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Mitotic Phosphorylation of Histone H3 Is Governed by Ipl1/aurora Kinase and Glc7/PP1 Phosphatase in Budding Yeast and Nematodes

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Summary

Phosphorylation of histone H3 at serine 10 occurs during mitosis and meiosis in a wide range of eukaryotes and has been shown to be required for proper chromosome transmission in *Tetrahymena*. Here we report that Ipl1/aurora kinase and its genetically interacting phosphatase, Glc7/PP1, are responsible for the balance of H3 phosphorylation during mitosis in *Saccharomyces cerevisiae* and *Caenorhabditis elegans*. In these models, both enzymes are required for H3 phosphorylation and chromosome segregation, although a causal link between the two processes has not been demonstrated. Deregulation of human aurora kinases has been implicated in oncogenesis as a consequence of chromosome missegregation. Our findings reveal an enzyme system that regulates chromosome dynamics and controls histone phosphorylation that is conserved among diverse eukaryotes.

Introduction

The faithful segregation of chromosomes is an essential step in the accurate execution of each cell cycle and requires the precise coordination of a large number of events governing chromosome and microtubule dynamics (reviewed in Nurse, 2000). One of these events is the ordered interconversion between extended interphase chromatin and highly compacted chromosomes during mitosis and meiosis. Considerable progress has been made in the identification of protein complexes that are required for this event. For example, the condensin complex, which includes the SMC proteins, is thought to facilitate chromosome condensation by imposing topological changes on the DNA template in an energy-dependent manner (reviewed in Hirano, 2000). However, the molecular mechanisms that link the in vitro activities of the condensin complex with those of other chromosomal components in vivo remain to be determined.

Phosphorylation of histone H3 and linker histone H1 has long been shown to correlate with chromosome condensation during mitosis (Bradbury et al., 1973; Gurley et al., 1974). However, since H1 phosphorylation, or even H1 itself, is dispensable for mitotic or meiotic chromosome condensation (Ohsumi et al., 1993; Shen et al., 1995), the role for H1 phosphorylation may not be essential for these processes. In contrast, phosphorylation within the amino-terminal domain of H3 is now well established to be important during mitosis and meiosis in a wide range of organisms (Hendzel et al., 1997; Wei et al., 1998). Genetic studies in *Tetrahymena* show that a point mutation of serine 10 (Ser-10) in H3 (S10A) leads to abnormal chromosome segregation and extensive chromosome loss during mitosis and meiosis in micronuclei (Wei et al., 1999). Moreover, inhibiting H3 phosphorylation, presumably by blocking the H3 mitotic kinase activity, prevents the initiation of chromosome condensation and entry into mitosis (Van Hooser et al., 1998; de La Barre et al., 2000), whereas inhibiting dephosphorylation of H3 with protein phosphatase inhibitors induces abnormally condensed chromosomes and abnormalities during exit from mitosis into interphase (Guo et al., 1995). Thus, both phosphorylation and dephosphorylation of H3 are important features of the normal chromosome condensation/decondensation cycle. However, a crucial gap in our understanding of this pathway is the identity of the protein kinase and phosphatase enzyme system responsible for regulating the balance of mitotic H3 phosphorylation.

Using budding yeast *Saccharomyces cerevisiae* and the worm *Caenorhabditis elegans* as models, we demonstrate that Ipl1/aurora kinase and its genetically interacting phosphatase Glc7/PP1 are the enzymes that govern the steady-state level of H3 phosphorylation during mitosis and meiosis. The *IPL1* gene encodes a serine/threonine kinase that was originally identified in a genetic screen for mutants that increase in ploidy (Chan and Botstein, 1993), and it was suggested that Ipl1p opposes the protein phosphatase 1 activity of Glc7p in budding yeast (Francisco et al., 1994). Homologs of Ipl1/aurora kinase have been reported in various organisms including *C. elegans* (Schumacher et al., 1998a, 1998b; Woollard and Hodgkin, 1999), *Drosophila* (Glover et al., 1995), and mammals (Kimura et al., 1997; reviewed in Bischoff and Plowman, 1999). Interestingly, human homologs of the Ipl1/aurora kinases (referred to as aurora kinases; see Bischoff and Plowman, 1999) are overexpressed in a variety of cancers (Gopalan et al., 1997; Bischoff et al., 1998; Tatsuka et al., 1998; Zhou et al., 1998), supporting the notion that these enzymes are important for maintaining chromosome stability (Bischoff

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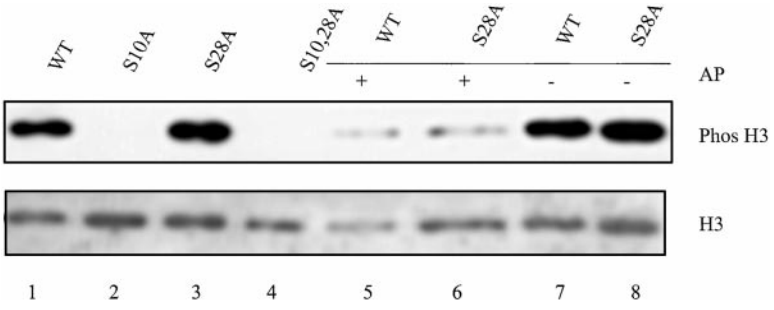

Figure 1. Histone H3 Is Phosphorylated at Serine 10 in *S. cerevisiae*

Histone proteins were extracted from wild type (WT, lanes 1, 5, and 7), S10A (lane 2), S28A (lanes 3, 6, and 8), and S10,28A (lane 4) cells, and those from WT and S28A cells were further treated with/without bacterial alkaline phosphatase (AP, lanes 5–8) as described previously (Sweet et al., 1996). Approximately 1.5 μg of proteins were resolved on a 15% polyacrylamide gel and analyzed by Western blot. Top: Western blot using Phos H3 Ab; bottom: the same membrane reprobed with general H3 Ab.

and Plowman, 1999; reviewed in Giet and Prigent, 1999). The results described below reveal that this highly conserved enzyme system also brings about the ordered addition and removal of phosphate from Ser-10 of H3 during entry and exit from mitosis and meiosis. Taken together, these findings reinforce an emerging theme that covalent modifications of histone proteins and chromatin remodeling in general have significant roles in human biology and in the progression of human diseases.

Results

Histone H3 Is Phosphorylated at Ser-10 in Budding Yeast

Histone H3 is phosphorylated at Ser-10 during mitosis and meiosis in a wide range of organisms from protozoan to human (Hendzel et al., 1997; Wei et al., 1998) and in mammalian cells is also phosphorylated at serine 28 (Ser-28) (Goto et al., 1999). However, H3 phosphorylation in *S. cerevisiae* was not observed in a previous study (Marian and Wintersberger, 1982). Given the strong evolutionary conservation of this histone modification, we reexamined this in yeast by using an antibody that is highly selective for phosphorylation at Ser-10 of H3 (Phos H3 Ab; Hendzel et al., 1997).

Histones were prepared from the nuclei of logarithmically growing cells, and the presence of phosphorylated H3 was assayed by Western blot with Phos H3 Ab. As shown in Figure 1, H3 from wild-type cells (JHY86) reacted strongly with Phos H3 Ab, suggesting that yeast H3 is indeed phosphorylated at Ser-10 as in other eukaryotes (lane 1). To confirm this conclusion genetically, we constructed serine-to-alanine mutations in H3 at Ser-10 (S10A), Ser-28 (S28A), or both together (S10,28A). Histone H3 from the S10A and S10,28A mutants failed to react with Phos H3 Ab (lanes 2 and 4), while that from the S28A mutant reacted strongly, giving a signal that was indistinguishable from that of wild-type H3 (lanes 1 and 3). The Phos H3 signals were due to phosphorylation since treatment with bacterial alkaline phosphatase (AP) virtually abolished the signals for both wild-type and the S28A strains (lanes 5 and 6). Thus, both biochemical and genetic results establish that H3 is phosphorylated at Ser-10 in yeast.

Histone H3 Is Phosphorylated at Ser-10 in a Cell Cycle–Dependent Manner during Mitosis and Meiosis

Next, we asked whether the levels of Phos H3 were linked to mitotic nuclear division and meiosis in yeast. Looking first at the vegetative cell division cycle, wild-type cells (CY1221) were arrested in G1 phase by treatment with α factor as described in Creanor and Toyne (1993) and then released into YPD medium to establish a synchronously growing culture. Whole-cell extracts (WCE) collected at 20 min intervals were assayed for Phos H3 by Western blot and for cell cycle distribution by flow cytometry as described in Smith (1991). As illustrated in Figure 2A, a low constitutive level of Phos H3 signal was detected at the earliest time points, and this signal increased between 100 and 140 min after release from the α factor block. This elevated Phos H3 signal coincides with the increasing proportion of cells entering G2/M phase during that interval (Figures 2B and 2C). Thus, the level of H3 phosphorylation in vegetative cells is cell cycle regulated, strongly increasing around the time of nuclear division. Interestingly, Phos H3 detected in interphase cells suggests that in yeast H3 might be phosphorylated in response to additional growth signals, such as the mitogen-induced H3 phosphorylation described in mammalian cells (Sassone-Corsi et al., 1999; reviewed in Thomson et al., 1999).

To test whether H3 is also phosphorylated during meiosis, diploid cells derived from the rapid sporulating SK1 strain were induced for synchronous sporulation (Bishop, 1994). Upon examining Phos H3 levels during the course of sporulation, a sharp peak of H3 phosphorylation was found at 4.5 hr after the induction of meiosis (Figure 2D). Judging by the progress of cells through meiosis (Figure 2E), the peak of this H3 phosphorylation initiates in late prophase and declines rapidly around the time that the meiotic divisions occur. To confirm the above conclusion, we performed double immunostaining of Phos H3 and Zip1, a component of the mature synaptonemal complex that serves to identify the pachytene stage of meiotic prophase when chromosomes reach maximal condensation (Dresser and Giroux, 1988; Sym et al., 1993). As shown in Figure 2F, weak Zip1 and Phos H3 staining appear concomitantly in early prophase (a–c), whereas by pachytene, when extended Zip1 structures are formed, 91% of the nucleoids are stained brightly with Phos H3 Ab (d–f). We further tested this correlation by examining Phos H3 levels in an *ndt80* mutant, which arrests before the first meiotic division with condensed chromosomes (Chu and Herskowitz, 1998). The *ndt80* mutant fails to undergo meiotic divisions and maintains Phos H3 at high levels (Figure 2D), correlating well with the persistent Zip1 staining observed in *ndt80* nucleoids (Figure 2F, g–i). Finally, wild-type Phos H3 pattern is observed in the *spo11* mutant (Figure 2D), indicating that the H3 phosphorylation does not require the initiation of meiotic recombination by forming DNA double-strand breaks, a process that does

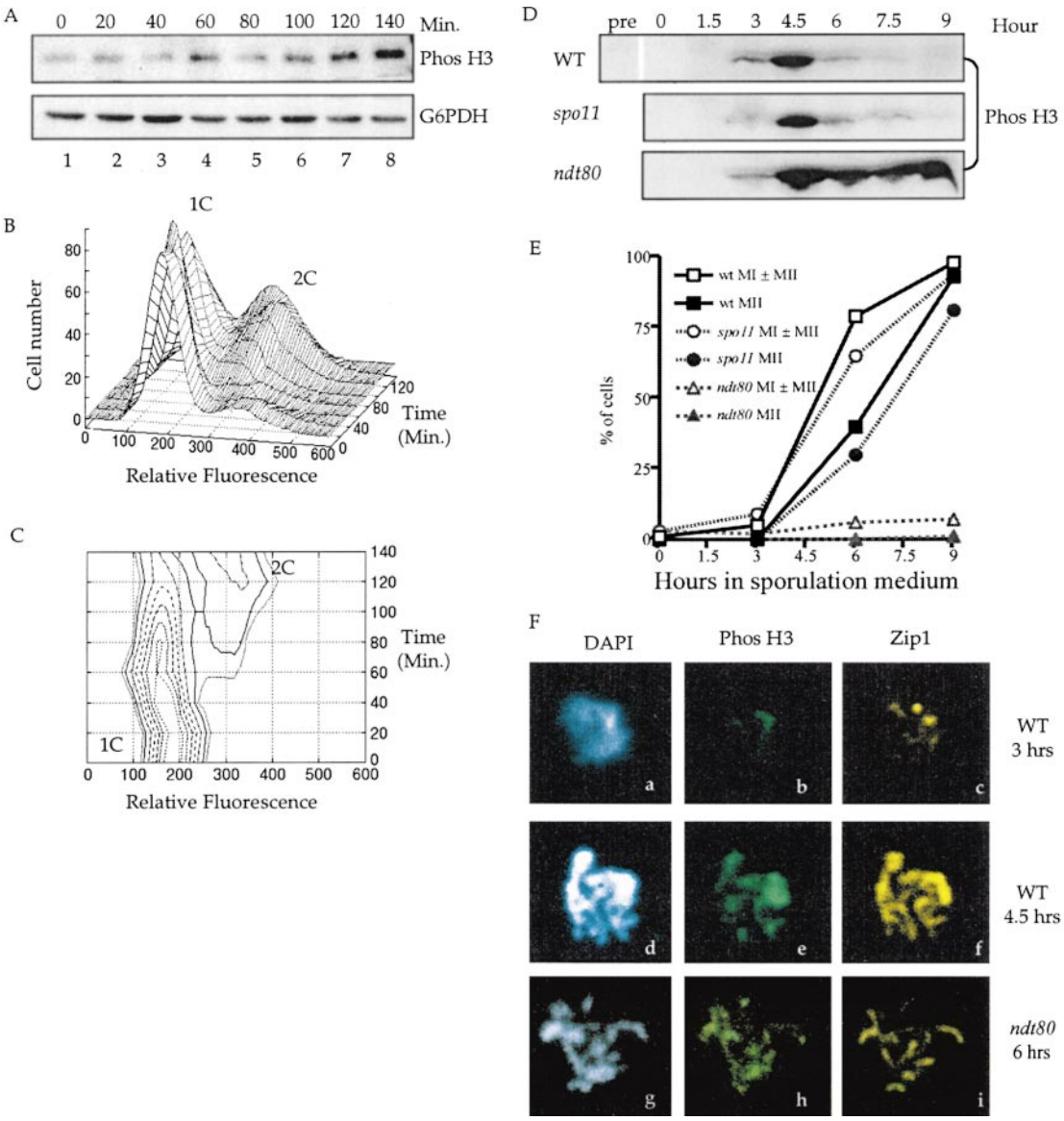

Figure 2. H3 Phosphorylation during Mitosis and Meiosis

(A) Western blot of samples collected from cells released from α factor block. Whole-cell extracts of cells collected at the indicated time points were probed with Phos H3 Ab. Protein loading was analyzed by G6PDH Ab.

(B) Fractions of cells from (A) were stained with propidium iodide and histograms of DNA content ("Relative fluorescence") were determined by flow cytometry for each time point. Individual histograms were then aligned to create a surface representation of the change in cell cycle distribution with time. A perspective view of this surface is shown, labeling the G1 ("1C") and G2 + M ("2C") peaks of DNA content.

(C) An alternative view of the flow cytometry data is shown as a contour plot. The isobars join points of identical cell counts in the histograms. Peaks corresponding to "1C" and "2C" DNA content are labeled.

(D) WT (DKB974), *ndt80* (DKB1478), and *spo11* (DKB10) diploid SK1 strains were induced to undergo synchronous meiosis. At the indicated time points, cells were resuspended in 2× SDS loading buffer and subjected to Western blot analysis. Equivalent loading was assessed by Coomassie staining of one parallel gel (data not shown). The premeiotic sample (pre) was late log phase cells taken immediately before transfer to meiotic medium.

(E) Aliquots of cells taken at the indicated time points were stained with DAPI and scored for meiotic progression (1 nuclear body = prophase, 2 = MI, 3 or more = MII).

(F) Meiotic chromosomes of WT (DKB974) and *ndt80* (DKB1478) diploid SK1 strains were spread on glass slides at the time points indicated above and examined for Zip1 and Phos H3 signals by double immunostaining. Representative WT nucleus from early (a–c) and late (d–f) prophase are shown, as well as a late *ndt80* nucleus from the 6 hr time point (g–i).

not occur in *spo11* mutants (Klapholz et al., 1985). These results demonstrate that H3 phosphorylation is tightly regulated during meiosis and is correlated with maximal chromosome condensation at pachytene. Thus, as in all other eukaryotes that have been examined to date, H3 in *S. cerevisiae* is also phosphorylated at Ser-10 during both mitosis and meiosis.

Ipl1p Is the Upstream Mitotic H3 Kinase in Budding Yeast

The highly regulated pattern of H3 phosphorylation during sporulation suggested that the activity of the H3 kinase might be regulated in a similar manner. Upon examining the yeast transcriptional program during sporulation (Chu et al., 1998), the Ipl1 kinase was found

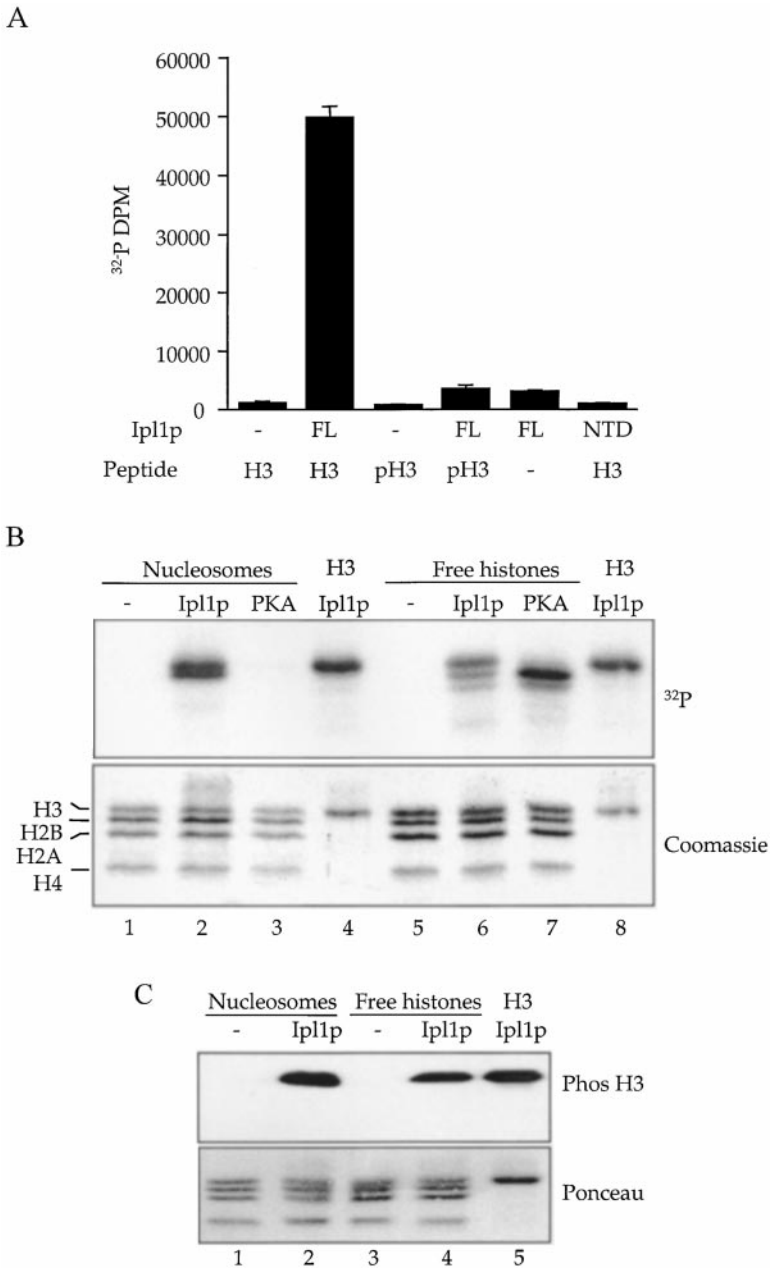

Figure 3. Phosphorylation of Histone H3 at Ser-10 by Ipl1 Kinase

In vitro kinase assays were performed in the presence of protein kinase A (PKA), His_{10}-tagged full-length (FL), or N-terminal domain (NTD) of Ipl1p. (A) Phosphorylation of amino-terminal H3 peptides (amino acids 1–20) that were either unmodified (H3) or singly phosphorylated at Ser-10 (pH3) was assessed by measuring incorporation of [γ-^{32}P]ATP using a filter binding assay. The indicated kinase activities were obtained from three independent experiments. Phosphorylation of chicken H3, mixtures of free histones, or mononucleosomes by the indicated enzymes were analyzed by autoradiography (B) or Western blot (C). Note that upon longer exposure, radioactivity can be detected in the H3 band in (B), lane 3.

to be one of the kinases that are induced between 2 and 5 hr in sporulation medium, corresponding to the peak of H3 phosphorylation (Figure 2D). Moreover, genetic defects in *IPL1* cause abnormal chromosome segregation and nondisjunction (Chan and Botstein, 1993), which resembles the phenotype observed in *Tetrahymena* bearing the S10A mutation in H3 (Wei et al., 1999). Together, these findings prompted us to investigate whether Ipl1p is the mitotic H3 (Ser-10) kinase in yeast.

To test this hypothesis biochemically, full-length (FL) and amino-terminal domain (NTD, amino acids 1–100 lacking the catalytic domain) of Ipl1p were both His_{10} tagged and purified from bacteria for kinase assays. As shown in Figure 3A, FL Ipl1p but not its NTD phosphorylates the unmodified H3 peptide (amino acids 1–20) but cannot phosphorylate a comparable peptide that is singly phosphorylated at Ser-10 (pH3) or when Ser-10 is replaced by alanine (data not shown). Moreover, mass spectrometry analyses confirm that Ser-10 is the only residue being phosphorylated in the unmodified H3 peptide under these assay conditions (data not shown).

In agreement, Ipl1p phosphorylates H3 and to a lesser extent H2B in a mixture of free histones (Figure 3B, lane 6). In contrast, an evolutionarily related kinase, protein kinase A (PKA; Francisco et al., 1994), displays a stronger preference for H2B over H3 (lane 7; also see Shibata et al., 1990). PKA has long been known to phosphorylate nucleosomal H3 in vitro (Taylor, 1982). When comparing the activity of Ipl1p and PKA toward nucleosomes, the more physiological substrate, Ipl1p, phosphorylates H3 much more effectively than PKA (Figure 3B, compare lanes 2 and 3). Interestingly, Ipl1p also phosphorylates nucleosomal H2B (see doublet, Figure 3B, lane 2), suggesting that a possible functional overlap

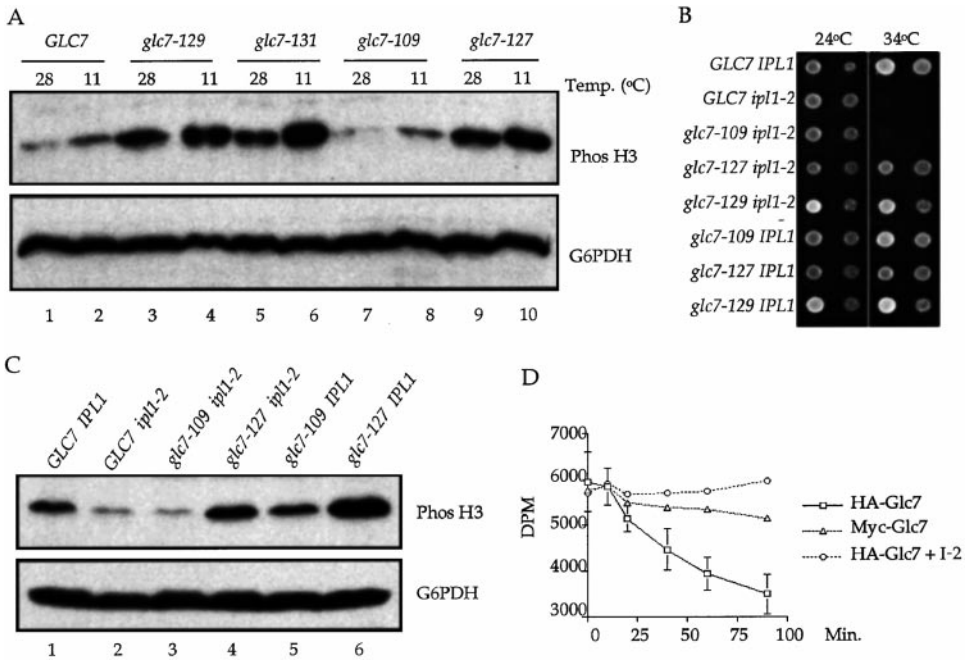

Figure 4. H3 Dephosphorylation Is Dependent on Glc7p

(A) Yeast cells containing alleles of *Glc7* were grown overnight to early log phase at 28°C. Half of each culture was collected for WCE preparation and the other half was added with prechilled (4°C) YPD medium then grown for an additional 24 hr at 11°C. WCE were prepared for Western blot analysis.

(B) Cultures of yeast strains with the designated relevant genotypes were serially diluted 10-fold, spotted onto YPD plates, and incubated at the indicated temperatures for 36 hr.

(C) Indicated strains from (B) were grown to early log phase at 24°C, then harvested for Western blot analysis.

(D) HA-tagged Glc7 proteins were immunoprecipitated by HA Ab (12CA5) from *HA-GLC7* (KT1207) or *Myc-GLC7* (KT1208) strains and assayed for phosphatase activity toward ^{32}P-labeled nucleosomes. Radioactivity remaining in the nucleosome substrates after reaction for 10, 20, 40, 60, and 90 min at 30°C was measured by the p81 filter binding method. Errors represent standard deviations of five individual experiments. Phosphatase activity of HA-Glc7p preincubated with inhibitor-2 (I-2) is also presented.

might exist between H3 and H2B phosphorylation by Ipl1p (see Discussion). Finally, Western blot analyses confirm that Ser-10 is the site of phosphorylation in all of the substrates tested (Figure 3C). These results demonstrate that Ipl1 kinase has the potential to act on chromatin substrates in vivo.

To determine if Ipl1p is involved in H3 phosphorylation in vivo, a temperature-sensitive lethal *ipl1* strain, *ipl1-2* (Francisco et al., 1994), was examined for its Phos H3 levels by Western blot. Strikingly, Phos H3 levels in the *ipl1-2* strain are dramatically reduced compared to that in a wild-type strain, even when growing at permissive temperature (compare Figure 4C, lanes 1 and 2). This result is consistent with a previous study showing that *ipl1-2* mutant cells have a normal growth rate at permissive temperature yet still exhibit an ~10 fold increase in the frequency of chromosome gain (Chan and Botstein, 1993). Thus, from our in vitro and in vivo studies, we conclude that Ipl1p is required for mitotic H3 phosphorylation, likely through a direct enzyme–substrate interaction, in yeast.

Glc7p Is the Upstream Mitotic H3 Phosphatase

The identification of Ipl1p as the mitotic H3 kinase provided a genetic clue to the identity of the corresponding mitotic H3 phosphatase. *IPL1* has been shown to interact genetically with *GLC7*, the only type 1 protein phosphatase in yeast (Francisco et al., 1994). These findings suggest that these two enzymes maintain the proper balance of phosphorylation in a set of shared targets. Therefore, we next examined if Glc7p could be the phosphatase responsible for H3 dephosphorylation in yeast using a collection of *glc7* mutants generated by alanine scanning (Baker et al., 1997). Glc7p is required in a diverse set of nuclear and cytoplasmic pathways, and its specificity is thought to be conferred by interactions with specific regulatory proteins (reviewed in Stark, 1996). Thus, if Glc7p is the H3 phosphatase, we reasoned that some but not all mutant alleles of *GLC7* should show elevated levels of Phos H3.

As shown in Figure 4A, the above prediction is correct. Mutants expressing *glc7-109* are defective for one or more cytoplasmic pathways that require PP1 phosphatase activity but are wild type for nuclear PP1 function. In contrast, *glc7-129* and *glc7-131* mutants are cold-sensitive lethal, defective for one or more nuclear pathways including chromosome segregation, and arrest growth at the G2/M boundary at the restrictive temperature of 11°C (Baker et al., 1997; Bloecher and Tatchell, 1999). Mutants expressing the *glc7-127* allele are also defective for one or more unknown nuclear functions but do not arrest growth at G2/M and can form colonies at 11°C (unpublished data). H3 from all three *glc7* mutants with defects in nuclear pathways showed elevated levels of phosphorylation relative to *GLC7* wild-type cells. In the case of the *glc7-129* and *glc7-131* mutants,

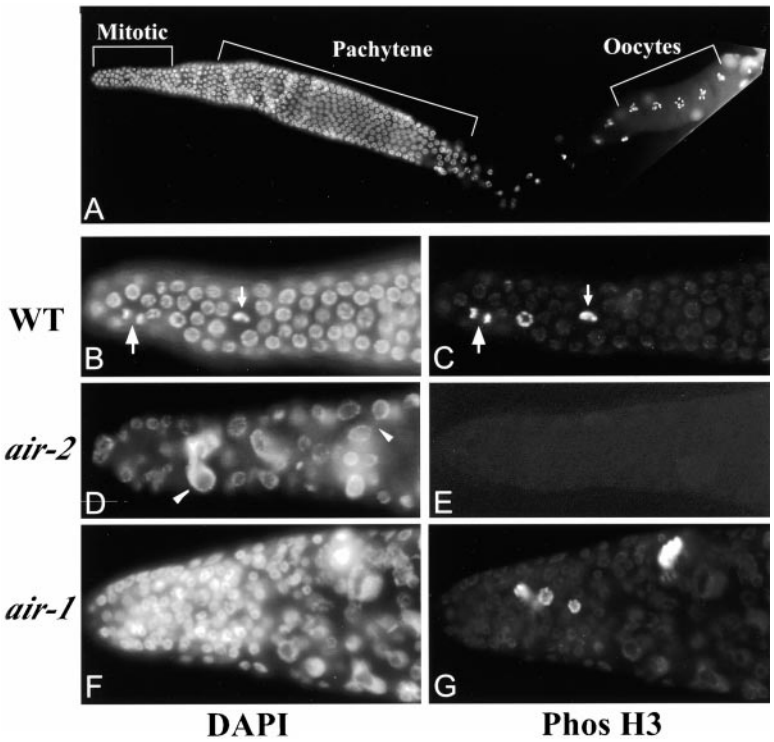

Figure 5. AIR-2 Activity Is Required for H3 Phosphorylation in *C. elegans*

Gonads were stained with DAPI to visualize nuclei (A, B, D, and F) or with Phos H3 Ab (C, E, and G). Gonads are orientated with the distal tip toward the left. Shown in (A) is a low magnification micrograph of an adult wild-type gonad. Nuclei at the distal region divide mitotically. Upon entering meiosis, nuclei progress through different stages of meiotic prophase I. (B–G) High magnification micrographs of mitotic gonads from wild-type (B and C), *air-2(RNAi)* (D and E), and *air-1(RNAi)* (F and G) animals. Small and large arrows in (B) and (C) point to nuclei in the metaphase and telophase, respectively. Arrowheads in (D) point to defects in chromosome condensation and segregation. Note that nuclei to the right are still connected with a thin chromatin bridge that is especially clear when viewed with adjacent images in stack forms (see web site: http://www.cell.com/cgi/content/102/3/279/DC1).

Phos H3 signals were further enhanced when growing at the restrictive temperature (Figure 4A, lanes 3–6). In contrast, the *glc7-109* mutant showed no increase in Phos H3 at either temperature (lanes 7–8). Since *glc7-127* cells do not arrest at the G2/M boundary, the increased levels of Phos H3 seen in the nuclear *glc7* mutants are most likely due to the loss of Glc7 phosphatase activity per se and not simply the arrest at mitosis. These results argue that H3 dephosphorylation in vivo is dependent on Glc7p in yeast.

The allele-specific defect in H3 phosphatase activity in *glc7* mutants leads to the prediction that *glc7* alleles giving high levels of Phos H3 should suppress the *ipl1-2* mutation, whereas *glc7* alleles giving normal levels of Phos H3 should not. We tested this prediction by assaying the growth rate of a set of congenic strains that contain mutations in *IPL1* and *GLC7*. As shown in Figure 4B, *glc7-109* fails to suppress *ipl1-2*, and the double mutant remains unable to grow at nonpermissive temperatures. In contrast, *glc7-127* and *glc7-129* partially suppress the temperature sensitivity of *ipl1-2*, allowing robust growth of *ipl1-2* cells at 34°C. Consistently, *glc7-127* but not *glc7-109* is able to suppress the defect in H3 phosphorylation conferred by *ipl1* mutation (Figure 4C, compare lanes 3 and 4). Thus, the correlation of *ipl1* suppression and high levels of Phos H3 provides further support for the hypothesis that Glc7p and Ipl1p act in opposition to affect the phosphorylation state of H3.

If Glc7p acts directly to dephosphorylate H3 in vivo, then purified Glc7p should be able to dephosphorylate H3 in vitro. To test this prediction, HA-tagged Glc7 protein (Sutton et al., 1991; Stuart et al., 1994) was purified by immunoprecipitation from yeast cell extracts using an HA antibody (12CA5). The immunoprecipitants were then assayed for their ability to dephosphorylate nucleosomal H3. Nucleosomes were ^{32}P-labeled on Ser-10 of H3 in vitro by PKA (Shibata et al., 1990). Although much less efficient than Ipl1 kinase, PKA has the advantage of uniquely labeling H3 without also labeling H2B (Figure 3B, see legend). As shown in Figure 4D, HA-Glc7p were able to dephosphorylate ^{32}P-labeled nucleosomal H3, and this activity was proportional to the amount of immunoprecipitants added to the reaction (data not shown). Furthermore, this H3 phosphatase activity was blocked by inhibitor-2 (I-2), which specifically inhibits type 1 protein phosphatases (Cohen et al., 1989). Using extracts from Myc-tagged Glc7p containing cells as a negative control for immunoprecipitation with anti-HA antibody, no H3 phosphatase activity was detected in the immunoprecipitants. Finally, gel analyses showed that the substrates were not degraded and Western blot confirmed that the phosphate was released from Ser-10 of H3 (data not shown). These results demonstrate that Glc7p is capable of direct dephosphorylation of nucleosomal H3 and together with the previous genetic data suggest that Glc7p regulates the level of phosphorylated H3, possibly through a direct enzyme–substrate interaction.

air-2 Is Required for Mitosis, Meiosis, and H3 Phosphorylation in *C. elegans*

The above results in *S. cerevisiae* suggest that the level of mitotic H3 phosphorylation is established by the balance of antagonistic activities: Ipl1 kinase and Glc7 phosphatase. We reasoned that the fundamental nature of mitosis and meiosis predicts that this system should operate in other eukaryotes. In *C. elegans*, two Ipl1/aurora kinase family members, AIR-1 and AIR-2, have been described and their loss of function assayed by RNA interference (RNAi) (Guo and Kemphues, 1995; Schumacher et al., 1998a, 1998b; Woollard and Hodgkin, 1999). To test whether either of these two kinases is the

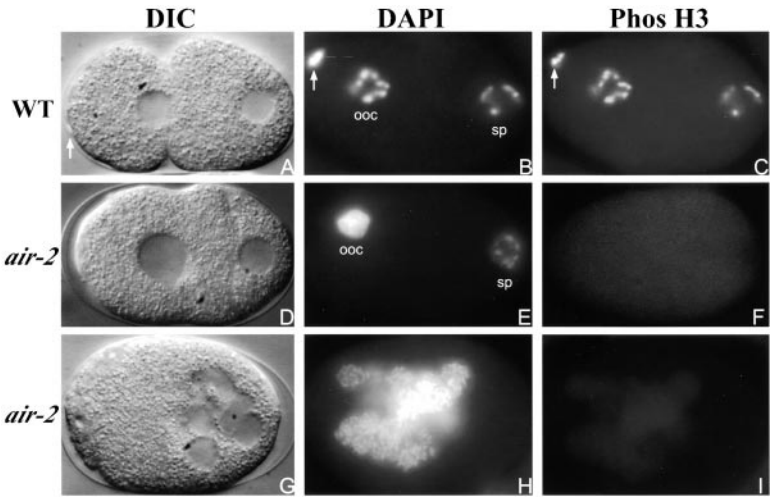

Figure 6. AIR-2 Is Required for Meiosis in *C. elegans*

(A), (D), and (G) are light micrographs of embryos taken with DIC optics. (B) and (C), (E) and (F), and (H–J) are fluorescence micrographs of embryos stained with either DAPI (B, E, and H) or Phos H3 Ab (C, F, and I). (A)–(F) are 1-cell embryos immediately prior to the fusion of sperm and oocyte pronuclei from either wild-type (A–C) or *air-2(RNAi)* (D–F) animals. (G)–(I) are *air-2(RNAi)* embryos roughly 300 min after fertilization. Wild-type embryos at 300 min consist of ~500 cells. Arrows in (A)–(C) point to polar bodies, which are not found in *air-2(RNAi)* embryos (D). Oocyte (ooc) and sperm (sp) pronuclei are indicated in (B) and (E). Note that the oocyte pronucleus in *air-2(RNAi)* embryos (E) has an abnormal DNA content compared to that of wild type (B). Embryos shown in the DIC column are independent of those shown in the DAPI and Phos H3 columns.

mitotic H3 kinase in worms, we reexamined the RNAi phenotypes for both genes paying particular attention to mitosis, meiosis, and the phosphorylation status of histone H3.

The adult gonad of *C. elegans* hermaphrodites provides an excellent opportunity to examine the developmental progression from mitosis to meiosis in germ cells. Briefly, the distal-most germ nuclei divide mitotically and serve as a stem cell population. Moving proximally along the gonad, germ nuclei exit the mitotic cycle and enter a progressive series of meiotic prophase stages. Eventually, the germ nuclei cellularize and develop into oocytes at diakinesis of the first meiotic division (Figure 5A). Upon fertilization, the oocyte nucleus resumes meiosis with each of the two meiotic divisions marked by extrusion of a polar body (Figures 6A and 6B). After fusion of gametic pronuclei, the first embryonic mitotic division cycle begins.

In wild-type animals, Phos H3 staining is associated with metaphase and telophase chromosomes in the mitotic region of the gonad (see arrows in Figure 5C; Kadyk and Kimble, 1998; Lieb et al., 1998). This staining disappears in adjacent meiotic nuclei and reappears as oocytes grow and enter diakinesis (Figures 7A and 7B). Staining continues after fertilization and becomes detectable in the sperm pronucleus immediately before fusion of two pronuclei (Figures 6B and 6C). During embryogenesis, Phos H3 staining of chromosomes is detected from prophase to anaphase during each mitotic cycle (data not shown; Lieb et al., 1998).

Strikingly, animals injected with double-stranded RNA (dsRNA) of *air-2* [hereafter *air-2(RNAi)*] do not show detectable Phos H3 staining in the gonad (Figures 5E and 7D) or in the RNAi embryos (Figures 6F and 6I), suggesting that *air-2* is required for H3 phosphorylation in *C. elegans*. Condensed nuclei are not detected in the distal gonads of *air-2(RNAi)* animals. Instead, abnormal mitotic divisions, characterized by decondensed chromatin or interconnecting chromatin bridges, are often observed (see arrowheads in Figure 5D). Embryos from *air-2(RNAi)* animals do not contain polar bodies and arrest at the 1-cell stage without undergoing cell divisions. DAPI staining reveals that nuclei in *air-2(RNAi)* embryos are polyploid, disorganized, and sometimes fragmented (Figures 6G and 6H; Schumacher et al., 1998b; Woollard and Hodgkin, 1999).

We examined whether the above phenotypes were a result of a failure in meiosis. In all cases where embryos are observed with two partially decondensed pronuclei (equivalent to postmeiotic stage in a wild-type zygote), one pronucleus always had an abnormally high DNA content (Figure 6E). These abnormal pronuclei were unequivocally identified as being of oocyte origin following careful examination of embryos that were retained in the uterus and could be oriented. Based upon the high DNA content in oocyte pronuclei and the lack of detectable polar body, we conclude that meiosis fails to occur in *air-2(RNAi)* embryos.

In contrast to what is observed with *air-2(RNAi)* animals, Phos H3 staining in *air-1(RNAi)* animals and embryos is similar to wild type, suggesting that *air-1* is not required for H3 phosphorylation (Figure 5G and data not shown). In addition, all *air-1(RNAi)* embryos extrude polar bodies indicating that the meiotic divisions occur properly (data not shown). However, the occurrence of abnormal mitotic divisions (Figure 5F) and 100% embryonic lethality suggest that *air-1* is required for other aspects of mitosis function that remain unclear (data not shown; Schumacher et al., 1998a).

RNAi with the *C. elegans* Orthologues of *GLC7* Suppress *air-2(RNAi)* Phenotypes

In light of our findings in yeast, we predict that the mitotic H3 phosphatase should genetically interact with *air-2* in *C. elegans*. Two open reading frames (ORFs), F29F11.6 and F56C9.1, encoding putative PP1 isoforms are >80% identical to Glc7 protein (hereafter referred to as CeGLC-7α and CeGLC-7β, respectively). Both isoforms are 90% identical within a 295 amino acid block and 76% identical within a 727 nucleotide region. To test if either of the CeGLC-7 isoforms is the opposing phosphatase for AIR-2, coinjection experiments were performed using *air-2* and either of these two *Ceglc-7* dsRNAs. Preliminary serial dilution experiments established that injecting 0.1 mg/ml of *air-2* dsRNA was sufficient to cause a complete loss of Phos H3 staining in mature oocytes and embryos, and 100% of these embryos arrested at the 1-cell stage (Figures 6D–6I, 7C, and 7D).

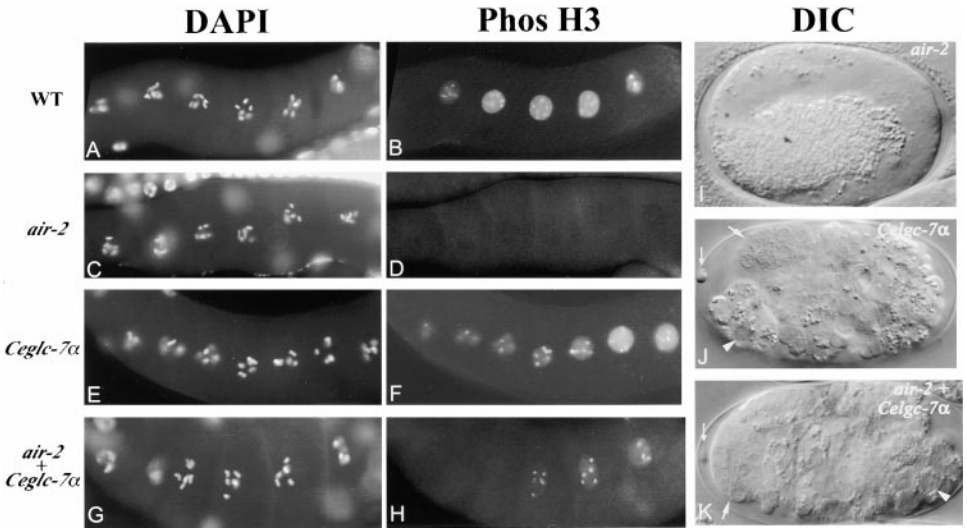

Figure 7. *air-2(RNAi)* Phenotype Can Be Suppressed by *Ceglc-7(RNAi)*

(A)–(H) are fluorescence micrographs of maturing oocytes stained with either DAPI (A, C, E, and G) or Phos H3 (B, D, F, and H): (A and B) wild type; (C and D) *air-2(RNAi)*; (E and F) *Ceglc-7α(RNAi)*; (G and H) *air-2(RNAi);Ceglc-7α(RNAi)*. All gonads are presented with the proximal end to the right. (I)–(K) are light micrographs of embryos taken with DIC optics showing arrest phenotypes of (I) *air-2(RNAi)*, (J) *Ceglc-7α(RNAi)*, and (K) *air-2(RNAi);Ceglc-7α (RNAi)* embryos. Small arrows in (J) and (K) point to polar bodies. Large arrows and arrowheads in (J) and (K) indicate differentiated pharyngeal and intestinal tissues, respectively. Epidermal and neuronal tissues are out of focal plane. *air-2* and *Ceglc-7α* dsRNAs used in the above experiments are 0.1 mg/ml and 1 mg/ml, respectively. Similar results show that H3 phosphorylation in *air-2(RNAi)* embryos can be restored by coinjecting *Ceglc-7β* dsRNA (see text for details).

Using the above concentration (0.1 mg/ml), coinjection of 1 mg/ml of either *Ceglc-7* dsRNA completely suppresses the loss of Phos H3 staining in the gonad of *air-2(RNAi)* animals and in *air-2(RNAi)* embryos (Figures 7G and 7H and data not shown). When a higher concentration of *air-2* dsRNA (1 mg/ml) was used, suppression was not observed, indicating that a low level of AIR-2 kinase is required to restore Phos H3 signal when the opposing phosphatase is inhibited. These data are consistent with the situation in yeast where growth defect of *ipl1-2* can be suppressed by specific *glc7* alleles only at semipermissive temperature (34°C; Figure 4B) but not at restrictive temperature (37°C; data not shown).

It has been shown that RNAi fails to distinguish family members with nucleic acid identity of 80% or greater (Tabara et al., 1998). To test if cross interference is observed between *Ceglc-7α* and *Ceglc-7β*, a series of single and double RNAi experiments were performed. Injection of 1 mg/ml *Ceglc-7α* dsRNA alone results in nearly 100% embryonic lethality with embryos arresting at a late stage exhibiting well-differentiated cell types including pharyngeal, intestinal, epidermal, and neuronal tissues (Figure 7J, arrow and arrowhead). Cells with abnormal DNA content or misshaped nuclei were observed (data not shown), suggesting that chromosome segregation was defective in some cells. In contrast, injection of the same concentration of *Ceglc-7β* dsRNA alone resulted in <50% embryonic lethality, and all of the dead embryos arrested at a 50- to 100-cell stage exhibiting cells of varying size, suggestive of severe cell division defects. However, when *Ceglc-7α* and *Ceglc-7β* dsRNA were coinjected, 100% embryonic lethality was observed, with all embryos arresting at the 50- to 100-cell stage. Our results demonstrate that injection of either dsRNA alone results in distinct phenotypes, while coinjection of both dsRNAs has a more severe phenotype than injection of either RNAi alone. Thus, we conclude that little, if any, cross interference occurs between *Ceglc-7α* and *Ceglc-7β*.

Embryos from animals coinjected with *air-2* and *Ceglc-7α* dsRNA developed beyond the hallmark 1-cell arrest stage of *air-2(RNAi)* embryos and arrested at a stage similar to that of *Ceglc-7α(RNAi)* (Figure 7K). Interestingly, embryos from animals coinjected with *air-2* and *Ceglc-7β* dsRNA, despite the restoration of H3 phosphorylation, arrest at a stage similar to that of *air-2(RNAi)* alone. These results suggest that CeGLC-7α and CeGLC-7β may share with AIR-2 distinct subsets of targets that remain to be determined. Despite these uncertainties, we conclude that both CeGLC-7α and CeGLC-7β are the *C. elegans* orthologues of Glc7p and that they, as in yeast, act in opposition to AIR-2 kinase in a highly conserved pathway required for phosphorylation of histone H3.

Discussion

In this report, we demonstrate that two members of the Ipl1/aurora family of protein kinases, Ipl1p in *S. cerevisiae* and AIR-2 in *C. elegans*, are kinases that regulate mitotic H3 phosphorylation in these two model eukaryotes. Similarly, two related type 1 phosphatases, Glc7p in *S. cerevisiae* and CeGLC-7 in *C. elegans*, are required for dephosphorylating H3. Collectively, these data indicate that the ordered addition and removal of phosphate from H3 at Ser-10 is brought about by a highly conserved enzyme system in diverse eukaryotes. In support, we note that Ipl1/aurora kinases evolved as early, if not founding, members of the AGC branch of cyclic nucleotide-regulated protein kinases (Bischoff and Plowman,

1999), underscoring their fundamental roles in mitosis and meiosis.

Ipl1p and Glc7p family members play an essential role in mitotic and meiotic chromosome transmission, presumably through the modification of multiple protein substrates. Several observations suggest that histone H3 may be one of the target substrates associated with this function. First, both the Ipl1p and Glc7p pathways and the specific modification of H3 Ser-10 have been evolutionarily conserved from fungi to humans. Second, the reversible phosphorylation of Ser-10 is temporally correlated with the mitotic and meiotic activities of Ipl1p and Glc7p, and H3 phosphorylation is specifically associated with condensed mitotic and meiotic chromosomes in both yeast and worms (Figures 2 and 5–7), as well as all other eukaryotic systems examined to date. Third, in *Tetrahymena*, the mutation of H3 Ser-10 results in defects in chromosome condensation and mitotic and meiotic transmission (Wei et al., 1999). As discussed below, this simple causal relationship between H3 phosphorylation and chromosome condensation and segregation is not established in *S. cerevisia*e or *C. elegans*; however, understanding the effect of this modification on chromosome structure in each organism is likely to be informative.

H3 Phosphorylation in *S. cerevisiae*

In yeast, as in other eukaryotes, H3 phosphorylation peaks during mitosis when chromosomes are in their most condensed mitotic state (Figure 2A). Likewise, in meiosis, the strongest peak in H3 phosphorylation is observed during pachytene when chromosomes are dramatically compacted (Figure 2F; Dresser and Giroux, 1988). However, the S10A, S28A, and S10,28A mutations do not result in a major defect in mitotic or meiotic chromosome transmission in *S. cerevisiae*. The generation times and cell cycle progression of the mutants, as measured by fluorescence microscopy and flow cytometry, were indistinguishable from the isogenic H3 wild-type strain, and diploids homozygous for the S10A mutation were able to sporulate (data not shown). Thus, the simple causal relationship between H3 Ser-10 phosphorylation and chromosome dynamics evident in *Tetrahymena* is likely more complex in *S. cerevisiae*. One potential explanation for this difference is that other histone modifications in *S. cerevisiae* may compensate for the loss of Ser-10 phosphorylation for proper chromosome dynamics. As shown in Figure 3B, Ipl1p phosphorylates both H3 and H2B in vitro when a nucleosomal substrate is used, suggesting the intriguing possibility that H2B phosphorylation may be functionally redundant with H3 phosphorylation in vivo. Consistent with this hypothesis, we note that in most eukaryotes, including yeast, H2B amino termini contain several serines that are embedded within stretches of basic residues that resemble the immediate environment of Ser-10 in H3. Interestingly, these serines are not present in *Tetrahymena* H2B.

H3 Phosphorylation in *C. elegans*

In *C. elegans*, *air-2* has been implicated in chromosome segregation (Woollard and Hodgkin, 1999) and cytokinesis (Schumacher et al., 1998b). Our analyses of the gonad of *air-2(RNAi)* animals suggest that like Ipl1p in yeast, AIR-2 is required for histone H3 phosphorylation and for proper chromosome dynamics. Failure to complete mitotic divisions underlies, at least in part, the *air-2* mutant phenotype in which the gonad does not proliferate and homozygotes grow to sterile adults without gonads (Woollard and Hodgkin, 1999). We also show that like Ipl1p in yeast, AIR-2 is required for meiotic divisions in *C. elegans*, and failure to complete meiosis likely contributes to the 1-cell arrest phenotype of *air-2(RNAi)* embryos. However, a difference appears to exist in the initiation of H3 phosphorylation during meiosis in worms and yeast. In yeast, Phos H3 staining is first detected in pachytene, while in *C. elegans*, it is first detected at the later stage of diakinesis. The significance of this difference for chromosome segregation remains to be determined. It is worth noting that in both yeast and worms, the peak of H3 phosphorylation occurs at pachytene in yeast and around the time of the meiotic divisions in worms, coinciding the stages at which chromosomes are maximally condensed.

Our data clearly demonstrate that AIR-2 is required for H3 phosphorylation, although loss of H3 phosphorylation cannot be the sole cause for the 1-cell arrest phenotype of *air-2(RNAi)* embryos. Restoration of H3 phosphorylation is not sufficient to suppress the 1-cell arrest phenotype as shown by coinjecting *Ceglc-7*β dsRNA. In addition to H3, AIR-2 is likely to phosphorylate other substrates including proteins involved in cytokinesis, a possibility supported by localization of AIR-2 on chromatin as well as on midbody microtubules during embryonic divisions (Schumacher et al., 1998b).

Recently, NCC-1, a Cdk1/Cdc2-related kinase, has also been implicated in phosphorylation of H3 during mitosis in *C. elegans* (Boxem et al., 1999). However, when directly compared to yeast Ipl1p, human Cdc2 kinase failed to phosphorylate an amino-terminal H3 peptide containing Ser-10 (Figure 3A and data not shown). Therefore, the loss of Phos H3 signal in the *ncc-1(RNAi*) animal is most likely a consequence of cell cycle arrest caused by the inactivation of *ncc-1*. We note that the noncatalytic NTD of both Ipl1p and AIR-2 contains putative consensus motifs for phosphorylation by CDKs. Indeed, Ipl1p has been found to be phosphorylated in vivo (Kim et al., 1999), although the upstream kinase and its effect on the kinase activity or chromosomal targeting remains to be determined.

Targeting H3 Kinase/Phosphatase Activities

Besides H3, both Ipl1/aurora kinases and Glc7/PP1 phosphatases must target additional substrates in multiple steps of mitotic division. For example, mutations in *IPL1* also confer defects in kinetochore function, and at least one target substrate for Ipl1p is likely to be the kinetochore protein Ndc10p (Biggins et al., 1999). In the case of the original *aurora* mutation in *Drosophila*, monopolar spindles were noted to arise from a failure in centrosome separation (Glover et al., 1995). Other family members, such as *C. elegans* AIR-1 (Schumacher et al., 1998a), *Xenopus* Eg2 (Roghi et al., 1998), mouse IAK (Gopalan et al., 1997), rat AIM-1 (Terada et al., 1998), and human AIK-1 (Kimura et al., 1997) have been shown to be associated with the centrosomes and/or mitotic spindle microtubules. In addition, centrosome separation and spindle formation phenotyes caused by perturbing aurora and Eg2 may be caused by defects in kinetochore–microtubule interactions that remain to be accurately defined. While AIR-2 has been reported to

associated with chromosomes (Schumacher et al., 1998b), the mechanism underlying how Ipl1/aurora kinases are targeted to chromosomes remains to be established.

Similarly, PP1 must also target a wide range of substrates through interaction with specific factors. In support of this view, more than ten Glc7p biding proteins have been found in yeast (Stark, 1996). Identification of the factor(s) that targets Glc7p to H3 will shed light on the mechanistic connections between histone modifications and chromosome structure and dynamics. In contrast to single PP1 gene in yeast, at least three isoforms of PP1 are known in mammals (reviewed in Shenolikar, 1994). Interestingly, only PP1δ appears to associate strongly with the mitotic chromosomes during metaphase (Andreassen et al., 1998), predicting that PP1δ is likely to be the mitotic H3 phosphatase. Strong evidence suggests that PP1 is important for mitotic chromosome transmission. For example, specific alleles of *glc7* in yeast are defective for kinetochore function (Bloecher and Tatchell, 1999; Sassoon et al., 1999). Defects in chromosome segregation and condensation have also been noted for PP1 mutants in *S. pombe* (Ohkura et al., 1989) and in *A. nidulans* (Doonan and Morris, 1989). Moreover, mutation of the *Pp1-87B* gene in *Drosophila* or inhibition of protein phosphatase activity in mammalian cells can cause severe mitotic defects and overcondensed chromosomes (Axton et al., 1990; Guo et al., 1995). Our data suggest that histone H3 is one of the physiologically relevant substrates for PP1 in yeast and nematode, although the causal relationship of H3 phosphorylation and chromosome condensation has not been firmly established.

H3 Phosphorylation and Genome Stability

Just as the discovery of histone acetylation and deacetylation complexes has led to a wealth of new insights into mechanisms of transcription (reviewed in Struhl, 1998), we anticipate that defining the roles of Ipl1p and Glc7p in governing the balance of H3 phosphorylation will pave the way for a better understanding of mitotic and meiotic chromosome transmission. Understanding the physiological targets of these enzymes is of paramount importance in understanding the processes that underlie normal cellular proliferation. The close association of human aurora kinases with oncogenesis reinforces an emerging view that covalent modifications of histones play a vital role in the regulation of proper chromosome dynamics with far-reaching implications for human biology and diseases.

Experimental Procedures

Yeast Strains, Worm Strains, and Culture Conditions

Genotypes of the yeast strains were as follows: CY1221 (*MAT*a *bar1 trp1 leu2 gal1*), JHY86 (*MAT*α *ura3-52 leu2-3,112 trp1-289 his3*Δ*1* Δ*(hht1 hhf1)* Δ*(hht2 hhf2)* pJH18[*CEN ARS TRP1 HHT2 HHF2*]). JHY87 (S10A), JHY88 (S28A), and JHY89 (S10,28A) were derived from JHY86. Diploid strains in SK1 background include: DKB974 (wild type), DKB1478 (*ndt80::KAN-MX4/ndt80::KAN-MX4*), and DKB10 (*spo11::hisG-URA3-hisG/spo11::hisG-URA3-hisG*). All strains containing alleles of *GLC7* are congenic to KT1112 (Stuart et al., 1994). *glc7-131* and *glc7-129* strains are described in Baker et al. (1997). *glc7-127* and *glc7-109* strains are described in Venturi et al. (2000). KT1207 and KT1208 are described in Stuart et al. (1994). Strains used in Figures 4B and 4C are spore clones derived from crosses between an *ipl1-2* strain KT1829 *(MAT*α *ura3-52 leu2 his3 ipl1-2*) and strains containing alleles of *GLC7*. The *ipl1-2* allele originated from a strain provided by Dr. Clarence Chan (CCY915-2B) and was serially backcrossed to KT1112. Yeast cells were grown in complete (YPD) or synthetic complete (SC) media as described (Sherman, 1991). Sporulation of SK1 cells was described by Bishop (1994). The wild-type N2 Bristol strain obtained from the *Caenorhabditis* Genetic Center was used throughout this study. *C. elegans* strains were cultured as described by Brenner (1974).

DNA Construction

To construct yeast strains containing serine to alanine mutations in H3, the ~1.8 kb SpeI–SpeI DNA fragment covering the *HHT2-HHF2* locus was first PCR amplified using genomic DNA of W303-1a strain as template. For cloning purpose, the SpeI site 3′ to the *HHT2* gene was engineered to become SalI site. This DNA fragment was cloned into SalI–SpeI sites of pRS314 (*CEN, TRP1*) to create pJH18. pJH15 [*hht2-3*(S10A)], pJH16 [*hht2-4*(S28A)], and pJH17 [*hht2-5*(S10,28A)] were derived from pJH18 by site directed mutagenesis. Each plasmid was then transformed into MX1-4C strain (*MAT*α *ura3-52 leu2-3, 112 trp1-289 his3*Δ*1* Δ*(hht1 hhf1)* Δ*(hht2 hhf2)* pMS327[*CEN ARS URA3 HHT1 HHF1*]) and cells that lost pMS327 spontaneously were selected by growing on 5-FOA containing medium.

Yeast Protein Extraction, Western Blot, and Immunofluorescence Staining

Yeast nuclei were first isolated as described by Almer and Hörz (1986), except that inhibitors of proteinase and protein phosphatase (1 μg/ml of aprotinin, leupeptin, and pepstatin A, 1 mM PMSF, 1 μM microcystin-LR, and 0.2 mM *p*-chloromercuriphenylsulfonic acid) were included in the solutions. Histones were extracted from nuclei by 0.4 N H_2SO_4 as described previously (Edmondson et al., 1996). For whole-cell extractions (WCE), yeast cells were collected and washed with water, and then resuspended in the breaking buffer (20 mM Tris-HCl [pH 7.9], 10 mM $MgCl_2$, 1 mM EDTA, 5% glycerol, 0.3 M $(NH_4)_2SO_4$, 1 mM DTT, and the inhibitors mentioned above). Cells were broken in the presence of acid-washed glass beads (425–600 μm, Sigma) using a mini-beadbeater (Biospec Products), and extracts were separated from the beads by centrifugation at 16,000 × g for 10 min. Supernatants were collected and protein concentrations were measured by the Protein Assay kit (Bio-Rad). Approximately 30–50 μg of WCE or 1.5 μg of yeast histone proteins were analyzed by SDS–PAGE on 12% or 15% gels followed by Western blot analysis as described elsewhere (Hendzel et al., 1997). The dilution power for the antibodies were as follows: Phos H3 Ab: 1:5,000; general H3 Ab: 1:250 (Wei et al., 1998), and G6PDH Ab: 1:100,000 (Sigma).

Yeast meiotic chromosome spread and immunostaining were performed as described in Bishop (1994). Spread nucleoids were costained with DAPI, rabbit Phos H3 Ab (1:1000 dilution in the presence of 50 mg/ml of H3 1–20 peptide), and mouse anti-Zip1 (1:1000 dilution, generous gift of S. Roeder), followed by secondary staining using Alexa 488–conjugated goat anti-rabbit IgG and Alexa 594–conjugated goat anti-mouse IgG (Molecular Probes, Eugene, OR). Equivalent amounts of phosphorylated H3 peptide eliminates bright Phos H3 staining (data not shown).

Protein Kinase Assays

The coding sequence of *IPL1* and its N-terminal domain (NTD, amino acids 1–100) were PCR amplified and inserted into NdeI–BamHI sites of the His_{10}-tagged expression vector pET-16b (Novagen), respectively. The fusion proteins were overexpressed in the *E. coli* strain BL21-Codon Plus (DE3)-RIL (Stratagene) at 30°C for 2 hr and purified on Ni-NTA agarose according to the manufacturer's instructions (Qiagen S.A.).

Kinase assays were performed in 25 μl of 20 mM Tris-HCl (pH 7.0), 5 mM $MgCl_2$, 1 mM EGTA, 90 μM ATP, 0.75 μCi of [γ-^{32}p]ATP, and 1 μg of recombinant His_{10}-Ipl1p, its NTD, or 1 unit of human $p34^{cdc2}$/cyclin B protein kinase (NEBiolabs) in the presence of 5 μg (2.1 nmoles) of the indicated H3 peptides. After 10 min incubation at 30°C, the reaction was stopped by removing 10 μl of the reaction mixture and spotted onto a P81 filter paper. The filter paper was then washed, dried, and subjected to scintillation counting according to

the S6 kinase assay kit procedures (Upstate Biotechnology). Alternatively, Ipl1p or 3.5 units of protein kinase A (PKA, Sigma) was incubated as above with either the reverse-phase HPLC-purified chicken H3 (0.5 μg), a mixture of acid-extracted chicken free histones (2.5 μg), or sucrose gradient-purified chicken mononucleosomes (1.5 μg) (for details see Sassone-Corsi et al., 1999). In this case, assay mixtures were resolved by SDS–PAGE (15% gels) and processed for autoradiography. For mass spectrometry analysis, 1 pmol of the H3 1–20 peptide, ARTKQTARKSTGGKAPRKQLC, that had been treated with Ipl1p was analyzed by nanoHPLC/μESI LC/MS/MS using an LCQ (Finnigan, San Jose, CA) (Shabanowitz et al., 2000). Directed MS/MS of the +6 and +5 charge states at m/z 395.6 and 474.4, respectively, produced spectra corresponding to the peptide phosphorylated at Ser-10 only. The phosphorylation site was further confirmed by comparison with MS/MS spectra of the synthetic H3 peptide in which Ser-10 is phosphorylated.

Protein Phosphatase Assay

Epitope-tagged Glc7p from KT1207 and KT1208 cells was immunoprecipitated according to the procedures described by Stuart et al. (1994), except that 100 ml of cells were grown, and after spinning down broken cell debris, the total supernatant was subjected to IP. Immunoprecipitants were washed three times with 50 mM Tris-HCl, 250 mM NaCl, 5 mM EDTA, 0.1% Triton X-100, and 1 mM PMSF. After the final wash, immunoprecipitants were resuspended in 1 ml of phosphatase reaction buffer (50 mM Tris-HCl [pH 7.0], 0.1 mM EDTA, and 0.1% β-mercaptoethanol) in 25% glycerol and stored at −20°C before use.

Monomeric chicken nucleosomes, purified as described (Sassone-Corsi et al., 1999), were ^{32}P-labeled by PKA (Shibata et al., 1990). Arbitrary amounts (50–200 μl) of the immunoprecipitants were aliquoted and washed two times with phosphatase reaction buffer prior to resuspension in 40 μl of the same buffer. Immunoprecipitants were preincubated with or without inhibitor-2 (1 μM, courtesy of D. Brautigan) at 30°C for 5 min, then supplemented with ~1 μg of the ^{32}P-labeled nucleosomes in 10 μl reaction buffer and incubated at 30°C. At the 0, 10, 20, 40, 60, and 90 min time points after the reaction was initiated, 5 μl of the reaction were taken, spotted onto a p81 filter, and air-dried immediately. The p81 filters were washed and the radioactivity was determined as described above.

RNA Interference

cDNA clones corresponding to *air-1* (yk483g8), *air-2* (yk274b8), *Ceglc-7α* (yk393h9), and *Ceglc-7β* (yk150g8) were provided by Yuji Kohara. For each clone, single-stranded RNA was prepared from either T7 or T3 promoter separately using the Promega Ribomax In Vitro Transcription kit. dsRNA was generated by mixing an equal amount of plus- and minus-strand RNA in 0.3 M NaOAc, incubating at 70°C for 10 min, followed by cooling to 37°C for 30 min. Yields of double-stranded RNA were estimated by spectrophotometry and by gel electrophoresis using a double-stranded DNA control (pGEM, Applied Biosystems).

Serial dilutions were used to determine the effective concentration range for each dsRNA. For *air-2*, the defects in the distal gonad were observed only with a dsRNA concentration ≥3 mg/ml, although 1 mg/ml dsRNA results in the loss of Phos H3 staining. Staining in the oocytes and RNAi embryos is affected with as low as 0.01 mg/ml *air-2* dsRNA. All injections were performed using 1 mg/ml dsRNA unless specified otherwise.

For coinjection experiments, various concentrations of *Ceglc-7* dsRNA were combined with various concentrations of *air-2* dsRNA to determine if coinjecting *Ceglc-7* dsRNA can suppress the embryonic lethality caused by *air-2(RNAi)* or vice versa. For each combination, 15 animals were injected and embryos were collected and analyzed as described above.

All RNAi experiments were performed by injecting dsRNA into both gonad arms of young adult hermaphrodites (Fire et al., 1998). Injected worms were allowed to recover for 12 hr at 20°C and transferred to individual plates. Embryos collected in the 12–22 hr window after injection were analyzed. Only worms that gave 100% dead embryos were fixed and stained with the Phos H3 Ab. In all cases, at least 40–80 injected animals were stained with Phos H3 Ab.

Antibody Staining and Microscopy

Embryos were processed for immunostaining as described previously (Lin et al., 1998), except that a different blocking solution (3% BSA, 2 mM $MgCl_2$, 0.1% Tween) was used. Hermaphrodite gonads were dissected and fixed for staining as described previously (Francis et al., 1995; Jones et al., 1996) with few modifications. The dissected gonads were fixed in 2% paraformaldehyde (Lin et al., 1998) for 1 hr followed by dimethylformamide (prechilled to −20°C) for 5 min. Fixed gonads were washed with Tris-tween three times, 10 min each, then incubated in the blocking solution for 20 min. After fixation, embryos or gonads were incubated at 4°C for 12 hr with the Phos H3 Ab (1:5,000 dilution). Alexa 488–conjugated goat anti-rabbit secondary Ab (1:250 dilution) was purchased from Molecular Probes. DAPI (0.05 μg/ml) was added to the Tris-tween in the final wash.

All images were analyzed with an Olympus Bmax 60F microscope equipped with epifluorescence and differential interference contrast (DIC) optics. Shutters, filter wheels, and focus motor were from Ludl Electronic Products, and a MicroMAX Kodak KAF 1400 or MicroMAX-512EBFT CCD camera were from Princeton Instruments. Image acquisition, intensity scaling, and automated optical sectioning were performed with custom software designed by James A. Waddle (*Jimage4D*, http://hamon.swmed.edu/~jwaddle/jimage4d.html). To analyze the defects resulting from RNAi, all photographs were collected as sequential images along the Z axis with 0.2 micron between adjacent images. On average, 80 image slices were taken per specimen; only representative slices are presented here. The entire stacks can be viewed at the web site: http://www.cell.com/cgi/content/102/3/279/DC1

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Note Added in Proof

De Souza et al. (Mitotic histone H3 phosphorylation by the NIMA kinase in *Aspergillus nidulans*, Cell *102*, 293–302), also published in this issue of *Cell*, have found that NIMA kinase is required for the mitotic H3 phosphorylation at serine 10 in *Aspergillus nidulans*. Further experiments are needed to determine the relationship, if any, between Ipl1/aurora and NIMA-type kinases.