from occurring precociously by inhibiting the CDK-1 (CDC2) kinase (Burrows et al., 2006). In contrast, in each case Ser2 phosphorylation was not detectable during diakinesis (data not shown), indicating that subsequent transcription steps were largely inhibited.

Events associated with oocyte development and maturation (H3P staining appearance, nucleolus disappearance, nuclear

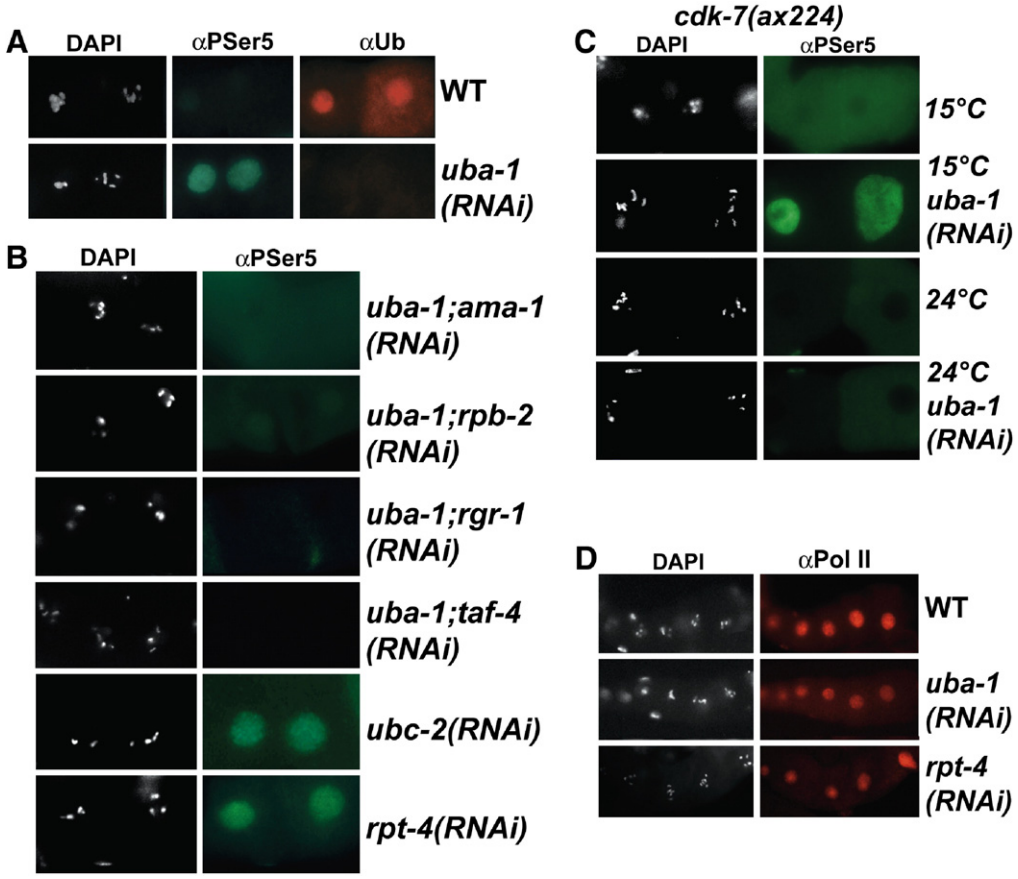

Fig. 4. Regulation of transcription-associated PSer5 accumulation by a ubiquitylation pathway. (A) Proximal oocytes of wild type (top row) or *uba-1(RNAi)* gonads stained with DAPI, and antibodies to PSer5 and mono-/poly-ubiquitinated proteins (αUb). (B) Proximal oocytes stained with DAPI and αPSer5 in the indicated RNAi backgrounds. PSer5 staining similarly appeared in proximal oocytes after RNAi knockdown of an additional proteasome component, *pbs-7* (data not shown). In each combinatorial RNAi experiment, input RNA amounts were carefully maintained by addition of a control dsRNA where appropriate (Materials and methods). (C) Proximal oocytes from a conditional *cdk-7* mutant (Wallenfang and Seydoux, 2002) at the permissive (15 °C) or non-permissive temperature (24 °C) were stained with DAPI and αPSer5. This *cdk-7* mutant was used in these *uba-1* experiments because *uba-1* and *cdk-7* RNAi effects become manifest in embryos at different times after injection. (D) Levels of the Pol II large subunit AMA-1 in oocytes lacking ubiquitylation or proteasome function. Columns show DAPI or an antibody to a non-CTD epitope of Pol II (ARNA3).

envelope breakdown) were not detectably altered after RNAi knockdown of *fcp-1*, *uba-1*, *uba-2* or *rpt-4* (data not shown), suggesting that the PSer5 staining seen in diakinetic oocytes under these circumstances did not result from precocious maturation. We examined this question further in *uba-1(RNAi)* animals because ubiquitylation influences many cellular processes. *uba-1(RNAi)* oocytes showed normal levels of maturation-associated MAPK activity, and maturation rates that were reduced, not increased (Figs. 5D, E). This indicates that maturation was not de-regulated by inhibition of *fcp-1* or *ubc-2*-dependent ubiquitylation, suggesting that these mechanisms normally prevent PSer5 from accumulating in maturing oocytes by regulating processes associated with transcription.

Appearance of phosphorylated CTD Ser5 in transcriptionally silent embryonic cells

To determine whether similar mechanisms affect CTD phosphorylation in the embryo before ZGA occurs at the four-cell stage (Seydoux and Dunn, 1997), we examined embryos produced by *fcp-1(RNAi)* hermaphrodites. In wild type one- and two- cell embryos, which are transcriptionally silent, Ser5 phosphorylation is normally restricted to two faint nuclear foci (Seydoux and Dunn, 1997). In contrast, at these stages *fcp-1 (RNAi)* embryos uniformly exhibited high levels of nucleoplasmic PSer5 staining (Fig. 6A; data not shown). About 30% of early *fcp-1(RNAi)* embryos showed abnormalities in polar body position or delayed cell division timing (data not shown) but in all *fcp-1(RNAi)* embryos the levels and localization of the germline-specific protein PIE-1 appeared normal (Figs. 6A, B; data not shown), suggesting that early asymmetries were intact (Tenenhaus et al., 1998). Interestingly, at the four-cell stage *fcp-1(RNAi)* embryos showed inappropriate nucleoplasmic PSer5 staining in the transcriptionally silent germ cell precursor, even though this cell appeared to contain normal levels of PIE-1 (Fig. 6B), a repressor that maintains transcriptional silence in the early embryonic germline (Batchelder et al., 1999; Seydoux et al., 1996). In contrast to this robust PSer5 staining, in *fcp-1(RNAi)* embryos PSer2 staining was undetectable prior to ZGA and dramatically reduced at later stages (Fig. 6C; Supplementary

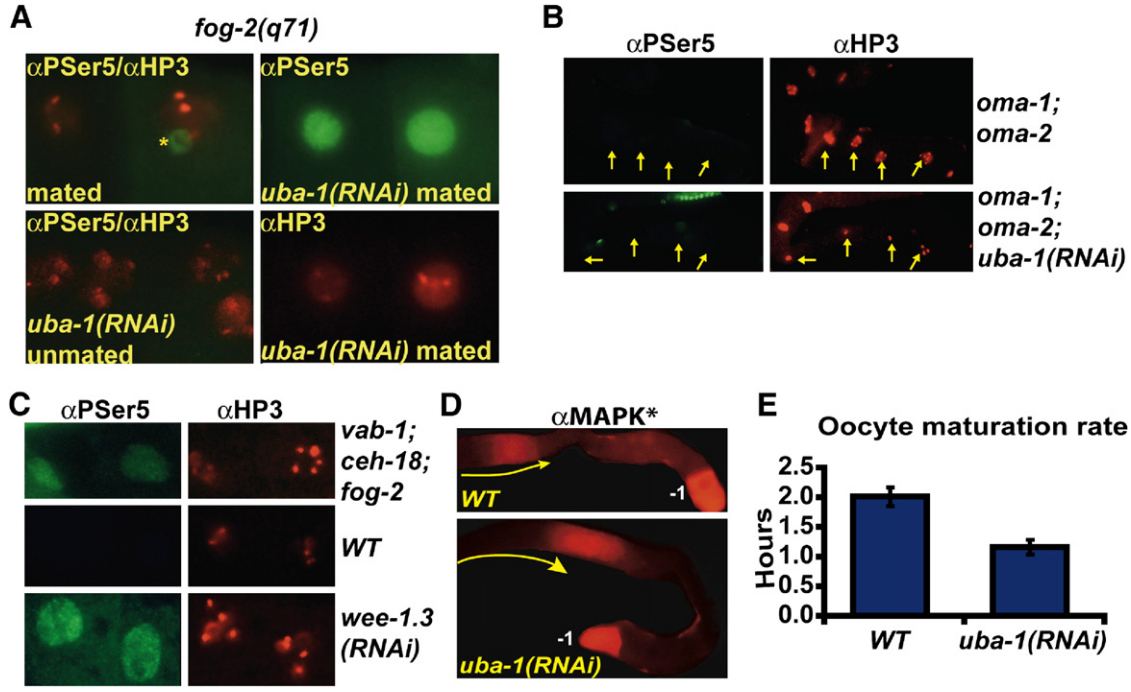

Fig. 5. Induction of CTD Ser5 phosphorylation by oocyte maturation signals. (A) *fog-2(q71)* proximal oocytes stained with αPSer5 (green) and αH3P (red) to mark oocytes in diakinesis. A somatic sheath cell nucleus is marked by an asterisk. The top and bottom panels on the right (mated *fog-2(q71)*; *uba-1(RNAi)*) show αPSer5 or αH3P staining of the same gonad. (B) *oma-1(zu405te33); oma-2(te51)* gonads were stained with αPSer5 and αH3P to mark oocytes in diakinesis. (C) Constitutive maturation promotes CTD Ser5 phosphorylation. The top row shows proximal oocytes from unmated *vab-1(dx31); fog-2(q71); ceh-18(mg57)* females. For *wee-1.3 (RNAi)* gonads (bottom) the -2 and -3 oocytes are shown. (D) Wild type (top) or *uba-1(RNAi)* (bottom) gonads were stained with an antibody to activated MAP kinase. Staining levels and patterns were comparable between wild type and RNAi gonads, although staining intensities varied among individuals. (E) Oocyte maturation is not accelerated by *uba-1* RNAi. Maturation rates in wild type and *uba-1(RNAi)* hermaphrodites. Error bars show standard deviation among independent experiments.

Fig. 5), suggesting that without FCP-1 most transcription was stalled after the initiation step. Accordingly, *fcp-1(RNAi)* embryos arrested development at about the 100-cell stage, similarly to *ama-1(RNAi)* embryos (Powell-Coffman et al., 1996), suggesting a broad defect in zygotic gene transcription (data not shown).

uba-1(RNAi) and *ubc-2(RNAi)* animals produced embryos that usually arrested development during the earliest cell divisions and uniformly exhibited high levels of PSer5 staining before the four-cell stage (Fig. 6A; data not shown). Western blotting similarly demonstrated that early *uba-1(RNAi)* embryos were highly enriched for PSer5 when compared to *apc-11 (RNAi)* embryos (Fig. 6D), which also arrest cell division primarily before ZGA (Frazier et al., 2004). The PSer5 staining seen in transcriptionally silent cells in *fcp-1(RNAi)* and *uba-1 (RNAi)* embryos depended upon *cdk-7*, *rgr-1* and *taf-4* (data not shown), as was observed in the corresponding diakinetic oocytes (Figs. 3A, 4B, C), indicating that this CTD phosphorylation occurred in the context of a transcription initiation complex. Thus, as seen in maturing oocytes, both *fcp-1* and a *ubc-2*-dependent ubiquitylation pathway are required to prevent Ser5-phosphorylated Pol II from being present in pre-ZGA embryonic nuclei.

Transcription is broadly blocked during somatic cell mitosis, through an incompletely understood process that involves inhibition of initiation and subsequent steps (Akoulitchev and Reinberg, 1998; Jiang et al., 2004). Ser5 staining was markedly reduced in wild type embryonic nuclei that have entered mitosis, but was uniformly present at high levels in H3P-positive mitotic nuclei in *fcp-1*, *uba-1*, *ubc-2* and *rpt-4(RNAi)* embryos, as seen in diakinetic oocytes (Fig. 6E; data not shown). This suggests that some mechanisms involved in regulating transcription silencing during oogenesis and meiosis may parallel processes that act during mitosis.

Discussion

Oocyte maturation is a complex process that prepares the oocyte to undergo dramatic changes rapidly upon fertilization (Greenstein, 2005; Masui and Clarke, 1979). Many hallmarks of maturation have been described for years, including nuclear envelope breakdown and cytoskeletal rearrangements, but the molecular and regulatory events associated with maturation are not well understood. Here we report that *C. elegans* oocytes shut down transcription independently of signals that stimulate maturation, as indicated by Ser5 and Ser2 phosphorylation being undetectable in diakinetic *fog-2* oocytes in the absence of sperm (Fig. 2; data not shown). Surprisingly, our findings also indicate that maturation signals induce the occurrence of early transcription steps in these transcriptionally "silent" cells (Fig. 7).

Firstly, we found that diakinetic oocytes accumulate Ser5-phosphorylated Pol II in response to maturation signals after RNAi knockdown of the CTD phosphatase FCP-1 (Fig. 3B). Importantly, the levels of this PSer5 staining characteristically increase as *fcp-1(RNAi)* oocytes develop and move distally-to-proximally (Fig. 1C, Supplementary Table 1), indicating that

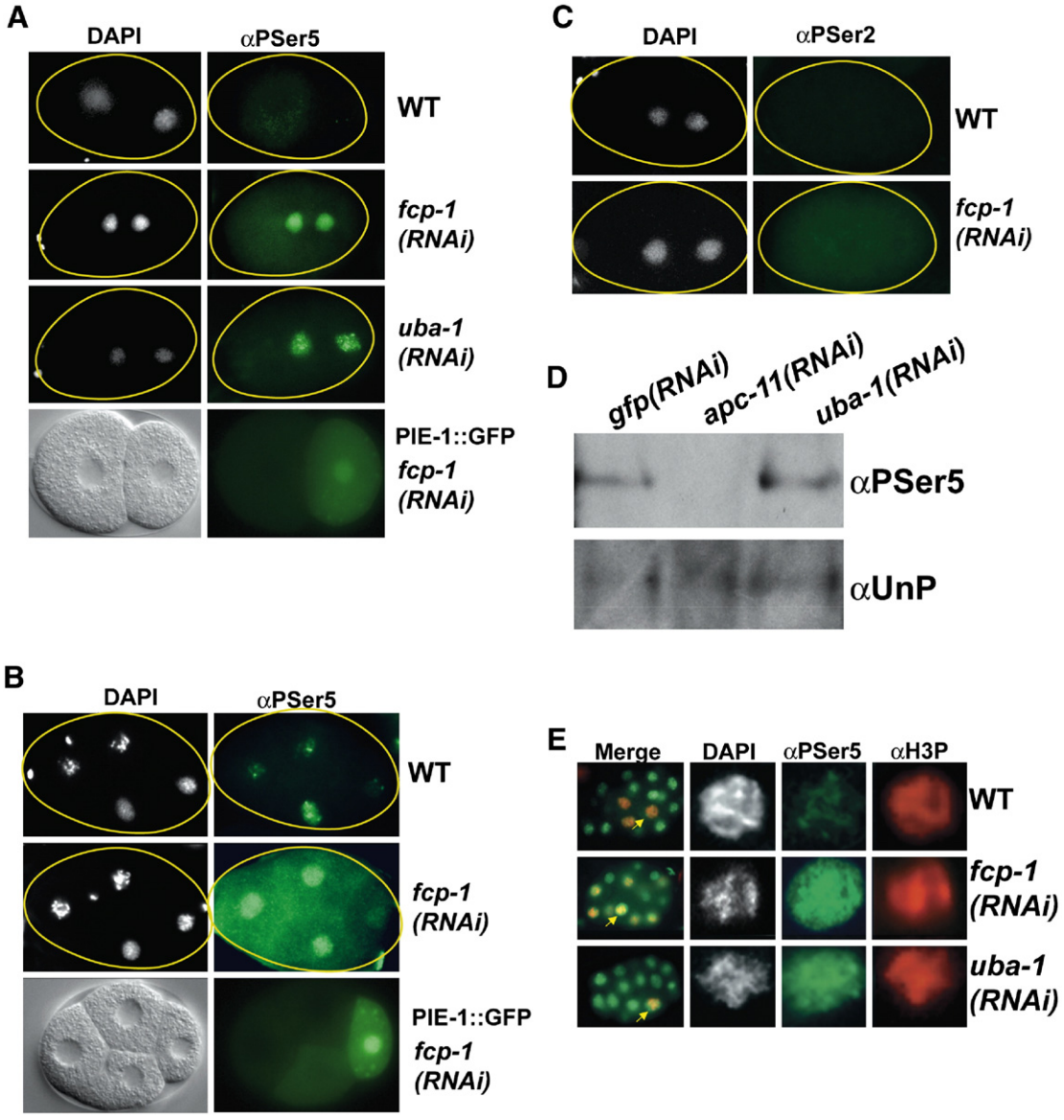

Fig. 6. *fcp-1* and a ubiquitylation pathway prevent PSer5 accumulation in transcriptionally silent embryonic cells. (A) Pre-ZGA embryos accumulate PSer5 after *fcp-1* or *uba-1* RNAi. Staining with DAPI (left column) or PSer5 (right) is shown in the top three rows. In multiple RNAi experiments the levels of PSer5 staining observed in one- and two-cell embryos were comparable to those seen in transcriptionally active cells in later stage embryos on the same slide (data not shown). The bottom row shows Nomarski (left) and epifluorescent (right) images of an embryo that expresses a translational fusion of PIE-1 to green fluorescent protein (PIE-1∷GFP) (Reese et al., 2000) and has been subjected to *fcp-1* RNAi. In panels A–C posterior is to the right. (B) Presence of nucleoplasmic PSer5 staining in the germ cell lineage in *fcp-1 (RNAi)* embryos. The top two rows show wild type or *fcp-1(RNAi)* embryos stained with DAPI and αPSer5. In the top panel, the two most anterior cells are entering mitosis. The bottom row contains Nomarski (left) or epifluorescent (right) images of a four-cell *fcp-1(RNAi)* embryo that expresses PIE-1∷GFP. The germline precursor P2 is to the right. (C) PSer2 staining is not detected in pre-ZGA *fcp-1(RNAi)* embryos. Wild type or *fcp-1(RNAi)* embryos were stained with DAPI and αPSer2. Robust PSer2 staining was apparent in later-stage embryos on the same slides (not shown). (D) High levels of Ser5 phosphorylation in pre-ZGA *uba-1(RNAi)* embryos. This western blot assayed extracts prepared from the same number of *gfp(RNAi)* embryos (ranging in age from 1 cell to around 250 cells), *apc-11(RNAi)* embryos (>80% arrested at less than 4 cell stage) and *uba-1(RNAi)* embryos (>90% arrested at less than 4 cell stage). αUnP (8WG16) recognizes the unphosphorylated Pol II CTD. (E) *fcp-1* and a ubiquitylation pathway prevent PSer5 accumulation during mitosis. The left column shows merged staining with αPSer5 and αH3P, which identifies mitotic cells. Arrows show nuclei expanded in the other panels. PSer5 staining uniformly appeared in mitotic cells in *fcp-1(RNAi)*, *uba-1(RNAi)* and *ubc-2(RNAi)* embryos at all stages (data not shown).

the bulk of this Ser5 phosphorylation does not perdure from earlier stages and is generated de novo in these diakinetic oocytes. This PSer5 staining depends upon multiple independent transcription initiation factors that associate with Pol II specifically at promoters (Fig. 3A), suggesting that this CTD phosphorylation is generated in the context of transcription initiation steps. The intensity of this PSer5 staining is comparable to or higher than that seen in transcriptionally active diplotene oocytes (Fig. 1C), indicating that a substantial level of this CTD phosphorylation occurs. Because this phosphorylation accumulates to high levels in maturing oocytes when the CTD phosphatase FCP-1 is lacking, we believe that this phosphorylation normally occurs during maturation but is rapidly turned over through dephosphorylation. In general, the

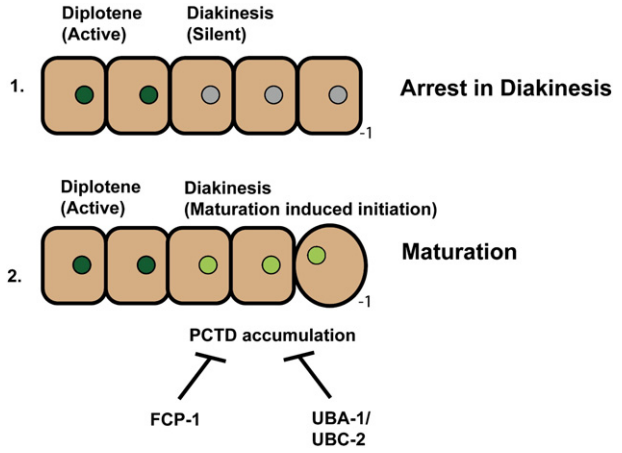

Fig. 7. Induction of transcription steps by oocyte maturation. During *C. elegans* oogenesis transcription ceases upon entry into diakinesis, in which meiosis arrests in the absence of maturation. Signals that trigger maturation release this arrest and induce incomplete transcription events in which CTD Ser5 is phosphorylated. The FCP-1 phosphatase and a *ubc-2*-dependent ubiquitylation pathway are required to prevent this phosphorylated Pol II from accumulating within the nucleoplasm.

transcription steps in which this PSer5 staining is generated do not appear to progress to the elongation phase, however, based upon the far lower levels of Ser2 phosphorylation detected in these diakinetic oocytes (Fig. 1D).

We also detected Ser5 phosphorylation that was associated with transcription and maturation when we inhibited *ubc-2*-dependent ubiquitylation (Figs. 4A–C, 5A, B). It will be difficult to identify the molecular target of this ubiquitylation pathway, particularly because numerous transcription factors undergo ubiquitylation (Muratani and Tansey, 2003; Somesh et al., 2005), but these results independently corroborate the occurrence of maturation-dependent transcription steps during diakinesis, as detected in *fcp-1(RNAi)* oocytes. It is intriguing that inhibition of this ubiquitylation mechanism did not result in a dramatic progressive increase in PSer5 levels as seen in *fcp-1 (RNAi)* oocytes (Fig. 1C). Perhaps the ubiquitin/proteasome pathway we have identified acts at an earlier initiation step, either to limit the rate of CTD Ser5 phosphorylation or promote Pol II recycling.

Finally, we detected robust Ser5 but not Ser2 phosphorylation in diakinetic oocytes in *vab-1(dx31); fog-2(q71); ceh-18(mg57)* and *wee-1(RNAi)* animals (Fig. 5C), which undergo maturation prematurely. These last experiments are significant because they allowed us to detect this CTD phosphorylation without manipulating regulatory mechanisms that directly influence transcription. Presumably, the presence of these maturation signals at inappropriate intensity or duration during late oogenesis stages resulted in accumulation of transcription-dependent PSer5 even in the presence of the FCP-1 phosphatase.

An important implication of our findings is that FCP-1 may function as a major Ser5 phosphatase in vivo, in addition to its previously described functions in Ser2 dephosphorylation (Cho et al., 2001; Meinhart et al., 2005). When FCP-1 was lacking, transcription-associated PSer5 that was generated in response to maturation accumulated throughout the nucleus, suggesting that this modified Pol II had dissociated from the DNA. Interestingly, after *fcp-1* RNAi we also observed nucleoplasmic PSer5 staining in embryonic germ cell precursors (Fig. 6B), in which PIE-1 has been proposed to repress transcription by acting at a post-initiation step (Batchelder et al., 1999; Zhang et al., 2003). We speculate that an important function of FCP-1 may be to recycle Ser5-phosphorylated Pol II that is produced during stalled or abortive transcription events, although we do not know whether in this context these events proceed to the point of generating incomplete mRNA transcripts. Importantly, *fcp-1* does not seem to be required for Ser5 phosphorylation levels to drop dramatically upon diakinesis entry in oocytes that do not proceed to maturation (*fog-2(q71); fcp-1(RNAi)* oocytes; Fig. 3A). This suggests that in *C. elegans*, oocyte transcription shutdown may not require *fcp-1* and therefore does not seem to involve a wave of FCP-1-mediated CTD dephosphorylation, as may occur in *Xenopus* (Palancade et al., 2001).

The evidence that maturation signals induce high levels of transcription-associated CTD phosphorylation suggests that, in these transcriptionally silent oocytes, many genes are accessible to and are bound by the transcription apparatus. We propose that beginning at diakinesis *C. elegans* oocytes initiate and maintain transcriptional silence largely through regulation of the transcription machinery, as opposed to rendering genes globally inaccessible through chromatin changes. Accordingly, prior to ZGA, embryos display histone modification markers that are associated with active chromatin (Bean et al., 2004; Schaner et al., 2003).

As in maturing oocytes, we also observed transcription-dependent PSer5 staining in pre-ZGA and mitotic embryonic cells after RNAi knockdown of *fcp-1*, *uba-1* or *ubc-2* (Figs. 6A, B, E). While we cannot rule out that this phosphorylated Pol II simply perdured from oogenesis, it seems more likely that it was produced de novo in these cells because their PSer5 levels were comparable to those seen in transcriptionally active embryonic nuclei, as indicated by immunofluorescence and western blotting assays (Figs. 6A, D, E; data not shown). We speculate that parallels may exist between how transcription is globally blocked during mitosis and meiosis, related cellular processes that each involve chromosome condensation, nuclear division and a subsequent rapid reactivation of transcription.

Why would oocyte maturation induce abortive transcription steps? During the transition from oocyte to zygote, the transcription machinery must shift between dramatically different gene expression programs (Baugh et al., 2003; Seydoux and Fire, 1994; Zeng and Schultz, 2005). By initiating early transcription steps at some promoters, oocyte maturation could facilitate their subsequent activation in the embryo. Recent studies suggest precedents for such a model. In mitotic mammalian cells, the *hsp70i* gene is "bookmarked" in a transcriptionally competent state, so that it can later be induced more rapidly by stress during G_1 (Xing et al., 2005). Similarly, during stationary phase in *S. cerevisiae* Pol II is bound upstream of >2500 genes that are silent, but will be induced within minutes after refeeding (Radonjic et al., 2005). We speculate that oocyte maturation may similarly poise the Pol II machinery for rapid induction of transcription at some promoters, and that

in some animals preparations for ZGA may begin before fertilization. Recent work has shown that maturation signals lead to "marking" of some maternally provided proteins for degradation in the early embryo (Stitzel et al., 2006). In light of those findings and our results, we believe that maturation signals not only lead to preparations for fertilization, but also may initiate processes that facilitate the transition from maternal to embryonic gene expression.

Note added in proof

Since the acceptance of the manuscript, it was reported that in mammalian embryonic stem cells tissue-specific gene loci are maintained in a transcriptionally competent state in which transcription and transcription factor occupancy are continuously suppressed by the 26S proteasome (Cell 127, 1375–1388, 2006). This dynamic state of activation may poise these loci for rapid induction upon differentation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ydbio.2006.12.039.

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